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DEVELOPMENT OF THE DIGESTIVE SYSTEM IN LARVAL YELLOWTAIL (*PLEURONECTES FERRUGINEA*) AND WINTER FLOUNDER (*PLEURONECTES AMERICANUS*)

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy & Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, PEI

December, 1997

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ABSTRACT

Yellowtail (*Pleuronectes ferruginea*) and winter (*Pleuronectes americanus*) flounder are two pleuronectid species that are considered aquaculture candidates in Atlantic Canada. Rearing practices typically use zooplankton that is costly and time consuming to maintain. Weaning to an artificial diet is successful only after several weeks of development. The ability to digest artificial diets and their effect on larval survival, growth and development is not well understood. This study provides information about digestive system ontogeny in both species reared on live food, weaned at day 15 or starved. The objective was to examine the morphological and functional status of the developing digestive tract and determine if different feeding regimes induced diet-related modifications of enzymatic activity.

Digestive tract ontogeny of larval yellowtail flounder fed a live diet was studied using light microscopy and mucous histochemistry. The undifferentiated tube at 3 days post hatch became regionally and epithelially differentiated into the buccal cavity, pharynx, esophagus, post-esophageal swelling (PES), intestine and rectum by day 10. Further regionalisation of the esophagus and PES and beginning of caecal development occurred at day 29. The presence of gastric glands (stomach formation) by day 36 is suggestive of the onset of metamorphosis. Supranuclear inclusions in the rectal epithelium from day 10 are indicative of intracellular protein digestion. Combinations of acid and neutral mucins were present in the undifferentiated epithelium at day 3 and subsequently at the brush border of the intestine and rectum. Neutral mucins were found in the PES/stomach epithelium whereas acid mucins were present in the buccal cavity, pharynx, esophagus and goblet cells. The variation of mucous histochemistry between these regions indicates functional differentiation for the mucins.

Digestive enzymes were studied in yellowtail and winter flounder larvae reared on three different diets using enzyme histochemical methods. Analysis was based on semi-quantitative ranking of color intensity. Specimens were freeze-dried and embedded in glycol methacrylate. Enzymatic intensity increased during development and fluctuations of intensity stabilized. Alkaline phosphatase (AP), dipeptidylpeptidase IV (DPP IV), aminopeptidase M (MAP) and esterase (E) were present at 3 days post hatch and became differentially distributed coinciding with morphological development. Acid phosphatase was not identified in either species. For fed larvae, AP activity was present in the intestine of both species, rectum of yellowtail flounder and in winter flounder, the PES. Activity was absent in starved winter flounder larvae. In feeding larvae, DPP IV and MAP were localized to the intestine of both species and rectum of yellowtail flounder. For both species, MAP was stronger in intensity and exhibited less variation compared to DPP IV. Esterase was present in the intestine and liver of both species and rectum of yellowtail flounder. A decrease in MAP and E activity occurred in starved yellowtail and winter flounder larvae. The effect of weaning on enzymatic activity was not fully evaluated as the larvae did not survive long enough after the introduction of the artificial diet to complete experimentation. Results suggest that by the time the larvae begin feeding exogenously (7 days post hatch), the digestive system is able to process live food.

ACKNOWLEDGEMENTS

I would first and foremost like to extend my sincerest gratitude to both of my supervisors Dr. Glenda Wright and Dr. Greg Goff for their support throughout the duration of this project, from the initial start-up to the numerous drafts of the final thesis. Additionally, I thank those on my supervisory committee, Dr. Amreek Singh, Dr. Bill Ireland and Dr. David Speare for their insight into the various phases of my project. The staff at the Huntsman Marine Science Centre, namely Joy Wade and Stacey Paul, who participated in the larval rearing and made my stay in St. Andrew's NB very enjoyable, I extend my warmest thanks. I also acknowledge the contribution of the Ocean Sciences Centre at Memorial University for supplying the larvae needed for the first part of this study on yellowtail flounder development as well as Harry Murray for his work during the initial phases.

The assistance and technical support of Kim Whitman, Dorota Wadowska, Molly Kibenge, Ruth MacPhail, Shelley Ebbett and Marie-Claire Galvin have been greatly appreciated. There are many people at the AVC whose continued friendship and support I value: Debby LeBlanc, Judy MacIntyre, Holly Beaman, Chris Campbell, John Ficele, Blair MacDuff, Joanne Dennis and Cheryl Wartman. Finally, this research would not have been possible without the financial assistance from the AVC Internal Research Fund and Fisheries Products International as well as personal support from an NSERC PGS A.

For my Mother

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A	- Acid mucins
a	- Anterior region
AB	- Alcian blue
AcP	- Acid phosphatase
Ant	- Anterior PES/stomach
AP	- Alkaline phosphatase
BB	- Columnar cells with a brush border
bb	- Brush border
BC. bc	- Buccal cavity
C	- Combination of acid and neutral mucins
Col	- Columnar
CR	- Cardiac region
Cu	- Cuboidal
Cu-gl	- Glandular cuboidal
CX	- Trials using transverse sections
CX(2)	- Repeated trials using transverse sections from CX
DPH	- Days post hatch
DPP IV	- Dipeptidylpeptidase IV
E	- Eye
es	- Esophagus
ESSC	- Esophageal surface secretory cells
Est	- Nonspecific esterase
fm	- Food mass
FDGE	- Freeze-dry glycol methacrylate
FR	- Fundic region
G	- Undifferentiated gut
G1	- Group 1
G2	- Group 2
G3	- Group 3
GC	- Goblet cells
Gly-Pro	- glycyl-prolyl
GMA	- Glycol methacrylate
HUFA	- Highly unsaturated fatty acid
I. Int	- Intestine
K	- Kidney
L	- Liver
L-leu	- L-leucine
LX	- Trials using sagittal sections
Main	- Main body of PES/stomach
MAP	- Aminopeptidase M
MNA	- Methoxy-2-naphthylamide

N	- Neutral mucins
PAS	- Periodic acid-Schiff
pan	- Pancreas
PES	- Post-esophageal swelling
PH, p	- Pharynx
Post	- Posterior stomach
PR	- Pyloric region
PS	- Pyloric sphincter
Rec, R, r	- Rectum
S	- Ileorectal sphincter
SCu	- Stratified cuboidal
SSq	- Stratified squamous
Sq	- Squamous
St	- Stomach
WF	- Winter flounder
y	-Yolk sac
YTF	- Yellowtail flounder
1	- Zone 1
2	- Zone 2

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1. GENERAL INTRODUCTION

The cultivation of marine fish for aquaculture has met with numerous problems. The main difficulty in culturing species such as Atlantic halibut (*Hippoglossus hippoglossus)* and cod (*Gadus morhua)* is optimizing the production of juveniles, where the major obstacle is still high mortalities during the larval phase (Kjorsvik & Holmefjord, 1995). This high mortality rate in many marine species has been linked to nutritionally inadequate food organisms, underdevelopment of the larval fish especially the digestive system, poor larval quality or poor water quality (Stottrup, 1993). To develop from the larval into the juvenile stage (metamorphosis), marine fish must evolve through a series of morphological, biochemical, and behavioural alterations while the development of the organ systems involved in nutrient metabolism and digestion undergo structural and functional changes (Segner & Verreth, 1995).

Obtaining adequate nutrition is important during these early larval stages and far more critical for most marine fish because they have limited yolk reserves, small mouths, and primitive digestive systems (Lavens *et al.*, 1995). One major difference between the larval and adult marine fish is the lack of gastric glands in the larvae. In species such as catfish (*Clarius gariepinus*), whitefish (*Coregonus lararetus*), and turbot (*Scophthalmus maximus*) (Segner *et al.*, 1993), the larvae lack a morphological and functional stomach (Govoni *et al.*, 1986) where gastric glands do not appear until near metamorphosis. Because first feeding larvae do not have a stomach, digestion starts in the intestine (Hjelmeland *et al.*, 1993) where cells in the hindgut carry out acid proteolytic digestion (Sarasquete *et al.*, 1993).

Two species of marine flatfish, the yellowtail flounder (*Pleuronectes ferruginea*) and the winter flounder (*Pleuronectes americanus*), are considered as candidates for cold water aquaculture in Atlantic Canada (Brown *et al.*, 1995). Their development within this industry, however, has met with limited success. High rates of mortality during the larval stage coupled with insufficient scientific knowledge about general larval biology such as their digestive system ontogeny, nutrient requirements and optimal rearing procedures have been major "bottlenecks".

One difficulty lies in the feeding of the larvae. While the use of live feed (rotifers and *Artemia*) is common practice and adequate results are usually obtained, the maintenance of food for larviculture is both time consuming and expensive. Presently, there is insufficient knowledge about the digestive organization, functions and capabilities of both these species at the larval stage to develop an alternate food supply for first-feeding larvae, such as dry feed that is frequently used for salmonid species (Rosenlund, 1997).

Although structure of the digestive system has been studied in the adult (Murray *et al.*, 1994 a,b; 1996), little is known about the ontogeny of the larval digestive system in either of these species. By investigating and comparing the effects of diet on the structural and functional development of the digestive system, a further understanding of larval nutritional requirements can be obtained. Ultimately, greater success during artificial rearing may eventually be achieved.

1.1 Pleuronectiformes

Flatfish belong to the order Pleuronectiformes which are marine bottom-dwelling fish that lie on their left or right side with this underside being white and the "eyed" or top side darkly pigmented. Both yellowtail and winter flounder belong to a family in this order, the Pleuronectidae or right-eyed flounders which inhabit the Arctic, Atlantic and Pacific Oceans. They display the characteristic asymmetry and life style of other flatfish: i.e. are carnivorous, with a laterally compressed oval body with and light brown to black pigmentation. Within this family, there are 41 genera comprising 99 species; of these, 8 have been reported in Atlantic Canada (as reviewed by Scott & Scott, 1988).

Flatfish larvae swim and live oriented in a bilaterally symmetrical fashion, with one eye on either side of the head. During metamorphosis, the eye on the blind side (underside) migrates to the upper side. In the Pleuronectidae, as described by Scott & Scott (1988), the eye on the underside migrates to the right side of the head with the fish lying on its left (blind) side. Coincident with ocular migration, the fish deepens in colour and the juvenile eventually turns completely over onto the blind underside and settles on the bottom to begin the demersal life of the adult.

1.2 Yellowtail and Winter Flounder

Yellowtail and winter flounder, although belonging to the same genus, do exhibit species variation in their distribution, range, natural diet and life history.

1.2.1 Distribution

The range for both these species is on the Atlantic Coast of North America where

they are common along the Newfoundland coasts and banks. The yellowtail flounder is found from the Gulf of St. Lawrence, south to the lower part of Chesapeake Bay (Scott & Messieh, 1976). In the Canadian region, the yellowtail occurs from Barge Bay, Straight of Belle Isle to the Newfoundland Grand Banks, Gulf of St. Lawrence, Scotian Shelf and Bay of Fundy (Scott & Scott, 1988). The winter flounder occurs commonly from Labrador south to Georgia (Scott & Messieh, 1976) with its Canadian range including the Gulf of St. Lawrence, Grand Banks, Scotian Shelf and into the Bay of Fundy (Scott & Scott, 1988).

Both the yellowtail and winter flounder differ in their preferred habitat range. The yellowtail flounder is most typically an offshore species which is found on the continental shelf, living at depths of 27 to 364 m, commonly at 37-91 m. It prefers a temperature of 2-6 ^{0}C but also can be found from -1.0 to 12.2 ^{0}C (Martin & Drewry, 1978). This species moves into shallower waters in the spring and deeper waters in the fall and winter (Scott & Scott, 1988). In contrast, the winter flounder is a more shallow-water, inshore species living at depths usually from 1.8-36.6 m. It can tolerate a wider range of temperatures (Scott & Scott, 1988), reported from -1.5 to 28 ^{0}C, avoiding water that is cooler than 0 ^{0}C or warmer than 14-15 ^{0}C (Martin & Drewry, 1978). Like yellowtail flounder, this species undergoes seasonal movements, tending to move offshore in the winter and onshore in the summer, possibly to spawn or feed (Scott & Scott, 1988).

1.2.2 Natural Diet

Both yellowtail and winter flounder ingest crustaceans (particularly amphipods) as

well as polychaetes. Both the species show reduced food intake during the winter: that is particularly true for the winter flounder (Scott & Scott, 1988; Martell & McClelland, 1994); one study noted that food was absent from the stomachs of these animals during February (Martell & McClelland, 1994).

Yellowtail flounder feed primarily on polychaete worms, crustaceans such as amphipods (Yazdani, 1969; Libey & Cole, 1979; Scott & Scott, 1988) and tunicates (Martell & McClelland, 1994) with cumaceans being the most important food item (Libey & Cole, 1979). Yellowtail flounder feed on the bottom as indicated by the common occurrence of plant detritus, sand, pebbles, sand dollars and sand-burrowing animals in their stomachs (Libey & Cole, 1979). It selects its prey on the basis of size; smaller fish consumed cumaceans, tunicates and amphipods while larger fish ingested polychaetes (Martell & McClelland, 1994).

Winter flounder, like the yellowtail flounder, consume a variety of organisms with polychaetes and crustaceans being the most important food items (Scott & Scott, 1988; Martell & McClelland, 1994). They have also been known to feed on capelin eggs, snails and soft shell clams (Scott & Scott, 1988). Selection of food organisms on the basis of size also occurs with this species; smaller winter flounder primarily ingest polychaetes whereas larger fish consume crustaceans associated with ectoproct colonies (Martell & McClelland, 1994).

1.2.3 Life History

The life history of both the yellowtail and winter flounder is like that of other

flatfish: a bilaterally symmetrical larvae is transformed at metamorphosis into a bottom-dweller, although species specific differences (e.g. the timing of events such as spawning, hatching, etc.) do occur.

1.2.3.1 Spawning

Yellowtail flounder is a batch or serial spawner (Zamarro, 1991) that release their eggs on or near the bottom (45-75 m; Martin & Drewry, 1978) from May to July (Scott & Scott, 1988). A single female can yield large numbers of eggs, approximately 350,000 to 4,570,000 (Scott & Scott, 1988) throughout the spawning season at a temperature of about 5-7 ^{0}C (Martin & Drewry, 1978). The eggs are fertilized and float to the surface where they may hatch in five days at 10 ^{0}C to 11.1 ^{0}C (50-55.5 degree days) (Scott & Scott, 1988).

Winter flounder spawn in shallow water (1.8-3.6 m; Martin & Drewry, 1978) in the late winter to late spring, from about February to early June. Spawning peaks when the water temperature is between 2-5 ^{0}C at a salinities from 11 to 33 ‰ (Martin & Drewry, 1978). Fecundity is less than that in the yellowtail flounder with about 500,000 eggs released in a single spawn. Eggs are adhesive, settling on the bottom once fertilized where they hatch in 15 to 18 days at 3 ^{0}C (Scott & Scott, 1988). Although hatching can occur at temperatures between -1.8-18 ^{0}C, lower success rates occur at temperatures above 10 ^{0}C. Successful hatching takes place at salinities between 4.5 and 40 ‰ but the optimum salinity for hatch lies between 15 and 35 ‰ (Martin & Drewry, 1978).

Flatfish eggs and the emergent larvae are considerably smaller when compared

with salmonid species. Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) eggs are 6.0 mm and 4.0 mm in diameter, respectively (Liewes, 1984). Yellowtail and winter flounder eggs, by comparison, are considerably smaller, 0.8-0.9 mm in diameter for yellowtail and average about 0.8 mm (between 0.71-0.96 mm) for winter flounder (Fahay, 1983; Scott & Scott, 1988).

1.2.3.2 Larval and juvenile stages

Yellowtail flounder larvae are approximately 2-3.5 mm long at hatching (Fahay, 1983). These yolk-sac larvae are pelagic and their movements being limited to those of the water mass (Scott & Scott, 1988). They are largely unpigmented but a slight colouration may be seen on the head or in one or two patches on the tail (Martin & Drewry, 1978). They are between 11.6 to 16 mm in length at the start of metamorphosis (Fahay, 1983). The length of juvenile yellowtail flounder is from about 14 mm to 26 cm (Martin & Drewry, 1978).

The newly-hatched larvae of the winter flounder are also pelagic and are about 2.4 mm in length (Fahay, 1983). They are almost transparent except for the eyes and a vertical pigment band on the tail. The larvae of this species are commonly found in depths less than 37 m and occur between a salinity of 3.5 to 27.7 ‰, with peak abundance occurring between 6-15 ‰ at a temperature between 4-13 ^{0}C (Martin & Drewry, 1978). Transformation starts to occurs when the larvae are at 7.0 to 13.0 mm in length (Fahay, 1983).

By comparison, the larvae of salmon are approximately 25 mm at hatch

(Rosenlund, 1997).The smallest juveniles occur from about 6 to 9 mm. Generally by 8 mm, the right side is completely pigmented and the left side has lost pigment except for a few scattered spots; by the time they reach 20 mm in length, all pigment is gone from its left side (Martin & Drewry, 1978).

1.2.3.3 Adults

In Canadian waters, yellowtail flounder measures about 27 cm at four years of age with a maximum length of 62.7 cm off Newfoundland and 59 cm off Sable Island Bank (Scott & Scott, 1988). In this same region (Newfoundland), 50% of males reach maturity by five years and females by six years (Martin & Drewry, 1978). Those that are taken commercially (off of the Grand Banks) are usually fish aged 4-10 years (Scott & Scott, 1988).

Winter flounder in this region of Newfoundland is mature by age nine; some males are mature by four years and some females by five years. In terms of length, 50% of the males are mature by 21 cm and 50% of all females by 26 cm (Martin & Drewry, 1978). They seldom exceed 45 cm in length or 1.4 kg in weight (Scott & Scott, 1988).

1.2.4 Commercial Importance

Both the yellowtail and winter flounder are economically important to the sport and commercial fisheries (Scott & Messieh, 1976; Scott & Scott, 1988). For yellowtail flounder, the total catch in 1973 was 67,000 mt (metric tons) of which 30,000 were landed in Canada (Scott & Messieh, 1976). In George's Bank from 1962-1976, the

average was 16,300 mt but this declined to 5800 mt between 1978-1981. Presently, it is estimated that total biomass is low on George's Bank (Fisheries Resource Conservation Council, 1997). This has continued to decline since 1985 and only 2300 mt were landed in 1993. Similar trends have been seen with yellowtail founder stocks in Southern New England, Cape Cod and Mid-Atlantic Tables, all three areas reaching record low levels in 1993 (Northeast Fisheries Science Centre, 1994).

Winter flounder is often described as being less commercially important (Scott & Scott, 1988) where in 1973, 3000 mt were landed in Canada (Scott & Messieh, 1976). Declining winter flounder stocks have been recorded in the Gulf of Maine, Georges Bank and Southern New England-Middle Atlantic Tables. Similar to the yellowtail flounder, near record low levels in all of these regions were reported in 1993 (Northeast Fisheries Science Centre, 1994). Generally in the past few years, all commercially exploited flatfish have shown a declining biomass (Canadian Stock Assessment Secretariat, 1996).

1.3 Alternative Species for Aquaculture

The decline in the wild flatfish stocks coupled with good market value (total value of flounder in 1996 for Prince Edward Island: $519,000; Fisheries and Oceans Statistical Services, 1996) makes yellowtail and winter flounder desirable candidates as potential aquaculture species. For the yellowtail flounder, important rearing parameters such as optimum temperatures and feeding strategies for the larvae and juveniles as well as brood stock nutrition have not yet been fully investigated.

1.3.1 Difficulties During the Larval Stage

Insufficient knowledge about basic developmental biology and nutrient requirements and the high cost of live food production are two of the main difficulties that can occur during the larval stage.

1.3.1.1 Live Food Production

The diets of many cultured fish species during the larval stage have traditionally involved a live diet. Algal, rotifer, or *Artemia nauplii* cultures are the most common live diets that are used with relatively good success. Success in aquaculture, however, not only involves maintaining an adequate and successful brood stock, but also requires accomplishing this in a cost-efficient manner.

Algal cultures for fish hatcheries are labour intensive and expensive. Additional problems also exist with respect to contamination and consistent nutritional quality. Algae do not constitute a major food source at the start of feeding for fish larvae, although they may be a source of micro-nutrients or condition the dietary quality of rotifers fed to the larvae in tanks (Lavens *et al.*, 1995).

Rotifer diets are administered as a starter diet in marine fish larviculture but the main constraints are still reliable and cost-effective procedures for continuous mass production. *Artemia* are used worldwide and sold commercially. They must be produced in relatively large quantities, enriched and continuously maintained. Rotifer and *Artemia* enrichment is necessary to increase their dietary value, primarily by addition of algae or dry products such as Culture Selco® or Protein Selco®. This allows for constant and

predictable levels of (n-3) HUFA enrichment (Lavens *et al.*, 1995) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are necessary for normal growth in marine finfish (Watanabe, 1993).

1.3.1.2 Weaning

Problems with the use of artificial diets are encountered with marine fish because the larvae are minute, sensitive to variations in their ambient environment, and rather poorly developed at hatching (Lavens *et al.*, 1995). These larvae display incomplete organogenesis at start of exogenous feeding (Segner & Verreth, 1995) and organ development continues until metamorphosis resulting in significant changes in both morphology and enzymology of the digestive system (Lavens *et al.*, 1995). Replacement diets such as commercial microparticulate diets (MPD), therefore, must fulfil special characteristics such as particle size, physical performance in water, attractability, digestibility, and nutritional composition in order to meet the requirements of the larvae, while still remaining cost effective (Lavens *et al.*, 1995).

Weaning (compound artificial diet substitution for live prey) can only be performed after some weeks of life in marine fish, often near the time of metamorphosis. This has been shown for many flatfish species such as turbot, (Kuhlmann *et al.*, 1981; Segner *et al.*, 1993); Dover sole, *Solea solea*, (Person-Le Ruyet, 1990); Japanese flounder, *Paralichthys olivaceus*.(Kurokawa & Suzuki, 1996) and Atlantic halibut (Brown *et al.*, 1995). Simply, weaning yellowtail flounder onto an artificial diet will likely only be successfully achieved after the larvae have undergone metamorphosis.

For most of these species, research into larval digestive system development is recent, including information about the ontogeny of digestive system functions, primarily the enzymes. Nutritional requirements, production of artificial diets and weaning will be better understood in these species when this information can be completed.

1.4 Digestive tract of adult teleost fish

The teleost digestive system consists of two functional units (Groman, 1982): (1) the alimentary tract as a compartment for the assimilation of food and (2) extramural organs for metabolic processing. Teleosts show a great diversity in the form and function of their digestive tracts. This seems to relate to the kind of food eaten and the amount of surface area needed to achieve absorption efficiently. This efficiency is accomplished by lengthening of the gut, often with elaborate folding, adding pyloric caeca, or internal ridging and folding (Smith, 1989).

In most adult teleost species, the digestive system is composed of the alimentary canal and digestive glands (gastric and intestinal glands, liver, and pancreas) (Takashima & Hibiya, 1995). The alimentary tract follows the general plan: oral (buccal) cavity, pharynx, esophagus, stomach, and intestine. The histological structure is composed of four basic layers: the mucosa (mucosal epithelium, basal lamina, lamina propria, and muscularis mucosae), submucosa, muscularis (circular and longitudinal muscle layers) and the serosa (Takashima & Hibiya, 1995).

1.5 Digestive tract of adult yellowtail and winter flounder

As noted recently by Murray *et al.* (1994a,b; 1996), the digestive tract of both the yellowtail and winter flounder follows the general teleost scheme. Histologically distinct regions of both the stomach and esophagus have been identified. The esophagus is further differentiated into the anterior (Zone 1) and posterior (Zone 2) esophagus. The stomach in both of these species possesses glandular (cardiac) and aglandular (pyloric) regions.

1.5.1 Posterior esophagus

The posterior esophagus of both yellowtail and winter flounder consists of a mucosa, propria-submucosa, muscularis externa, and serosa. The posterior esophageal mucosa contains branching folds with the epithelium consisting of stratified cuboidal cells (surface cuboidal cells termed ESSC's- esophageal surface secreting cells) interspersed with goblet cells. The increase in surface area from these folds may reflect a digestive function whereas the stratified nature of the epithelium supports the numerous goblet cells. Secretory granules of the ESSC's may with these other features suggest some digestive capacities (Murray *et al.*,1994b).

1.5.2 Stomach

The stomach of both species is divided into a cardiac (glandular) region and pyloric (aglandular) region (Murray *et al.*, 1994a). The mucosa, organized into folds, is composed of compact connective tissue lamina propria and epithelium. The epithelia of the glandular region are divided into three zones: zone 1 containing surface mucous cells,

zone 2 mucous neck cells, and zone 3 gland (oxyntic-peptic) cells. This division is unusual where, in most teleost species, mucous neck cells are not distinguished from the surface mucous or gland cells. In addition, enteroendocrine-like cells are found in the epithelium of the gastric mucosa (Murray *et al.*, 1994a).

1.5.3 Intestine, pyloric caeca and rectum

Intestinal, pyloric caecal, and rectal epithelia in both species is defined by two cell types- columnar and goblet cells. The luminal surface of the intestine and pyloric caeca is characterized by numerous microvilli (Murray, 1993).

1.6 Gastrointestinal mucins

While enzymatic secretions catalyze reactions of digestive processes, the obvious functional relevance for the production and secretion of mucins from mucous-secreting cells or goblet cells along the alimentary canal is less clear.

Mucus in the gastrointestinal tract, besides being a moisturized shield, acts as a lubricant, a trap for bacteria and parasites, a trap for physical immobilization of enzymes, a digestive zone, a permeability barrier in the intestine and a protective secretion in the stomach (Forstner & Forstner, 1986). This mucous gel is comprised mainly of water with these many functional properties attributed to one component, mucins.

Mucins are viscous glycoproteins that are made, stored and secreted from goblet cells (Forstner & Forstner, 1986). Generally, mucins consist of hexosamine sugars covalently bound to a lipid or protein (Bancroft & Cook, 1984). The presence of free

hexose groups, acidic moieties or sulphation of the hexosamine molecule (Cook, 1990) allows for histochemical demonstration of different types of mucins.

1.6.1 Neutral and acid mucins

Mucins can be divided into two groups: acidic and neutral mucins. Neutral mucins have no acid radical but a free hexose group is usually present. Acid mucins can be divided into three groups: sulphated acidic mucins that can be strongly or weakly acidic, carboxylated acid mucins that contain a sialic acid molecule, and sulphated sialomucins (Bancroft & Cook, 1984).

The distribution of these mucosubstances within regions of the teleost digestive tract shows considerable variation. In the esophagus, mucous (goblet) cells can contain neutral (Grau *et al.*, 1992), acid (Bucke, 1971; Groman, 1982; Anderson, 1986) or combinations of neutral and acid mucins (Hirji, 1983). In the stomach, neutral mucins in the epithelium are predominant (Reifel & Travill, 1978; Groman, 1982; Osman & Caceci, 1991; Grau *et al.*, 1992) although combinations can be present (Clarke & Witcomb, 1980). Gastric glands contain neutral mucins (Osman & Caceci, 1991) as well as cellular combinations (Grau *et al.*, 1992). In the intestine, goblet cells are acidic (Reifel & Travill, 1979; Sis *et al.*, 1979; Groman, 1982; Anderson, 1986) and the epithelium neutral (Clarke & Witcomb, 1980; Groman, 1982).

The presence of mucins has been examined in various studies of the larval digestive system. Sarasquete *et al.* (1996) found the epithelial digestive system in the Senegal sole, *Solea senegalensis*, contained both neutral and acid mucins. In the larval

gilthead sea bream, *Sparus aurata*, esophageal mucous cells, intestinal goblet cells, epithelial columnar cells in the stomach and enterocytes of the digestive epithelium were rich in carboxylated, sulphated and/or neutral mucosubstances (Sarasquete *et al.*, 1995).

1.6.2 Distribution in adult yellowtail and winter flounder

In the esophagus of both of these species, epithelial and goblet cells showed positive staining reactions for mucosubstances (Murray *et al.*, 1994b). In the winter flounder goblet cells and ESSC's were positive for only sulphated acid mucins. In the yellowtail flounder, goblet cells were positive for both acid nonsulphated and sulphated mucins and the epithelium was positive for nonsulphated acid mucins. The functions of these secretions in this part of the digestive tract are unknown (Murray *et al.*, 1994b).

In the stomach of winter flounder surface mucous cells, mucous neck cells, and gastric gland cells stained positive for neutral mucins. In the yellowtail flounder, surface mucous cells were positive for neutral and nonsulphated acid mucins. Mucous neck cells of the cardiac stomach stained strongly for neutral mucins and weakly for sulphated and nonsulphated acid mucins. The glandular cells stained weakly for neutral mucins (Murray *et al.*, 1994a). The functional significance of different staining reactions between species, as proposed by Murray *et al.* (1994a), suggests differences in the gastric lumen like variations in the pH or enzyme secretions.

Differences could also be seen between these two species with respect to mucous histochemistry in the intestine, pyloric caeca, and rectum. According to Murray *et al.* (1996), goblet cells in these regions of winter flounder contained acid mucins and those

of the yellowtail flounder contained cellular combinations. The differences seen indicated different post-gastric luminal chemistry and may correlate with different digestive functions. Neutral mucosubstances can indicate an absorptive function (Grau *et al.*, 1992). Generally, mucosubstances may provide cofactors needed for digestive enzymes (Anderson, 1986).

1.7 Digestive tract of larval fish

The digestive system of larval marine fish does not follow the general teleost plan typically seen in the adult. Regional and epithelial differentiation or the initial presence of accessory organs like the liver and pancreas are not seen until later stages of development.

The larval digestive system is described as simply organized and shorter than its adult counterpart (Lauff & Hofer, 1984). Smith (1989) divides the early digestive capabilities of teleost fish into two broad categories. Generally, fish with sinking eggs and large amounts of yolk produce large, well-developed larvae that have digestive tracts that are relatively complete and functional when yolk absorption occurs (e.g. salmonids). In comparison, species that have small floating eggs and little yolk material start feeding early with minimal digestive capability (e.g. herring and flatfish).

Several common characteristics of marine fish larval digestive systems that define it as incompletely developed. The lack of a functional stomach (Govoni *et al.*, 1986; Hjelmeland *et al.*, 1993), low production of digestive enzymes at first feeding (Lauff & Hofer, 1984; Govoni *et al.*, 1986), and incomplete organogenesis at start of exogenous feeding are key differences between the adult and the larvae. Because the digestive

system of larval marine fish is not morphofunctionally developed, they may rely on the digestive enzymes introduced with their food; that is, the status of enzymes in their food greatly affects the digestive enzyme activity in the gut (Lauff & Hofer, 1984; Bone *et al.*, 1995). These exogenous dietary enzymes may contribute to the digestive capability of the larval gut. In addition, acid digestion of proteins and the ability to absorb nutrients differ between exogenous-feeding larvae and fully developed adults (Sarasquete *et al.*, 1993). In the larvae, supranuclear vacuoles in the gut epithelium represent pinocytic absorption of macromolecules from the gut lumen. The presence of a functional stomach during later stages facilitates the protein uptake by secretions from the gastric gland, namely, pepsin and HCl (Govoni *et al.*, 1986). In marine fish, metamorphosis is associated with the morphological appearance of functional stomach as well as pyloric caeca (Govoni *et al.*, 1986; Hjelmeland *et al.*, 1993).

1.7.1 Embryology of the digestive tract

The digestive tract develops from a simple tube derived from the endoderm. The endodermal cells form the epithelial lining of the pharynx, esophagus, stomach, intestine and rectum. Buds from this tube will form the accessory organs- the liver, gallbladder and pancreas. The mesodermal (splanchnic) mesenchyme surrounding this tube provides the muscles (muscularis externa and muscularis mucosae), connective tissue (lamina propria) (Hopper & Hart, 1985; Collins, 1995; Gilbert, 1997) and angiogenic tissue (Collins, 1995). This close association between the epithelium and the mesenchyme has been demonstrated to be critically important for the correct organ placement and functional

development of the entire gastrointestinal system (Hopper & Hart, 1985; Aufderheide & Ekblom, 1988; Haffen *et al.*, 1989; Menard & Calvert, 1991; Collins, 1995; Gilbert, 1997).

Based on studies with zebrafish embryos, *Danio rerio*, it is known that the teleost digestive system develops from cells that initially lie above the yolk at the marginal zone of the blastoderm (Kimmel *et al.*, 1990; Warga & Kimmel, 1990; Ho, 1992). Gastrulation occurs when these cells involute underneath the margin towards the yolk cell surface forming the hypoblast, the rudiment of the endoderm and mesoderm. Those cells involuting early will form the endoderm, the digestive tract anlage.

In a recent study on the development of zebrafish digestive organs, Pack *et al.* (1996) concluded the gastrointestinal system matures in a mode that is similar to that in higher vertebrates. In mammals, a continuous tube of endodermal cells initially covering the yolk sac constricts and becomes regionally segmented into the esophagus, stomach and intestines. The intestinal multilayered epithelium eventually becomes polarized columnar composed of different cell types (Grand *et al.*, 1976). Similarly, the gut (intestine) of the zebrafish begins as a cord of radially aligned cells that undergoes (cyto)differentiation into goblet cells and enterocytes. Polarization of the enterocytes becomes complete, the microvillus brush border forms and digestive enzymes become histochemically demonstratable.

1.7.2 Ontogeny of the digestive tract

Govoni *et al.* (1986) provided a generalized overview of the derivation, sequence

and timing of digestive organs in typical larval fishes. This allows a comparison between many different species, including members of family Pleuronectidae:

Yolk-sac larva- final yolk absorption	→ Larva	→ Transformation	→ Juveniles & adults
			Esophagus
	Foregut		Stomach
Incipient gut	Midgut		Anterior intestine
	Hindgut		Posterior intestine

The digestive tract in marine finfish develops through a series of histotypic events that differs temporally amongst species. In newly-hatched fish, the digestive tract is straight, usually composed of one cell type that becomes differentiated by the end of yolk absorption when larval nutrition changes from endogenous yolk to exogenous food (Otake *et al.*, 1995). Further differentiation at later ontogenetic stages often includes segmentation into the buccopharynx, fore-, mid- and hind-gut (reviewed by Govoni *et al.*, 1986). Functional development occurs at or near the time of metamorphosis, indicated primarily by the appearance of gastric glands and pyloric caeca whereby the larval digestive system assumes its final adult form.

Sarasquete *et al.* (1993) noted that the digestive tract of the developing sea bream consisted, at hatching, of a straight tube with no folds or muscular layers and no anterior

or posterior openings. The same observations have recently been made for several Pleuronectiform species, including the Japanese flounder (Kurokawa & Suzuki, 1996), Atlantic halibut (Pittman *et al.*, 1990), Senegal sole, *Solea senegalensis* (Sarasquete *et al.*, 1996), turbot (Segner *et al.*, 1994) and summer flounder, *Paralichthys dentatus* (Bisbal & Bengtson, 1995). The time of regional partitioning is similar amongst these species, ranging from 1 to 3 days post-hatch. The intestinal tract over the next several weeks also shows similar developmental trends including an increase in gut length and folding, mucosal differentiation and an increase in stratification and further appearance and differentiation of the liver, gallbladder and pancreas.

The larval foregut can be arbitrarily divided into a buccal cavity, pharynx, esophagus and stomach. Similar to the adult digestive system, the larval buccal cavity and pharynx are composed of squamous (Bisbal & Bengtson, 1995; Sarasquete *et al.*, 1995) or cuboidal epithelium dispersed with mucous cells (Govoni *et al.*, 1986). Posteriorly, the mucosa of the esophagus ranges from multilayered squamous epithelium (Kjorsvik *et al.*, 1991; Bisbal & Bengtson, 1995) to simple cuboidal (Sarasquete *et al.*, 1996) interdispersed with goblet cells. The stomach starts off as an anlage that is histologically distinct from both the esophagus and intestine. The mucosa is composed of columnar epithelium devoid of a brush border and goblet cells (Bisbal & Bengtson, 1995; Sarasquete *et al.*, 1996).

A characteristic feature at this developmental stage is the absence of gastric glands or pyloric caeca during the first month of life; true to most teleost species, a functional stomach and pyloric caeca tend to appear late in larval life (Sarasquete *et*

al.,1993) and are usually not observed until metamorphosis (Pedersen & Falk-Pedersen, 1992; Segner *et al.*, 1994; Bisbal & Bengtson, 1995). The development of both of these structures constitutes the last morphological change in the alimentary canal (Govoni *et al.*, 1986).

The intestine and rectum (anterior and posterior intestine) in the larvae are similar in structure and function to those of the adult. Developing from the mid- and hind-guts respectively, these regions are specialized for nutrient digestion and absorption, both containing a single layer of columnar cells with a striated border of microvilli. Cytologically, supranuclear vacuolar structures in the intestine and rectum epithelia are proposed digestive mechanisms after luminal hydrolysis of lipids and proteins, respectively (Govoni *et al.*, 1986).

Accessory organs appear to be differentiated by the time of first feeding. The pancreas of the summer flounder is differentiated at hatching and pancreocytes begin to organize into acini by four days after hatching; the liver is morphologically complete by day 10 (Bisbal and Bengtson, 1995).

1.8 Digestive enzymes

Enzymes are classified according to the types of chemical reactions they catalyze into six classes- (1) oxidoreductases, (2) transferases, (3) hydrolases, (4) lysases, (5) isomerases and (6) ligases (Lojda *et al.*, 1979). Digestive enzymes are hydrolases; that is, the food material (substrate) is broken down by the process of hydrolysis, the splitting of a compound by addition of a water molecule. The hydrolases can be further

classified into the phosphatases, sulphatases, esterases, glycosidases, and proteolytic enzymes. Enzymes are characterized by four numerals, the first of which indicates to which of the six classes it belongs. The second indicates the subclass, the third the sub-subclass and the fourth the original number of the enzyme within the sub-subclass (Lojda *et al.*, 1979).

The phosphatases catalyze the hydrolysis of esters of phosphoric acid (3.1.3.-) and phosphorous containing acid anhydrides (3.6.1.-). This group includes acid and alkaline phosphatase and glucose-6-phosphatase. These enzymes are found in the endothelium of blood vessels, brush border of renal convoluted tubules, and luminal margin of intestinal epithelium; acid phosphatase is found mainly in lysosomes (Lojda *et al.*, 1979).

The esterases (3.1.1.-) split esters of carboxylic acid, generally hydrolysing short chain fatty acids (Oliver *et al.*, 1991). In many animals esterase activity has been reported to be high in the liver, intestine and kidney (Lojda *et al.*, 1979).

The sulphatases (3.1.6.-) hydrolyse sulphuric esters. Of the 14 known sulphatases, only arylsulphatase can be demonstrated histochemically. Enzymes for this group are located in the liver and kidney (Oliver *et al.*, 1991).

Gylcosidases (3.2.-) hydrolyze glycoside bonds in glycosides, oligosaccharides, glycoproteins, glycolipids and glycosaminoglycans and are found in numerous plant and animal tissues (Lojda *et al.*, 1979; Gossrau *et al.*, 1991).

The proteolytic enzymes (3.4.-) catalyze the splitting of peptide and amide bonds and are vital in protein digestion. They are divided into two groups: exopeptidases (3.4.11 to 3.4.19) require the presence of terminal carboxyl or amino group on one side of the

peptide bond and include the aminopeptidases and dipeptidylpeptidases. Endopeptidases (3.4.21 to 3.4.24, 3.4.99) hydrolyze peptide bonds within a protein chain and are described as the proteolytic enzymes of the digestive tract. Examples of this group include trypsin, chymotrypsin and enteropeptidase (Lojda *et al.*, 1979; Lojda *et al.*, 1991).

1.8.1 Distribution of digestive enzymes

These enzymes are present at various regions along the digestive tract, but it is the intestine where most of them are found. The luminal membrane of enterocytes is characterized by the abundance of microvilli forming the brush border. Microvillar hydrolases such as alkaline phosphatase, aminopeptidase, enteropeptidase, dipeptidylpeptidase IV, and γ-glutamyltranspeptidase play a role in the final digestion of the nutrients and allow transport across the microvillar membrane (Noren *et al.*, 1986).

In higher vertebrates, the activity of various proteinases has been found along the length of the digestive tract. Dipeptidylpeptidase (DAP) IV has been found in the epithelial cell surface membrane of the esophagus and DAP I and II in their lysosomes. In the small intestine aminopeptidase (AP) A and M (microsomal); γ-glutamyltranspeptidase; DAP I, II, and IV; trypsin; endopeptidase; and chymotrypsin were found associated with the microvillus brush border. Endocrine cells in the gastric glands of the stomach showed positive staining for APM while DAP I and II and APA was seen in the chief cells (Gossrau, 1981).

1.8.2 Ontogeny of digestive enzymes

The functional secretions of the developing digestive tract have not been studied in either yellowtail or winter flounder but have been in other marine larvae with aquaculture potential. Protein digestibility, linked to the high mortality rate of larval marine fish (Pedersen, 1993) has led to an increase in the research of food digestibility and localization of hydrolytic enzymes during various stages of development (Kolkovski *et al.*, 1993; Segner *et al.*, 1993; Lindner *et al.*, 1995; Moyano *et al.*, 1996).

The apparent intensities (activity of enzymatic reaction based on a color reaction) of digestive enzymes increase as development continues. In a review of digestion in fish larvae, Govoni *et al.* (1986) indicate that digestive enzymes increase in their activity during the larval period before transformation. It was proposed that the general increase in enzyme activities after yolk absorption and first feeding is because of enzymes inherent in the live food. Further, enzyme production may be a response to initial food consumption and increasing ration size.

The amount of enzymes detected in the digestive tract seems to be dependent on the feeding of the larvae. Ueberschar (1993), studying herring (*Clupea harengus*) and turbot larvae, reported on the higher portion of trypsin in young feeding herring larvae than in starving larvae, indicating that perhaps exogenous trypsin-like enzymes may influence the measurement. In coregonid larvae (*Coregonus lavaretus* L.) trypsin and maltase activities were higher in larvae fed dry diets than those feed zooplankton whereas aminopeptidase was higher in larvae fed zooplankton. In starved larvae trypsin, maltase, and alkaline phosphatase showed a negative response (Segner *et al.*, 1989).

1.9 Research objectives

Knowledge of ontogenetic changes in yellowtail and winter flounder before, during and after metamorphosis will heighten our basic understanding of these two Pleuronectid species. Basic biological fundamentals are necessary to achieve a more systematic approach to their rearing. Knowledge of digestive system ontogeny, including the ontogeny of digestive enzymes, may improve rearing strategies of these species by providing initial information on digestive system capabilities associated with different feeding regimes.

The research objectives of this study were:

1. To examine the structural changes of the digestive system during development of yellowtail flounder using light microscopy and mucous histochemistry.

2. To examine the ontogeny of digestive function by studying the cellular localization of the activity of digestive enzymes using enzyme histochemistry.

3. To compare the ontogeny of digestive function on the basis of diet: a standard live (rotifer and algae) diet, weaning from live food to a selected quality microparticulate diet, and starving larvae.

Ontogeny of the digestive tract during larval development of yellowtail flounder: a light microscopic and mucous histochemical study

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Journal of Fish Biology, 1997, 51: 120-134.

Published for The Fisheries Society of the British Isles by Academic Press

Candidate's Contribution:

The candidate's contribution included data analysis and description of the result and was responsible for the preparation of the manuscript for publication including writing the introduction, materials and methods, results, discussion and references.

2. DIGESTIVE TRACT ONTOGENY AND MUCOUS HISTOCHEMISTRY OF LARVAL YELLOWTAIL FLOUNDER

2.1 Introduction

The functional life history of larval stages for many of our marine flatfish species are poorly understood, particularly with respect to morphological development and the ontogeny of digestive systems. This is probably due to the small size of these free living animals (Timmermans, 1987) and the difficulties encountered initially during rearing procedures. The indirect development (Balon, 1984) which this group of species displays provides an added aspect of complexity associated with the dramatic metamorphic changes occurring at the end of the larval stage.

The yellowtail flounder *Pleuronectes ferruginea* (Storer) occurs between Labrador and New England in cool ocean water with a preferred temperature range for adults between 2-6 ^{0}C (Scott & Scott, 1988) and 4.1-8.9 ^{0}C for larvae (Martin & Drewry, 1978). It is a valuable commercial marine species whose abundance has suffered the recent decline experienced by many species in the western North Atlantic (Hutchings & Meyers, 1995).

Yellowtail flounder is a candidate for aquaculture development (Brown *et al.*, 1995) despite slower growth at lower temperatures in comparison with other species of marine flatfish successfully cultured such as turbot, *Scophthalmus maximus*. In Canadian waters, yellowtail flounder measures 27 cm at year 4 (Scott & Scott, 1988) but turbot, in the warmer waters of areas such as the North Sea, reaches 40-45 cm at the same age

(Person-Le Ruyet, 1990). The earlier life stages of turbot have a high growth rate obtained experimentally over a temperature range of 10-20 ^{0}C with the optimum being 18-19 ^{0}C (Jones *et al.*, 1981; Danielssen *et al.*, 1990; Gaumet, 1994). Optimum conditions of artificial rearing for yellowtail flounder have not yet been investigated extensively.

The digestive system in flatfish, subsequent to the juvenile stage, was thoroughly reviewed by De Groot (1971). Smith (1989) provided a thorough analysis of digestive function in teleost fish and Govoni *et al.* (1986) outlines the physiology of larval fish digestion based on morphological development, digestive mechanisms, enzymology, histochemistry, peristalsis, digestion rates and assimilation. Recent morphological studies comparing the alimentary canal in adults of pleuronectid species including yellowtail flounders are provided by Murray *et al.* (1994a,b; 1996). However, the basic knowledge of the morphological development of the gut in larval yellowtail flounder is unknown.

Research on gut development of larval stages of related marine flatfish is available for summer flounder, *Paralichthys dentatus* (L.), (Bisbal & Bengtson, 1995), the European turbot (Cousin & Baudin-Laurencin, 1985; Cousin *et al.*, 1987; Segner *et al.*, 1994), Atlantic halibut, *Hippoglossus hippoglossus* (L.), (Blaxter *et al.*, 1983; Pittman *et al.*, 1990; Kjorsvik & Reiersen, 1992; Murray *et al.*, 1993), and the Dover sole, *Solea solea* (L.), (Boulhic & Gabaudan, 1992) and allows a comparison with the developmental changes occurring in the gut of larval yellowtail flounder examined in this study.

The objective of this study was to determine the structural changes during the development of the gut of larval yellowtail flounder from 3 days post-hatch to a stage

when they were metamorphosing (46 days post-hatch), to provide a foundation for future studies on the ontogeny of digestive function with respect to the localization and activity of digestive enzymes.

2.2 Materials and methods

2.2.1 Yellowtail flounder larval culture and maintenance

Wild yellowtail flounder brood stock were collected aboard the research vessel W Templeman in 1992 and brought to the Ocean Sciences Centre in St. John's, Newfoundland. They were held in 2.0 metre diameter tanks with running ambient seawater and fed a mixed diet of frozen caplin and shrimp. The mature fish were conditioned naturally in seawater of approximately 31 ‰ and ranged in size from 26 to 32 cm. This brood stock spawned in 1993 and again in the summer of 1994 during the normal spawning season. Eggs were stripped manually from obviously swollen females and sperm was collected from running males. The eggs were fertilized and incubated in 7 to 8 ^{0}C seawater for approximately 6 days until hatch.

Larvae were uniform in size of 2.8 mm at hatch. These were reared in 70 litre polyethylene tubs at a density of 50 larvae per litre in 8-10 ^{0}C water at 31 ‰. The water flow through these tubs was 1.0 to 1.5 litre min^{-1}: aeration was provided to older larvae. The larvae were first offered food at 3 days post-hatch. The 3, 7 and 10 day old larvae were fed a diet of live rotifers enriched with Super Selco® and DHA Selco® (INVE Aquaculture Inc., Grantsville, Utah) along with the algae *Isochrysis*. At day 29, twenty-four-hour-old *Artemia nauplii* were introduced to the larval diet. The 46 day old larvae

received only *Artemia*. Each larval rearing container was fed twice daily. Rotifers and *Artemia* were fed at a rate of 4 to 6 per ml; one litre of *Isochrysis* (density unknown) was added each day to the larval rearing container. The containers were examined for mortalities 1 to 2 times daily any mortalities were removed using a siphon.

Batches of larvae sampled for histological purposes were of good quality, characterized by survival from hatch to metamorphosis which ranged from 10-25%. Metamorphosis in these groups of fish started at 40-45 days post-hatch and was complete in one to two weeks. Subsequent performance as juveniles confirmed the high quality of these animals which grew to an average standard length of 1.96± .11 cm by 10 weeks post-hatch and 3.43± 0.08 cm by 20 weeks post-hatch.

2.2.2 Tissue Sampling, processing and histochemistry

Yellowtail larvae of six different ages post-hatch were sampled based on availability of the animals at the time of study. Groups of five animals at days 3, 7, 10, 29, 36 and 46 post-hatch were anaesthetized in tricane methanesulfonate and fixed *in toto* in Bouin's fluid at room temperature for 24-48 h, dehydrated through an ethanol series and embedded in paraffin. Serial transverse and sagittal sections, 8 μm thick, were cut and stained with alcian blue (AB)/periodic acid-Schiff reagent (PAS) pH 2.5 (Bancroft & Cook, 1984). Positive reactions for mucous cell histochemistry at AB/PAS pH 2.5 were: blue for acid mucins, red for neutral mucins and reddish purple for a combination of neutral and acid mucins.

All animals were maintained and killed according to the guidelines set by the Canadian

Council of Animal Care (Olfert *et al.*, 1993).

2.3 Results

2.3.1 Light microscopy

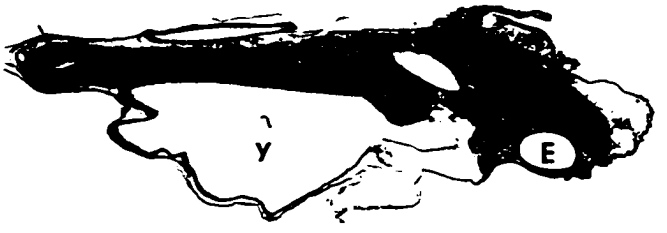
At 3 days after hatching, the digestive tract is a straight, undifferentiated tube composed of columnar epithelium, 18-22 μm thick, closed at the oral end by a thin layer of tissue. A large yolk reserve ventral to the digestive tube extended posteriorly two-thirds the length of the larvae and terminated just before the caudal end of the digestive tube (Fig. 2.1).

Segregation of the digestive tract occurred between days 3 and 7 and was indicated by regional partitioning and epithelial differentiation. Five morphologically distinct regions could be defined: the buccal cavity, pharynx, esophagus, post-esophageal swelling (PES), and intestine (Fig. 2.2a). By day 10, the rectum was distinguishable from the intestine (Fig. 2.2b). The PES was identified as a stomach by the glandular organization observed in 36 day old larvae.

2.3.1.1 Buccal cavity

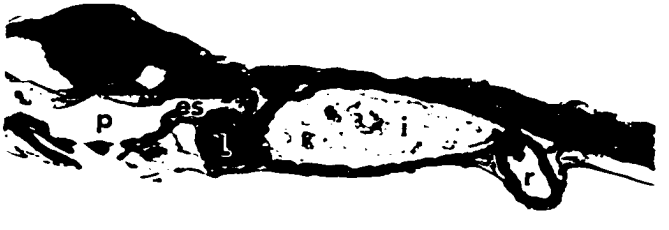
Between 3 and 7 days after hatching the mouth had opened and at day 7, oral valves were present, defined by dorsal and ventral epithelial folds that were evaginations of connective tissue. Both the oral valves and buccal cavity were composed of simple squamous epithelium, which was 2-4 μm thick in the buccal cavity. Goblet cells interdispersed within the epithelium appeared to increase substantially in numbers

Figure 2.1-2.2. Larval yellowtail flounder 3, 7 and 10 days post-hatch.
Figure 2.1. Sagittal section of a 3 day post-hatch yellowtail flounder larva showing the undifferentiated digestive tube (arrows). Bar = 94 μm.
Figure. 2.2. (a) Regional segregation of the digestive tract is evident in a 7 day post-hatch larva. Buccal cavity is not present in this section. Bar = 94 μm. (b) Sagittal section of the digestive tract of larvae 10 days post-hatch showing differentiation between the intestine (i) and rectum (r). Buccal cavity is not present in this section. Bar = 130 μm.
E, eye; es, esophagus; i, intestine; L, liver; asterisk, post- esophageal swelling; p, pharynx; r, rectum; y, yolk sac.



2.1

2.2a



2.2b

posteriorly toward the pharynx and as development proceeded. By day 29, stratification of the epithelial layer was evident and appeared thickest posteriorly, with a thickness ranging from 4 to 12 μm.

Stratification of the oral mucosa became more pronounced with age and by day 46, taste buds subtended the epithelial layer both dorsally and ventrally (not shown). Tooth development occurred between days 36 and 46 where by day 46, teeth were observed to penetrate the premaxillary epithelium (Fig. 2.3). The transitory region from buccal cavity to pharynx contained a thick layer of circular muscle (84-120 μm thick) at the first gill filament.

2.3.1.2 Pharynx

The pharyngeal region is distinguished from the buccal cavity by the development of the gill structure evident by 7 days post-hatch. At this stage, the pharynx is lined with squamous epithelium, 2-7 μm thick, interdispersed with goblet cells. The pharynx continues posteriorly past the last gill where it constricted, becoming the esophagus.

Increased mucosal stratification and number of goblet cells were apparent by 29 days post-hatch and was most pronounced posteriorly (Fig. 2.4) with a range of 4-6 μm anteriorly and 14-28 μm posteriorly. Circular muscle overlayed by a serous membrane was more evident in the dorsal wall of the pharynx. Taste buds were located within the mucosal epithelium on the dorsal surface (not shown). Pharyngeal teeth were also present but did not penetrate the mucosae. Epithelial folding in the posterior two- thirds of the pharynx was very pronounced at 46 days after hatching and the muscularis consisted of circular and an inner layer of longitudinal muscle.

Figs. 2.3-2.5. Figure 2.3. Sagittal section through the buccal cavity of yellowtail flounder 46 days post-hatch showing tooth development (arrowheads). Bar = 94 μm.
Figure 2.4. Sagittal section through a portion of a 29 day post-hatch larva showing mucosal folding and goblet cells (arrows) in the posterior pharynx (p) near Zone 1 (1) of the esophagus (es). asterisk, anterior region of post-esophageal swelling; L, liver; 2, Zone 2. Bar = 72 μm.
Figure 2.5. Pharynx (p) and a portion of the esophagus (es) of a larva 10 days post-hatch. arrow, goblet cell; L, liver; pan, pancreas. Bar = 31 μm.

2.3

2.4

2.5

2.3.1.3 Esophagus

The esophagus of the 7 day post-hatch larvae was lined with stratified cuboidal epithelium, 4-8 μm thick. A few goblet cells were observed by 10 days post-hatch (Fig. 2.5). At day 29, the esophagus could be further differentiated into two morphologically distinct zones (Fig. 2.4). Zone 1 (anterior) consisted of a stratified epithelium with a few goblet cells. Zone 2 (posterior) was further defined by a more dense population of goblet cells and mucosal folding. The distinction between zone 2 of the esophagus and the anterior most aspect of the PES was defined by an abrupt transition from a stratified epithelium with numerous goblet cells to a simple columnar epithelium devoid of goblet cells (Fig. 2.4). A distinct muscularis was observed by day 36. A slight increase in epithelial folding and a marked increase in goblet cell density was seen in zone 2 by day 36. By day 46, a thicker and well-developed muscularis composed of circular muscle was distinct.

2.3.1.4 PES/stomach

At day 7, a constriction at the posterior end of the esophagus followed by a swelling defined the PES which was lined with simple, low columnar epithelium, 4-16 μm thick, devoid of a brush border and goblet cells (Fig. 2.2a). In 10 day-old larvae, there was evidence of some mucosal folding. By day 29, the shape of the PES had changed and consisted of a short, narrow anterior region lined with simple columnar epithelium, which led into a distinctly wider diameter main region also lined with simple columnar epithelium (compare Fig. 2.2a with Figs. 2.4, 2.6). Epithelial folding was

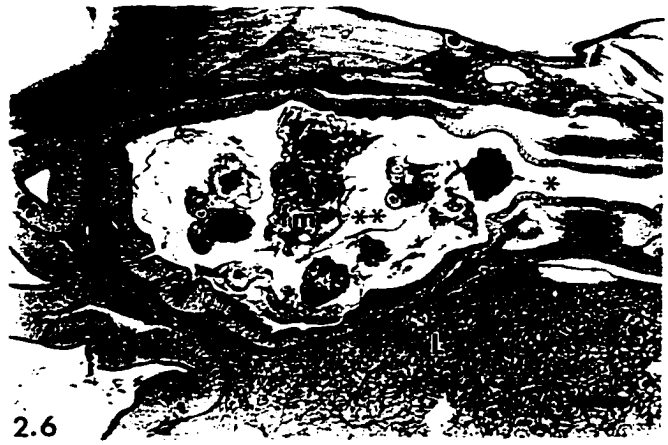
pronounced in the main region.

The development of multicellular glands had occurred by day 36, allowing the PES to be defined as a stomach. An absence of glands in the anterior end closest to the esophagus and at the posterior end of the stomach near the intestinal constriction allowed the differentiation of an anterior non-glandular region (cardiac), main glandular region (fundic), and posterior non-glandular region (pyloric) (Fig. 2.7). The glands were more commoner within the mucosa of the dorsal wall of the fundic region, where they were acinar in shape, and lined with a simple cuboidal epithelium (Fig. 2.7). Therefore, two distinct gastric epithelia could be distinguished- surface columnar and glandular cuboidal. By 46 days post-hatch, the gastric glands were distributed both dorsally and ventrally and the muscularis consisted of circular muscle.

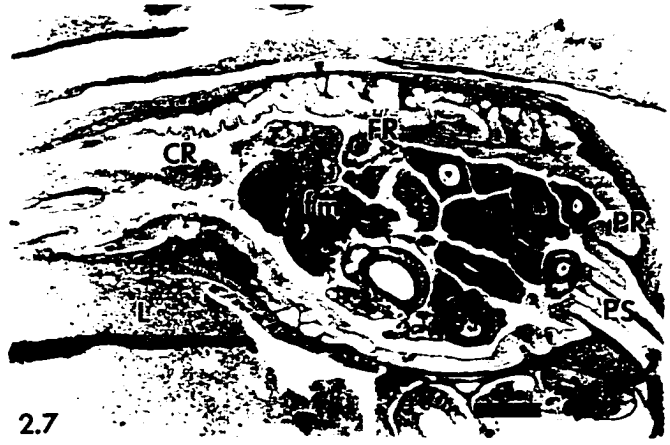
2.3.1.5 Intestine

The intestine was defined in 7 day-old larvae by a constriction at the caudal end of the PES. The mucosal epithelial layer, 8-18 μm thick, was composed of columnar cells with a distinct microvillus brush border and a few goblet cells. The intestine, although distinct, was not coiled and folding not evident. By day 10, the mucosa exhibited slight folding on the ventral wall that was continuous along the length of the intestine (Fig. 2.2b). The intestine was highly coiled by day 29 and the mucosal folding uniform (Fig. 2.6). Folding became more pronounced by day 36, and goblet cell density appeared to have increased by day 46. The musculature was less defined than the esophagus or stomach (PES) although by 29 days the musculature surrounding the intestinal (pyloric)

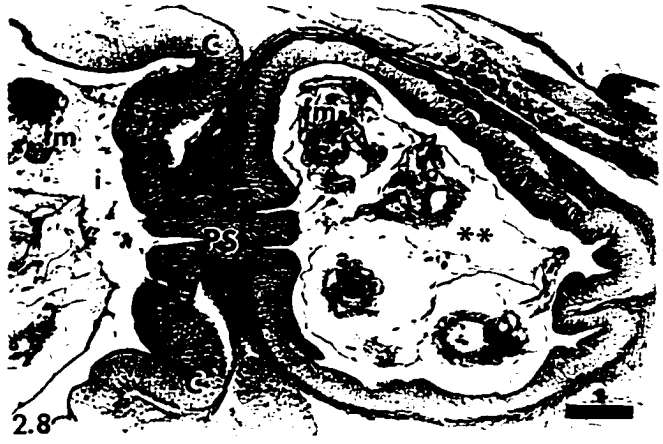
Figures 2.6-2.8. PES and stomach of larval yellowtail flounder.
Figure 2.6. Sagittal section of a larva 29 days post-hatch showing anterior (*) and posterior (**) regions of the PES. Note the food mass in the posterior PES. i, intestine. Bar = 72 μm.
Figure 2.7. Regional differentiation of the stomach of larvae 36 days post-hatch into cardiac (CR), fundic (FR) and pyloric (PR) regions based on development of glands (arrowheads) and glandular distribution. Note food mass (fm) in the stomach. PS, pyloric sphincter. Bar = 140 μm.
Figure 2.8. Junction of the posterior PES (**) and intestine (i) in a 29 day post-hatch larva showing pyloric sphincter (PS) and developing caeca (C). fm, food mass. Bar = 61 μm.



2.6



2.7



2.8

sphincter was well-developed. The intestinal mucosa immediately posterior to the pyloric sphincter showed a pronounced folding, indicating the beginning of caecal development by 29 days (Fig. 2.8).

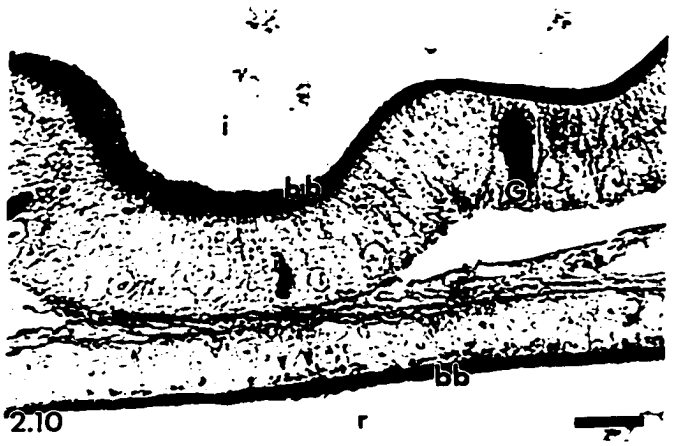
2.3.1.6 Rectum

By day 10, the rectum was differentiated from the intestine posteriorly by an ileorectal sphincter where there was a definite increase in musculature (Fig. 2.9). The rectum, like the intestine, was composed of columnar epithelium 8-14 μm thick with a distinct brush border (Fig. 2.10). A few goblet cells were present by day 10. A striking difference between rectal and intestinal epithelium at 10 days was the presence of dense inclusions common in the apical cytoplasm of the rectal epithelium (Fig. 2.10). These inclusions continued to be a prominent feature of the rectal epithelium through days 29-46. An increase in mucosal folding and diameter of the muscularis (inner circular; outer longitudinal) and absence of goblet cells and brush border was observed as the digestive tract neared termination at the anal opening.

The timing of regional partitioning and epithelial differentiation is summarized in Table 2.1.

Figs. 2.9- 2.10. Intestinal and rectal segments of larval yellowtail flounder.
Figure 2.9. The rectum is divided from the intestine (i) in 10 day post hatch larvae by an ileorectal sphincter (S). Bar = 31 μm.
Figure 2.10. Dense inclusions (arrowheads) are present in the apical cytoplasm of the epithelial cells lining the rectum (r) but not in the cells lining the intestine (i) of larvae 29 day post-hatch.
bb, brush border; G, goblet cell. Bar = 19 μm.

2.9



2.10

Table 2.1. Regional partitioning and epithelial differentiation of the digestive tract of larval yellowtail flounder

Day	Buccal cavity	Pharynx	Esophagus	PES/Stomach	Intestine	Rectum
7	Sq. GC	Sq. GC	SCu	Col	BB. GC	-
10	Sq. GC	Sq. GC	SCu. GC	Col	BB. GC	BB
			Zone1 Zone2	Ant Main		
29	SSq. GC	SSq. GC	SCu GC SCu GC	Col Col	BB. GC	BB. GC
				Ant Main Post		
36	SSq. GC	SSq. GC	SCu GC SCu GC	Col Col Col Cu-gl	BB. GC	BB. GC
46	SSq. GC	SSq. GC	SCu GC Scu GC	Col Col Col Cu-gl	BB. GC	BB. GC

Ant: Anterior PES/stomach; BB: columnar cells with a brush border; Col: columnar; Cu: cuboidal; Cu-gl: glandular cuboidal; GC: goblet cells; Main: main body of PES/stomach; Post: posterior stomach; SCu: stratified cuboidal; SSq: stratified squamous; Sq: squamous

2.3.2 Mucus histochemistry

The digestive tract of larval yellowtail flounder showed positive results for acid and neutral mucins as well as combinations of acid and neutral mucins (Table 2.2). At day 3, the apical surface of the epithelium of the undifferentiated digestive tract was positive for combinations of acid and neutral mucins (Fig 2.11). The luminal layer of stratified epithelium in the buccal cavity, pharynx and esophagus was weakly positive for acid mucins from day 7 onward (Fig. 2.12 a,b). The apical cytoplasm of the columnar epithelium of the PES/stomach demonstrated a weakly positive reaction for neutral mucins from day 10 onward. The glandular cuboidal epithelium present at days 36 and 46 showed no staining for mucins (Fig 2.13). The brush border of the intestinal epithelium was positive for combinations of acid and neutral mucins from day 7 and the rectal epithelium from day 10 onward (Fig. 2.14). All goblet cells stained positive for acid mucins throughout development (Fig. 2.12b).

Table 2.2. Distribution of acid and neutral mucins during development of the digestive tract of larval yellowtail flounder

	Buccal cavity	Pharynx	Esophagus	PES/Stomach	Intestine	Rectum
Day 7*	A	A	A	-	C	-
Day 10-46	A	A	A	N	C	C

A: acid mucins; N: neutral mucins; C: combination of acid and neutral mucins

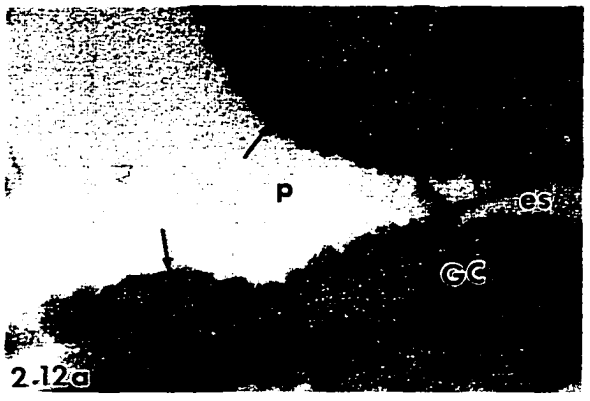
-: no staining

*Undifferentiated digestive tube positive on day 3 for cellular combinations

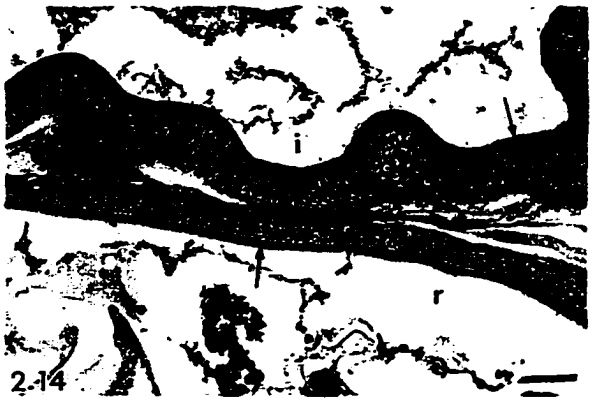
Figs 2.11-2.14. Mucous histochemistry of yellowtail flounder larvae.
Figure 2.11. The luminal surface of the undifferentiated gut at 3 days post-hatch is positive for cellular combinations (arrows). Bar = 24 μm.
Figure 2.12. The (a) pharynx and (b) esophagus of a larva at 29 days post hatch showing positive staining for acid mucins (arrows). Bar = 30 μm.
Figure 2.13. Gastric epithelium and gland (arrowheads) of a larvae at 36 days post-hatch. Only the epithelium is positive for neutral mucins (arrows). Bar = 22 μm.
Figure 2.14. AB/PAS staining of the intestinal (i) and rectal (r) epithelium of a larvae at 29 days post-hatch showing cellular combinations (arrows). Bar = 30 μm. GC, goblet cell, p; pharynx; es, esophagus; i, intestine.


2.11

2.13



2.12a



2.14

2.12b

2.4 Discussion

The differentiation of the digestive tract of larval yellowtail flounder into six morphologically distinct regions- the buccal cavity, pharynx, esophagus, PES, intestine and rectum was established by 10 days post-hatch. The last major morphological change occurred between days 29 and 36 with the development of the gastric glands and pyloric caeca. Recent light microscopic studies of digestive tract development in larval summer flounder (Bisbal & Bengtson, 1995) and ultrastructural and light microscopic descriptions of the adult digestive tract of three species of flatfish including the yellowtail flounder (Murray *et al.*,1994a,b; 1996) allows a comparison with that of the yellowtail flounder larvae in this investigation.

The mucosae of the posterior esophagus in adult yellowtail flounder is composed of a stratified cuboidal epithelium (Murray *et al.*, 1994b) as are those seen in the yellowtail larvae from day 7 post-hatch onward. In larval summer flounder, the esophagus is initially lined with a simple cuboidal epithelial cells that differentiates into stratified squamous by 6 days after hatching (Bisbal & Bengtson, 1995). A notable difference is seen in the histomorphology of halibut larvae where the foregut is composed of columnar epithelium (Kjorsvik & Reiersen, 1992), suggesting an absorptive function.

The distinction between an anterior and posterior esophagus observed in the larval yellowtail is based on the distribution of goblet cells which appear by 10 days post-hatch and on mucosal folding. In adult yellowtail, the mucosa of the posterior esophagus is a stratified layer of cuboidal cells interdispersed with goblet cells (Murray *et al.*, 1994b). The esophageal surface secretory cells (ESSC) stain positive for acid mucins and the

ultrastructural features of these cells suggest they are serous-like secretory cells. Murray *et al.*, (1994b) suggest the posterior esophagus functions in pregastric digestion based on a combination of morphological features including that of the ESSCs. A similar mucus histochemistry and epithelial morphology first occurring in the larval esophagus between 10 and 29 days post-hatch suggests the larval esophagus may be functioning in pregastric digestion by 10 days post-hatch. Increased epithelial stratification in correspondence with the greater number of goblet cells in the posterior esophagus also eludes to a supportive function of the mucosae.

The distinction between the PES and stomach in larval yellowtail is based on the development of gastric glands between days 29 and 36. Often, the lack of glandular organization is noted but no separation made, although in some instances a stomach anlage is distinguished (Segner *et al.* 1989; Segner *et al.*, 1994). At this stage, the stomach of yellowtail flounder is divided into three regions- an anterior, non-glandular (cardiac) region, a glandular main (fundic) region, and a posterior non-glandular (pyloric) region that is similar to that of larval summer flounder (Bisbal & Bengtson, 1995) and larval turbot (Segner *et al.*, 1994). Contrary to this, the stomach of adult Atlantic halibut is entirely glandular and could correlate to this species consuming large whole fish and the consequential increased volume of digestive secretions (Murray *et al.*, 1994a). Neutral mucins weakly observed in the surface epithelium of the PES of 10 day-old larvae may play a role in digestive activity at this stage by absorption of easily digested substances such as disaccharides and short-chain fatty acids as seen in many adult species of teleosts (Reifel & Travill, 1978; Osman & Caceci, 1991; Grau *et al.*, 1992). This is inconsistent

with that observed in the adult yellowtail where neutral mucins were present in gland cells and cellular combinations in surface and mucous neck cells (Murray *et al.*, 1994a). This co-existence in the adult may be a function of the level of mucus maturity (Elbal & Agulleiro, 1986) and occurs beyond the 46 day post-hatch period examined in this study.

The lack of gastric glands and presumably a functional stomach in larval yellowtail flounder up to and including day 29 may prevent weaning onto a commercial diet during this early larval stage. Attempts at weaning turbot larvae have proven difficult and is successful only after the stomach has become functional (gastric glands; subdivision into cardia, pylorus, etc.; acid luminal milieu; and pepsin digestion) (Segner *et al.*, 1993) during metamorphosis between 20 and 35 days after hatch (Segner *et al.*, 1994). It has been hypothesized that stomach formation is important because of an improved mechanical and enzymatic digestion of food and that all or certain nutrients may be easily accessible from live diets but released from dry diets only after enforced digestion (Segner *et al.*, 1993). To farm turbot successfully, it is desirable to wean the larvae on to cheaper artificial diets at an early age (Bromley, 1978) and thus the maintenance of live food remains necessary until a dry diet can be introduced. Weaning is important for flatfish aquaculture because it is economically advantageous (Lee & Litvak, 1996) where the labour, cost and time involved in rearing and dispensing live food can be eliminated (Bromley, 1981).

Pyloric caecal formation by the pouch-like foldings in the intestinal junction occurs between days 29 and 36, the same time that gastric glands form in the stomach. The latter development of these structures in both larval yellowtail and summer flounder

holds true for teleosts that posses a stomach where these structures appear late in metamorphosis (Sarasquete *et al.*, 1993). It is believed that the pyloric caeca is involved in nutrient absorption (lipid) (Buddington & Diamond, 1987; King, 1995) and digestion, primarily by an increase in surface area (Barrington, 1957) without increasing the length or thickness of the intestine itself (Buddington & Diamond, 1987). Pyloric caeca facilitate digestion by transport of absorbable nutrients to the blood stream before passage of the food bolus to the intestine for further breakdown and absorption. They may also neutralize the acid bolus entering the intestine from the stomach which is supported by the absence of pyloric caeca in stomachless fish (Gawlicka *et al.*, 1995).

The folded mucosal epithelium of the intestine and rectum between days 7 and 10 consisted of absorptive columnar cells with a distinct brush border interdispersed with mucus-producing goblet cells. Intestinal coiling between days 10 and 29 increased intestinal length thereby increasing absorptive surface area. Mucosal folding may aid in mixing the food with hepatic and pancreatic enzymes as well as with mucus secreted by goblet cells (Grau *et al.*, 1992). The mucus histochemistry of the larval intestinal and rectal epithelium stained positive for combinations of acid and neutral mucins and is similar to that described in the adult (Murray *et al.*, 1996). The presence of neutral mucosubstances in the rectal brush border may serve to lubricate the faecal mass and that in the intestine may function in digestion (Bucke, 1971). The adult intestine and rectal goblet cells also contained combinations of neutral and acid mucins (Murray *et al.*, 1996) but those of the larvae contained only acid mucins. Again, this co-existence in the adult may be an indication of mucus differentiation with maturity. Dense inclusions in the

rectal apical cytoplasm allows a further distinction between the intestine and rectum in both the adult (Murray *et al.*, 1996) and larval yellowtail flounder and suggests a function of intracellular rectal digestion of proteins (Watanabe, 1984) that is especially important before gastric gland differentiation. In the larval summer flounder, these (supranuclear) inclusions are present in the cytoplasm of the anterior, medial, and posterior intestine although they are more prominent in the posterior intestine and similarly function in the pinocytic uptake of nutrients (Bisbal & Bengtson, 1995).

In conclusion, the gut ontogeny of larval yellowtail flounder follows closely the pattern in many marine species described thus far. The lack of a glandular stomach in larvae up to and including day 29 may prevent weaning onto a dry starter diet until after metamorphosis. Enzyme histochemical analysis of the developing digestive tract of yellowtail flounder will give an actual indication of the ontogenetic functionality of the various regions. Comparing ontogenetic development of larvae fed different diets (ie. dry vs. live feed) will enable a better understanding of the required feeding regime and thus increase their performance in cultured conditions.

3. DIGESTIVE ENZYME DISTRIBUTION IN LARVAL YELLOWTAIL AND WINTER FLOUNDER

3.1 Introduction

The simple, undifferentiated digestive tract of a newly-hatched marine fish larvae undergoes structural and functional changes over the first month of life, where by the time metamorphosis has ended, the digestive system is complete and the juvenile is able to assimilate artificial diets like the adult. The developmental time-frame of the major morphological events associated with the digestive system, such as regional differentiation and gastric gland formation, has been well studied in species like summer flounder (Bisbal & Bengtson, 1995) and Atlantic halibut (Pittman *et al.*, 1990). The general functional development occurring concomitantly with these morphological changes was reviewed by Govoni *et al.* (1986), including information on the enzymology of the developing alimentary canal in a number of marine and freshwater species. An increase in enzymatic activity during the period before transformation (Kawai & Ikeda, 1973) supports the temporal relationship of several carbohydrolytic and proteolytic enzymes. Other than the reports on histochemistry of alkaline phosphatase in the developing gut (Ikeda, 1959; Prakash, 1961), no precise regionalisation of these enzymes had been reported.

More recently, investigations dealing with the histochemical detection of digestive enzymes in fish larvae with respect to developmental localization and variation in dietary regime have complimented earlier studies dealing with morphology (Lauff &

Hofer, 1984; Segner *et al.*, 1989; Sarasquete *et al.*, 1993; Cahu & Zambonino Infante, 1994; Segner *et al.*, 1994; Zambonino Infante & Cahu, 1994; Gawlicka *et al.*, 1995; Segner & Verreth, 1995; Gawlicka *et al.*, 1996; Moyano *et al.*, 1996). Results of these studies have shown that digestive enzymes such as trypsin, esterase, acid and alkaline phosphatases and aminopeptidases are usually present by the time the larvae start to feed and that activity increases with age. In this investigation, activity of an enzyme is based on the intensity of the reaction observed. Higher intensities, implying an increase in enzymatic activity, are seen in the intestine, mostly localized in the anterior intestinal segments (Segner *et al.*, 1989; Sarasquete *et al.*, 1993). It has been suggested that this ontogenetic increase within the intestine is accompanied by an increase in the volume of intestinal mucosa (Segner *et al.*, 1989).

The digestive enzyme activities of larvae fed different dietary regimes (i.e. a dry diet, a live diet or starvation conditions) are both enzyme and species dependent. Cahu & Zambonino Infante (1994) noted that in sea bass larvae (*Dicentrarchus labrax*), neither diet nor dietary change (i.e. weaning) affected trypsin activity but amylase activity was induced by the high starch content of a microparticulate diet. In striped bass (*Morone saxatilis)* larvae, the specific activity of digestive enzymes (including trypsin and amylase) was not influenced by diet for the first 12 days (Baragi & Lovell, 1986). All enzymatic activities in sea bream larvae that were starved for a period of 10 days decreased (Moyano *at al.*, 1996).

Although the optimum developmental stage for weaning fish larvae from a live diet onto an artificial dry diet is also species dependant (Zambonino Infante & Cahu,

1994), in marine larvae (including Pleuronectiformes), diet substitution is difficult and usually successfully accomplished at or shortly after metamorphosis (Person Le Ruyet *et al.*, 1993) when the stomach differentiates and becomes functional (Miwa *et al.*, 1992; Segner *et al.*, 1993; Segner *et al.*, 1994; Bisbal & Bengtson, 1995; Kurokawa & Suzuki, 1996). The immature digestive tract of yolk-sac larvae may require the presence of exogenous enzymes inherent in live food (Lauff & Hofer, 1984; Baragi & Lovell, 1986; Kolkovski *et al.*, 1993). In sea bream larvae, addition of exogenous enzymes to a microdiet has a positive effect on assimilation of that diet (Kolkovski *et al.*, 1993). Munilla-Moran and co-workers (1990) concluded that for exogenous digestive enzymes played an important role in digestion for turbot larvae. Other researchers have reported, however, that larvae are not dependent on exogenous enzymes for digestion of food (Kurokawa & Suzuki, 1996) nor do these enzymes contribute to the digestive enzyme capacity of the larvae (Zambonino Infante *et al.*, 1996). Rather, factors that stimulate digestive enzyme secretion may be found in natural zooplankton (Kurokawa & Suzuki, 1996). In addition, it has been proposed that early weaning may actually delay larval development, particularly the onset of pancreatic secretory functions (Cahu & Zambonino Infante, 1994).

The temporal and spatial development of digestive enzymes in either yellowtail or winter flounder larvae have not been studied to date. While rearing practices typically utilize cultured zooplankton, the effects of weaning on survival, growth and development have not been investigated in either of these species. The purpose of this study was to compare the development of digestive enzyme activity in two species of pleuronectids,

yellowtail and winter flounder, under three different dietary conditions: a live (rotifer and algal) diet, weaning onto a compound diet or starved.

3.1.1 Phosphatases

Both acid and alkaline phosphatase are capable of hydrolyzing phosphomonoesters (3.1.3.-) and are distinguished from each other on the basis of their pH optimum, that of alkaline phosphatase being higher than acid phosphatase. Alkaline phosphatase (3.1.3.1) catalyzes the breakdown of esters of orthophosphoric acid:

O-phosphoric acid monoester + H_2O = alcohol + orthophosphate

This enzyme is primarily found in cell membranes where active transport takes place like the brush border of enterocytes and proximal tubule cells of the kidney as well as in hepatocytes and capillaries (Lojda *et al.*, 1979). Although according to Borgers and co-workers (1991) the role of this enzyme in physiological processes remains to be elucidated, it is described as being of great importance for membrane digestion in fishes (Kuz'mina & Smirnova, 1992). It has been correlated with fat absorption and may serve as a marker of the intensity of nutrient absorption (Govoni *et al.*, 1986; Segner *et al.*, 1989), particularly in catalyzing the transfer of glucose through the intestinal epithelium.

Acid phosphatase (3.1.3.2) also cleaves esters of orthophosphoric acid but liberates phenol as the alcohol. It is localized in lysosomes with highest activity in the spleen, kidney, liver, intestine and adrenals (Lojda *et al.*, 1979). Like alkaline phosphatase, the actual *in vivo* biochemical function of acid phosphatase remains obscure (Borgers *et al.*, 1991). In fish larvae, it has been localized in the epithelium of stomach

mucosa, striated border of intestinal enterocytes and supranuclear granules of the enterocytes of the posterior intestine. This enzyme appears during exogenous feeding in the intestine of sea bream larvae (Sarasquete *et al.*, 1993).

3.1.2 Nonspecific esterases

Nonspecific esterases (3.1.1.-) belong to a large group of enzymes, carboxylester hydrolases, that include carboxylesterases, arylesterases and acetylesterases. Generally, esterases hydrolyze carboxylic esters of alcohols, phenols, naphthols and lipoproteins, as shown by the general equation (Lojda *et al.*, 1979; Hirji & Courtney, 1983):

$$RCOOR' + H_2O \quad \rightleftharpoons \quad RCOOH + R'OH$$

In teleosts, the nonspecific esterases have been found in the kidney, stomach, intestine, rectum, exocrine pancreas, liver and yolk syncytium. They are believed to function as lipoproteolytic enzymes (Hirji & Courtney, 1983; Segner *et al.*, 1989). Nonspecific esterases have been localized both in the cytoplasm and brush border of enterocytes (Hirji & Courtney, 1983; Segner *et al.*, 1989). Esterase activity is located in the glandular and epithelial cells of the stomach (Segner *et al.*, 1989) where in the perch, this activity was present in the surface and chief cells (Hirji & Courtney, 1983). The hydrolysis of carboxylic esters by esterase is extracellular and is initiated in the stomach and completed in the intestine. It has been suggested that esterases are responsible for intracellular hydrolysis of absorbed simple carboxylic esters (Hirji & Courtney, 1983).

3.1.3 Proteases

Peptidases are proteolytic enzymes that catalyze the hydrolysis of proteins and peptides according to the general reaction (Lojda *et al.*, 1979; Lojda *et al.*, 1991):

$$R\text{-}CO\text{-}NH\text{-}R' + H_2O \quad = \quad R\text{-}COOH + R'\text{-}NH_2$$

Generally, proteins are important in providing an energy source and in the production of muscle tissue and enzymes (Clark *et al.*, 1985). Proteolytic activity starts in the stomach with the breakdown of proteins by the action of pepsin. The final digestion of proteins occurs in the intestine (Hirji & Courtney, 1982) with the sequential cleavage of peptides by exopeptidases and absorption of free amino acids from the gut lumen (Clark *et al.*, 1987). In fish larvae, pepsin activity is not detectable until gastric gland formation following metamorphosis (Baragi & Lovell, 1986; Zambonino Infante & Cahu, 1994), suggesting other proteases (i.e. exoproteases) are important during pre-metamorphic stages of digestive system development.

Aminopeptidases release amino acids from the amino end of peptide chains (Lojda *et al.*, 1979) and therefore participate in the terminal digestion of proteins (Lojda *et al.*, 1991). There are both cytosolic (cytosol aminopeptidase) and membrane-bound (microsomal aminopeptidase- 3.4.11.2) aminopeptidases (Lojda *et al.*, 1991). In fish larvae, they have been localized in the undifferentiated digestive tube and subsequently in the posterior intestine, kidney tubules and pyloric caeca and bile canaliculi of the liver (Cousin *et al.*, 1987; Segner *et al.*, 1989; Gawlicka *et al.*, 1995). Based on their localization in the supranuclear cytoplasm of the enterocytes of intestine II (posterior intestine) of *Coregonus lavaretus*, this group of (general) exopeptidases is believed to

participate in digestive processes within supranuclear vacuoles of this part of the digestive tract (Segner *et al.*, 1989).

Dipeptidyl peptidases (DPP's) hydrolyze dipeptides from the amino end of the peptide chain (Lojda *et al.*, 1979) and include several members: DPP's I, II, and IV. They play a role in the terminal digestion of proteins in the brush border of enterocytes and cells of proximal kidney tubules (Lojda *et al.*, 1991). DPP IV has been localized in the enterocyte brush border of the anterior and spiral intestine of sturgeon larvae (*Acipenser transmontanus*) where there was an apparent increase in activity during later stages of development. Increased activity of this enzyme, together with aminopeptidase M and γ-glutamyltranspeptidase, indicate that the larvae possesses enzymes required to complete protein digestion and show complete differentiation of the enterocytes (Gawlicka *et al.*, 1995).

3.1.4 Histochemical demonstration

Methods frequently used in enzymology are based on the quantification of a particular enzyme, usually either by radioimmunoassay or spectrophotometry (Hummel, 1959; Hjelmeland & Jorgensen, 1985; Oozeki & Bailey, 1995; Segner & Verreth, 1995). Localization and quantification of trypsin in adult and larval Atlantic cod (*G. morhua*) has been determined by radioimmunoassay of homogenates of the liver, gut, pyloric caeca, whole gallbladder, and stomach from adult fish, as well as homogenates of the entire larvae (Hjelmeland & Jorgensen, 1985). These methods, however, do not allow for precise cellular localization of trypsin activity, maintenance of spatial tissue relationships

or accurate determination of the origin of the digestive tract enzymes (Clark *et al.*, 1986).

More accurate procedures of determining the spatial and temporal relationship of enzymes in various tissues have been developed one of which is simultaneous coupling. This procedure has two steps: (1) splitting of the substrate and (2) a coupling and precipitation reaction (Lojda *et al.*, 1979). Generally, this method is done by reacting the enzyme in question with an appropriate substrate, usually a naphthol derivative. The primary reaction product formed is not visible and must be coupled to another substance (such as a diazonium salt). This final reaction product is visible as a coloured precipitate localized at the site of enzymatic activity (Lojda *et al.*, 1979; Brancroft & Cook, 1984).

The lead capture method is commonly used for the enzymatic detection of phosphatases. It involves three steps: (1) splitting of the substrate (usually a natural substrate), (2) a precipitation reaction (involving Ca^{2+}, Pb^{2+}, Cu^{2+} or Ba^{2+}) and (3) visualization (Lojda *et al.*, 1979). It uses the presence of a metal ion (originally Pb^{2+}) present in the incubation medium to capture and precipitate the inorganic phosphate as soon as it is released from the substrate (Steps 1 and 2). The lead-phosphate complex formed from this initial reaction is then converted to another product to render it visible, usually by precipitation with ammonium sulphide (Step 3) to form lead sulphide. This yields a brown/black color wherever enzymatic activity is present (Lojda *et al.*, 1979; Borges *et al.*, 1991).

Both of these methods not only allow the detection of enzymatic activity at various sites and in various tissues, but based on the intensity of the staining, provide a qualitative measure of the amount. These procedures are suitable for determining the

following in larval fish: (1) the different regions along the digestive tract where the enzyme is being expressed, (2) the approximate degree of expression, and (3) the establishment of a developmental time-frame for these enzymes.

3.2 Materials and Methods

All larval rearing and collection was done at the Huntsman Marine Science Centre (HMSC) in St. Andrew's, New Brunswick.

3.2.1 Yellowtail flounder larval culture and maintenance

Wild, sexually mature yellowtail flounder females were collected aboard the research vessel WB SCOTT from the Passamaquoddy Bay in July, 1996. A single, spawning female was transferred to a holding tank at HMSC. Eggs were collected immediately after capture and again three days later; both times the eggs were fertilized with sperm from a male brood stock maintained at HMSC. Both batches of eggs were incubated in 10L static black buckets at a salinity of 31‰ and a temperature range between 6-9 ^{0}C. Fifty percent water exchange was performed on a regular basis until hatch nine days later. After hatch, the larvae were evenly distributed into 6-10L circular buckets (two replicates for each feeding regime) maintained on a wet bench at a constant water temperature. Flow rate through the buckets averaged 1L every 7.5 minutes. Larval were distributed according to three feeding regimes: (1) live food, (2) switching to microparticulate diet (Bio Kyowa- particle size 250 µm; Bio Kyowa, Inc., St. Louis, MO) at 15 days post-hatch and (3) no food (Table 3.1). The larvae were between 2.5 and 3.0 mm at hatch.

Table 3.1. Yellowtail flounder larval feeding schedule

	Group 1	Group 2	Group 3
Algae[#]	0-3 days post-hatch	0-3 days post-hatch	-
Rotifers[•]	10/ml, 4-7 times/day at 100ml/ feeding (from day 3)	10/ml, 4-7 times/day at 100ml/ feeding (days 3-15)	-
Biokywa[¶]	-	2-3 times daily[§]	-

[#] *Chlorella* sp. or *Isochrysis* sp. used as feed for the rotifers as well as early food supplement for the larvae (density unknown)

[•] *Brachionus* sp. were enriched with either Protein Selco® or Culture Selco® on the evening prior to feeding on a bi-nightly basis

[¶] Switched to artificial diet at 15 days post hatch

[§] Pinch per feeding (≈ 0.1g)

3.2.2 Winter flounder larval culture and maintenance

Winter flounder brood stock were collected in the spring of 1996 aboard the WB SCOTT in the Passamaquoddy Bay. They were maintained under ambient sea water conditions in 3.0 M circular tanks at HMSC. The brood stock spawned during the 1996 season between early May and late June. Ova and sperm were collected and the fertilized eggs (embryos) were incubated in static containers (with frequent water changes) on a wet bench at 6-7 ^{0}C until they hatched 12-13 days later. For each of three successive hatches, the larvae were evenly divided into three groups (Groups 1, 2 and 3) and reared in static containers; 50% water exchange occurred on a daily basis. Larval distribution was based on three feeding regimes from day of hatching: (1) live food, (2) switching to microparticulate diet (Bio Kyowa- particle size 250 μm) at 15 days post-hatch and (3) no food (Table 3.2).

3.2.3 Tissue sampling and processing

Twelve larvae from each of the three groups, unless otherwise indicated, were sampled and individually processed for enzyme histochemistry (Table 3.3). Sample larvae were taken approximately 1-2 hours after the first feeding of the day. Larvae were randomly sampled at various times of the day and viewed under a dissecting scope to assess if they were feeding. Visual observations of rotifers and pigmentation due to algal content within the gut gave an indication of the presence of food.

Each sampled anaesthetized (MS-222; dose = 0.04g/L) animal was placed on a strip of aluminum foil, inserted into a plastic ultracentrifuge tube (1.5 ml) and immersed

Table 3.2 Winter flounder larval feeding schedule

	Group 1	Group 2	Group 3
Algae[#]/Rotifers[*]	10/ml, 3-4 times/day at 80ml/ feeding (from day 3)	10/ml, 3-4 times/day at 80ml/ feeding (days 3-15)	-
Biokywa (day 15)[¶]	-	1 time daily[§]	-

[#] *Chlorella* sp. used as feed for the rotifers as well as early food supplement for the larvae (density unknown)

[*] *Brachionus* sp. were enriched with either Protein Selco® or Culture Selco® on the evening prior to feeding on a bi-nightly basis

[¶] Switched to artificial diet at 15 days post hatch

[§] Pinch per feeding (≈ 0.1g)

Table 3.3. Number of yellowtail and winter flounder larval samples taken for enzyme histochemistry.

Days post-hatch	Group 1		Group 2		Group 3	
	YTF	WF	YTF	WF	YTF	WF
3	12	12	12	12	12	12
7	12	12	12	12	12	12
10	12	12	12	12	12	12
15	12	12	12	12	-	-
20	12	-	11	-	-	-
29	12	-	-	-	-	-
36	8	-	-	-	-	-

YTF, yellowtail flounder; WF, winter flounder; -, no larvae available for sampling

in liquid nitrogen contained within Dewar flasks (1-50L and 1-25L). The samples were stored in the liquid nitrogen for approximately 3 to 6 weeks until they could be further processed.

3.2.3.1 Freeze-drying and glycol methacrylate embedding

Samples were removed from the liquid nitrogen and immediately placed onto the pre-chilled platen (temperature approximately -45 ^{0}C) of a batch freeze-dryer (Labconco-Stoppering Tray Dryer, Labconco Corporation, Kansas City, MO) and desiccated for 16-16.15 hours. The samples were then removed from the freeze-dryer and infiltrated in chilled, glycol methacrylate (GMA) catalyzed solution A (JB-4 Embedding Kit, Polysciences, Inc., Warrington, PA) prepared according to manufacturer's instructions. Infiltration was for a total of 72 h at 4 ^{0}C: 2 x 24 h plus 24 h under vacuum. After infiltration, the samples were embedded in GMA using gelatin capsules (size no. 4; J. B. EM Services, Inc., Dorval, Québec) and polymerized at -20 ^{0}C for 24 hours under UV light (long wavelength). Polymerization at this temperature prevented internal heating of the tissue that can be caused by the exothermicity of the GMA polymerization reaction. After polymerization was complete, the capsules were removed and the blocks were trimmed around the sample and mounted onto pre-labelled Epon resin (J. B. EM Services, Inc.) blocks in the desired orientation for cutting. Blocks were stored at 4 ^{0}C to prevent any deterioration of enzymatic activity (Teh & Hinton, 1993).

To allow for maximum use of digestive tissue in the small larvae of yellowtail and winter flounder, serial transverse and sagittal 3.0 μm thick sections were generated on a

Reichert-Jung Ultracut E ultramicrotome using dry glass knives and placed on clean glass slides (5 sections/slide). Thinner sections easily folded and crumpled. These were air dried at 4 ^{0}C and subsequently stored in slide boxes at this temperature until used. Only those slides containing region(s) of the digestive tract were used for enzyme histochemical staining. Other slides were stained with toluidine blue (1% in 1% sodium borate solution) to assess histological quality; only samples displaying adequate histological preservation were used for histochemistry.

3.2.4 Enzyme histochemistry

A total of 12 trials for each enzyme was performed for yellowtail flounder and eight trials for winter flounder (Table 3.4). The number of trials was based on samples with adequate tissue preservation. Slides were incubated in the same batch to minimize variation in staining intensity. That is, one trial consisted of consecutively staining all slides of both species for a given enzyme. Each trial used one sample (larvae) per age and group (i.e. 1, 2 and 3) for all five enzymes.

Negative controls included omission of the substrate and inhibitors for differentiation between phosphatases. Positive controls included sections (3 μm) of rat (adult Zucker rats) and fish intestine (juvenile rainbow trout, *Oncorhynchus mykiss*, obtained from the Cardigan Fish Hatchery, Prince Edward Island) in complete incubation medium. Control tissue was processed for glycol methacrylate embedding as described above except desiccation was 3.5-4 hours. Summaries for each enzyme procedure are listed in Table 3.5. All incubations were carried out at room temperature and slides cover

Table 3.4. Number of enzyme histochemistry trials for yellowtail and winter flounder larvae.

Species	AP	AcP	DPP IV	MAP	Est
YTF	LX: 4	LX: 4	LX: 4	LX: 4	LX: 4
	CX: 4	CX: 4	CX: 4	CX: 4	CX: 4
	CX(2): 4	CX(2): 4	CX(2): 4	CX(2): 4	CX(2): 4
WF	LX: 4	LX: 4	LX: 4	LX: 4	LX: 4
	CX: 2	CX: 2	CX: 2	CX: 2	CX: 2
	CX (2): 2	CX (2): 2	CX (2): 2	CX (2): 2	CX (2): 2

AP, alkaline phosphatase; AcP, acid phosphatase; DPP IV, dipeptidyl peptidase IV; MAP, aminopeptidase M; Est, nonspecific esterase; YTF, yellowtail flounder; WF, winter flounder; LX, trials using sagittal sections; CX, trials using transverse sections; CX(2), repeated trials using transverse sections from CX.

slipped in glycerin jelly (prepared according Bancroft & Stevens (1990) except 1 ml of phenol was added).

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

3.2.4.1 Alkaline phosphatase (3.1.3.1)

The incubation solution was prepared according to Bancroft & Cook (1984) except that the solution was not filtered and the pH was adjusted using HCl. Fast Blue B (o-Dianisidine, tetrazotized) was used as the diazonium coupling salt. EDTA (≈ 10 mM) was used to inhibit alkaline phosphatase in control sections by pre-incubation for 30 minutes in 0.2M Tris-HCl buffer containing EDTA followed by incubation in full solution containing EDTA (Borgers *et al.*, 1991). Enzymatic activity is a purple.

3.2.4.2 Acid phosphatase (3.1.3.2)

The incubation medium was prepared according to Lojda *et al.* (1979). The metal salt procedure was followed without modification. $CuSO_4$ (0.25%) was used as an inhibitor in control slides. Color of localized activity is black/brown.

3.2.4.3 Dipeptidyl peptidase IV (3.4.14.4)

The incubation medium was prepared according Lojda *et al.*, (1979) but not filtered. The simultaneous azo-coupling procedure was followed except the slides were post-incubated for five minutes in 2% $CuSO_4$ instead of 1% OsO_4 and not incubated in 4% formaldehyde. Enzymatic activity yields a reddish/orange color.

3.2.4.4 Aminopeptidase M (3.4.11.2)

The incubation medium was prepared according to Lojda *et al.*, (1979) but the incubation solution was not filtered. The simultaneous azo-coupling method was followed except that the slides were not placed in 4% formaldehyde. Activity is reddish/orange.

3.2.4.5 Nonspecific esterase (3.1.1.-)

The incubation medium was prepared according to Lojda *et al.* (1979) except that the solution was not filtered. The simultaneous azo-coupling procedure followed without post-incubation of the slides in 4% formaldehyde. Fast Blue B was used as the diazonium coupling agent. Enzymatic activity is black.

3.2.5 Evaluation of enzymatic activity

Evaluation was based on a semi-quantitative ranking scheme (Segner *et al.*, 1989; Gawlicka *et al.*, 1995; Gawlicka *et al.*, 1996). Briefly, enzymatic activity was analyzed and further described according to the intensity of the color reaction observed: -, no enzymatic activity; +/-, weak (barely detectable) reaction; +, clearly present reaction; ++, strong reaction. Increase in enzymatic activity are based on visual assessments of an increase in color intensity.

Table 3.5. Summary of substrate, buffer, pH and length of incubation used for enzymological procedures.

Enzyme	Buffer	Substrate	pH	Time (min)
Alkaline phosphatase[#¶] (3.1.3.1)	0.2M Tris-HCl	Naphthol AS-BI phosphate	8.3	45
Acid phosphatase* (3.1.3.2)	0.1M acetate	β-glycerophosphate	5.0	120
Dipeptidyl peptidase IV*[¶] (3.4.14.4)	0.1M phosphate	Gly-Pro- 4-MNA	7.2	120
Aminopeptidase M*[¶] (3.4.11.2)	0.1M phosphate	L-Leu-4-MNA	6.5	120
Nonspecific Esterase*[¶] (3.1.1.-)	0.1M phosphate	α-naphthyl acetate	7.3	120

MNA, methoxy-2-naphthylamide; Gly-Pro, glycyl-prolyl; L-leu, L-leucine.

References: [#] Bancroft & Cook (1984); * Lojda *et al.* (1979); [¶]Gawlicka *et al.* (1995).

3.3 Results

Negative controls exhibited no color reaction except in the inhibition tests for acid phosphatase. Inhibition of acid phosphatase by 0.25% $CuSO_4$ occurred selectively in sections of rat intestine; complete inhibition occurred in the cytoplasmic pinocytic vesicles but not in the brush border. Positive controls generally yielded a strong staining reaction.

A summary of the development of enzymatic distribution in yellowtail and winter flounder larvae is given in Table 3.6.

At 3 days post hatch, the gut could be arbitrarily divided into two distinct regions that allows regionalization of the enzymes to be clearly defined. In yellowtail flounder larvae, an anterior segment was defined by the head, gill filaments, narrow lumen and lower epithelial height. Posteriorly, the lumen widened and epithelium became distinctly columnar. In winter flounder, similar observations were made except that gill filaments were not clearly identified.

3.3.1 Alkaline phosphatase (3.1.3.1)

The pattern of staining and regionalisation of this enzyme were similar for both species (Tables 3.7 and 3.8, compilation of trials).

Day 3

By 3 days post hatch, differential distribution of alkaline phosphatase had occurred. No staining was associated with the epithelium of the anterior regions (i.e.

Table 3.6. Distribution of enzymes in the developing digestive tract of yellowtail and winter flounder (intensities not assessed).

DPH	AP		AcP		DPP IV		MAP		Est	
	YTF	WF	YTF	WF	YTF	WF	YTF	WF	YTF	WF
Day 3 G1	G,L	G,L	-	-	G	G	G	G	G	G
G2	G,L	G,L	-	-	G	G	G	G	G	G
G3	G,L	G,L	-	-	G	G	G	G	G	G
Day 7 G1	I,L	P,I,L	-	-	I	I	I	I	I,L	I
G2	I,L	P,I,L	-	-	I	I	I	I	I,L	I
G3	I,L	P,I,L	-	-	I	I	I	I	I,L	I
Day 10 G1	I, R,L	P,I,L	-	-	I,R	I	I,R	I	I,L	I
G2	I, R,L	P,I,L	-	-	I,R	I	I,R	I	I,L	I
G3	I, R,L	-	-	-	I,R	I	I,R	I	I,L	I
Day 15 G1	I, R,L	P,I,L	-	-	I,R	I	I,R	I	I,L,R	I
G2	I, R,L	P,I,L	-	-	I,R	I	I,R	I	I,L,R	I
Day 20 G1	I, R,L	x	-	x	I,R	x	I,R	x	I,L,R	x
G2	I, R,L	x	-	x	I,R	x	I,R	x	I,L,R	x
Day 29 G1	I, R,L	x	-	x	I,R	x	I,R	x	I,L,R	x
Day 36 G1	I, R,L	x	-	x	I,R	x	I,R	x	I,L,R	x

-, no enzymatic reaction; x, larval sample not available; DPH, days post-hatch; AP, alkaline phosphatase; AcP, acid phosphatase; DPP IV, dipeptidylpeptidase IV; MAP, aminopeptidase; Est, nonspecific esterase; YTF, yellowtail flounder; WF, winter flounder; G1, group 1; G2, group 2; G3, group 3; G, undifferentiated gut; P, post-esophageal swelling; I, intestine; R, rectum; L, liver;

Table 3.7. Distribution and range of intensities of alkaline phosphatase in yellowtail flounder larvae.

DPH	BC	Ph	Eso	PES/ St	Intestine	Rectum
Day 3*						
Day 7 G1	-	-	-	-	-(1) +(11)	**
G2	-	-	-	-	-(2) +(10)	**
G3	-	-	-	-	-(3) +/-(2) +(6) ¶(1)	**
Day 10 G1	-	-	-	-	-(7) +/-(2) +(2) ¶(1)	-(2) +/-(2) +(4) ¶(4)
G2	-	-	-	-	-(4) +/-(2) +(3) ¶(3)	-(3) +/-(2) +(1) ¶(6)
G3	-	-	-	-	-(7) +/-(2) +(2) ¶(1)	-(7) +/-(1) +(2) ¶(2)
Day 15 G1	-	-	-	-	-(7) +(4) ¶(1)	-(4) +/-(1) +(4) ¶(3)
G2	-	-	-	-	-(7) +(1) ¶(4)	-(6) +(3) ¶(3)
Day 20 G1	-	-	-	-	-(6) +/-(1) +(5)	-(6) +/-(2) +(3) ¶(1)
G2	-	-	-	-	-(5) +/-(3) +(3) ¶(1)	-(5) +/-(2) +(4) ¶(1)
Day 29 G1	-	-	-	-	-(1) +/-(1) +(8) ++(1) ¶(1)	-(3) +/-(3) +(6)
Day 36 G1	-	-	-	-	+/-(1) +(10) ++(1)	-(1) +/-(3) +(4) ++(1) ¶(3)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for alkaline phosphatase.

** region not present at this age

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis. number of trials (out of a total of 12) reaction was observed; ¶,number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

Table 3.8. Distribution and range of intensities of alkaline phosphatase in winter flounder larvae.

DPH	BC	Ph	Eso	PES	Intestine
Day 3*					
Day 7 G1	-	-	-	¶ (8)	++(1) ¶(7)
G2	-	-	-	¶ (8)	-(2) +/-(1) +(3) ¶(2)
G3	-	-	-	+(1)¶(7)	+(5) ++(1) ¶(2)
Day 10 G2	-	-	-	+(2) ¶ (6)	-(4) +(1) ++(2) ¶(1)
G3	-	-	-	-(2) ¶(6)	-(3) ¶(5)
Day 15 G1	-	-	-	-(2) +(2) ¶(4)	-(3) +(2) ++(3)
G2	-	-	-	-(2) +(2) ¶(4)	+(8)

*Posterior-most aspect of the digestive tract at 3 days post hatch positive for alkaline phosphatase

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 8) reaction was observed: ¶, number of trials region not present/identified

Intensity: -, no reaction: +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

future buccal cavity and pharynx). The columnar epithelium brush border in the posterior region of the digestive tract was positive for alkaline phosphatase (Figures 3.1a.b). In winter flounder, alkaline phosphatase was also associated with the outer-most layer of connective tissue associated with the anterior portion of the digestive tract; reaction terminated at the liver. Alkaline phosphatase was also seen in the developing kidney and eyes (retina); a weak reaction also occurred in the liver. In winter flounder, a reaction was seen in the brain. For both species, no difference occurred between the three treatment groups at this age.

Day 7

Differential distribution of alkaline phosphatase occurred by 7 days after hatching for both species, concomitant with regional differentiation of the digestive tract into the buccal cavity, pharynx, esophagus, PES and intestine.

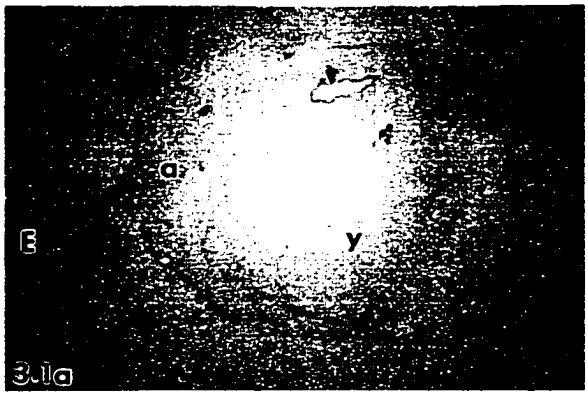
Alkaline phosphatase was primarily localized to the brush border of the intestinal epithelium and the distribution was continuous along its length (Figure 3.2a). In yellowtail flounder, some staining was also associated with the food mass within the lumen of the intestine and similar to those in day 3, reactions were also seen in the kidney, eye, liver and kidney with a slight increase in intensity.

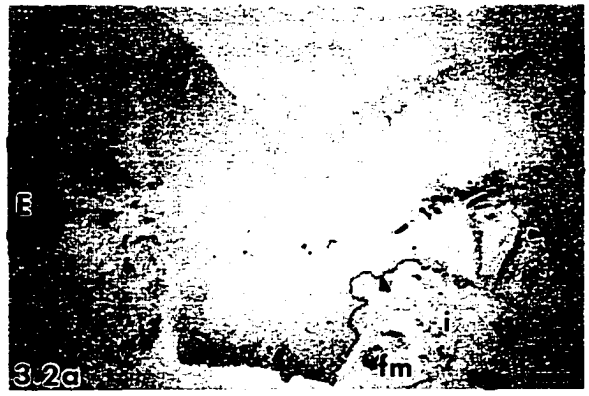
Distribution and intensity were slightly different in winter flounder where alkaline phosphatase was also seen in the PES beginning immediately past the esophageal junction (Figure 3.2b). An increase in alkaline phosphatase activity from day 3 was observed. As compared to yellowtail flounder, distribution was also seen in the intestine, eye, brain and liver. The intestine was similar in appearance as described in day 3; it was

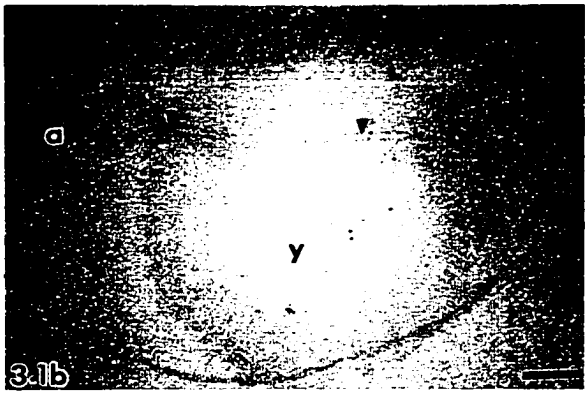
Figs 3.1-3.2. Spatial and temporal development of alkaline phosphatase in yellowtail and winter flounder larvae.

Figure 3.1. Posterior region of the digestive tract at 3 days post hatch positive for alkaline phosphatase activity (arrowheads) in (a) yellowtail (Bar = 40 μm) and (b) winter flounder larvae (Bar = 60 μm).

Figure 3.2. Differential distribution of alkaline phosphatase activity at 7 days post hatch. (a) Alkaline phosphatase distribution along the brush border of the intestine (arrowheads) of larval yellowtail flounder. Food mass (fm) in lumen also positive. Bar = 50 μm. (b) Alkaline phosphatase in winter flounder larva occurred in the PES (asterisk) and intestine (arrowheads). Note the positive reaction in the kidney (arrow) and liver (L). Bar = 42 μm. a, anterior region; E, eye; i, intestine; y, yolk sac.


a
E
y
3.1a


E
i
fm
3.2a


a
y
3.1b


i
L
3.2b

straight with no folding or coiling and a lumen was not as evident as in yellowtail flounder. Staining was also found in the connective tissue on the ventral surface of the esophagus, terminating at the liver.

No difference was seen between the three feeding groups.

Days 10-36

The distribution of alkaline phosphatase in yellowtail and winter flounder larvae was consistent from day 10 onward. In yellowtail flounder, activity was localized to the brush border of the intestine and rectum. The rectum exhibited a stronger staining intensity (Figure 3.3a). An increase in staining intensity was seen in the intestine as it continued posteriorly towards the rectum. An increase in enzymatic activity occurred within the liver. No clear difference was observed between the three treatment groups. Intensity in the intestine was variable (presence or absence) between days 10, 15 and 20 although the rectum was generally positive. By day 29 (group 1 only), a slight increase in intensity was seen in both regions (Figure 3.3b) and a strong reaction was observed in the brain.

The distribution of alkaline phosphatase in winter flounder at 10 days post hatch was the same as for yellowtail flounder (the rectum could not be defined from the intestine in this study) and the activity, based on intensity within the intestine, increased between day 7 and 10 (compare Figure 3.2b and Figure 3.4a). By 15 days post hatch, the intensity of alkaline phosphatase activity in the intestine had decreased slightly from that seen in day 10 (compare Figure 3.4b and 3.4a). At this age, the intestine also had a wider lumen and distinct mucosal folding. There was no staining of any of the regions in the

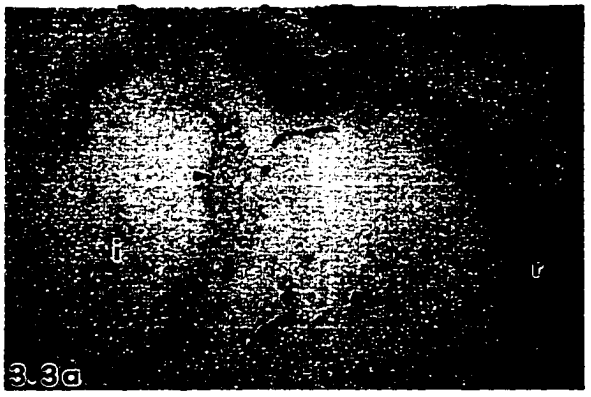
Figs 3.3-3.5. Alkaline phosphatase activity (arrowheads) in yellowtail and winter flounder.

Figure 3.3. (a) Alkaline phosphatase in yellowtail flounder larvae at 10 days post hatch in the intestine (i) and rectum (r). Note the stronger staining reaction in the rectum. Bar = 48 μm. (b) An increase in intensity was observed by day 29 in the intestine, kidney (arrow) and liver (L). Bar = 78 μm.

Figure 3.4. Activity of alkaline phosphatase changed during subsequent stages of development in winter flounder. (a) Strong alkaline phosphatase activity along the brush border of the intestine (i) at 10 days post hatch. Bar = 74 μm. (b) Transverse section of winter flounder larva at day 15 showing a decrease in activity in the intestine. Bar = 28 μm.

Figure 3.5. Sagittal section of winter flounder larvae that were starved. Note the absence of any staining along the intestine (i) (arrowheads). Bar = 70 μm.

E, eye; p, pharynx; es, esophagus; fm, food mass; asterisk, post-esophageal swelling.



3.3a

3.4a

3.3b

3.4b

3.5

starved winter flounder larvae (Figure 3.5).

3.3.2 Acid Phosphatase (3.1.3.2)

No acid phosphatase was observed in yellowtail or winter flounder larvae at the various stages of development or between the three groups.

3.3.3 Dipeptidylpeptidase IV (3.4.14.4)

The activity of dipeptidylpeptidase IV within the developing digestive tract of yellowtail and winter flounder larvae are summarized in Tables 3.9 and 3.10, respectively.

Day 3-36

Dipeptidylpeptidase IV in both yellowtail and winter flounder was localized to the columnar epithelium brush border of the posterior region of the gut at 3 days post hatch (Figure 3.6a,b). For both species, the intensity was generally weaker than that of the other enzymes assayed except acid phosphatase. The presence/absence of this enzyme throughout the stages of development (i.e. day 7, 10, 15 and 20 for yellowtail flounder) was not consistent between the trials or groups.

Differential distribution of dipeptidylpeptidase IV activity occurred in the intestinal brush border by 7 days post-hatch and in the rectum by 10 days post-hatch (yellowtail flounder larvae only). The reaction continued to be both variable and weak in intensity.

The intensity and regularity of the appearance of dipeptidylpeptidase IV activity

Table 3.9. Distribution and range of intensities of dipeptidylpeptidase IV in yellowtail flounder larvae.

DPH		BC	Ph	Eso	PES/St	Intestine	Rectum
Day 3*							
Day 7	G1	-	-	-	-	-(9) +(2) ¶ (1)	**
	G2	-	-	-	-	-(7) +/-(3) +(2)	**
	G3	-	-	-	-	-(7) +/-(2) +(1)	**
Day 10	G1	-	-	-	-	-(8) +/-(3) ¶(1)	-(6) +/-(1) ¶(5)
	G2	-	-	-	-	-(9) +/-(1) ¶(2)	-(8) ¶(4)
	G3	-	-	-	-	-(10) +/-(1) ¶(1)	-(7) ¶ (5)
Day 15	G1	-	-	-	-	-(6) +/-(3) +(2) ¶(1)	-(11) ¶(1)
	G2	-	-	-	-	-(7) +/-(1) +(2) ¶(2)	-(7) +(1) ¶(4)
Day 20	G1	-	-	-	-	-(6) +/-(5) ¶(1)	-(11) ¶(1)
	G2	-	-	-	-	-(7) +/-(3) +(2)	-(7) +(3) ¶(2)
Day 29	G1	-	-	-	-	-(6) +/-(1) +(5)	-(5) +/-(3) +(2) ¶(2)
Day 36	G1	-	-	-	-	-(1) +/-(1) +(8) ¶(2)	-(3) +/-(3) +(2) ¶(4)

* Posterior-most aspect of the digestive tract at 3 days post hatch weakly positive for dipeptidylpeptidase IV

** region not present at this age

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 12) reaction was observed; ¶, number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction

Table 3.10. Distribution and range of intensities of dipeptidylpeptidase IV in winter flounder larvae.

DPH	BC	Ph	Eso	PES	Intestine
Day 3*					
Day 7 G1	-	-	-	-	+/- (1) ¶(7)
G2	-	-	-	-	-(5) ¶(3)
G3	-	-	-	-	-(4) +/-(1) +(1)
Day 10 G2	-	-	-	-	-(4) +/-(1) +(1)
G3	-	-	-	-	-(2) ¶(6)
Day 15 G1	-	-	-	-	-(4) +/-(2) +(1) ++(1)
G2	-	-	-	-	-(4) +/-(1) +(2) ¶(1)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for dipeptidylpeptidase IV

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 8) reaction was observed; ¶, number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

increased during subsequent developmental stages. By 15 days post-hatch, a stronger reaction was observed in both species (compare Figures 3.6a,b and Figure 3.7). In yellowtail flounder, the reaction was stronger in the intestine. Increases in the activity of this enzyme in yellowtail flounder larvae also occurred at days 20 and 29 (Group 1 only) (Figure 3.8a,b) at which time the difference in activity between the intestine and rectum was negligible. A strong reaction was also associated with a granular staining pattern within the apical cytoplasm of the enterocytes. At these later stages, the enzyme was almost always present although some variability occurred with respect to intensity. Weak activity in the food mass within the gut was noted in a few specimens.

3.3.4 Aminopeptidase M (3.4.11.2)

The intensity and distribution of aminopeptidase M in the digestive tract of yellowtail and winter flounder changed during the course of development. Similarities and differences are summarized in Tables 3.11 and 3.12 for all of the trials performed.

Day 3

In yellowtail flounder larvae 3 days after hatching, aminopeptidase M was present along the columnar epithelium brush border in the posterior region (Figure 3.9). Differential distribution occurred at this early age where staining intensity increased posteriorly. Distribution in winter flounder was similar although the intensity of aminopeptidase M staining was less than that of yellowtail flounder.

Days 7 and 10

With the differentiation of the digestive tract between day 3 and 7,

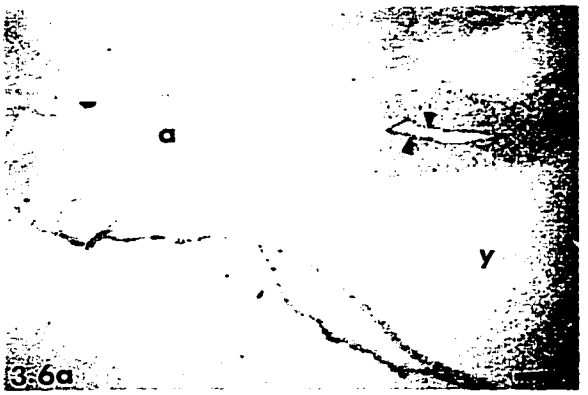
Figs 3.6-3.8. Localization of dipeptidylpeptidase IV (arrowheads) in yellowtail and winter flounder larvae.

Figure 3.6. The brush border in the posterior region of the digestive tract is positive at 3 days post hatch in (a) yellowtail (Bar = 44 μm) and (b) winter flounder larva (Bar = 40 μm).

Figure 3.7. Dipeptidylpeptidase IV activity in the brush border of the intestine (i) of winter flounder larvae at 15 days post hatch. Bar = 66 μm.

Figure 3.8. Dipeptidylpeptidase IV activity in the intestine (i) and rectum (r) epithelial brush border stabilized during later stages of development. (a) Yellowtail flounder larva at 20 (Bar = 62 μm) and (b) 29 days post hatch (Bar = 86 μm). Note the presence of granular cytoplasmic staining (insert).

fm, food mass; E, eye; y, yolk sac; a, anterior region; asterisk, post-esophageal swelling; es, esophagus; L, liver.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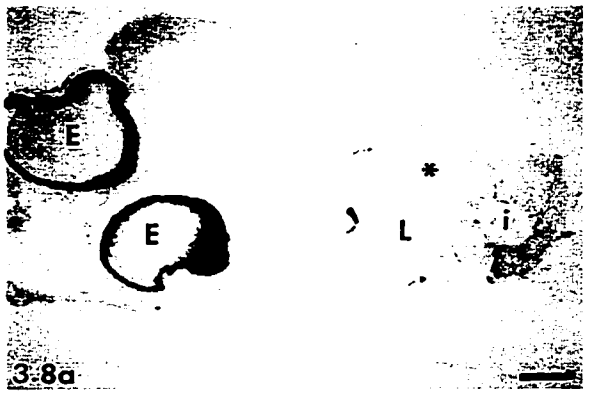
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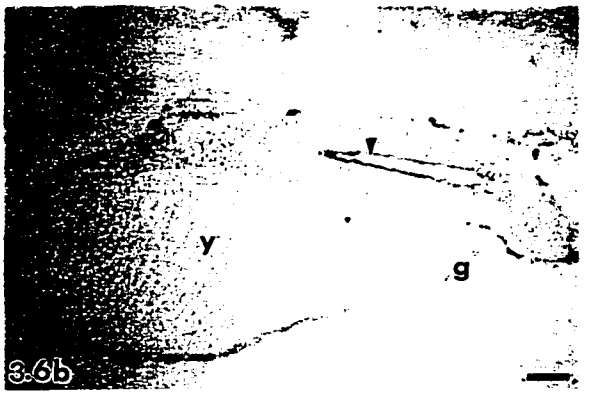
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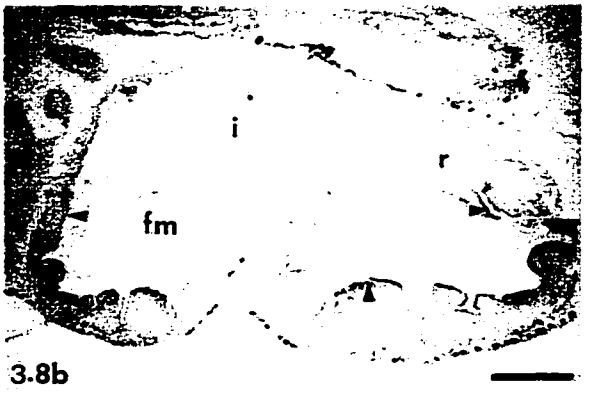
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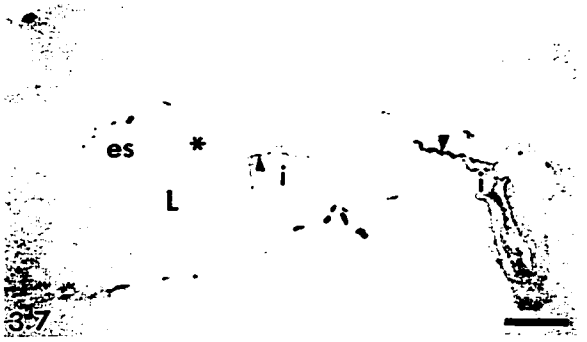
es
L
i
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Table 3.11. Distribution and range of intensities of aminopeptidase M in yellowtail flounder larvae.

DPH	BC	Ph	Eso	PES/St	Intestine	Rectum
Day 3*						
Day 7 G1	-	-	-	-	-(1) +/-(1) +(9) ++(1)	**
G2	-	-	-	-	-(1) +(11)	**
G3	-	-	-	-	-(1) +/-(2) +(8) ¶(1)	**
Day 10 G1	-	-	-	-	+/-(2) +(9) ¶(1)	-(2) +/-(2) +(3) ¶(5)
G2	-	-	-	-	+/-(3) +(3) ++(2) ¶(4)	-(3) +/-(2) +(2) ¶(5)
G3	-	-	-	-	-(7) +/-(3) ¶(2)	-(4) +/-(3) ¶(5)
Day 15 G1	-	-	-	-	-(1) +(8) ++(2) ¶(1)	+/-(3) +(8) ¶(1)
G2	-	-	-	-	+(5) ++(3) ¶(4)	+/-(2) +(4) ++(2) ¶(4)
Day 20 G1	-	-	-	-	+/-(1) +(3) ++(8)	+(6) ++(3) ¶(3)
G2	-	-	-	-	++(12)	+(5) ++(5) ¶(2)
Day 29 G1	-	-	-	-	+(1) ++(10) ¶(1)	+(4) ++(8)
Day 36 G1	-	-	-	-	+(2) ++(9) ¶(1)	-(1) +(4) ++(4) ¶(3)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for aminopeptidase M

** region not present at this age

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 12) reaction was observed; ¶, number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

Table 3.12. Distribution and range of intensities of aminopeptidase M in winter flounder larvae.

DPH		BC	Ph	Eso	PES	Intestine
Day 3*						
Day 7	G1	-	-	-	-	+(1) ¶(7)
	G2	-	-	-	-	-(2) +(2) ¶(4)
	G3	-	-	-	-	+/-(1) +(6) ¶(1)
Day 10	G2	-	-	-	-	-(3) ++(2) ¶(2)
	G3	-	-	-	-	-(2) ¶(6)
Day 15	G1	-	-	-	-	+/-(1) +(4) ++(2) ¶(1)
	G2	-	-	-	-	+/-(1) +(3) ++(3) ¶(1)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for aminopeptidase M

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 8) reaction was observed; ¶, number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable); + clearly present reaction; ++ strong reaction

aminopeptidase M activity became restricted to the brush border of the intestine in both flounder species. A general increase in enzyme intensity occurred by 7 days post hatch in yellowtail flounder although the strength of this reaction was variable. Although activity increased slightly throughout development in winter flounder. differences between the three groups was not observed.

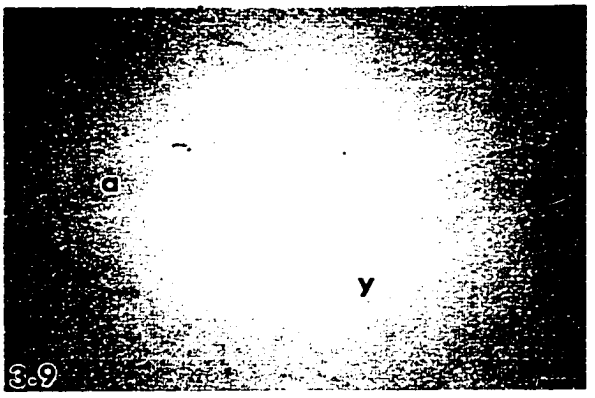
Activity was observed in the brush border of the rectal epithelium by 10 days post hatch in yellowtail flounder. While no consistent difference was observed between the animals of groups 1 and 2. a decrease in this enzyme within these regions occurred in the starved group of larvae. At this age. variability in the intensity of aminopeptidase M between the larvae of both species and between the trials occurred.

Days 15-36

In yellowtail flounder. aminopeptidase activity continued to increase from 10 days post hatch through days 15 to 36 and was present in both the intestine and rectum (Figure 3.10). In winter flounder. a stronger and more consistent reaction was seen in the intestine at 15 days post hatch than day 10 (Figure 3.11a.b).

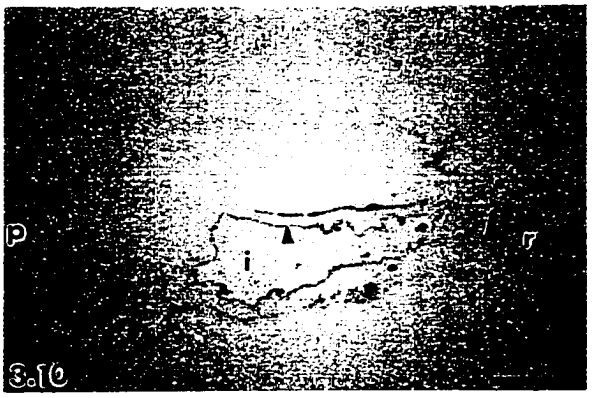
Subsequent to day 15 in yellowtail flounder, enzymatic activity within the intestine and rectum reached a maximum by 29 days post hatch (Figure 3.12a) and remained strong through day 36 (Figure 3.12b) for group 1. A stronger reaction occurred in the intestine epithelium as compared to the rectum in some specimens. No staining at either the pyloric or ileorectal sphincter was present. The presence of aminopeptidase associated with food mass in the lumen of the intestine and rectum increased in frequency from 15 days post hatch.

Figs 3.9-3.12. Aminopeptidase M activity (arrowheads) in yellowtail and winter flounder.
Figure 3.9. The columnar epithelium of the posterior region of the gut in yellowtail flounder larva at 3 days post hatch is positive. Bar = 50 μm.
Figure 3.10. Differential distribution of aminopeptidase M in the brush border of the intestine (i) and rectum (r) of yellowtail flounder larvae at day 15. Bar = 80 μm.
Figure 3.11. Distribution and intensity aminopeptidase M activity in winter flounder. (a) Transverse section of a winter flounder larva at 10 days post-hatch; activity is localized to the intestinal (i) brush border (Bar = 30 μm) (b) Sagittal section of a larvae at 15 days post hatch shows an increase in intensity in this same region (compare to Figure 3.10) (Bar = 40 μm).
Figure 3.12. Aminopeptidase M activity yellowtail flounder. (a) By 29 days post hatch, strong activity is seen in the intestine (i) and rectum (r) (Bar = 32 μm). (b) Reaction continued to be strong through day 36. No staining was associated with the ileorectal sphincter (S) (Bar = 80 μm).
y, yolk sac; a, anterior region; p, pharynx; asterisk, PES; fm, food mass.



3.9

3.11b



3.10

3.12a

3.11a

3.12b

3.3.5 Nonspecific esterases (3.1.1.-)

Activity and distribution of nonspecific esterase in developing yellowtail and winter flounder larvae are summarized in Tables 3.13 and 3.14, respectively.

Day 3

For both yellowtail and winter flounder, the brush border of the columnar epithelial cells in the posterior region of the gut was positive for nonspecific esterase (Figure 3.13a); no enzymatic activity was found in the anterior-most aspect of the gut although intensity increased posteriorly. In yellowtail flounder, no reaction could be observed in the most caudal intestinal region. Although the liver was present at this stage, no clear reaction was observed there. No difference in the activity of nonspecific esterase was seen between the three feeding group animals.

Day 7

In yellowtail flounder, the distribution of nonspecific esterase at 7 days post hatch was mainly localized to the columnar epithelial brush border of the intestine (Figure 3.13b). Nonspecific esterase appeared in the liver as isolated focal points but the intensity was not as strong as the activity in the intestine. Reaction was slightly more intense than day 3 and in some cases, the reaction in the intestine was found in the cytoplasm and the brush border. There was no reaction in the posterior most aspect of the intestine (i.e. future rectum). Esterase activity in winter flounder, like yellowtail, was restricted to the intestine. In this species, intensity was increased from day 3 and was stronger than the reaction in yellowtail flounder.

No clear difference between the animals from different feeding trials was

Table 3.13. Distribution and range of intensities of nonspecific esterase in yellowtail flounder larvae.

DPH	BC	Ph	Eso	P/St	Intestine	Rectum
Day 3*						
Day 7 G1	-	-	-	-	-(1) +/-(1) +(10)	**
G2	-	-	-	-	-(1) +/-(1) +(9) ++(1)	**
G3	-	-	-	-	-(1) +/-(1) +(7) ++(2)	**
Day 10 G1	-	-	-	-	-(1) +/-(1) +(8) ++(1) ¶(1)	-(3) +/-(3) ¶(6)
G2	-	-	-	-	+(7) ++(1) ¶(4)	-(1) +/-(1) +(4) ¶(6)
G3	-	-	-	-	-(3) +/-(5) +(2) ++(1) ¶(1)	-(3) +/-(5) +(1) ¶(3)
Day 15 G1	-	-	-	-	+(6) ++(5) ¶(1)	-(2) +/-(4) +(5) ¶(1)
G2	-	-	-	-	+(6) ++(3) ¶(3)	-(4) +/-(1) +(1) ¶(6)
Day 20 G1	-	-	-	-	+(5) ++(7)	-(6) +/-(1) +(2) ++(2) ¶(1)
G2	-	-	-	-	+(8) ++(4)	-(3)+/-(2) +(3) ¶(4)
Day 29 G1	-	-	-	-	+(3) ++(9)	-(4) +(5) ¶(3)
Day 36 G1	-	-	-	-	+(2) ++(8) ¶(2)	-(5) +(3) ¶(4)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for nonspecific esterase

** region not present at this age

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; P/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 12) reaction was observed

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

Table 3.14. Distribution and range of intensities of nonspecific esterase in winter flounder larvae.

DPH		BC	Ph	Eso	PES	Intestine
Day 3*						
Day 7	G1	-	-	-	-	+(2) ¶(6)
	G2	-	-	-	-	+(1) ++(4) ¶(3)
	G3	-	-	-	-	+(2) ++(5) ¶(1)
Day 10	G2	-	-	-	-	-(2) ++(3) ¶(3)
	G3	-	-	-	-	-(2)+/- (1) ¶(5)
Day 15	G1	-	-	-	-	+(3) ++(5)
	G2	-	-	-	-	+(2) ++(6)

* Posterior-most aspect of the digestive tract at 3 days post hatch positive for nonspecific esterase

DPH, days post hatch; BC, buccal cavity; Ph, pharynx; Eso, esophagus; PES/St, post-esophageal swelling/stomach; G1, group 1; G2, group 2; G3, group 3; parenthesis, number of trials (out of a total of 8) reaction was observed; ¶, number of trials region not present/identified

Intensity: -, no reaction; +/- weak (barely detectable) reaction; + clearly present reaction; ++ strong reaction

observed for either species.

Day 10

Similar to that in day 7, the reaction in the intestinal brush border was strong and a distinct increase in the reaction was seen in the liver as compared to day 7. There was also a reaction in the musculature along the body wall as well as in the brain and kidney. The activity within the intestine of this enzyme was not as strong in group 3 as in groups 1 and 2 (Figure 3.14 a.b); in some cases, no associated enzymatic activity was seen in group 3 larvae.

In winter flounder, an increase in intensity was seen in the intestine, yielding a very strong reaction (Figure 3.15a), with an increase from anterior to posterior. Esterase activity within the intestine is both the brush border and cytoplasmic and this decreased just anterior to the posterior opening.

Day 15-36

In yellowtail flounder larvae, the intensity of esterase activity in the liver and intestine increased between days 10 and 15 (Figure 3.16 a). The brush border activity of the enzyme was retained in the intestine and cytoplasmic activity was evident, primarily within the supranuclear region. The reaction in the rectal epithelium was very weak and confined to the cytoplasm. Generally, a correlation between intestinal and rectal activity was noted where strong intestinal activity increased the incidence of esterase activity in the rectum (Figure 3.16b). Over the next several stages of development (i.e 20, 29 and 36 days post-hatch), a gradual increase in intensity occurred within all of these regions (Figure 3.17 a.b). The incidence of esterase activity in the rectal epithelium by day 29 in

Figs. 3.13-3.15. Nonspecific esterase activity (arrowheads) in yellowtail and winter flounder.

Figure 3.13. Esterase activity in yellowtail flounder. (a) Caudal-most aspect of the posterior region of the gut at 3 days post hatch (Bar = 40 μm). (b) Esterase activity by day 7 in localized to the intestinal (i) brush border (Bar = 54 μm).

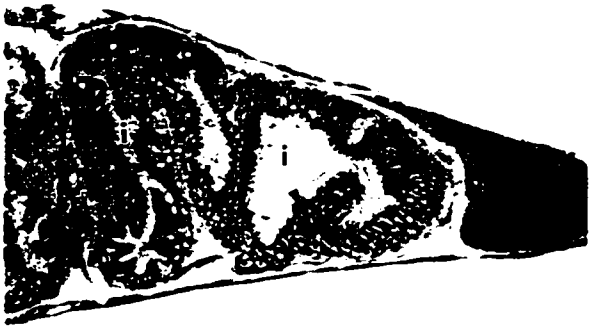
Figure 3.14. In yellowtail flounder, differences in the intensity of esterase activity occurred at 10 days post hatch in group 3 larvae. (a) Esterase activity (arrowheads) is strongly located in the intestine (i) of a group 2 larva. Bar = 36 μm. (b) This activity is less intense in group 3 larvae that had not received any food. Bar = 60 μm.

Figure 3.15. (a) Strong esterase activity in a winter flounder larva at 10 days post hatch is localized to the brush border (arrowheads) and cytoplasm (arrow) of the intestine (i). Bar = 74 μm. (b) At 15 days post hatch, activity in the intestine decreased from day 10. Bar = 44 μm.

es, esophagus; E, eye; asterisk, PES; r, rectum; L, liver; y, yolk.

3.13a

3.14b



3.13b

3.15a

3.14a

3.15b

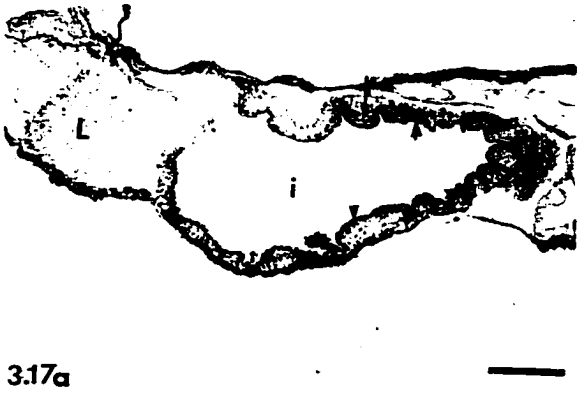
Figs 3.16-3.17. Esterase activity (arrowheads) increased gradually during the development of yellowtail flounder.

Figure 3.16. Esterase in yellowtail flounder at day 15 (a) Strong activity in the intestine (I): reaction in intestine is also cytoplasmic (arrows). A weak reaction was seen in the rectum (r). Bar = 30 μm. (b) No reaction is associated with the rectal epithelium when the intestinal reaction is weaker. Note that the food mass (fm) in the rectum is positive. Bar = 80 μm.

Figure 3.17. (a) At 20 days post hatch, a consistent reaction was observed in the brush border and a weaker, diffuse reaction in the cytoplasm (arrow) of the intestine (I). Bar = 90 μm. (b) By 36 days post hatch, the activity was stronger in the intestine and a weak reaction in the brush border of the rectum (r). Cytoplasmic activity is also strong (arrows). Bar = 98 μm.

asterisk, PES: S, ileorectal sphincter; PS, pyloric sphincter; L, liver.

3.16a



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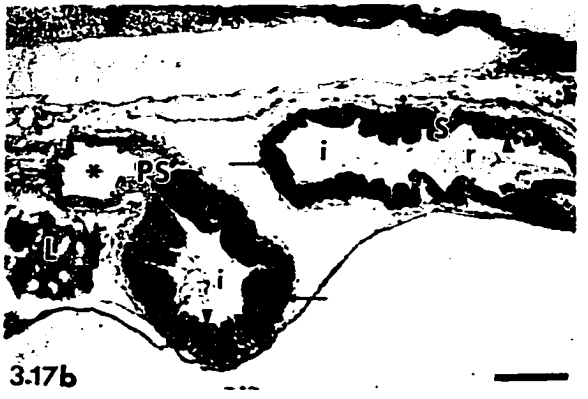
3.16b



3.17b

group 1 was increased in both the cytoplasm and brush border, although it was weaker than that in the intestine. No staining was associated with either the pyloric or ileorectal sphincters.

The distribution of esterase activity in winter flounder larvae did not change from 10 to 15 days post hatch; activity was distributed along the length of intestine with an increase from anterior to posterior (Figure 3.15b). The intensity of the reaction was not as high as in the earlier ages. Distribution changed from both cytoplasmic and brush border to just being localized at the brush border.

3.4 Discussion

Investigations dealing with the ontogeny of digestive enzymes in marine fish are increasing in number. Many studies are now dealing with the response of these enzymes to diet and the influence of exogenous sources of dietary enzymes on digestive capacity. Little information, however, is available dealing with this aspect of flatfish ontogeny. The present investigation describes the temporal and spatial development of digestive enzymes in larval yellowtail and winter flounder on different dietary regimes using histoenzymological methods. Generally, digestive enzymes were present at the earliest age examined- 3 days post hatch. Alkaline phosphatase, aminopeptidase M, dipeptidylpeptidase IV and nonspecific esterase were present in varying intensities at this time in the posterior region of the digestive tract of both species. By 7 days post hatch, concomitant with regionalisation of the gut, these enzymes were localized primarily in the intestine. Nonspecific esterase and aminopeptidase M increased in intensity during

developmental stages subsequent to day 7. The regularity of expression (i.e. presence or absence of enzymatic activity) of alkaline phosphatase and dipeptidylpeptidase IV increased during later ages. A moderate increase in dipeptidylpeptidase IV activity was also observed while acid phosphatase was not detected in either species. For a summary of the spatial and temporal development of enzymatic activity in yellowtail and winter flounder, refer to Figures 3.18 to 3.21.

The results obtained suggest that digestive enzymes in both yellowtail and winter flounder were present, under experimental conditions before the larvae start to feed exogenously at approximately day 7 and that morphological differentiation of the digestive tract corresponded with specific regionalisation of the digestive enzymes.

The correlation between morphological differentiation based on histological observations and functional differentiation based on digestive enzyme activity has been noted by several researchers (Segner *et al.*, 1989; Gawlicka *et al.*, 1995; Oozeki & Bailey, 1995). Early development of digestive enzymes before or during the transformation from endogenous to exogenous food is not unusual. In striped bass, digestive enzymes (except pepsin) are present on day 4, one day before they started to feed (Baragi & Lovell, 1986). In sea bass, all enzymes examined (except γ-glutamyl transpeptidase) increased between day 4 and 6, coinciding with mouth opening (Zambonino Infante & Cahu, 1994). In sea bream larvae, no enzymatic activity is observed between hatching and 3 days post hatch (Sarasquete *et al.*, 1993). During exogenous feeding, this activity generally increased (Sarasquete *et al.*, 1993). Early development of digestive enzymes is observed in turbot larvae where the activities of alkaline phosphatase and aminopeptidase are present at one

Figure 3.18a. Distribution of alkaline phosphatase activity in the developing digestive tract of yellowtail flounder. Note that there is no difference between the three feeding groups.

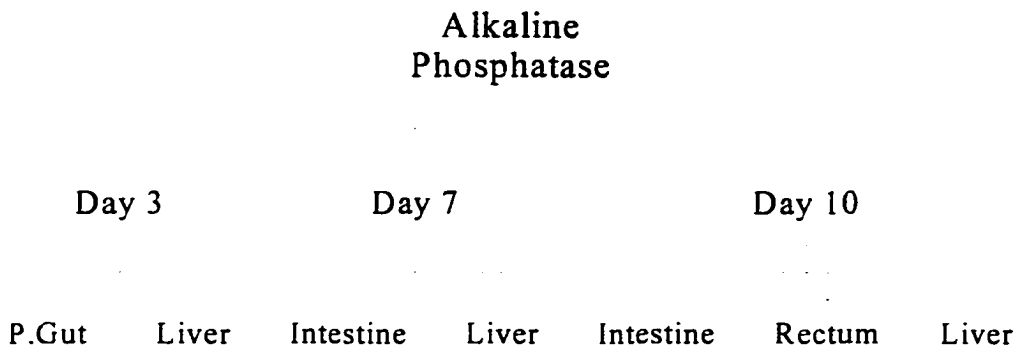


Figure 3.18b. Distribution of alkaline phosphatase activity in the developing digestive tract of winter flounder. Note that group 3 larvae showed no staining and by day 15, the reaction had decreased.

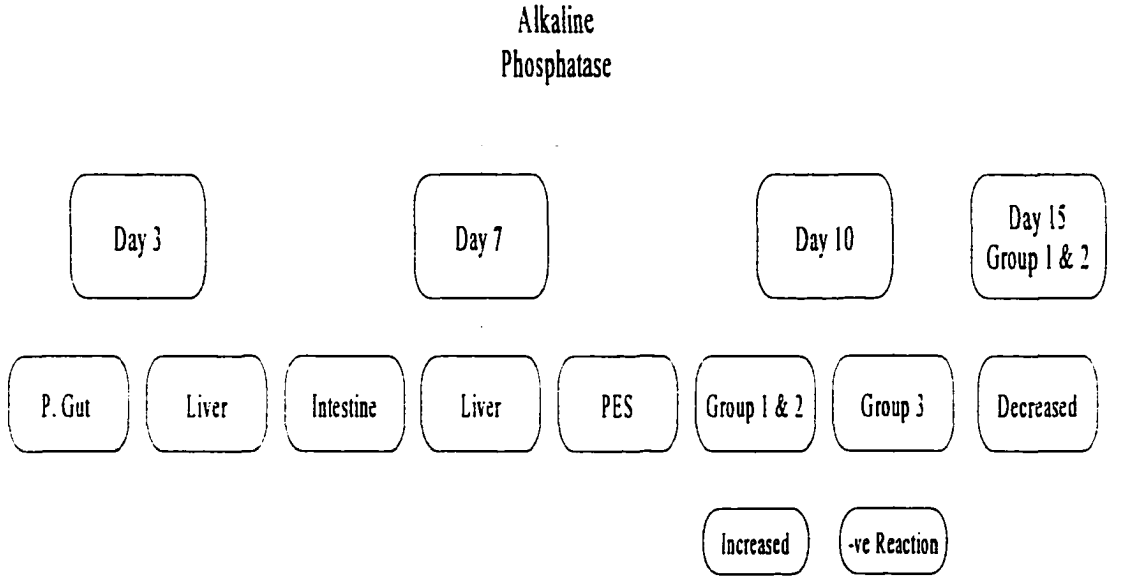


Figure 3.19a. Distribution of dipeptidyl peptidase IV (DPP IV) activity in the developing digestive tract of yellowtail flounder. Note that there is no difference between the three feeding groups.

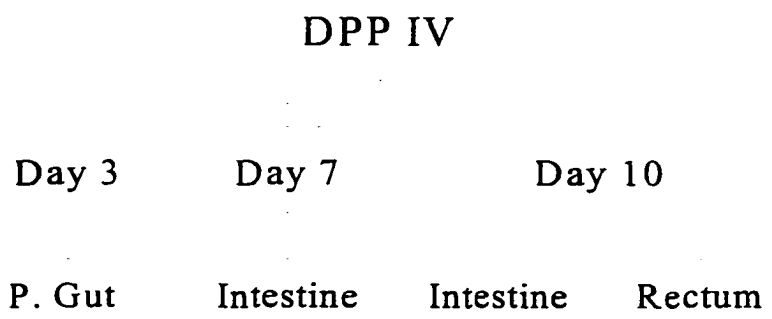


Figure 3.19b. Distribution of dipeptidyl peptidase IV activity in the developing digestive tract of winter flounder. Note the difference between group 3 and that intensity increased with age.

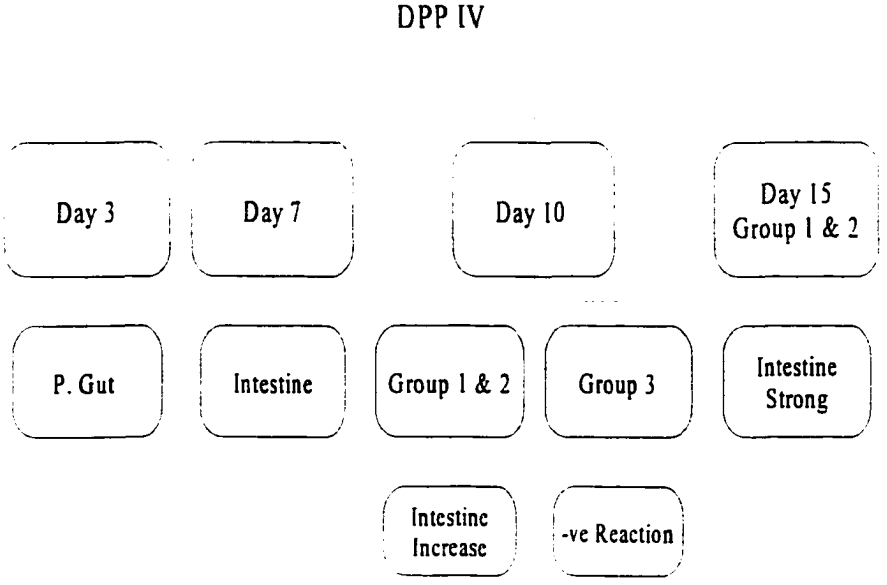


Figure 3.20a. Distribution of aminopeptidase M activity in the developing digestive tract of yellowtail flounder. Note the reaction in group 3 was weaker and that intensity increased with age.

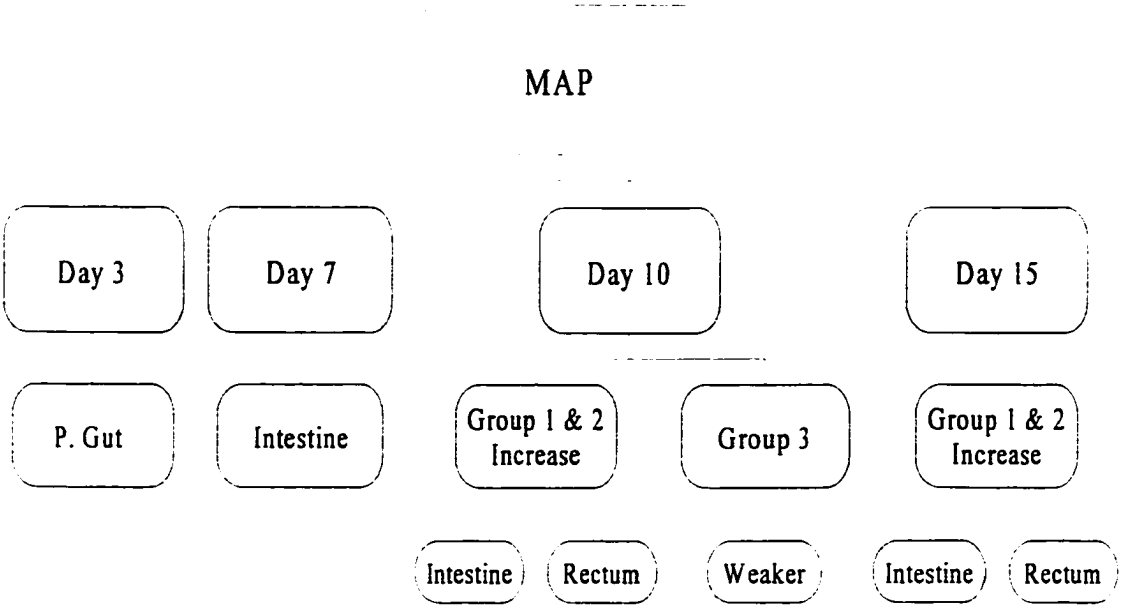


Figure 3.20b. Distribution of aminopeptidase M activity in the developing digestive tract of winter flounder. Note the reaction in group 3 was weaker and that intensity increased with age.

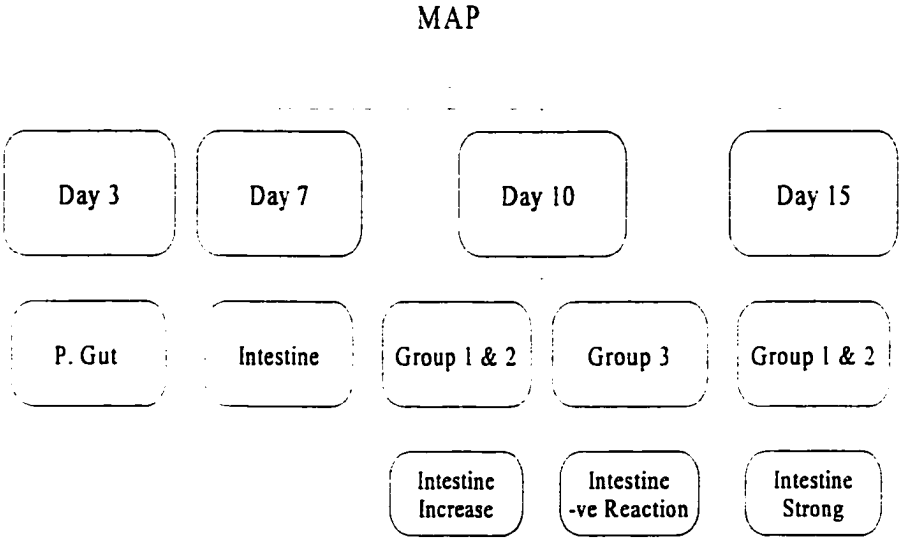


Figure 3.21a. Distribution of nonspecific esterase activity in the developing digestive tract of yellowtail flounder. Note the reaction in group 3 was weaker.

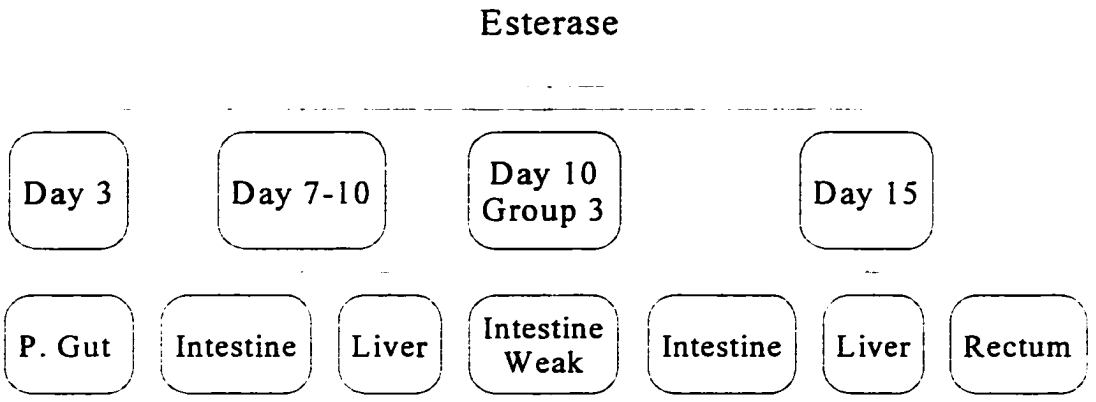
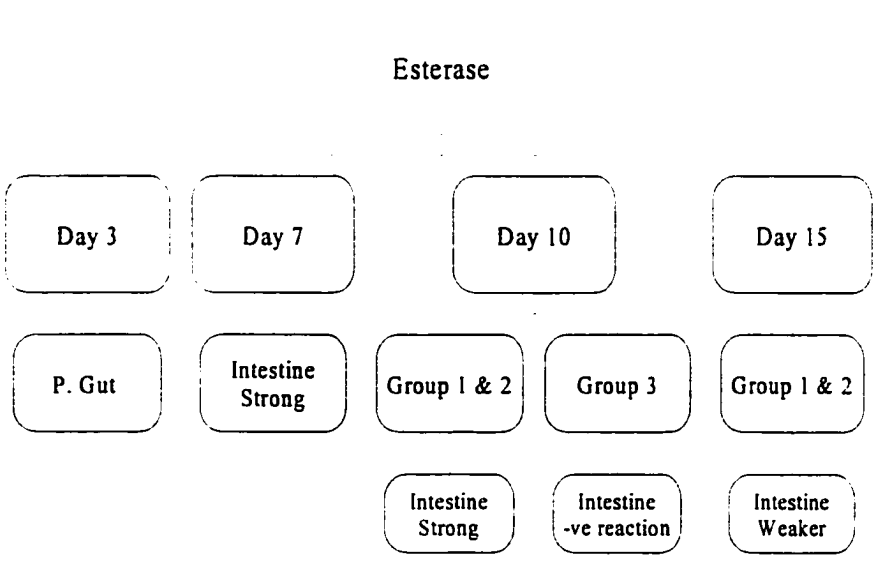


Figure 3.21b. Distribution of nonspecific esterase activity in the developing digestive tract of winter flounder. Note the reaction in group 3 was weaker and by day 15, intensity had decreased.



day after hatching at the distal end of the digestive tube. At mouth opening and the transfer to exogenous feeding (day 2-3), these enzymes are found throughout the intestine. Also, it is at this time that nonspecific esterase and protease activities became apparent (Cousin *et al.*, 1987). In a similar study of larval digestive physiology by Segner and colleagues (1993), the larval digestive system at first feeding for whitefish (*Coregonus lavaretus*), catfish (*Clarias gariepinus*) and turbot exhibited activity for various pancreatic and enterocytic microvilli enzymes such as maltase, aminopeptidase and alkaline phosphatase. Cousin and co-workers (1987) suggested that at the time of mouth opening, the digestive system appears to be functional even though gastric glands were not present and thus presumably without pepsin activity. The general lack of digestive capacity, therefore, is not true for first-feeding larvae (Segner *et al.*, 1993). From the results of this study, similar inferences could also be made for both yellowtail and winter flounder. The early activity of various hydrolytic digestive enzymes indicates that the larvae should be able to, even if to a limited extent, to process ingested food when they switch from an endogenous to exogenous food supply at around seven days post hatch.

The extent to which exogenous dietary enzymes of live prey contribute to the expression of enzymes in the digestive tract of fish larvae and whether this contribution is essential for larval survival, especially during the switch to live food, is not certain. It was noted in this study that food in the lumen of the gut, especially in yellowtail flounder, stained positively for several of the enzymes in question. This indicates that these enzymes (nonspecific esterase, alkaline phosphatase, aminopeptidase M and dipeptidyl

peptidase IV) are present in the live food (i.e. rotifers) and remained active even after passage through the gut. A similar observation was noted by Cousin and colleagues (1987) where live prey fed to turbot larvae contained enzymes such as amylase, aminopeptidase and acid and alkaline phosphatase that may contribute to digestion. This suggests that exogenous enzymes may be needed for digestion of food (Oozeki & Bailey, 1995). The importance of exogenous enzymes has also been speculated by other researchers (Lauff & Hofer, 1984; Oozeki & Bailey, 1995) and appears to be a significant aspect of prey quality for the larvae. From the time of first feeding, exogenous enzymes seem to play a key role in larval digestion (Munilla-Moran *et al.*, 1990). Exogenous digestive enzymes may also be activators of zymogens to help complete the digestive process (Oozeki & Bailey, 1995). Caution, however, should be exercised in simply stating that digestive enzymes contribute to the digestive enzyme capacity in larval yellowtail and winter flounder based on the information presented.

As indicated by Oozeki & Bailey (1995), consideration should be given to the optimum enzyme temperatures for both the fish species and the zooplankton used as prey. In their study on walleye pollock (*Theragra chalcogramma*), where larvae were reared at approximately 6 ^{0}C, the authors pointed out the optimum temperature of rotifer culture is much higher, usually between 30-34 ^{0}C, and thus their optimum enzymatic temperatures are also likely to be higher. Therefore, enzymes of rotifers reared artificially may not actually contribute significantly to the digestive capacities of the fish as compared with those in naturally occurring prey at ambient temperatures. Similar inferences can be made regarding the rearing conditions of the larvae in our study. Consequently, the actual

contribution of digestive enzymes from the live prey remains unresolved.

The differences observed in intensities during the early stages (day 3) and the variation in these intensities during subsequent stages is not unusual among marine fish larvae. Zambonino Infante & Cahu (1994) studied digestive activities in sea bass using spectrophotometric determination and noted that digestive enzymes appeared at different times after hatching and that their activity levels fluctuated greatly during the first three weeks. Alkaline phosphatase, leucine aminopeptidase and γ-glutamyltransferase were present early (by day 4), increased up to day 23 and then fell to a constant value until day 40. In yellowtail flounder larvae, alkaline phosphatase was initially present at a constant level between day 3 and 7. This varied over the next three weeks until reaction stabilized at day 29 and 36. Similar observations were made for dipeptidylpeptidase IV in both species. The literature provides several explanation for this fluctuation. According to Zambonino Infante & Cahu (1994), this decline could indicate greater degradation or lower synthesis of the enzyme. Reduced enzyme synthesis for a period during early development in herring larvae has also been suggested by Pederson *et al.* (1987) for the fluctuation of trypsin and trypsinogen. Based on comparison with a similar study on cod larvae by Hjelmeland *et al.* (1984), Pederson *et al.* (1987) concluded that oscillations in enzyme content may be a common phenomenon in marine fish larvae, independent of whether the larvae were offered food or not. As a result, this reduced enzymatic capacity in the developing gut may depress digestive function and contribute to the high mortalities often encountered during artificial rearing. The presence of food in the gut, however, has been known to stimulate gastrointestinal secretions in higher vertebrates

(Hopfer, 1992) and has been proposed as a stimulant for pancreatic secretions in Japanese flounder (Kurokawa & Suzuki, 1996). Introduction of live food to the larvae well in advance of yolk absorption may increase survival by stimulating the synthesis and secretion of hormones and/or enzymes need for digestion.

Many of these studies used more sensitive quantification techniques such as radioimmunoassay to obtain an analysis of enzyme content. While radioimmunoassay may allow more minute quantities to be assayed, the exact spatial distribution of digestive enzymes cannot be accurately determined by this method. The purpose of this investigation was to provide an *in situ* indication of the developmental timing of enzymatic activity and distribution. The enzyme histochemical techniques used in this study may not have been sensitive enough to detect very low levels of certain enzymes.

In the enzymes assayed for yellowtail and winter flounder, no obvious histological difference could be detected between the group of larvae fed the live diet and those weaned to an artificial diet. The larvae in the weaned group likely did not survive long enough past the introduction of the artificial diet for this feed source to induce any modifications of enzymatic activity, likely due to poor consumption. The live diet was not flushed out of the rearing containers at the time of weaning, and consequently the larvae in this group could have ingested the live diet until it was exhausted. Once the live food was not available, the larvae may simply not have been feeding on the artificial food and subsequently starved and died. The higher number of mortalities in the weaned group could be indicative of poor digestibility, attractability or palatability to the artificial diet. Switching the food this early in the developmental sequence was most likely the

contributing factor behind the higher incidence of mortalities within this group.

Weaning is generally not successful until metamorphosis (Segner *et al.*, 1993), i.e. after the stomach becomes functional. Weaning in marine fish is often described as a period of mortality sometimes greater than 50% (Rosenlund, 1997). It has been suggested that the requirement for live food is related to the absence of pepsin digestion during this early rearing period (Verreth *et al.*, 1993). A functional stomach is necessary to utilize dry feed as efficiently as live feed (Segner *et al.*, 1993). To obtain higher survival of both yellowtail and winter flounder during weaning, switching to an artificial diet during later ontogenic stages, at least once the stomach forms, may decrease mortality. It could be assumed that in this study, the switch made was too early for maximum survival of the larvae. Results, therefore, were not conclusive for enzymatic differences on the basis of diet because of poor survival.

The strong overall increase in intensity observed for aminopeptidase M and nonspecific esterase and the moderate increase noted for dipeptidylpeptidase IV in yellowtail as well as winter flounder is consistent with that found in other ontogenetic studies. For example, in larval Coregonids, there was ontogenetic enhancement of enzyme staining intensities for trypsin, aminopeptidases, maltase and alkaline phosphatase but not for nonspecific esterase (Segner *et al.*, 1989). Similar trends were also seen in white sturgeon (Gawlicka *et al.*, 1995) and gilthead sea bream (Moyano *et al.*, 1996). The temporal development of enzymatic activity is, therefore, also enzyme dependant.

The distribution of esterase activity in the liver and intestine in both larval flatfish

and to a limited extent in the rectum of older yellowtail flounder is similar to that in the other species in marine and freshwater. Distribution of esterase among fish species is varied with activity present in the esophagus, intestine, liver, pancreas pyloric caeca, stomach and gall bladder (adapted from Cousin *et al.*, 1987). This enzyme has lipolytic properties (Oliver *et al.*, 1991). Correlation has been made in several fish species between the presence of this enzyme with fat digestion (Chakrabarti *et al.*, 1995) and lipid absorption (Gawlicka *et al.*, 1996). Further, this enzyme may be of particular importance because fish utilize lipid/fat as their main nutritional source rather than carbohydrate and protein (Chakrabarti *et al.*, 1995). It may account for the strong staining intensity of this enzyme compared to the others analyzed.

Esterase activity was found in both the brush border and within the cytoplasm of the intestine of yellowtail and winter flounder. In the yellow perch (*Perca fluviatilis*), however, this activity was absent from the brush border and thus it was concluded that esterases were not responsible for the initial entry of carboxylic esters into the columnar cells (Hirji & Courtney, 1983). Based on cellular distribution, this does not seem to be the case for the flounder species. Nonspecific esterase localized to the brush border may facilitate entry of carboxylic esters into the enterocytes where they can undergo further degradation.

The lower activity of nonspecific esterases in the rectum corresponds to analogous distribution in the perch where activity in the lower intestine and rectum was weak (Hirji & Courtney, 1983). In the larvae of several fish species, including sturgeon (Gawlicka *et al.*, 1995), whitefish (Segner *et al.*, 1989) and turbot (Cousin *et al.*, 1987), esterase

activity was absent or negligible in the posterior-most region of the digestive tract (i.e. regions corresponding to the rectum in flounder). This differential distribution could reflect differences in function between the two post-gastric regions. For example, the intestine is generally associated with lipid absorption based on the appearance of lipid-containing vacuoles while intracellular protein digestion occurs in the rectum (Kjørsvik *et al.*, 1991; Segner et al., 1994; Bisbal & Bengtson, 1995; Sarasquete *et al.*, 1995).

Morphological signs of lipid digestion were found in the intestine of adult yellowtail and winter flounder (Murray, 1993; Murray *et al.*, 1996). Generally, lipids are absorbed following luminal hydrolysis to fatty acids and monoglycerides (Kjørsvik *et al.*, 1991), to which nonspecific esterases may contribute, particularly during the transition phase to exogenous feeding. The absence of esterase in the anterior intestine of sturgeon larvae led Gawlicka *et al.* (1995) to conclude that hydrolysis of carboxylic esterase by nonspecific esterase (occurring during lipid and carbohydrate digestion) may not occur in this region of the gut. Minimal activity in the rectum of yellowtail flounder suggests the rectal epithelium has negligible esterolytic activities as well.

Alkaline phosphatase has a wide distribution in developing tissues. In turbot, it is found in the nervous system, developing retina, intestine, pancreas, liver, connective tissues, kidney, heart, gills and spleen (Cousin *et al.*, 1987). Consistent localization is also found within intestinal segments, (Segner *et al.*, 1989; Kuz'mina & Smirnova, 1991; Gawlicka *et al.*, 1995) including that of larval yellowtail and winter flounder. Inconsistency between the two flounder species, however, may reflect intraspecific variation. Winter flounder had higher alkaline phosphatase activity up to day 10 as

compared to yellowtail flounder. By day 15, this activity in winter flounder had decreased while that of yellowtail showed no clear difference. While localized in the intestine, kidney, liver and developing eye in both species, activity was also found in the stomach anlage in winter flounder. The activity of alkaline phosphatase within the PES of winter flounder is not easily explained and this enzyme has not been observed within the stomach anlage in other larval fish.

Functions within the teleost digestive tract for alkaline phosphatase have been proposed. For example, according to Lojda *et al.* (1979), this enzyme is found primarily in cell membranes where active transport takes place. Therefore, it has been speculated that this enzyme is important in the absorptive process in turbot (Cousin *et al.*, 1987) and is considered to be a general marker of nutrient absorption (Segner *et al.*, 1989). Presence in the intestinal and rectal mucosa in flounder identifies this tissue as a site of active nutrient absorption, and suggests that the stomach anlage of winter flounder may be functioning in some sort of absorptive process.

Exoproteases such as aminopeptidase and dipeptidylpeptidase appear to be important in the digestion of proteins during early development. Protein digestion is considered inadequate in the underdeveloped larval digestive tract and is linked to high mortalities occurring during first feeding and subsequent weaning. Trypsin remains one of the best-investigated proteolytic enzymes in fish larvae (Ueberschar, 1993) and it is generally accepted that pepsin is not present until the stomach forms (Baragi & Lovell, 1986; Segner *et al.*, 1993; Segner *et al.*, 1994). The importance of intestinal proteases during early development has aroused interest among researchers that has led to their

histochemical demonstration in other species (Cousin *et al.*, 1987; Segner *et al.*, 1989; Segner *et al.*, 1993; Cahu & Zambonino Infante, 1994; Gawlicka *et al.*, 1995).

It was demonstrated in this study that two exoproteases- aminopeptidase M and dipeptidylpeptidase IV- are present at three days after hatching in both yellowtail and winter flounder and are found in the intestine and rectum (yellowtail flounder) from day 7 and 10, respectively. The aminopeptidase had greater activity than dipeptidylpeptidase IV. The activity of dipeptidylpeptidase IV was initially quite variable but became stable in its expression in older animals. The variation in dipeptidylpeptidase IV seen in yellowtail and winter flounder during the earliest ontogenetic stages may reflect low peptic activity. Gawlicka and colleagues (1996) examined the influence of a carrageenan microbound diet on the activity of digestive enzymes in white sturgeon larvae and found the activity of the enzymes was decreased. Further, they speculated that even though pepsin activity was not measured, this reduced activity of dipeptidylpeptidase IV observed in these larvae may be associated with delayed functional development of gastric glands.

These enzymes are important in the terminal digestion of peptides that result from the initial action of pancreatic and gastric peptidases (Norén *et al.*, 1986). Some authors correlate the presence of exoproteases with maturation of the intestine. Cahu & Zambonino Infante (1995) indicated that high activities of brush border enzymes, leucine aminopeptidase, γ-glutamyltransferase and maltase revealed the beginning of maturation of the intestine. Similarly, Gawlicka and co-workers (1995) indicated that increased activity of aminopeptidase M, dipeptidylpeptidase IV and γ-glutamyltransferase coincide with active feeding. Because these brush border enzymes (as well as lactase) have been

used in mammals as markers of enterocyte differentiation (Smith, 1992), their increased presence in fish intestine confirms the presence of more functionally developed enterocytes. The presence of two of these enzymes in the digestive tract of yellowtail and winter flounder, with increasing regularity and expression with age, illustrates the developing functional state of the gut.

The absence of acid phosphatase in both these species was an unexpected finding because of the regularity of expression in sections of control tissue and demonstration in other larval fish species. In turbot, acid phosphatase activity was found in pharynx, esophagus, intestine and stomach where this activity increased with age (Cousin *et al.*, 1987). In sea bream, activity was located in the striated (brush) border of enterocytes, the epithelium of the stomach mucosa as well as in the supranuclear vacuoles of the posterior intestine (Sarasquete *et al.*, 1993). It was therefore expected that (based on the presence of similar supranuclear vesicles observed in the rectum of yellowtail flounder larvae) activity of this enzyme would also be located in the rectum. However, acid phosphatase may simply not be found in either of these species until later on in development (i.e. beyond the time-frame of this study). Alternatively, this enzyme may have been lower than the others that were assayed and the histochemical technique used may not have been sensitive enough to visualize minute quantities.

The procedures used in the present study are based on adaptation and modifications on freeze-drying glycol methacrylate embedment (FDGE) without prior fixation and chemical dehydration of the tissue (Teh & Hinton, 1993). This technique has been successfully used in the studies on digestive enzymes in larval sturgeon by Gawlicka

and colleagues (1995, 1996). This method has several advantages over traditional procedures used in enzyme histochemistry; for instance denaturation of the enzymes is prevented by avoiding the use of harsh fixative or dehydrating agents. Glycol methacrylate (GMA) used to embed the specimens is an acrylic resin that is water-miscible. Therefore complete dehydration of the tissue is not necessary and will allow good penetration by hydrophilic staining reagents (Gerrits & Horobin, 1996). Because of these properties, the resin does not have to be removed prior to staining.

Despite the noted advantages of this procedure, there are technical aspects that could have affected the results seen in this study and account for some of the variability observed between fish. However, to minimize any variation in staining intensities between yellowtail and winter flounder, slides containing sections of digestive tract for both species were incubated concurrently (i.e. in the same batch) in the enzyme incubation solution. This batch control application minimized variability of the intraspecific intensities that were seen cannot be attributed to alterations that inherently come with separate batches of incubation medium.

The biggest disadvantage of this procedure is the tissue damage that often occurs because of the absence of tissue fixation and/or damage caused by the freeze-drying procedure. Since samples for this study were frozen directly and subsequently stored in liquid nitrogen before being transferred immediately to the pre-chilled platen of the batch freeze dryer, damage caused by severe temperature fluctuations was negligible. The drying time under vacuum must be optimum. Poor drying time, either too long or too short, generally results in poor cellular preservation. As described by Teh & Hinton

(1993), a "honeycombed" appearance of the tissue is due to extraction of nuclear and cytoplasmic material. This is not an uncommon problem with this procedure and has been reported by other researchers (Munda *et al.*, 1992).

While a drying time of 16 hours was determined experimentally using winter flounder larvae, they were all about the same age and consideration should have been given to the increase larval size corresponding with increasing age. It was noted during this study that as the animals aged (especially the older yellowtail flounder), the consistency of cellular preservation between blocks improved. Basically, tissue in the older animals was better preserved, suggesting that the size of the tissue used in this procedure is important (Teh & Hinton, 1993). Therefore, different drying times should have been established for the different sized tissues to prevent inconsistency in the tissue quality.

Poor cytological preservation could have accounted for some of the variability of enzyme expression seen. This occurred with some specimens even though regions of the digestive tract remained identifiable. A positive reaction for the enzymes was generally present in both adequately and poorly preserved specimens. The loss of cellular constituents could have affected the cytological demonstration of enzymes such as nonspecific esterase and perhaps to a lesser extent, the more firmly bound brush border enzymes.

To summarize, evidence presented from this study suggests that digestive enzymes are synthesized and functional during the earliest stage of post-embryonic development, the yolk-sac stage, in the two species of pleuronectids examined. The

spatial and temporal distribution exhibited by alkaline phosphatase, aminopeptidase M, dipeptidylpeptidase IV and nonspecific esterase indicates that the digestive tract becomes functionally segmented, coinciding with morphological development for optimum processing of nutrients even though major organs have yet to fully develop. Delaying the time of weaning until stomach formation is completed may increase survival rates of larval marine flatfish. Continuing the investigation of digestive enzymes through the juvenile developmental stages may provide a better indication of diet-related modifications to digestive enzymes that were not clearly established in this study.

While the FDGE method yielded adequate histochemical results, refinement of the technique (i.e. drying time) may clarify some of the variability that occurred with the data. The lack of digestive capacity in the underdeveloped digestive tract is most probably not the only contributing factor to the high mortality characteristic of the larval stage. Further investigation into rearing parameters, nutritional status of the live food, and nutrient requirements of these species may clarify the current problems encountered during attempts at artificial cultivation.

4. GENERAL DISCUSSION

4.1 Rationale

The main objective of this project was to provide information on the basic developmental biology of the digestive tract of two important species of cold-water pleuronectids: yellowtail and winter flounder. They are considered candidate species for aquaculture (Brown *et al.*, 1995; Wiseman & Brown, 1997), in part, because of their commercial importance and declining wild stocks, but also because of the desire to broaden the base of aquaculture, creating jobs within Atlantic Canada (Brown *et al.*, 1995). Research into the rearing of yellowtail flounder (Wiseman & Brown, 1997) has examined larval densities, flow rates, light intensity and photoperiod as related to larval survival and brood stock management.

The high mortalities occurring during cultivation of these species, the intensive nature of their rearing and desire to diversify the aquaculture industry have fuelled interest into basic and applied scientific studies of pleuronectids. For example, this project has direct relevance to the aquaculture industry on a fundamental level. At present, feeding regimes for yellowtail and winter flounder larvae require live prey. The development of an alternative food supply would dramatically reduce the present costs and therefore make commercial production more economically feasible. Formulation of an alternative food supply, however, can only begin with a thorough understanding their digestive system's structure and ontogeny. It was the intent of this study to examine the development of the digestive system in yellowtail flounder reared on a traditional live diet

and to provide information on the spatial and temporal distribution of digestive enzymes in both yellowtail and winter flounder under varied dietary regimes.

4.2 Morphological and functional development

The morphological development of the digestive tract in yellowtail flounder and the functional ontogeny of digestive enzymes is similar to other species observed thus far. Morphologically, the initially undifferentiated digestive tube undergoes cyto- and histological differentiation to become regionally segmented. Sarasquete and colleagues (1993) noted that the digestive tract of the developing sea bream (*S. aurata* L.) consisted at hatching of a straight tube with no folds or muscular layers and no anterior or posterior openings. The same observations have recently been made for several Pleuronectiformes, including Japanese flounder (Kurokawa & Suzuki, 1996), Atlantic halibut (Pittman *et al.*, 1990), Senegal sole (Sarasquete *et al.*, 1996), turbot (Segner *et al.*, 1994) and summer flounder (Bisbal & Bengtson, 1995). The intestinal tract also shows similar developmental trends over several weeks subsequent to hatching including an increase in gut length and folding, mucosal differentiation and/or an increase in stratification and the subsequent differentiation and appearance of the liver, gallbladder and pancreas.

The formation of a functional stomach is probably the single most important ontogenetic event in the development of the digestive system in larval marine species. In first-feeding turbot larvae, a small stomach anlage could be recognized by the end of Stage 1d (4 days after hatching) as a short segment located between the esophagus and intestine (Segner *et al.*, 1994). In the sole, it is described at hatch as a little pocket

(Sarasquete *et al.*, 1996). We have described the stomach anlage in yellowtail flounder as a post-esophageal swelling which forms between 3 and 7 days after hatching (Baglole *et al.*, 1997). A fully functioning stomach in most marine fish coincides with the formation of gastric glands, a developmental event that occurs around the time of metamorphosis (turbot: Segner *et al.*, 1994; summer flounder: Bisbal & Bengtson, 1995; Japanese flounder: Kurokawa & Suzuki, 1996).

Histologically, the epithelium of the stomach anlage in Japanese flounder is simple cuboidal to columnar. During metamorphosis, this epithelium begins to form folds and crypts. The cells at the bottom of the crypts formed simple alveolar or acinar structures that will proliferate and mature into functional gastric glands. According to Miwa and colleagues (1992), the regulatory mechanism for gastric gland formation in fish is poorly understood. In their studies of the control of metamorphosis in Japanese flounder by the pituitary-thyroid axis, it was hypothesized that the development of gastric glands of flounder stomach may be controlled by thyroid hormones. The administration of thyroxine (T_4) to late premetamorphic flounder larvae stimulated differentiation of gastric glands and the appearance of pepsinogen-like immunoreactivity. This suggests that thyroid hormones induce morphological and biochemical changes in epithelial cells of the alimentary tract and stimulate their differentiation and proliferation into pepsinogen-producing cells (Miwa *et al.*, 1992). Based on the relatively close taxonomic relationship of Japanese flounder to the Pleuronectidae, and the fact that gastric glands are not formed until metamorphosis in yellowtail flounder, similar regulatory mechanisms may play a part in stomach formation for this species.

The secretions of the stomach, pepsin and hydrochloric acid, are needed for digestion of proteins, a process that is completed in the intestine by pancreatic and enterocytic enzymes. Larval fish that are stomachless, however, do not have this digestive capability. The absence of a fully operational stomach has led numerous researchers to investigate how digestive processes are carried out in the larval digestive system, especially in terms of protein digestion. Several studies have noted the presence of pinocytic structures in the intestinal and rectal epithelium of larval gilthead sea bream (Sarasquete *et al.*, 1995), Senegal sole (Sarasquete *et al.*, 1996), turbot (Segner *et al.*, 1994) and summer flounder (Bisbal & Bengtson, 1995). Analogous supranuclear inclusions were observed within the rectal epithelium of yellowtail flounder, coinciding with formation of the rectum at 10 days post hatch, that indicate intracellular protein digestion (Watanabe, 1984).

Absorption of macromolecules in the hindgut epithelium occurs through 5 successive stages: (1) pinocytosis, (2) transport, (3) accumulation, (4) digestion and (5) extinction (Govoni *et al.*, 1986). Pinocytosis occurs along the microvilli plasma membrane. Once in the cells, pinocytic vesicles move towards the nucleus, accumulating in supranuclear inclusion bodies by the coalescence of the vesicles. Lysosomes become associated with these inclusion bodies and by the result of lysosomal action become extinct. The associated activity of acid phosphatase with the supranuclear vesicles in the study on sea bream larvae (Sarasquete *et al.*, 1993) suggests that the enzyme may participate in enzymatic degradation of proteins in this region of the digestive tract. In fact, localization of this enzyme is primarily within lysosomes (Lojda *et al.*, 1979). Little

is known about the actual biochemical function *in vivo* (Borgers *et al.*, 1991). It is likely, based on the degradative function of enzymes within lysosomes (Darnell *et al.*, 1990), that this enzyme may participate in the acid degradation of proteins within the rectal epithelium.

Generally, supranuclear inclusions containing proteins were observed in the posterior intestinal epithelium (i.e. rectum) while the anterior intestinal epithelium contained absorbed lipid droplets. These structures usually appeared concomitantly with exogenous feeding. Additionally, an increase in the number of lipid inclusions in older larvae suggests ontogenetic development of lipid digestion, as found in a study of larval cod development (Kjorsvik *et al.*, 1991). Such lipid structures have been observed in adult yellowtail and winter flounder (Murray, 1993; Murray *et al.*, 1996) and supranuclear inclusions were also noted in the rectal epithelium of yellowtail flounder larvae (Chapter 2 of this study and Baglole *et al.*, 1997).

The presence of chylomicra in the intestine of adult yellowtail and winter flounder was described in an ultrastructural study by Murray (1993). These structures contain products of lipid digestion (Murray, 1993) and therefore indicate intracellular processing of lipids. Analogous structures were not observed in the intestinal epithelium in larval yellowtail flounder. Chylomicra lipid inclusions cannot be resolved in light microscopic studies and could account for the discrepancy between larval and adult yellowtail flounder.

The importance of digestive enzymes during morphological development was determined by the spatial and temporal distribution of digestive enzymes observed in

larval yellowtail and winter flounder. Similar distributions were seen in other species: turbot (Segner *et al.*, 1994), Dover sole (Clark *et al.*, 1986) and sea bass (Zambonino Infante and Cahu, 1994). The results from this study suggest that at the time of exogenous feeding the larval digestive system is differentiated and functional enough to obtain nutrients from the live prey of rotifer and algae. The ability of yellowtail and winter flounder larvae to adapt their enzymatic capacity to different diets was not identified in this study. From comparable studies, there is indication that early weaning with a compound diet can induce a delay in the maturation of intestinal enzymatic digestion (Cahu & Zambonino Infante, 1995a), retard larval development and, the earlier the weaning, the lower the larval growth obtained (Cahu & Zambonino Infante, 1995b). Measurements of alkaline phosphatase in brush border membrane fractions of sea bass larvae weaned onto a microparticulate diet revealed depressed activity, indicating malnutrition (Cahu & Zambonino Infante, 1994). The reduced and variable activities of alkaline phosphatase in yellowtail and winter flounder beginning by 10 days post hatch could indicate a similar condition, regardless of diet.

The switch from endogenous to exogenous feeding (i.e. first-feeding) is an important, if not critical, stage in the life cycle of marine fish (Laurence, 1977; Fyhn, 1989; Hagen, 1993; Sarasquete *et al.*, 1995). It is known if feeding is initiated too late, the energy supply of the body will be used for maintenance, the digestive system may degenerate and the larvae may have irreversibly lost their ability to digest food (Lien & Holmefjord, 1992). The high mortalities occurring in aquaculture may not be due to the limited digestive capacities but the rather inability to initiate first feeding. Such indices

have also been described for the Japanese eel (*Anguilla japonica*) (Kurokawa *et al.*, 1995). The fluctuation in alkaline phosphatase activity shortly after first feeding in yellowtail and winter flounder and the death of the starved animals around day 13 may indicate poor nutritional status of the larvae.

4.3 Future directions of research

This research provides knowledge of a time frame in yellowtail and winter flounder for the formation of digestive organs, such as the stomach, and allows direct comparison to the ontogeny in other marine species. It also provides basic information for more applied studies on digestive function within several lines of research. First, studies into the nutrient requirements of the fish through different stages of its life history need to be pursued. Largely, the necessity of carbohydrates, proteins and fatty acids to normal larval growth and survival is not known. On the heels of this research, the formulation of an artificial diet which can incorporate these nutrients in the optimum proportions, and the optimum time of introduction can build upon the present state of knowledge provided by this project. It would also be interesting to examine parameters such as RNA/DNA ratios, midgut cell height or nuclear diameter of hepatocytes (Segner *et al.*, 1989) that would assess the overall condition of the larvae under varied dietary regimes.

4.4 Conclusions

1. The digestive system in larval yellowtail flounder develops from an undifferentiated digestive tube that undergoes regional partitioning and epithelial differentiation during subsequent ontogenetic stages to form the buccal cavity, pharynx, esophagus, PES/stomach, intestine and rectum.

2. The last major morphological changes to occur during ontogeny, indicating the onset of metamorphosis and the final adult form of the animal, are the formation of the pyloric caeca and gastric glands. A functional stomach (containing gastric glands) is likely needed to successfully wean yellowtail flounder from live food to an artificial diet.

3. The mucous histochemistry of the digestive tract of yellowtail flounder larvae indicated the presence of acid, neutral mucins and combinations of acid and neutral mucins that may have specific roles related to their spatial distribution within the gut. A similar distribution was described in the adult.

4. The early presence of digestive enzymes in the undifferentiated digestive tract of yellowtail and winter flounder indicates that the larvae have digestive capability before the onset of exogenous feeding. The presence of enzymes in the live food was demonstrated in this study. The actual contribution of these exogenous enzymes to the digestive capacity of the larvae, however, was not clear.

5. The ontogenetic increase in digestive enzyme activity as well as changes in spatial distribution occur around the time of first feeding. This indicates a correlation between morphological and functional differentiation. The absence of acid phosphatase from the digestive tract of both species and the presence of alkaline phosphatase in the PES of

winter flounder larvae is unique among larval fish species and is suggestive of unusual functions for these regions in these species.

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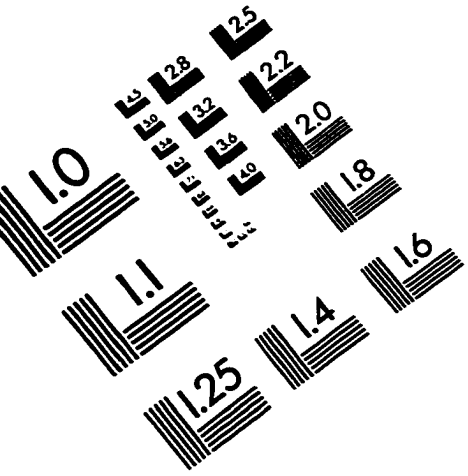
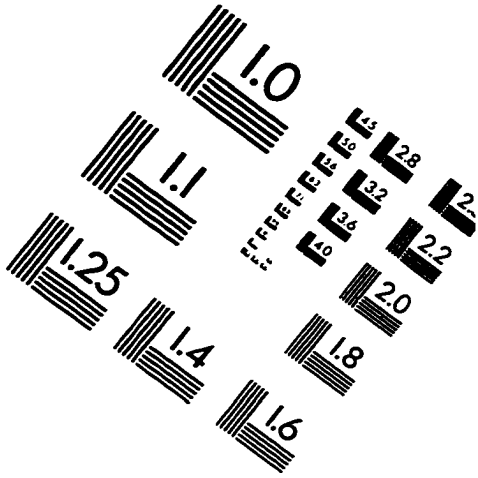
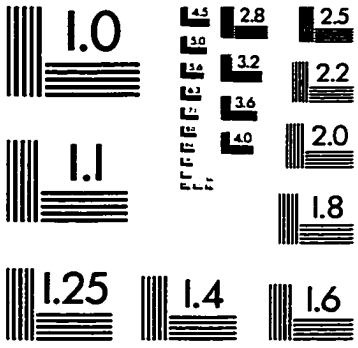
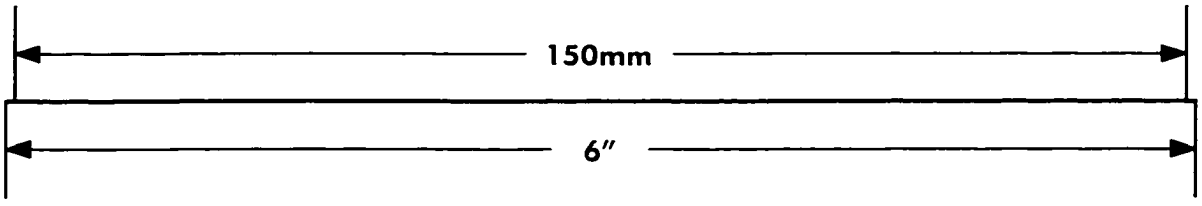
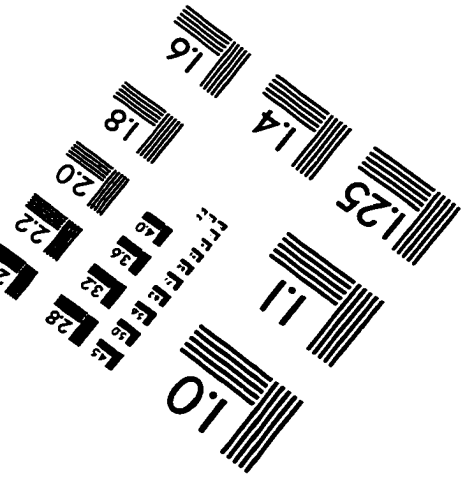
IMAGE EVALUATION TEST TARGET (QA-3)

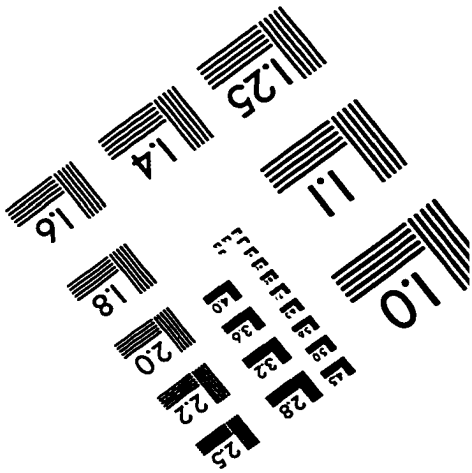












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