DIGESTIVE ENZYME EXPRESSION IN THE EXOCRINE PANCREAS DURING THE ONTOGENY OF THE WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*).

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EXTENDED ABSTRACT ONLY-DO NOT CITE

Introduction

One of the major challenges to successful rearing of marine fish is providing high quality nutrition at reasonable expense, especially during early larval life. Live feeds such as rotifers and *Artemia* give the best larval growth and survival, however these feeds are difficult and expensive to produce at commercial levels. Considerable effort has been put into the development of relatively cheap, high quality formulated diets. Unfortunately, these diets generally do not support the same level of larval growth and survival as live feeds. This may be due to improper diet formulation stemming from a lack of information on the dietary requirements for many species. To determine specific dietary requirements in part requires an understanding of digestive physiology. We are using histological and molecular techniques to describe the functional development of

the exocrine pancreas in the winter flounder with specific reference to trypsinogen, amylase and bile salt activated lipase (BAL).

Methods

Fish Rearing

Winter flounder larvae were reared at the Institut des Sciences de la Mer de Rimouski (ISMER), Quebec, Canada.

In situ Hybridisation

Sectioning

Winter flounder larvae at hatch, 5, 10, 20, and 35 days post-hatch (dph) and newly metamorphosed were fixed in 10 % formalin for 6-8 hours, dehydrated through ethanol series, cleared in xylene, infiltrated in paraffin and embedded for sagittal section. Seven micron sections were cut from one to two blocks and placed on silane coated microscope slides, dried briefly and then baked overnight at 60 ^{o}C. Fish from duplicate blocks were serial sectioned and stained in haematoxylin and eosin for general histology.

Probe Preparation

Probes specific for winter flounder trypsinogen, amylase and BAL were prepared by *in vitro* transcription of linearized cDNA clones using the DIG RNA labelling kit (Roche Applied Science, Laval, PQ, Canada) and T3 and T7 RNA polymerase. Following synthesis, probes were hydrolysed to 250 bp, precipitated and then resuspended in *in situ* hybridisation buffer (Murray et al., 2002). The specificity of the antisense (AS, cRNA sequence) probes was verified using the sense (S, mRNA sequence, control) probes.

Hybridization and marker detection

Hybridization conditions were modified from Murray et al., (2002). Briefly, deparaffinised, rehydrated and equilibrated tissue sections were hybridised with 50 µl of probe overnight at 45º C in a closed humid incubation chamber. Digoxigenin was detected using sheep anti-DIG-alkaline phosphatase conjugated antibodies following a 30 minute block in 0.1 M Tris pH 7.5, 150

mM NaCl containing 1% BSA and 10% lamb serum. Antibody was diluted to 1:250 with the above buffer and 500 µl was added to each slide. Slides were incubated for 30 minutes at room temperature in a humid chamber. Slides were then washed for a further 30 minutes in fresh buffer without lamb serum.

Antibody was detected by incubating slides for 10-15 minutes in a chromogenic buffer containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The reaction was stopped in 1x fish saline, and slides were rapidly dehydrated through ethanol series, cleared in xylene and mounted in cytoseal for observation using differential interference contrast microscopy. Images were captured using an Optronics VI-470 camera and Simple *PCI* software and grouped into plates using Adobe Photoshop 6.0.1.

Results

The pancreas was first identified in winter flounder larvae shortly following hatch and appears as a small compact structure situated just dorsal and slightly posterior to the liver (Figure 1A). As the fish develop toward metamorphosis the pancreas becomes diffuse, eventually spreading throughout the mesentery surrounding the stomach, the upper intestine and later the pyloric caecae (Figure 1B).

Hybridization conditions and reaction times were identical for all probes. Winter flounder trypsinogen, amylase and BAL all showed expression from about 5 dph (mouth opening) (Figure 2 ACE). Sense controls consistently showed no staining reaction (not shown). Trypsinogen gave the strongest reaction of all probes and remained as such through metamorphosis (Figure 2 AB). Amylase produced a strong reaction at mouth opening but gradually decreased in fish of later stages (Figure 2 CD). BAL gave a light reaction at mouth opening but gradually became more intense towards metamorphosis (Figure 2 EF).

Conclusions

In winter flounder the development of the pancreas through ontogeny is similar to that described for the Japanese flounder by Kurokawa and Suzuki (1996). *In situ* hybridization results for trypsinogen, amylase and BAL

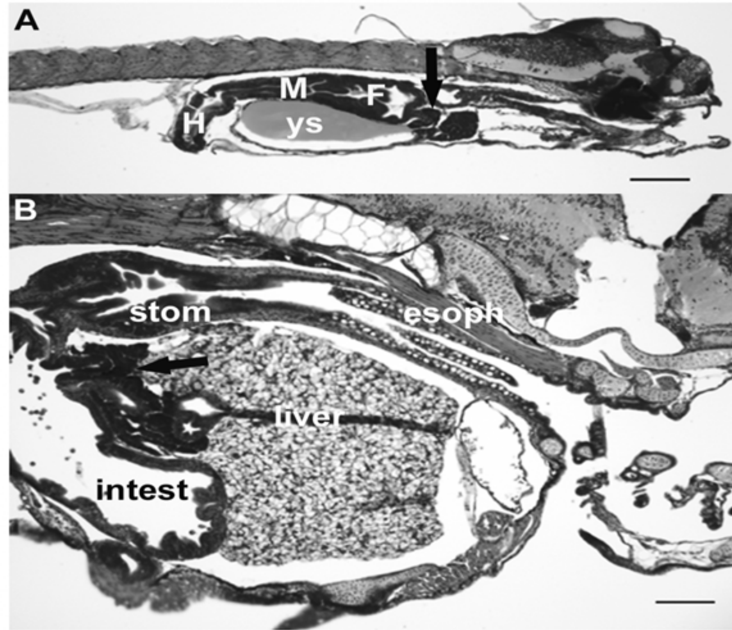

Figure 1. Sagittal sections through winter flounder larvae at mouth opening (A) and at metamorphosis (B) showing distrubution of the pancreas (arrows). stom, stomach; esoph, esophagus; intest,intestine; H, hindgut; M, midgut; F, foregut. Ys, yolksac. Scale bar = 100 µm.

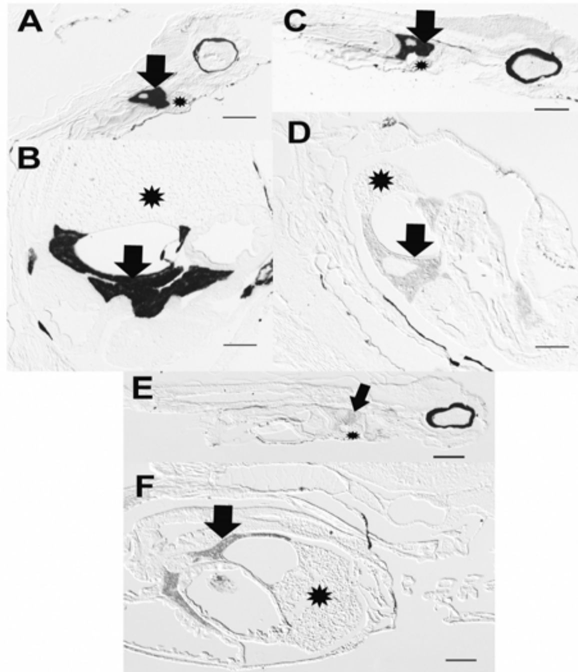

Figure 2. Sagittal sections through winter flounder larvae at mouth opening (A,C,E) and at metamorphosis (B,D,F) following *in situ* hybridization with cRNA probes for either trypsinogen, (A,B) amylase (C, D), or bile salt activated lipase (E,F). Note specificity of the hybridization reaction for the pancreas (arrows) and the differences in the distribution of the pancreas between a 5-dph fish and one at metamorphosis. Asterisk shows the position of the liver relative to the pancreas. Scale bar = 100 µm.

show that the exocrine pancreas actively expressed these genes at the time of mouth opening. The expression of trypsinogen at such an early age suggests that winter flounder are prepared to digest proteins long before the stomach is functional. The intense chromogenic reaction associated with the trypsinogen probe relative to the others, suggests that this gene is important in digestion at the start of exogenous feeding. The strong reaction for amylase transcripts at mouth opening suggests that this enzyme is important in early development. BAL is expressed at low levels at mouth opening with an increase in reaction intensity through ontogeny. Expression of these genes at mouth opening indicates that at the start of exogenous feeding, this species has potential for digesting a wide range of nutritionally important items including proteins, lipids and carbohydrates. We are presently verifying this expression data with biochemical analysis.

References

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