Histological, Histochemical and Morphological Development of Striped Trumpeter Larvae and the Effect on Larval Diet.

Ann Goodsell, David Wikeley and Lance Searle (Project Supervisor).

Final Report to the Fisheries Research and Development Corporation.

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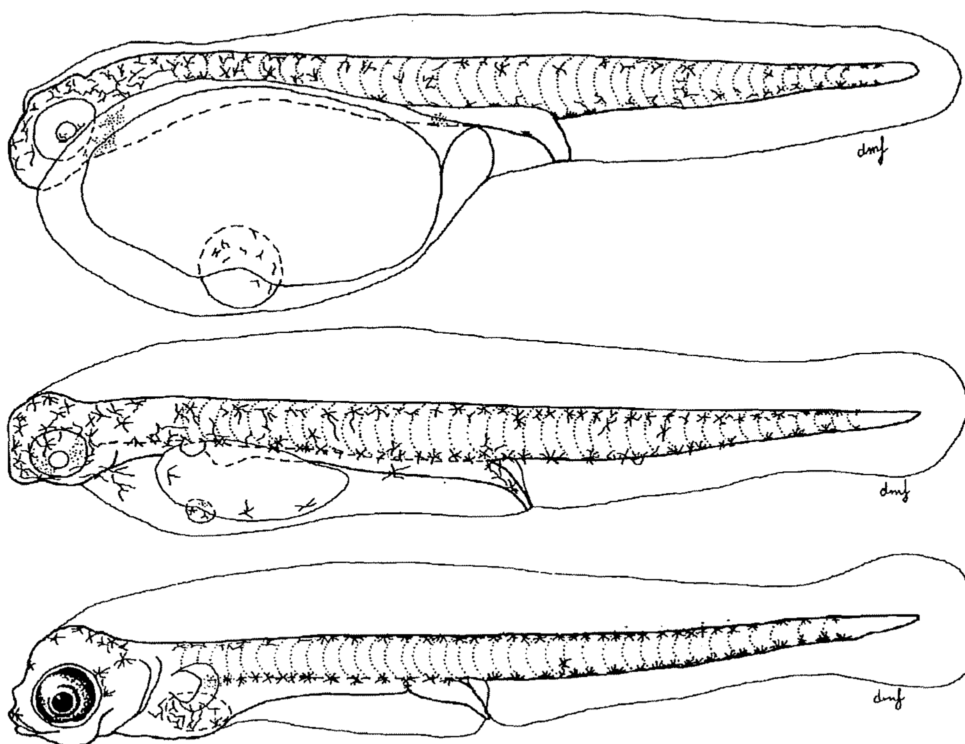

Marine Resources Division

Marine Research Laboratories, Taroona

Department of Primary Industry and Fisheries, Tasmania

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Histological, Histochemical and Morphological Development of Striped Trumpeter (*Latris lineata*) Larvae and the Effect of Larval Diet.

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Executive Summary

In November 1992, this project commenced with the development of methods for the evaluation of striped trumpeter (*Latris lineata*) larvae cultured by the Finfish Development Program (FDP). The first year was spent in the selection of digestive enzymes for investigation and the modification of methods for their detection. In addition to the digestive enzymes selected, lipids and glycogen deposits were also studied to provide an indication of the status of larval energy uptake and storage. The project used the observation and measurement of samples of larvae to provide an assessment of growth, development and the general health of larvae. The methods developed by the project have been detailed in a Technical Report published by the DPI&F.

Preserved samples of larvae were processed, thin-sectioned and stained to provide information on the development of the swimbladder and the gut with its associated organs, as well as the presence of digestive enzymes at different stages of growth. The timing and method of swimbladder inflation, as well as the development of the digestive tract prior to metamorphosis, was investigated, providing valuable basic biological information on the species. This information was used to modify larviculture systems and techniques and resulted in the successful culture of juvenile striped trumpeters, banded morwong and greenback flounder in the 1994 rearing season.

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Abstract

An understanding of the basic biology of a species is essential for its successful culture. The striped trumpeter (*Latris lineata*) is a native Tasmanian marine teleost, which has been designated a primary candidate for aquaculture, however, little is known of the species. This project used morphological assessment, as well as histological and histochemical techniques, to investigate the basic biology of larval striped trumpeter produced by the Finfish Development Program.

Histological analysis determined that primary inflation of the swimbladder occurs prior to first feeding and that inflation permanently alters the appearance of the swimbladder itself, enabling the accurate determination of the status and stage of inflation. The development of the gut, onset of enzyme activity and the status of energy uptake and storage were assessed using histology and enzyme histochemistry. The stomach was not present in larvae from either the 1992 or 1993 seasons, while the enzyme reaction strengths were variable in larvae from the 1993 season. Histological and Histochemical methods were developed or modified for use with resin processed tissues. The techniques developed by the project are applicable to the study of other species with aquaculture potential.

1 Introduction

Developmental work by the then Division of Sea Fisheries (DSF) in the early 1980's led to the establishment of a significant aquaculture industry based on seacage culture of salmonids. Both the Tasmanian Government and industry considered that diversification of the species cultured was necessary to ensure the continuing viability of the industry. To support this, the DSF commenced a major research program (Finfish Development Program - FDP) in 1989 to assess the feasibility of culturing several native finfish species.

The Program concentrated on striped trumpeter (*Latris lineata*), while working on other species out of season. Early rearing trials demonstrated a lack of swimbladder inflation, failure of larvae to thrive and total mortalities. In 1989, larvae survived to Day 45 post hatch, while in 1990, fish were cultured to Day 60 post hatch, but survival was poor and the incidence of swimbladder inflation was low. During the

1991 season, IFREMER researchers worked in collaboration with FDP staff; a nutritional deficiency was identified and new techniques were used which produced a high rate of swimbladder inflation. Although the lifecycle had not been closed, the FDP team were confident that rapid progression in the development of the species could be achieved over subsequent seasons.

Results from previous larval rearing seasons indicated that it was essential to have an understanding of the basic biology of striped trumpeter larvae in order to achieve successful larval rearing. Some of this information could be gained from growth trials and some from histological and histochemical studies of larvae. In addition, histological studies could be used to determine the mode and timing of swimbladder inflation and possible causes of mortalities (e.g. bacterial overload of the gut, energy budget problems - low glycogen stores due to lack of functional swimbladder or dietary inadequacy). In response to this need, a proposal to study the growth of striped trumpeter larvae under controlled conditions using morphological, histological and histochemical methods was submitted to the Fisheries Research & Development Corporation in 1991 (Original Application, see Appendix 1)

The project commenced in November 1992, with the first year spent selecting enzymes for study and developing, modifying and assessing methods for their detection. These techniques proved successful in monitoring the growth and development of the larval striped trumpeter, providing vital basic biological information on the species. However, during late 1993, the DPI&F underwent an evaluation of its research activities and species priorities. The Department considered that the striped trumpeter may have been technically too difficult to culture and that continued allocation of resources to this species may not have been warranted. FRDC was advised that the project was under review and may not continue as originally proposed.

A workshop on "Aquaculture Research Needs in Tasmania" with delegates representing industry and other research institutions was held in early 1994. It was determined that the development of striped trumpeter for aquaculture should remain the focus of activity for the FDP. In addition, it was recommended that greenback flounder *(Rhombosolea tapirina)* and banded morwong (*Cheilodactylus spectabilis*) should be regarded as prime candidates for aquaculture development. FRDC was advised of the workshop recommendations and the DPI&F sought to continue the original evaluation project until 31st December, 1994, enabling a thorough analysis of the previous striped trumpeter seasons results.

Application of information from the 1993 season to the 1994 larval rearing season resulted in the production of a small number of banded morwong juveniles, over 100,000 juvenile green back flounder in pilot-scale commercial trials and a significant number of striped trumpeter juveniles.

2 Background

The digestive organs of many fish larvae are similar to those of stomachless fish. Most possess an undifferentiated digestive tract at hatching and until metamorphosis, rely on exogenous food to supply some of the enzymes required to digest and absorb food (Buddington, 1985; Watanabe and Sawada, 1985; Dabrowski and Culver, 1991; Hjelmeland *et al.*, 1993). At metamorphosis, larval teleosts undergo a transition from larval morphology and digestive processes to those of the juvenile and assume new feeding habits and diet (Buddington, 1985).

The pattern of digestive enzyme development and activity is closely related to the different stages of growth and the onset of activity may be programmed genetically, independent of fish mass. The alkaline proteolytic enzymes trypsin and chymotrypsin are usually found in the intestine during the early larval stages when fish lack morphological and functional stomachs. The activity of these alkaline proteases decreases as the stomach epithelium differentiates and the pH drops, while pepsin activity increases (Walford and Lam, 1993). Munilla-Moran *et al.* (1990) estimated that between 89-94% of the total esterase activity and 43-60% of protease activity in the gut of cultured turbot larvae at Day 3 was derived from the live diets. The size and quality of the live food may be critical for larval growth and survival, as the prey size may be inadequate to stretch the oesophagus or intestine and stimulate the release of digestive enzymes (Hjelmeland *et al.*, 1988) or as suggested by Walford and Lam (1993), that mechanical damage by the pharyngeal teeth is necessary to allow the rapid autolysis of rotifers in the intestine of larval sea bass.

After a thorough literature review (See Appendix 2), the enzymes selected for study were:

<u>Acid Phosphatase (ACP)</u>

ACP's are hydrolytic enzymes involved in the breakdown of metabolites. They act at acid pH's (optimal around pH 5, varying with tissue of origin) (Kachmar and Moss, 1976). In fish larvae, this enzyme is active in yolk sac membrane - probably due to involvement in the breakdown and transfer of yolk to the circulation system (Cousin *et al.*, 1987).

<u>Alkaline Phosphatase (ALP)</u>

ALP's are hydrolytic enzymes involved in the breakdown of metabolites, however, these are active at alkaline pH's (optimal pH around 10) (Kachmar and Moss, 1976). In larval fish, they are active in many tissues (kidney, brush border of enterocytes, liver, gills & heart) and are implicated in membrane transport. They are used to indicate the degree of differentiation of enterocytes and to monitor the development in intestinal villi (renewal of cells occurs at the base of folds where activity is weakest) (Cousin *et al.*, 1987; Stroband *et al.*, 1979).

Aminopeptidase - M (AMP-M)

AMP-M is microsomal bound and is located in the brush border of enterocytes. It is an exopeptidase hydrolysing the terminal peptide bonds of large peptide chains to form amino acids and /or smaller peptides (Lojda *et al.*, 1979).

Lipase

Lipases catalyse the hydrolysis of long chain fatty acids and glycerol. They can only act on emulsions of water insoluble substrates and the breakdown rates vary e.g. the rate of hydrolysis of triglycerides is faster than that of mono glycerides (Kachmar and Moss, 1976). They may not be detected in the early larval period and levels vary between species according to diet and degree of activity (Cousin *et al.*, 1987; Mankura *et al.*, 1984).

Non-specific esterase (NSE)

NSE's hydrolyse esters of alcohols, naphthols and lipoproteins and are located in the mucosa of the intestine, especially in the brush border (Lojda *et al.*, 1979).

Maltase

Maltase is a disaccharidase localised in the brush border of the enterocytes which acts in the intestine, splitting maltose to glucose (Lojda *et al.*, 1979).

Trypsin

Trypsin is an endopeptidase specific for peptide bonds involving the carboxyl groups of arginine and lysine. It is pancreatic in origin and is secreted as the inactive trypsinogen. It is activated in the gut by enterokinase or trypsin already present in the gut, and attacks denatured proteins more rapidly than native proteins (Kachmar and Moss, 1976).

These enzymes are involved in breakdown of a variety of organic substances and as a group, provide an estimate of the function of the larval digestive tract and its ability to digest various compounds.

Other substances selected for study were:

Lipids

Lipids of various types act as a supply of energy for larval fish, as well as functioning in cell membranes. They are absorbed in the midgut region in early

larvae and digested intracellularly. Some storage of lipids occurs in the enterocytes of larvae (Segner *et al.*, 1989; Deplano *et al.*, 1991).

Glycogen

Glycogen acts as a store of energy and the major site of storage is in the form of granules found in the hepatocytes of the liver. It is also found in other tissues (Caraway, 1976).

The study of these two substances provides an indication of the uptake of energy and the status of larval energy stores.

3 Objectives

The project aimed to :

- characterise histological, histochemical and morphological aspects of larval and juvenile striped trumpeter growth and development under controlled culture and dietary conditions.
- determine the "boosted" nutritional profile of rotifers and *Artemia* used as live feeds.
- identify any nutritional problems encountered during larval rearing and evaluate solutions.

4 Methodology

The first twelve months of the project were spent assessing which digestive enzymes were to be studied and developing the staining methods for their detection. The available methods of enzyme detection for use on fresh, frozen and paraffin processed sections had to be adapted for use with resin processed sections. Paraffin sections are cut at 4-5μm, while frozen sections are cut at 7-10μm. The thickness of the section determines the resolution of the cellular and intracellular details in the tissue. Frozen sections, while demonstrating good preservation of enzyme activity and lipid droplets, do not provide sections with good cellular detail, as the section is one to two cells thick and resolution is lost by the overlapping of cells and the subsequent obscuring of detail. Paraffin sections are approximately one cell thick and give better resolution of detail than frozen sections, however, lipids are extracted by solvents used in processing and enzyme activity is lost due to fixation and the heat required for paraffin infiltration. Resin sections can be cut 0.5-3.0 μm thick, providing sections less than one cell thick and hence very good resolution of cellular and intra-cellular detail. For this reason, resin processing of tissues was selected. In addition, only one set of processing and sectioning equipment was

required for the preparation of sections for both histological and histochemical methods. However, the thinner sections resulted in decreased intensity and quantity of stain reaction due to fewer available enzyme binding sites, which required the adaptation of methods by varying the concentrations of stains, substrates and buffers to produce consistent quality of results.

Special precautions were required during the preparation and handling of stains, as most chemicals were either toxic or carcinogenic and caused skin irritation. Some of the methods required modification to optimise staining in resin processed tissues.

4.1 Sample collection

Twenty larvae were sampled daily from the total water column of each tank by siphoning with a clear 10 mm diameter plastic tubing. Sampling time was the same each day, to ensure that larvae had adequate time to feed to satiation if they desired. Ten of the larvae were used for morphological measurements (Total Length (T.L.), Standard Length (S.L.), Depth at Operculum (D.O.), Depth at Vent (D.V.), Oil Droplet Diameter (O.D. diam.), Yolk Sac Length (L) and Height (H)) and development assessment (stage of swimbladder inflation, folding of gut lining, presence/absence and type of food animals in the gut), while the remaining 10 were fixed for histological and histochemical examination. The volume of the yolk sac and oil droplet were calculated using the following formulae taken from Avila and Juario (1987). (Volume results are in mm^3)

$V_y = \pi/6 \, LH^2$ (Volume of a spheroid, where "L" is the length and "H" is the height of the yolk sac)

$V_{O.D.} = 4/3 \, \pi \, r^3$ (Volume of a sphere, where "r" is the radius)

Graphs of measurements were prepared using Statview® 4.0 (Abacus Concepts). A limitation of the programme was the inability to set the scale on the Y-axis, resulting in different scales on graphs of the same parameter for different tanks and cohorts. The 10 larvae used for histological examination were separated with minimal damage by filtering the sample using a 47 mm Millipore - filter funnel fitted with a 47 mm Whatman - No. 1 filter paper/adsorbent pad set. When all 10 larvae were collected, the filter paper was transferred to a 50 mm petri dish half filled with cold fixative. This was placed in the refrigerator until the sample was rigid (30-60 minutes). The samples were then transferred to a labelled 1.2 ml or 2.0 ml cryovial filled with cold fixative and returned to the refrigerator (stored flat to keep the larvae straight until adequately fixed). The samples were then processed as per Wikeley

and Goodsell (1994). All 10 larvae from each tank were embedded in the one mould.

4.2.1 Sample processing - Light Microscopy

Three processing methods were evaluated: frozen, wax and resin. While the activity of some enzymes was reduced by fixation, longer incubation times were used to compensate and the cellular detail provided by the resin sections was superior to that of both frozen sections and paraffin sections, confirming results by Higuchi *et al.* (1979). During the adaptation of methods, no enzyme activity could be detected in striped trumpeter tissue. Frozen sections of fresh fixed flounder and banded morwong tissue were used to check the enzyme method.

It was found that the time between sample collection and the cutting of sections should be kept to a minimum (one week maximum). Not only was enzyme activity found to decrease with time spent in fixative and infiltration solutions, but the localisation and stain reaction strength of enzymes and other substances (lipid and glycogen) was also reduced. Enzymes and glycogen are water miscible and diffuse away from the site of activity or storage under the concentration gradient in fixative and to some extent in infiltration solutions. While the resin infiltration solutions are water miscible, they also act as solvents and extract lipids from the tissues (Cope and Williams, 1969; Gerrits *et al.*, 1987).

Technical Report No. 49 (Wikeley and Goodsell, 1994) contains a full description of tissue processing, histological and histochemical methods and precedes the Results section.

4.2.2 Sample processing - Electron Microscopy

Three larvae from the two tanks containing fish with abnormal epithelium were processed by the schedule in Table 1. Blocks were cut with a glass knife. Thick sections (1μm) were stained with Richardson's Stain to enable further trimming of the block and to provide orientation during the scanning of thin sections in TEM. Thin sections were mounted on grids and stained using Uranyl Acetate and Lead Citrate before viewing with a JEOL 100C TEM.

Table 1. Processing Schedule for Electron Microscopy Samples

Procedure	Solution	Time	Temp.
Fix	Buffered formalin/seawater	18 months	10°C
Wash	0.1M Phosphate buffer (X3)	10 min. each wash	4°C
	0.05M Phosphate buffer	10 min.	4°C
Post-fix	1% Osmium tetroxide in Phosphate buffer	60 min.	4°C
Wash	0.05M Phosphate buffer (X3)	10 min. each wash	4°C to Room Temperature (RT)
en bloc staining	Saturated uranyl acetate in acetone/water (50:50)	15 min. (one larvae only from each tank)	RT
Dehydrate	Acetone/water (50:50)	15 min. (remaining two larvae)	RT
	Acetone/water (95:5)	15 min.	RT
	Acetone (100%)	15 min.	RT
	Dry Acetone	15 min.	RT
Infiltrate	Spurrs resin/dry acetone (50:50)	60 min.	RT
	Spurrs resin (100%)	60 min.	RT
Place and orient larvae in "bottle neck" Beem capsule			
Embed	fresh Spurrs resin (100%)	overnight	70°C

4.3 Live Feed

Analysis of live feeds (rotifer and *Artemia*) used in rearing trials were to be performed by external laboratories on a user pays basis. Samples prepared during 1993 were destroyed due to the isolation of disease at the facility and quarantine requirements. While replacement samples were prepared in 1994, the early completion date of the project resulted in the cancellation of this aspect of the project due to a lack of funds. The FDP has subsequently funded analysis of these samples and preliminary results have been received. (See Appendix 3).

4.4 Determination of nutritional problems and evaluation of solutions

Several authors (O'Connell, 1976; Watanabe, 1985; Dabrowski *et al.*, 1989; Oozeki *et al.*, 1989; Theilacker and Watanabe, 1989; Rösch and Segner, 1990) have used histological techniques to define the nutritional status of larval fish. Sagittal sections of four to six larvae from each tank of the 1993 season at age 12,16 and 20 days post hatch were scanned and measured using an Image Analysis System. The system was comprised of a Nikon Optiphot compound microscope mounted with a Panasonic CL-WV300 video camera, which was connected to a Macintosh Quadra. The programme NIH Image 1.53b23 was used to scan sections and measure the enterocyte height from the brush border to the basement membrane. The system was calibrated at the magnification used (X20 objective and X16 TV relay lens) with a stage micrometer.

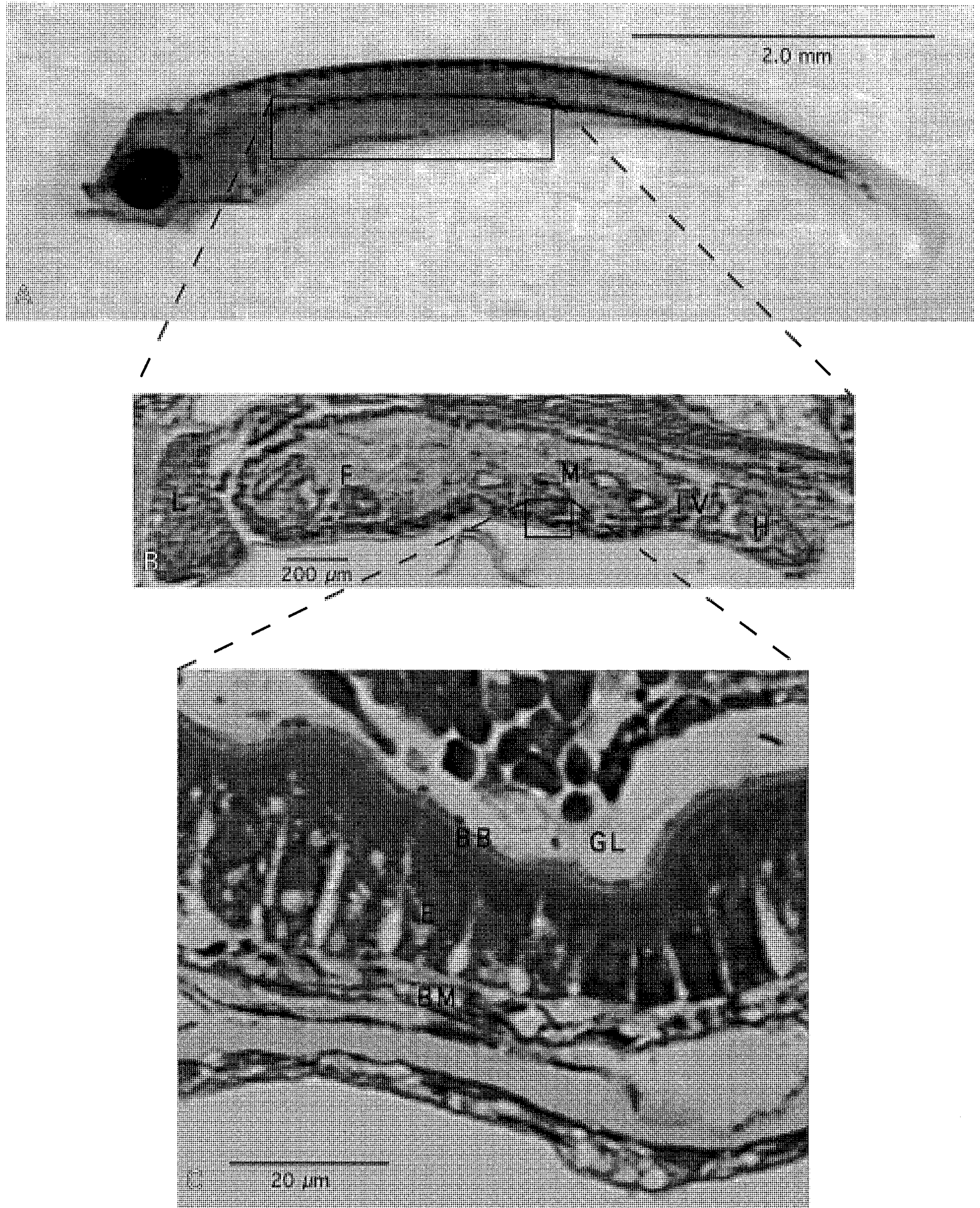

Figure 1: Demonstration of area scanned for enterocyte heights in larvae.

A - Striped trumpeter larva age 19 days post hatch.

B - Sagittal section of larval gut showing - L, liver; F, foregut; M, midgut; IV, intestinal valve; H, hindgut.

C - Ventral surface of gut lumen - GL, gut lumen; BB, brush border of enterocytes; E, enterocyte; BM, basement membrane.

Sections showing dorsal midgut with both dorsal and ventral epithelium were scanned and the enterocytes were measured on the ventral surface (see Figure 1). Measurements were made in micrometers (μm) and were entered directly in to an Excel 4.0 spreadsheet for later analysis to detect any significant differences between tanks and days within cohorts. While 14 age cohorts were produced during 1993, only cohorts 1, 2, 3, 5, 6, 7, 9 and 10 were tested, due to the poor survival of the remaining cohorts. Cohorts 6, 7, 9 and 10 were reared in two or more tanks, the remainder were reared in one tank only. Differences between cohorts were not tested due to limited replication and differences in tank volumes.

4.5 Larval Rearing

During the 1992 rearing season, the larval rearing system was comprised of 1 x 60 Litre, 2 x 400 Litre and 10 x 1000 Litre conical based tanks with white bases and black sides. All tanks shared the same recirculation system and received the same water flow (relative to tank volume) and quality. Each tank was enclosed with a black plastic curtain and the photoperiod and light intensity for each tank was individually controlled. Temperature commenced at 11.5°C and was increased by stages to 17.5°C on Day 6. Approximately 48,500 eggs from the same age cohort were stocked into each tank. Water flow to each tank was set at 25% of total tank volume per hour. Each tank had an outlet screen size of 250μm and photoperiod of total dark until first feed (Day 6). Skimming to clear surface debris commenced on Day 8 and by Day 11, significant mortalities (≥ 50%) occurred in all tanks. Numbers continued to decline and trials were terminated when less than 100 larvae survived in each tank. The 1000L tanks were labelled in pairs, 1A and 1B through to 5A and 5B. Their water inflow was via a central opening in the base of the tank and outflow was via a side exit, covered by a screen. The 2 x 400L tanks 6A and 6B, were hydrodynamically different, with a side inflow of water and a central standpipe with a screen for the outflow.

During the 1993 rearing season, the tank sizes were varied: 2 x 400L, 9 x 1000L and one 4000L tanks were used. As in 1992, all tanks received water from the same recirculation system and hence had similar water quality, however, incubation until first feeding (Day 8) was carried out at 12-14°C, while larval culture from Day 8 onwards was carried out at 15-16°C. Water flows in all tanks were equivalent based on % flows per hour (range 15-25% of tank volume per hour). The photoperiod and lighting for each tank was individually controlled. Onset of surface skimming varied for each tank, as did the addition of an anti-foaming agent (Sigma Chemicals, Antifoam A, silicon base). Twelve age cohorts were produced and trialled during the

1993 season. Tank 6 was 4000L, square in shape and dark grey on the sides and base, while the remainder of the tanks were conical, with white bases and black sides as in the 1992 season. Tanks 11 and 12 were 400L, with a side inflow and central standpipe for outflow, while the remaining nine tanks were 1000L, with central base inflow and side screen outflow.

4.6 Statistical Analysis of Results

Results of the Diet Trials run during 1993 were analysed by Bryce Graham and Ross Corkrey (Biometrics Department, DPI&F). Analysis was performed on an IBM compatible PC using Minitab® 10.0. A t-test was used to determine if differences in enterocyte heights were significant.

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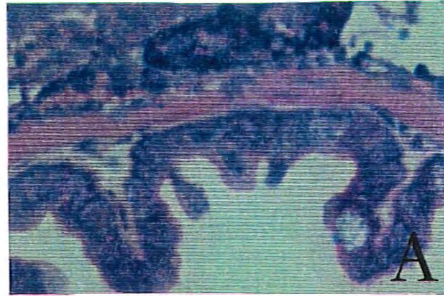
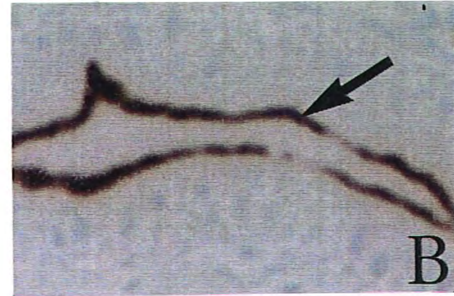
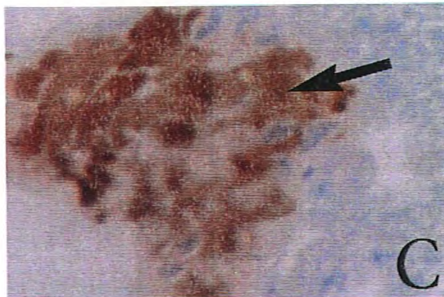
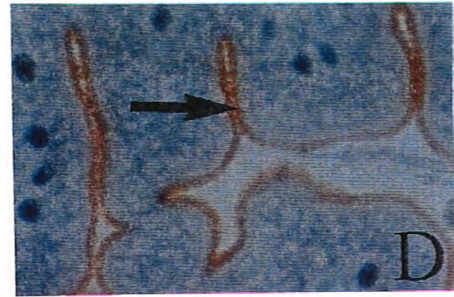
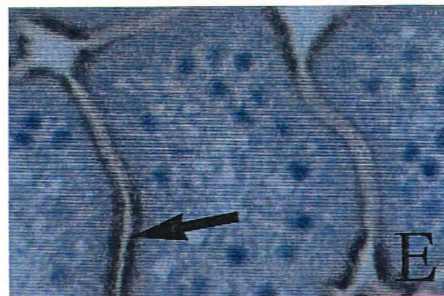
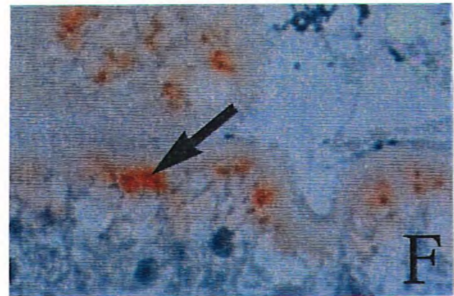


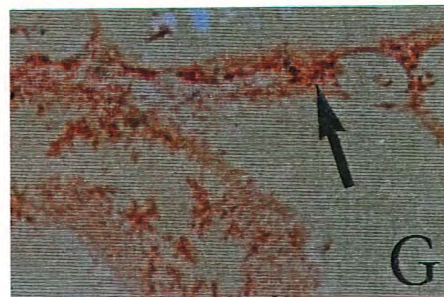












Plates: all plates taken with a Nikon Optiphot microscope 20X objective.

$\rightarrow$ = enzyme reaction site.

A: Lee's Methylene Blue / Basic Fuchsin.

B: Alkaline Phosphotase.

C: Acid Phosphotase.

D: Aminopeptidase-M.

E: Non-Specific Esterase.

F: Trypsin.

G: Maltase.

Manual of Histological and Histochemical Methods used for Larval Evaluation.

David M. Wikeley and Ann Goodsell

ABSTRACT

The development of a new species of marine fish for aquaculture requires the understanding of the life cycle, especially the development and function of the larval digestive system. The methods in this manual have been developed to enable the assessment of digestive tract development and enzyme activity in larval and juvenile fish and hence their ability to progress to the next stage of feed, whether live or artificial, as well as determining the causes of mortalities in larval rearing runs.

1 INTRODUCTION

The successful rearing of fish larvae requires an understanding of their morphological and functional development, as well as their nutritional needs (Kjørsvik *et al.*, 1991). The histology of the digestive tract of larval and some juvenile forms differs from that of the adult form. Altricial larvae, which hatch from small, positively buoyant eggs, lack a morphological and functional stomach (Baragi and Lovell, 1986; Govoni *et al.*, 1986; Mähr *et al.*, 1983; Verreth *et al.* 1992)). During endogenous feeding, larval fish possess a gut lined with simple columnar epithelium (Aronovich *et al.*, 1975; Blaxter, 1969; Govoni *et al.*, 1986; Smith, 1989). Prior to the onset of exogenous feeding, the development of the pharyngeal and rectal sphincters separates the gut into the bucco-pharyngeal section, fore-, midgut section and hindgut (Avila and Juario, 1987; Govoni *et al.*, 1986; Smith, 1989). At metamorphosis, which may occur days, weeks or months after hatch, the larva transforms in to the adult form and begins the juvenile phase of life. At this time in most species, a functional stomach and pyloric caecae develop from the foregut, allowing peptic digestion to commence and the animal should be able to digest artificial diets.

At each life stage, the enzymes present and the type of digestion, differ. In yolk-sac larvae, digestive enzymes are not found prior to the onset of exogenous feeding, while in exogenously feeding larvae, Aminopeptidase-m, Non-specific Esterase, Alkaline Phosphatase, Acid Phosphatase and Trypsin are some of the enzymes detected. At this stage, the digestive enzymes of ingested live foods are thought to assist the digestion of prey (Walford and Lam, 1993).

The methods in this manual were developed to allow the rapid monitoring of digestive tract structure and function and hence the best time to wean the larvae onto the next food item - live or artificial. They are also used to elucidate the basic biology of striped trumpeter and the reason for mortalities in larval rearing runs. Resin sections, as opposed to paraffin sections, were used, as these can be cut at 2 μm and give very good cellular detail and are also suitable for use with histochemical stains, eliminating the need for frozen sections and thus, two sets of sectioning equipment. While Historesin™ has been used for these methods, other resins which are also suitable for these methods exist and users should see which resin best suits their requirements.

PART A: PROCESSING

2 Tissue Fixation

Proper preparation of fish tissue is essential for accurate histological diagnosis.

2.1 Collection of specimens

- with minimum handling and stress
- only live animals should be sampled (or recently dead within last few minutes)
- complete history should be included
- frozen specimens are **not** suitable for histology

2.2 Fixation of specimens

- the main objective is to precipitate the protoplasm and thus prevent further cell and tissue changes
- specimens should be placed in fixative **as soon as possible**
- if the specimen is too large, small pieces should be cut (2.5 mm^3 - no thicker than 5 mm)
- a minimum of approximately 10 times their volume of fixative should be used for each specimen.

Fixative of choice depends upon the chosen method for viewing the tissue once it has been processed.

2.2.1 Fixative of choice for routine and histochemistry resin sections:

2% Paraformaldehyde in 0.1 M Phosphate buffer (store at 4°C) (Bancroft and Stevens, 1990):

Solution A

Dissolve 4 g paraformaldehyde (**methanol free**) in 100 ml of 0.2 µm filtered sea water by heating to 60°C with continuous stirring in a covered beaker to avoid evaporation.

Add 1 M sodium hydroxide dropwise with stirring until the solution clears. 1-5 drops should be sufficient.

Cool rapidly under running cold water (or in an ice bath).

*Solution **B***

Mix 100 ml of 0.2 M phosphate buffer; 19 ml 0.2 M sodium dihydrogen orthophosphate (M.W. 156) and 81 ml 0.2 M disodium hydrogen orthophosphate (M.W. 142). Check pH and adjust to pH 7.4 if necessary.

Method:

The fixative is prepared by mixing equal volumes of **A** and **B**, which gives 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The solution may need to be filtered before storage.

To make 1 litre of fixative:

20 g paraformaldehyde in 500 mls of 0.2 µm filtered seawater.

95 mls 0.2 M sodium dihydrogen orthophosphate (3.12 g in 100 mls of 0.2 µm filtered seawater).

405 mls 0.2 M disodium hydrogen orthophosphate (14.2 g in 500 mls of 0.2 µm filtered seawater).

2.2.2 Fixative of choice for electronmicroscopy:

0.1M Phosphate (or Cacodylate) Buffered Glutaraldehyde (Bancroft and Stevens, 1990) :

25% (aqueous) glutaraldehyde	25 ml
0.2 M phosphate buffer	50 ml
0.2 µm filtered sea water	40 ml

Final glutaraldehyde concentration 2.5% (for routine use). Store in a clean, glass stoppered bottle at 4°C. Diluted fixative is stable for about a week at 4°C. pH should be maintained and no precipitates should form.

2.2.3 Other fixatives that can be used:

1% osmium tetroxide (OsO_4) (Drury and Wallington 1980) :

1 ampoule osmium tetroxide crystals	0.5 or 1 g
0.2 µm filtered sea water	50 or 100 mls

Method:

Remove the label and all traces of gum from the tube with water. Dry the tube with a clean cloth, score with a glass file and break in the middle, dropping the two halves with contents into a dark-glass bottle containing the appropriate amount of 0.2 µm filtered seawater. Sonicate for at least 5 minutes. The bottle should have a glass stopper. Store at 4°C.

1% osmium tetroxide in 0.12 M phosphate buffer (Millonig, 1962)

Stock solutions:

A 2.26% sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$).

B 2.52% sodium hydroxide (NaOH).

Method:

Mix 41.5 ml of solution **A** with 8.5 ml of solution **B**. Check pH and adjust to 7.3-7.4 if necessary.

Add to 0.5 g of osmium tetroxide.

This fixative is reported to give a more uniform fixation than other osmium mixtures and is said to preserve more glycogen and protein. It is stored frozen and will keep for several weeks in this state. If stored in 2 ml aliquots, repeated thawing of the whole solution, which may decrease its effectiveness, is avoided.

3 Resin Embedding Method.

3.1 Note: While the methods in this publication have been developed using HistoResin™ and HistoResin Plus™, other similar resins eg: JB4, Ladd HEMA, may be used successfully.

3.2 Room Temperature Polymerising (Glycol Methacrylate) (HistoResin™)

3.2.1 Infiltration Solution

50 ml basic resin

1 packet of activator

Stir until dissolved (can be stored at 4°C for several months), to avoid bubble formation, a magnetic stirrer is recommended.

To ensure even infiltration, two changes of infiltration solution (100%) are used. Infiltration is complete when the specimen appears slightly translucent and sinks to the bottom of the infiltration vessel (1.2 ml cryovial for small samples, 2.0 ml cryovial for larger samples). The mimimum times used are:

1st solution - 3 hours at 4°C

2nd solution - 3 hours at 4°C

3.2.2 Embedding Medium

15 ml infiltration solution

1 ml hardener (store at room temp), see table below:

3.2.3 Embedding mould trays:

	Small Embedding Mould Trays		Large Embedding Mould Trays	
Number of wells	Resin volume (ml)	Hardener volume (ml)	Resin volume (ml)	Hardener volume (ml)
20	15.0	1.0		
19	14.25	0.95		
18	13.5	0.9		
17	12.75	0.85		
16	12.0	0.8		
15	11.25	0.75		
14	10.5	0.7		
13	9.75	0.65		
12	9.0	0.6		
11	8.25	0.55		
10	7.5	0.5	11.25	.75
9	6.75	0.45	10.13	.68
8	6.0	0.4	9.0	.6
7	5.25	0.35	7.88	.53
6	4.5	0.3	6.75	.45
5	3.75	0.25	5.63	.38
4	3.0	0.2	4.5	.3
3	2.25	0.15	3.38	.23
2	1.5	0.1	2.25	.15
1	0.75	0.05	1.13	.08

Mix and use immediately (use a syringe to dispense the resin into the moulds, this helps in reducing contact with oxygen). Note: polymerisation proceeds more rapidly in larger batches.

3.2.4 Blocking out

Place the Histomold™ in the air tight container, position the sample in the Histomold™, then completely fill the mould with resin / hardener mixture, as the lid is being placed on flood the inside of the container with nitrogen. Prepare no more than 10 moulds at a time as the resin starts to "set-off" within 20 minutes of the hardener being added. Allow to polymerise for 2 - 4 hours at room temperature (or overnight if time allows).

3.3 Cold Temperature '4°C' Polymerising (Glycol Methacrylate) (HistoResin Plus™)

3.3.1 Infiltration Solution

100 ml basic resin

1 packet of activator (0.6 g)

Stir until dissolved (can be stored at 4°C for 4 weeks), to avoid bubble formation, a magnetic stirrer is recommended.

To ensure even infiltration, two changes of infiltration solution (100%) are used. Infiltration is complete when the specimen appears slightly translucent and sinks to the bottom of the infiltration vessel (1.2 ml cryovial for small samples, 2.0 ml cryovial for larger samples). The mimimum times used are:

1st solution - 3 hours at 4°C

2nd solution - 3 hours at 4°C

3.3.2 Embedding Medium

15 ml infiltration solution

0.5 ml hardener (store at room temp), see table below:

3.3.3 Small enbedding mould trays:

Number of wells	Resin volume (ml)	Hardener volume (ml)
20	15.0	0.5
18	13.5	0.45
16	12.0	0.4
14	10.5	0.35
12	9.0	0.3
10	7.5	0.25
8	6.0	0.2
6	4.5	0.15
4	3.0	0.1
2	1.5	0.05

Mix and use immediately (use a syringe to dispense the resin into the moulds, this helps in reducing contact with oxygen). Note: polymerisation proceeds more rapidly in larger batches.

3.3.4 Blocking out

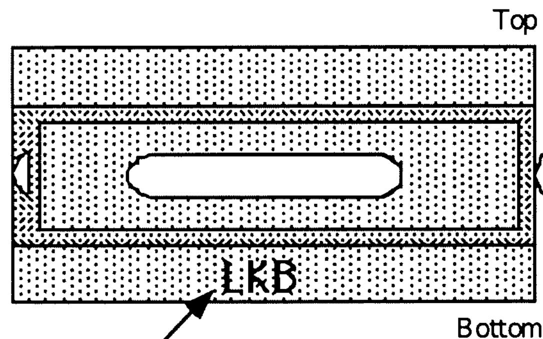
Place the Histomold™ in the air tight container, position the sample in the Histomold™, then completely fill the mould with resin / hardener mixture, as the lid is being placed on flood the inside of the container with nitrogen. Prepare no more than 10 moulds at a time as the resin starts to "set-off" within 20 minutes of the hardener being added. Allow to polymerise for 4-6 hours at 4°C (or overnight if time allows).

3.4 Mounting Medium

1 part powder (1 g)
1 part liquid (1 ml)
1 part powder + 1 part liquid = sufficient volume for 2 moulds.
Stir well and use immediately.

3.5 Mounting

Cover the upper depression of the Histomold™ with a thin layer of mounting medium and place the mounting block in the mould. The block is ready for sectioning after 10-15 minutes.
Store blocks in desiccator at 4°C to preserve enzyme activity until needed.



This must be at the bottom of the block for consistent orientation.

3.6 Double Embedding for Transverse Sections

The normal sections produced by resin embedding are usually cut in the sagittal plain, however if transverse sections are required then the specimen has to be double embedded. This is achieved by blocking the specimen out in a 2 cm length of silicon tube (the tube diameter needs to be slightly larger than the sample to be embedded) and the ends sealed with paraffin wax, then allowed to polymerise. After the block has polymerised it is taken out of the silicon tube, trimmed so that the desired area can be re-embedded and placed vertically into the mould tray normally used for sagittal sections. This is then filled with resin / hardener mixture and allowed to polymerise as in the usual method (LADD silicon moulds used for electron microscopy can also be used in place of the silicon tubes, with exclusion of oxygen by the use of silicon foils).

4 Section Cutting

4.1 Types of Knives

There are three types of knives suitable for resin sectioning in the range of 1-3 µm. All the methods in this manual have been developed using 2µm sections.
The three types are:

'D' profile steel knife.

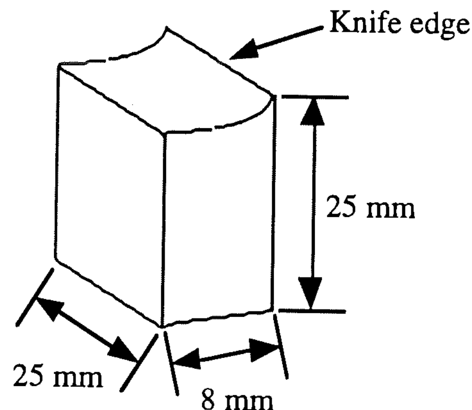
'C' profile tungsten carbide edged knife.

Ralph - Bennett glass knife.

All these knives have been tried and the best sections produced by a Ralph - Bennett glass knife with a cutting edge 25 or 38 mm long (the knife length depends on the width of the glass blank used, these come in standard widths of 25 or 38 mm) and 1 - 2 mm high.

4.1.1 Ralph - Bennett Glass Knife

These knives were first decribed by Bennett *et al.* (1976) and have become the standard type of knife for sectioning Glycol Methacrylate resin. Although used extensively overseas, most glass knives used for resin sectioning in Australia are of the triangular type used for electron microscopy. Ralph - Bennett knives enable resin blocks larger than those used in electron microscopy to be cut. It is important that the raised knife edge is no-more than 1 - 2 mm above the flat part of the knife block; if it is larger then the knife edge becomes too brittle, while a smaller edge causes cutting of the resin to become impossible.



4.2 Sectioning

Resin blocks are sectioned on a Microm 340 microtome (or any modern microtome with a retracting specimen holder), using a dry glass knife, with a standard specimen clamp and a Ralph knife holder fitted. Using a clearance angle of 4 - 7° for glass knives or 10 - 15° for metal knives, cutting sections of 0.5 - 2 µm in thickness (2 - 3 µm sections or thicker can be cut on either glass or disposable steel knives, sections thicker than 3 µm can be cut on D - type steel knives). Use a cutting speed of 1 - 20 cm/s, depending on the specimen and knife used,

and pick up the leading edge of the section with a pair of fine forceps. Once cut, the section is floated out in a water bath filled with distilled water at room temperature.

4.3 Mounting Sections

Pick up sections on acid washed slides by inserting the slide at a slant into the water bath and bring the slide up to the surface under the section. A number of sections can be placed on a single slide if the sections are oriented across the width of the slide, this enables either serial sections or sections from different blocks to be mounted on the one slide. By using frosted end slides details of the sections on the slide can be written in pencil, which is not erased during subsequent staining. The slides are air dried at room temperature overnight, then stored until needed for staining in a dust free slide box.

5 General Techniques

5.1 General

The time between the acquisition of the sample and the cutting and staining is of importance as the longer the interval the less likely a good staining result will be, especially when using some of the enzyme stains. If the sample can be processed (aquisition of sample to staining and mounting) in the same month then the stain reaction will be bright and very localised, the ideal result.

5.2 Fixation and Infiltration

To separate a larval sample from the water with minimal damage use a 47 mm Millipore® filter funnel fitted with a 47 mm Whatman® glass microfibre filter / adsorbent pad set. When all the larvae are separated out transfer the glass microfibre filter to a 50 mm petri dish half filled with cold fixative. Place the petri dish in the refrigerator until the sample is rigid (minimum time of 30 minutes). When the sample is adequately fixed, transfer the sample to a labelled 1.2 ml or 2.0 ml cryovial filled with cold fixative. Return to refrigerator, laying the cryovial flat to preserve the larvae as straight as possible, until adequately fixed (minimum time 2 hours).

When fixation is complete, decant the fixative from the cryovial, replacing with the first infiltration solution, returning the cryovial to the refrigerator for infiltration of the sample (minimum time 3 hours). Repeat procedure with the second infiltration solution.

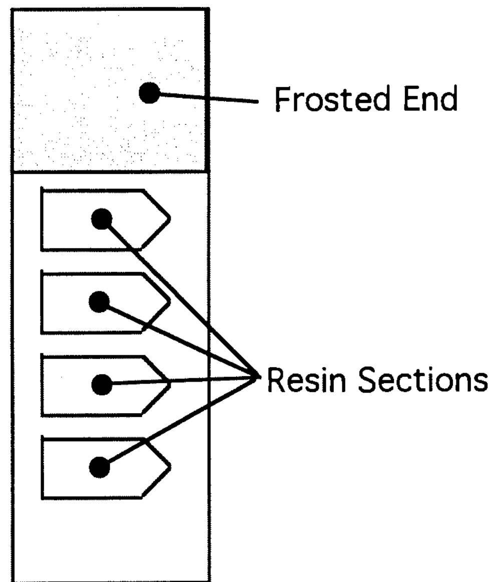
5.3 Embedding

Because oxygen is a strong inhibitor of the polymerisation process, polymerise all of the resins in an air tight box flooded with nitrogen. The use of gaseous nitrogen solves the problem of incomplete polymerisation of the resins.

Place the Histomold™ in the air tight container, position the sample in the Histomold™, then completely fill the mould with resin / hardener mixture, as the lid is being placed on flood the inside of the container with nitrogen, this is to exclude oxygen from the polymerising resin. Prepare no more than 10 moulds at a time as the resin starts to "set-off" within 20 minutes of the hardener being added.

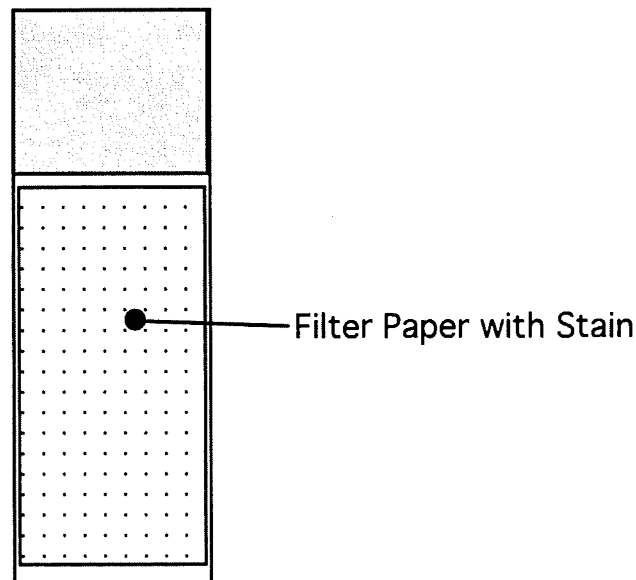
5.4 Cutting

Use fine forceps to pick up each section from the knife, position each section on the slide with the help of a fine probe. The slides are then stood on-end to allow excess water to drain off.



5.5 Staining

To minimise the amount of staining solution required it is necessary to cover the sections with pre-cut filter paper (25 mm x 50 mm). The stain solution can then be placed onto the filter paper, minimising the drying out of the stain during incubation.



All enzyme stains are incubated in a flat tray covered by a light proof lid, which also helps to reduce evaporation while excluding dust.

PART B: MORPHOLOGICAL STAINS.

6 Haematoxylin and Phloxine

6.1 Method Reference

Modified after Bancroft and Cook, 1984.

6.2 Method

There is no need to hydrate prior to immersion in haematoxylin.
Stain with haematoxylin for 5 minutes.
Rinse in distilled water.
Blue in saturated lithium carbonate for 15 seconds.
Wash in running water for 5 minutes.
Counter stain with phloxine / glacial acetic acid (GAA) for 2 minutes.
Rinse in distilled water.
When staining is finished, blot dry with filter paper, then air dry at room temperature.
Mount coverslip using DPX.
Air dry at room temperature overnight, or oven at 37°C for 3 hours.

This staining routine has been modified from a standard eosin / phloxine routine, it has been found that there is no need to include eosin as part of the stain.

6.3 Solutions

Haematoxylin:

Haematoxylin	5 g
Absolute Ethanol	50 ml
Potassium or Ammonium Alum	100 g
Distilled water	950 ml
Mercuric Oxide	2.5 g
GAA	40 ml

Dissolve the haematoxylin in the alcohol using gentle heat and dissolve the alum in the distilled water using heat with continuous stirring (use a 2 or 3 litre flask). Whilst the aqueous alum solution is still hot, add the alcoholic haematoxylin solution and bring to the boil stirring continuously. Turn off heat just before adding the mercuric oxide, as the resultant effervescence may cause spillage. Cool quickly by plunging the flask in cold water, then add the acetic acid and filter. The solution is ready for immediate use but will need refiltering before each use.

Phloxine:

Phloxine B	1 g
Absolute Ethanol	100 ml
GAA	1 ml

Dissolve Phloxine B in the alcohol, then add the GAA, filter and store in a brown bottle with a crystal of thymol.

6.4 Results

Nuclei - blue, background - pink.

7 Toluidine Blue

7.1 Method Reference

Modified after Bancroft and Cook, 1984.

7.2 Method

Stain with Toluidine Blue ; use either : 1.0% ----- 5 secs; or 0.5% ----- 15 secs; or 0.1% ----- 40 secs.

When staining is finished, blot dry with filter paper, then air dry at room temperature.

Mount coverslip using DPX.

Air dry at room temperature overnight, or oven at 37°C for 3 hours.

7.3 Results

Nuclei - deep blue, background - pale blue.

---

8 Polychrome Methylene Blue

8.1 Method Reference

Morris, 1947.

8.2 Method

Stain with polychrome methylene blue for 20 seconds.

When staining is finished, blot dry with filter paper, then air dry at room temperature.

Mount coverslip using DPX.

Air dry at room temperature overnight, or oven at 37°C for 3 hours.

8.3 Solutions

Methylene blue	1 g
Potassium carbonate	1 g
GAA	3 ml
Distilled water	300 ml

Place the distilled water in a large (1 litre) flask and add, with mixing, the methylene blue and potassium carbonate. Boil for 10-15 minutes. Whilst still hot add the GAA drop by drop shaking vigorously, until the formed precipitate is dissolved. Continue boiling until the volume of fluid is reduced to 100 ml. Cool and filter. Allow to stand 4 weeks prior to use. Add a crystal of thymol to the storage container to inhibit fungi.

8.4 Results

Nuclei components	dark blue.
Nuclei	light blue.
Background	various shades of blue-red-purple.

Polychroming of methylene blue is achieved by potassium carbonate when various azure's are formed. Ageing of the solution will accentuate this process.

9 Lee's Methylene Blue - Basic Fuchsin.

9.1 Method Reference

Modified after Bennett *et. al.*, 1976.

9.2 Method

Stain with Lee's methylene blue - basic fuchsin for 10 seconds.

Rinse in water, blot dry with filter paper, then air dry at room temperature.

Mount coverslip with DPX.

Air dry at room temperature overnight, or oven at 37°C for 3 hours.

9.3 Solutions

Stock Solution A

Methylene blue	0.13 g
Distilled water	100 ml

Stock Solution B

Basic Fuchsin	0.13 g
Distilled Water	100 ml

Staining solution

Methylene blue	30 ml
Basic Fuchsin	40 ml
0.05M Phosphate buffer	60 ml
Ethanol (100%)	45 ml

9.4 Note

Prepare staining solution just prior to use for best staining results.

9.5 Results

(Plate 1 A,)

Nuclei and amino acids - shades of blue

Background and cytoplasmic granules - shades of pink.

This stain gives good differentiation of cell types in the anterior pituitary and between smooth muscle and collagen. It demonstrates many of the secretory cells in the intestinal and stomach epithelium and displays C-cells of the thyroid as well as elastic fibres.

PART C: HISTOCHEMICAL STAINS.

10 Acid Phosphatase (Azo-dye coupling).

10.1 Method Reference

Drury and Wallington, 1980. p. 306.

10.2 Reagents

10-20 mg	Sodium α-naphthyl phosphate.
20 ml	0.1 M Acetate buffer, pH 5.0.
1.5 g	Polyvinyl pyrrolidone.
20 mg	Fast Garnet GBC salt.

Mix thoroughly and allow to dissolve, then filter. Cover reaction vessel against light.

10.3 Incubation:

30 to 60 minutes at 20°C.

10.4 Post treatment

Wash in running water 5 minutes.
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

10.5 Appearance

(plate 1 C)
Sites of ACP activity are reddish-brown, while nuclei are blue.

10.6 Localisation:

Organs with high activity are: spleen, kidney, liver and intestine. ACP is mainly localised in lysosomes, although there are some extra-lysosomal ACP's in endoplasmic reticulum which are identified by their differing affinity for aliphatic and aromatic esters of orthophosphoric acid (Lojda *et al.*, 1979). They are sensitive to inhibitors.

10.7 Chemistry

ACP releases α-naphthol which couples with a diazonium salt to form a coloured azo-dye. ACP catalyses the hydrolysis of esters of orthophosphoric acid as well as pyrophosphate compounds, producing various alcohols and phenols:

Monoester of *o*-phosphoric acid + water ⟶ Alcohol (phenol) + orthophosphate.

10.8 Optimum pH

pH 4 - 5

10.9 Inhibition

Cu^{2+} and tartrate ions, alloxan and formaldehyde; as well as the general inhibitors fluoride and phosphate ions.

10.10 Controls

(+ve) Run kidney, liver or spleen, **(-ve)** Put section through same procedure, but omit substrate from incubation medium.

10.11 Additional References

Lojda *et al.*, 1979. p 79.

11 Alkaline Phosphatase (Tetrazolium method).

11.1 Method Reference

Lojda *et al* 1979. p 61-62.

11.2 Reagents

5 mg	5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine
10 ml	0.1 M Tris buffer, pH 9.4
6 mg	Nitro B, tetrazolium salt
0.2 ml	N,N-dimethylformamide

Dissolve substrate in dimethylformamide, add Nitro BT then buffer, mix thoroughly and filter. Use immediately. Cover reaction vessel against light.

11.3 Incubation

30 - 90 minutes at 20°C

11.4 Post Treatment

Pour off incubation medium
Rinse in distilled water
Place in 4% formaldehyde (optional) for 2 hours
Wash in running water 5 minutes
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

11.5 Appearance

(plate 1 B)
Sites of activity are blackish brown. Tissues with high activity are kidney, small intestine and liver. Nuclei are blue.

11.6 Localisation

ALP facilitates the transfer of metabolites across the cell membrane and is associated with lipid transport as well as bone synthesis. It occurs in cell membranes where active transport takes

place, particularly in the proximal tubules of the kidney, the brush border of enterocytes and the bile pole of hepatocytes.

Activity is also found in Golgi bodies, pinocytic vesicles, endoplasmic reticulum and the lysosomes of enterocytes.

11.7 Chemistry

ALP catalyses the breakdown of esters of orthophosphoric acid, producing various alcohols and phenols. The formazan deriving from tetra-nitro BT is very stable and gives precise localisation, with few artifacts. However some naturally occurring pigments may be mistaken for reaction products. Formaldehyde fixation does not interfere with the method.

11.8 Optimum pH

pH 9.2 - 9.8 depending on the type and concentration of substrate and the buffer.

11.9 Activation

Magnesium, zinc and cobalt activate the enzyme.

11.10 Inhibition

Phosphate and arsenate anions, various alcohols, l-cysteine and tetramisol, inhibit activity.

11.11 Controls

(+ve) run liver or intestine sections, **(-ve)** put section through same procedure, but omit substrate from incubation medium.

11.12 Additional References

Grossrau, 1981., Segner *et al.*, 1989.

12 Aminopeptidase-M (Simultaneous azo-coupling).

12.1 Method Reference

Modified after Lojda *et al*, 1979. p. 191.

12.2 Reagents

10 mg	L-leucine-4-methoxy-β-naphthylamide.
0.5 ml	N,N-dimethylformamide.
10 ml	0.05 M Phosphate buffer, pH 7.4.
10 mg	Fast Blue BB.

Dissolve substrate in 0.5 ml N,N-dimethylformamide, mix all reagents together, filter. Use immediately. Cover reaction vessel against light.

12.3 Incubation

60 - 120 minutes at 20°C, longer in larvae less than 30 days old.

12.4 Post treatment

Rinse in distilled water.
Place in 2% copper sulfate for 5 minutes (chelation of azo-dye to stabilise complex).
Rinse in distilled water.
Place in 4% formaldehyde (optional) for 2 hours.
Wash in running water 5 minutes.
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

12.5 Appearance

(plate 1 D)
Reaction site stains red-orange. In larvae less than 30 days old the colour may be shades of pink to red.

12.6 Note

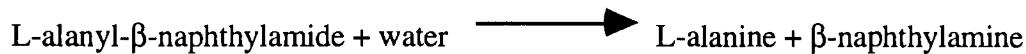
In larvae less than 30 days old colour product fades out overnight - check colour same day or photograph for record.

12.7 Localisation

Aminopeptidase-M occurs in microsomes, especially in the brush border of enterocytes and cells of renal proximal tubules.

12.8 Chemistry

Aminopeptidase-M hydrolyses various peptides and arylamides e.g. L-leucyl and L-alanyl-b-naphthylamide.

L-alanyl-β-naphthylamide + water  L-alanine + β-naphthylamine

The methoxy derivative gives precise localisation due to fast coupling. The Fast Blue BB produces an almost amorphous azo dye irrespective of pretreatment.

12.9 Optimum pH

The enzyme is active over a wide range of pH (6 - 9) and the optimum is species and organ specific.

12.10 Inhibition

EDTA strong inhibitor; formaldehyde is less inhibitory than glutaraldehyde. Inhibition increases with increase in pH. Methanol in the fixative is an extremely strong inhibitor.

12.11 Controls

(+ve) Run kidney or intestine sections, (-ve) Put section through the same procedure, but omit substrate from incubation medium.

12.12 Additional References

Segner *et al.*, 1989.

13 Maltase (Simultaneous azo-coupling).

13.1 Method reference

Modified after Lojda *et al*, 1979, p. 181.

13.2 Reagents

10 mg	β-naphthyl-α-D-glucoside (β-naphthyl-α-D-glucopyranoside)
0.5 ml	N,N-dimethylformamide
10 mg	Fast Garnet GBC salt
10 ml	0.05 M Phosphate buffer pH 7.4

Dissolve substrate in dimethylformamide, add salt and dissolve, add buffer, mix well and filter. Use immediately. Cover reaction vessel against light.

13.3 Incubation

60 - 120 minutes at 20°C.

13.4 Post treatment

Rinse in distilled water.
Place in 4% formaldehyde (optional) for several hours at room temperature to reduce gas bubbles in sections.
Wash in running water 5 minutes.
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

13.5 Appearance

(plate 1 G)
Sites of activity appear pink-red. Tissues of high activity are small intestine and kidney. Nuclei are blue.

13.6 Localisation

Enzyme is localised in the brush border both of enterocytes in the small intestine and cells of the proximal tubule in the kidney.

13.7 Chemistry:

Maltase catalyses the hydrolysis of maltose and some synthetic substrates.

Maltose + H_20 2 Glucose.

13.8 Optimum pH

pH 6.5 - 7.0.

13.9 Controls

(+ve) Run intestine or kidney sections, **(-ve)** put section through same procedure, but omit substrate from incubation medium.

13.10 Additional References

Segner *et al.*, 1989.

14 Non-Specific Esterases

14.1 Method Reference

Modified after Lojda *et al*, 1979, p. 117.

14.2 Reagents

5 mg	α-Naphthyl acetate.
0.5 ml	N,N-dimethylformamide.
20 ml	0.05 M Phosphate buffer, pH 7.4.
50 mg	Fast Blue BB.

Dissolve substrate in dimethylformamide, add coupling agent then buffer, mix well and filter. Use immediately. Cover reaction vessel against light.

14.3 Incubation

30 - 90 minutes at 20°C.

14.4 Post treatment

Rinse in distilled water.
Place in 4% formaldehyde (optional) for several hours at room temperature.
Wash in running water 5 minutes.
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

14.5 Appearance

(plate 1 E)

Enzyme sites are black, while the background of sections is stained green or yellowish brown. The inadequate coupling of stable diazonium salts with a-naphthyl in highly active organs (kidney, liver intestine) gives rise to diffusion artefacts of red or greenish brown precipitates outside enzyme-active sites. In such cases increase the pH to 7.8 or reduce the substrate amount by half. Nuclei are blue.

14.6 Localisation

Ubiquitous in plant and animal tissues; especially high activity is found in liver, kidney and intestine. Intracellular activity occurs in the endoplasmic reticulum, and lysosomes (possibly also mitochondria and hyaloplasm).

14.7 Chemistry

Non-specific esterases hydrolyse aromatic, aliphatic and aromatic esters of lower fatty acids.

14.8 Optimum pH

pH 5 - 8.

14.9 Inhibition

Glutaraldehyde fixation reduces activity. Activity is inhibited by 0.01 mM eserine, diisopropyl fluorophosphate and B-esterases as well as by 0.01 mM diethyl-p-nitrophenyl phosphate.

14.10 Controls

(**+ve**) Run liver, intestine or kidney sections, (**-ve**) put section through same procedure, but omit substrate from incubation medium.

14.11 Additional References

Segner *et al.*, 1989.

15 Trypsin

15.1 Method Reference

Modified after Segner, 1989.

15.2 Reagents

7 mg	N-CBZ-diglycyl-L-arg-4-methoxy-β-naphthylamide.
0.5 ml	N,N-dimethylformamide.
10 ml	0.05 M Phosphate buffer, pH 7.4.
10 mg	Fast Blue BB.

Dissolve substrate in dimethylformamide, add coupling agent, then buffer and mix. Filter and use immediately. Cover reaction vessel against light.

15.3 Incubation

120 - 180 minutes at 20°C.

15.4 Post treatment:

Rinse in distilled water.
Place in 2% copper sulfate for 5 minutes.
Rinse in distilled water.
Wash in running water 5 minutes.
Counterstain in Harris's haematoxylin, 90 seconds.
Rinse in distilled water.
Blue in lithium carbonate 15 seconds.
Wash in running water 5 minutes.
Dry with filter paper.
Mount coverslip with DPX.

15.5 Appearance

(plate 1 F)

Enzyme activity pink-red. Nuclei stain blue.

15.6 Localisation

Activity is found in the lumen of the intestine as well as along the brush border of enterocytes.

15.7 Chemistry

Trypsin is an endopeptidase which hydrolyses the peptide bonds involving α-carboxyl groups of the amino acids arginine and lysine. It is synthesised in the acinar cells of the pancreas in an inactive form as the pro-enzyme trypsinogen and stored as zymogen granules. Trypsin is secreted into the duodenum under stimulus from the vagal nerve or the intestinal hormone cholecystokinin - pancreozymin and is activated by enterokinase to the active form, trypsin. Active trypsin may also be secreted. Activity is stimulated by Ca^{++}, Mg^{++} and to a lesser extent, Co^{++} and Mn^{++}. Trypsin attacks denatured proteins more rapidly than native proteins. (Tietz, 1976).

15.8 Optimum pH

pH 7.8

15.9 Inhibition

Alkaline pH causes rapid destruction. Enzyme also auto-digests and activity falls off at optimum pH. Inhibiting substances: Cyanide, sulfide, citrate, fluoride and heavy metals. Egg white contains trypsin inhibitor.

15.10 Controls

(+ve) run intestine, **(-ve)** put section through the same procedure, but omit substrate from incubation medium.

15.11 Additional References

Lojda *et al.*, 1979. p 191.

16 Alcian Blue - Periodic Acid Schiff.

16.1 Method Reference

Modified after Bancroft and Cook, 1984

16.2 Reagents

1% alcian blue in 70% alcohol with 3% acetic acid, pH 2.5

1% aqueous periodic acid

Schiff's reagent

0.1% Diastase solution

Sulphite Wash (reducing agent):

Distilled water	92.5 ml
20% Potassium metabisulphite	2.5 ml
1N HCl	5.0 ml

16.3 Method

Stain with alcian blue solution for 20 minutes.

Wash in running water for 1 minute.

Oxidise for 5 minutes in 1% aqueous periodic acid.

Wash in running water for 1 minute.

Place in Schiff's reagent for 15 minutes.

Transfer to sulphite wash No. 1 for 3 minutes

Transfer to sulphite wash No. 2 for 5 minutes.

Counterstain in Harris's haematoxylin, 1 minute.

Rinse in distilled water.

Blue in lithium carbonate 15 seconds.

Wash in running water 5 minutes.

Dry with filter paper.

Mount coverslip with DPX.

16.4 Note

ALWAYS PERFORM PAS WITH AND WITHOUT DIASTASE. Schiff's reagent has a limited life, up to 12 months if stored at 4°C, and should be discarded if it becomes pink.

16.5 Diastase Control

For diastase control, treat with 0.1% diastase for 30 minutes before starting, then rinse for 5 minutes in water.

16.6 Appearance

Acid mucins blue. Neutral mucins (including glycogen) red or magenta. Nuclei pale blue.

16.7 Chemistry

Glycogen is the only important PAS positive tissue component that is removed by diastase, so that diastase digestion converts the PAS reaction into a specific test for glycogen.

17 Lipids

17.1 Method Reference

Modified after Gerrits *et al*, 1987.

17.2 Reagents

1% Osmium Tetroxide

Saturated Sudan Black B in 70% alcohol +1% GAA.

0.5% Acidified Neutral Red:

Neutral red	0.5 g
GAA	1.0 ml
Distilled water	100 mls

Dry Acetone:

Acetone can be dehydrated by adding anhydrous calcium chloride (20% calcium chloride: 80% acetone; v:v), allowing to stand for two days before use. Glassware must be dry and sections must be allowed to dry in air after immersion of the slides into anhydrous actetone.

17.3 Method

60 minutes in 1% Osmium Tetroxide

Rinse in running water for 1 minute

60 minutes in Sudan Black B at 20°C.

Wash in running water 1 minute

Counterstain with Neutral Red for 2 minutes

Rinse in water and mount coverslip in Immu-mount™.

17.4 Appearance

Lipids stain black or blue if present in sufficient quantity, and may be brownish-black indicating lipid or lipoprotein. Nuclei stain red.

17.5 Localisation

Lipids occur in adipose tissue, fat droplets, mitochondria, pancreas, liver, intestine and cartilage.

17.6 Controls

(**+ve**) run salmon pyloric caecae with fat attached (or similar fatty tissue), (**-ve**) run salmon pyloric caecae with fat attached (or similar fatty tissue) but remove hydrophobic lipids with dry acetone at 4°C for 1 - 8 hours depending upon the tissue.

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APPENDIX I: BUFFERS

1 Phosphate Buffer (0.05 M pH 5.8 - 8.0)

Stock solutions:

Stock A: 0.1M sodium dihydrogen orthophosphate (M.W. 156)
3.9g sodium dihydrogen orthophosphate.
250 ml distilled water.

Stock B: 0.1M disodium hydrogen orthophosphate (M.W. 142)
3.55 g disodium hydrogen orthophosphate.
250 ml distilled water.

Composition of buffer: **x** ml of A + **y** ml of B made up to 100 ml with distilled water.

pH	ml soln. A	ml soln. B
5.8	46.0	4.0
6.0	43.8	6.2
6.2	40.7	9.3
6.4	36.7	13.3
6.6	31.2	18.8
6.8	25.5	24.5
7.0	19.5	30.5
7.2	14.0	36.0
7.4	9.5	40.5
7.6	6.5	43.5
7.8	4.2	45.8
8.0	2.6	47.4

From Bancroft and Cook, 1984. p.696.

2 TRIS - HCl Buffer (0.1M pH 7.2 - 9.0)

Stock Solutions:

Stock A: 0.2M TRIS (M.W. 121.0)
2.42 g TRIS (hydroxymethyl) aminomethane.
100 ml distilled water.

Stock B: 0.2M HCl (M.W. 36.46)
1.7 ml hydrochloric acid.
100 ml distilled water.

Composition of buffer:
25 ml of **A** + x ml of **B**, made up to 100 ml with distilled water.

pH	x ml of B
7.2	22.1
7.4	20.7
7.6	19.2
7.8	16.3
8.0	13.4
8.2	11.0
8.4	8.3
8.6	6.1
8.8	4.1
9.0	2.5

From Bancroft and Cook, 1984. p.698., also Lojda *et. al.*, 1979. p.309.

3 ACETATE BUFFER (WALPOLE) (pH 3.5-5.6)

Stock solutions

Stock A : 0.2 M acetic acid (M.W. 60.0)
1.2 ml glacial acetic acid in 100 ml distilled water.

Stock B : 0.2 M sodium acetate
1.64 g sodium acetate anhydrous (M.W. 82) or 2.72 g sodium acetate trihydrate (M.W. 136) in 100 ml distilled water.

Composition of buffer:

pH	mls of Solution A	mls of Solution B
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

From Bancroft and Cook, 1984. p. 259.

4 PIPES Buffer (0.2M pH 7.3) (stock solution)

Warning: Care must be taken while handling PIPES compound as one of the intermediates for the synthesis of aminosulphonic acid buffer is potentially carcinogenic.

Method:
PIPES 15.12 g.
Distilled water 100 ml.
Sonicate the mixture about 5 minutes (the mixture has a milky appearance).
To this mixture 1 N NaOH is added dropwise under pH monitoring till the pH 7.3 is reached.
The resulting solution should be clear.
Pour into a volumetric flask, adjust with distilled water to obtain 250 ml.
Add 6.25 g of sucrose to obtain 600 mOs.
Label, date and store at 4°C.
discard buffer if there is any turbidity.

For marine tissue substitute 0.2µm filtered seawater for distilled water.

To obtain 0.1 M PiIPES pH 7.3 working solution, add equal volume of 0.2 M PIPES buffer to an equal amount of distilled water.

From Baur and Stacey, 1977. p. 315

5 Cacodylate Buffer (0.1 M pH 5.0 - 7.4)

Stock Solutions:

Stock A: 0.2 M sodium cacodylate (M.W. 214.4)
4.28 g sodium cacodylate.
100 ml distilled water.

Stock B: 0.2 M HCl (M.W. 36.46)
1.7 ml hydrochloric acid.
100 ml distilled water.

Composition of buffer: 25 ml of **A** + **x** ml of **B** made up to 100 ml with distilled water.

pH	x ml of B
5.0	23.5
5.2	22.5
5.4	21.5
5.6	19.6
5.8	17.4
6.0	14.8
6.2	11.9
6.4	9.2
6.6	6.7
6.8	4.7
7.0	3.2
7.2	2.1
7.4	1.4

From Bancroft and Cook, 1984. p. 695.

APPENDIX II: CHEMICAL SAFETY

Chemical	Classification	Cautions / First Aid
Sodium α-naphthyl phosphate		
Sodium diethyl barbituric acid 0.1M HCl-veronal buffer pH 5.0	Harmful substance	Harmful by inhalation and if swallowed. In case of contact with eyes, rinse with plenty of water immediately and seek medical advice.
Acetate buffer		
Polyvinyl pyrrolidone		
Fast Garnet GBC salt	Harmful substance	Harmful by inhalation, in contact with skin and if swallowed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. In case of a spill, remove contaminated clothing and after contact with skin, wash with plenty of water.
5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine	Toxic or very toxic substance possible danger of cumulative effects	Toxic by inhalation, in contact with skin and if swallowed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. In case of a spill, remove contaminated clothing after contact with skin wash immediately with plenty of water.
Tetrazolium salt, Nitro BT	Harmful substance	Harmful if swallowed. Avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If swallowed, seek medical advice immediately.

N,N-dimethylformamide	Harmful substance,	Harmful by inhalation and contact with skin, irritating to eyes. Wear suitable protective clothing and apparatus. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If you feel unwell, seek medical advice immediately.
L-alanyl-4-methoxy-β-naphthylamide		
0.1M Cacodylate Buffer		
Fast Blue BB	Harmfull substance,	Harmful by inhalation, in contact with skin and if swallowed. Wear suitable protective clothing. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If you feel unwell, seek medical advice immediately.
2-naphthyl-α-D-glucoside		
0.1M citric acid-phosphate buffer pH 7.0		
1N and 0.1N NaOH	Irritating and corrosive substance	In case of contact with eyes, rinse with plenty of water immediately and seek medical advice. If you feel unwell, seek medical advice immediately.
α-naphthyl acetate		
0.1M phosphate buffer pH 7.4		
N-CBZ-diglycyl-L-arg-4-methoxy-β-naphylamide		
0.1M cacodylate buffer pH 6.5-7.5		
2% copper sulphate	Harmful substance,	Toxic by inhalation and if swallowed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If you feel unwell, seek medical advice immediately.

4% formaldehyde (10% formalin)	Toxic substance, Flammable	Toxic by inhalation, in contact with skin and if swallowed. May cause sensitisation by skin contact. Wear suitable protective clothing. In case of contact with eyes, rinse with plenty of water immediately and seek medical advice. After skin contact, wash with plenty of water immediately. If you feel unwell, seek medical advice.
Periodic Acid	Oxidising and corrosive substance	Contact with combustible material may cause fire. Causes burns. Avoid contact with skin and eyes. Remove immediately all contaminated clothing. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If you feel unwell, seek medical advice immediately.
Schiff's reagent.		
Saturated Sudan Black B in 70% alcohol		Do not inhale dust and avoid contact with skin and eyes. Store at RT. Wear suitable protective clothing. In case of contact with eyes, rinse with plenty of water immediately and seek medical advice. If you feel unwell, seek medical advice immediately.
Mayer's Carmalum 1% aqueous neutral red.		
	Resin Embedding chemicals.	
Basic Resin; Glycol methacrylate monomer (GMA) Polyethylene glycol 400 (PEG) Hydroquinone		May cause irritation
Activator; Benzoyl peroxide (BPO) Plasticizer	Flammable, strong oxidiser. Heat may cause fire or explosive decomposition	Causes irritation.

Hardener; Derivative of Barbituric acid DMSO	DMSO may cause irritation.	DMSO - handle with care and *use gloves.*
Mounting medium:		
Powder; Polymethylmethacrylate (PMMA) Benzoyl peroxide (BPO) Plasticizer	Flammable, strong oxidiser. Heat may cause fire or explosive decomposition	Causes irritation.
Liquid; Methylmethacrylate Dimethylparatoluidin	Flammable, may cause irritation	
Immu-Mount; Polyvinyl alcohol resin Glycerol Less than 1% amino alcohol and quaternary ammonia chloride.	Harmful substance	Harmful if in contact with eyes or skin. If in contact with skin or eyes, wash with plenty of water immediately.

APPENDIX III:CHEMICALS

Chemical	Quantity	Supplier	Product Number
5-Bromo-4-Chloro-3-Indoyl-Phosphate-p-Toluidine	500 mg	Sigma	B8503
α-Naphthyl Acetate	5 g	Sigma	N8505
Alcian Blue (C.I. 74240)	25 g	Sigma	A3157
Aluminium Potassium Sulphate (Potassium Alum)	500 g	BDH	10009 3E
Basic Fuchsin (C.I. 42510)	25 g	BDH	34032 4J
Chloroform (GPR)	1 l	BDH	27710 5X
Copper Sulphate (Anhydrous)	500 g	BDH	27850 4G
Diastase	25 g	BDH	39123 3N
Disodium Hydrogen Orthophosphate	500 g	BDH	10249 4C
DPX	500 ml	BDH	36029 4H
Ethanol (Dehydrated)	20 l	CSR	SG
Fast Blue BB (C.I. 37175)	25 g	BDH	34117-2P
Fast Garnet GBC Salt (C.I. 37210)	5 g	Sigma	F8761
Glacial Acetic Acid	500 ml	AJAX	2-500ml
Glutaraldehyde (Aqueous 25%)	100 ml	BDH	36080 2F
Haematoxylin (C.I. 75290)	25 g	Serva	24420
Historesin Activator	10 * 0.5 g	Leica	530-772300
Historesin Basic Resin	500 ml	Leica	530-772300
Historesin Hardener	40 ml	Leica	530-772300
Historesin Mounting Medium (Liquid)	250 ml	Leica	530-772500
Historesin Mounting Medium (Powder)	500 g	Leica	530-772400
Historesin Plus Activator	5 * 0.6 g	Leica	530-773220
Historesin Plus Basic Resin	500 ml	Leica	530-773220
Historesin Plus Hardener	30 ml	Leica	530-773220
Hydrochloric Acid	1 l	BDH	28507 8C
Immu-Mount	20 ml	Shandon	67690003
L-Leucine-4-Methoxy-ß-Naphthylamide	100 mg	Sigma	L1136
Lithium Carbonate	100 g	Sigma	L 3876
Mercuric Oxide (Red)	100 g	BDH	29168 3G
Methanol (GPR)	500 ml	BDH	29192 4E
Methylene Blue (C.I. 52015)	25 g	BDH	34048 4B
N,N-Dimethylformamide	250 ml	Sigma	D8654
N-CBZ-Gly-Gly-Cyl-L-Arg-4-Methoxy-ß-Naphthylamide	25 mg	Sigma	C5770
Nitro B Tetrazolium Salt	1 g	Sigma	N6876

Neutral Red (C.I. 50040)	25 g	Sigma	N6634
Osmium Tetroxide	100 mg	BDH	36219 2K
Paraformaldehyde	500 g	BDH	29447 4L
Periodic Acid	100 g	BDH	10432 4Q
Phloxine B (C.I. 45410)	25 g	BDH	34064 4W
Piperizine (PIPES)	100 g	BDH	44120 4M
Polyvinyl Pyrrolidine	100 g	Sigma	PVP-10
Potassium Carbonate	500 g	BDH	10196 4H
Potassium Metabisulphite	500 g	Sigma	P2522
Schiff's Reagent	500 ml	Sigma	395-2-016
Silica Gel	5 kg	Selby	936993X
Sodium α Napthyl Phosphate	100 mg	Sigma	N7255
Sodium Acetate (Anhydrous)	250 g	BDH	10236 3P
Sodium Cacodylate	100 g	BDH	30118 3V
Sodium Dihydrogen Orthophosphate	500 g	BDH	10245 4R
Sodium Hydroxide	500 g	BDH	10252 4X
ß-Naphthyl-α-D-Glucoside (glucopyranoside)	100 mg	Sigma	N4504
Sudan Black B (C.I. 26150)	25 g	BDH	34210 3F
Thymol (Crystaline)	100 g	BDH	30433 2F
Toluidine Blue (C.I. 52040)	25 g	BDH	34077 4Y
TRIS (Hydroxymethyl) Aminomethane	500 g	BDH	45205 4C

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5 Results

5.1 Morphological studies

Results have been split into cohorts for the 1992 season, while those for the 1993 season have been split in to tanks within cohorts and cohorts. Cohorts were based on the date of hatch and origin of eggs. Generally, a batch of eggs produced on the same day and stocked into several tanks hatched within a 24 hour period.

5.1.1 Standard Lengths

The maximum survival indicated in Tables 2 and 3 differ from those in Figures 2 to 9 when the final sample from a tank was not taken at the termination of the experiment.

The 1992 season commenced late August and the last cohort hatched in mid November. Seven age cohorts were trialled during the season and all, except for cohorts 3 and 4, were run in one tank. See Table 2 for details of cohorts, tanks and survival.

Table 2. Details of 1992 cohorts and culture systems

Cohort	Date of hatch	Tank	Volume (L)	Maximum survival (days)
1	29.8.92	1B	1000	20
2	21.9.92	2	60	8
3	3.10.92	1A	1000	6
3	3.10.92	1B	1000	9
3	3.10.92	2A	1000	24
3	3.10.92	2B	1000	23
3	3.10.92	3A	1000	11
3	3.10.92	3B	1000	6
3	3.10.92	4A	1000	23
3	3.10.92	4B	1000	23
3	3.10.92	5A	1000	23
3	3.10.92	5B	1000	23
3	3.10.92	6A	1000	23
3	3.10.92	6B	1000	6
4	3.11.92	2B	1000	4
4	3.11.92	3B	1000	22
4	3.11.92	4B	1000	26
5	6.11.92	5B	1000	38
6	12.11.92	1B	1000	36
7	15.11.92	2B	1000	32

Standard lengths for cohorts from the 1992 rearing season are presented in Figure 2. While the maximum survival, and hence growth, of cohorts 1 and 2 was poor (20 and 8 days respectively), cohorts 3 and 4 survived for 24 and 26 days, while cohorts 5, 6 and 7 demonstrated better survival of 38, 36 and 32 days respectively.

The 1993 season commenced in mid September and the last cohort hatched in mid November. Twelve cohorts were trialled during the season and cohorts 6, 7, 9 and10 were run in two or more tanks. See Table 3 for details of cohorts, tanks and survival.

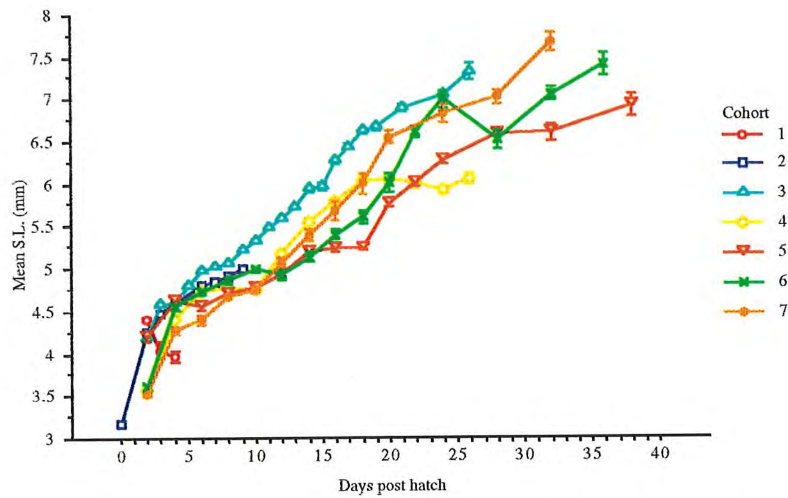

Figure 2. 1992 Standard lengths for all cohorts, demonstrating growth and survival. Each data point represents the Mean ± SE for 10 to 28 larvae.

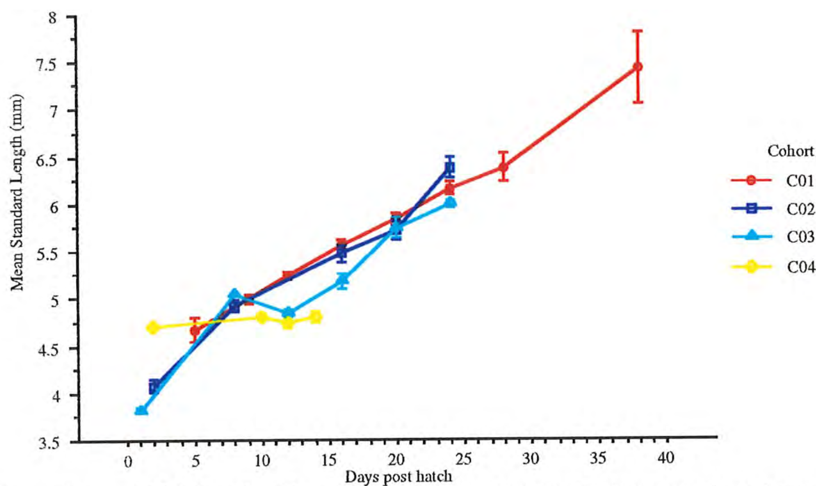

Figure 3. 1993 Standard lengths for cohorts demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

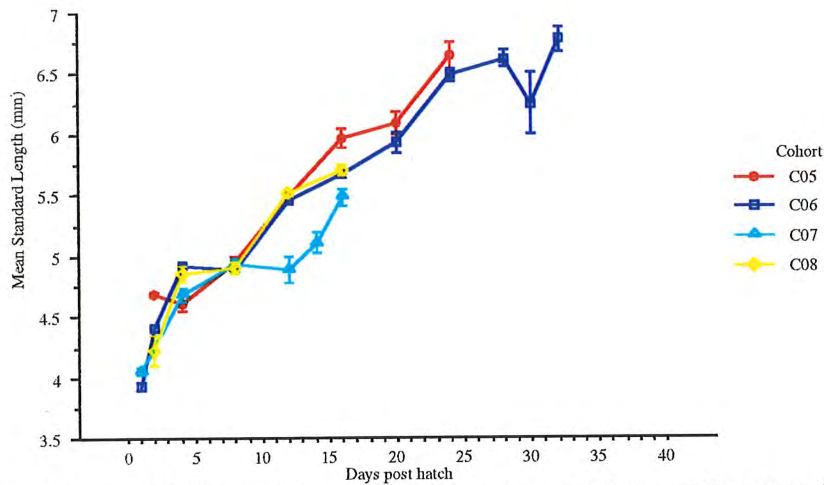

Figure 4. 1993 Standard lengths for cohorts demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

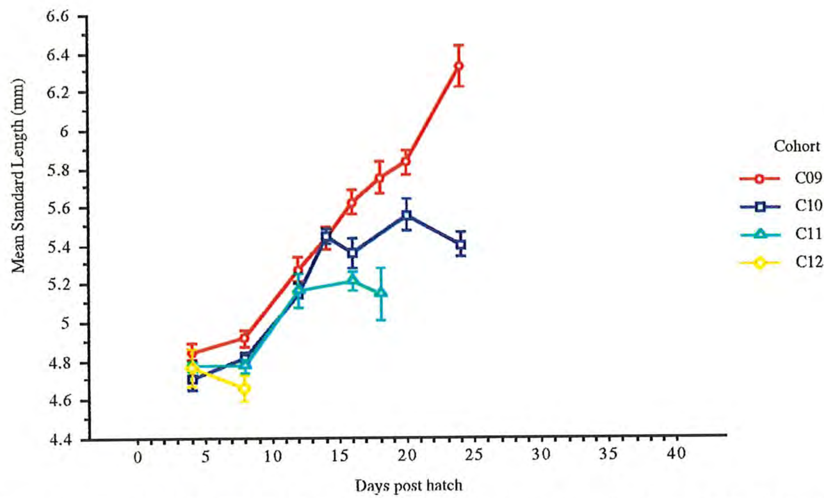

Figure 5. 1993 Standard lengths for cohorts demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

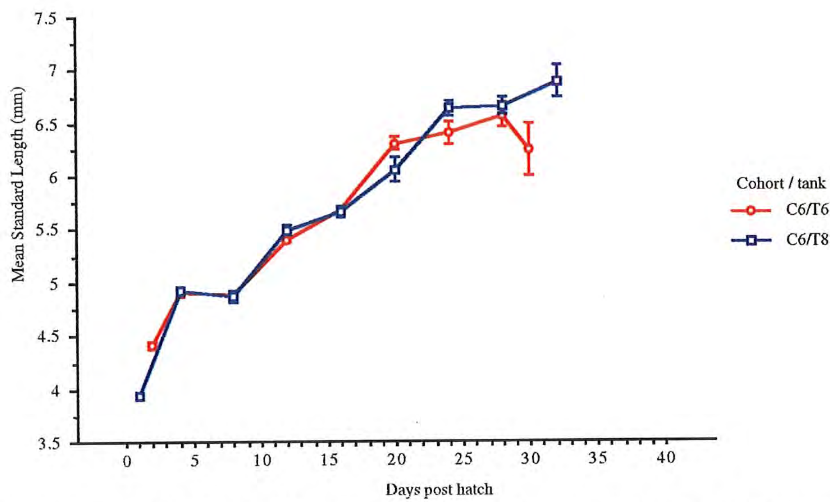

Figure 6. 1993 Standard lengths for tanks containing larvae from the same cohorts demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

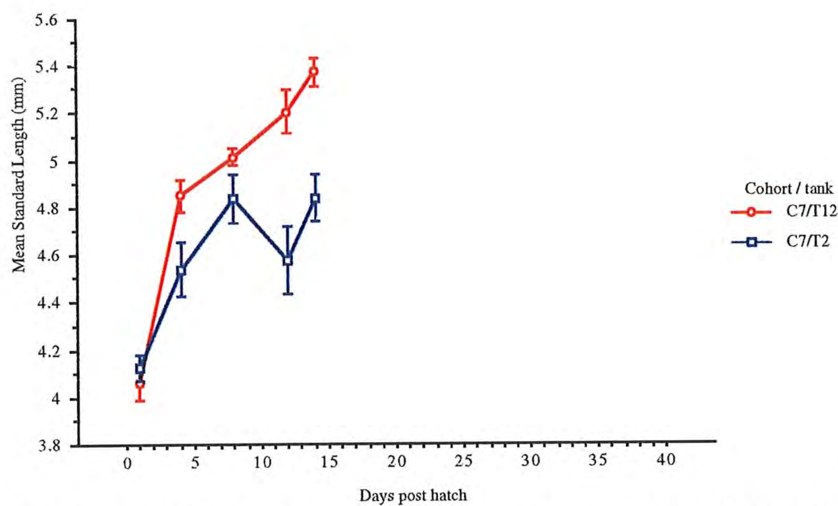

Figure 7. 1993 Standard lengths for tanks containing larvae from the same cohorts, demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

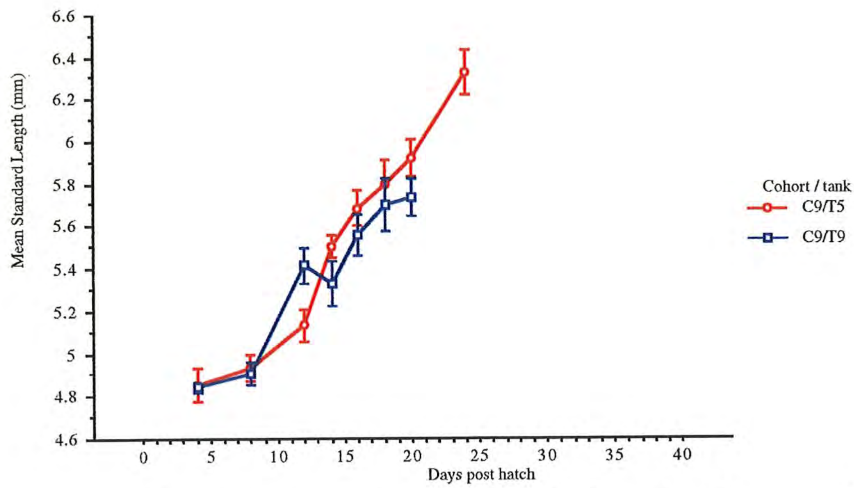

Figure 8. 1993 Standard lengths for tanks containing larvae from the same cohorts, demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

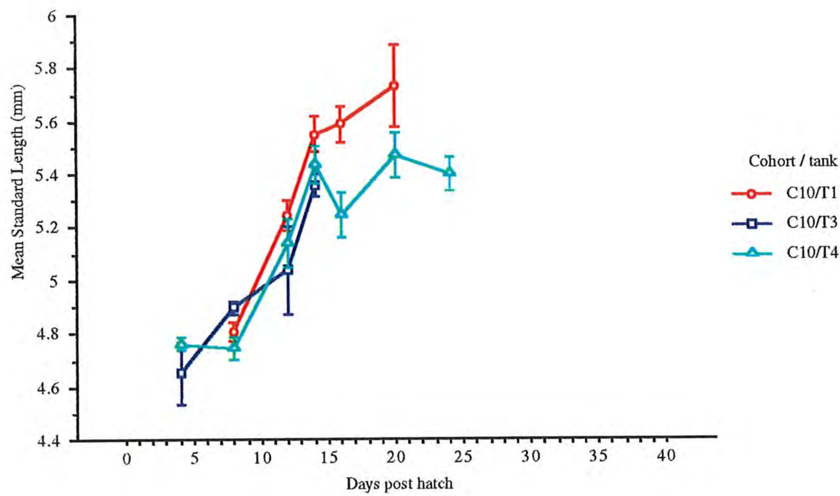

Figure 9. 1993 Standard lengths for tanks containing larvae from the same cohorts, demonstrating growth and survival. Each data point represents the Mean ± SE of 4 to 10 larvae.

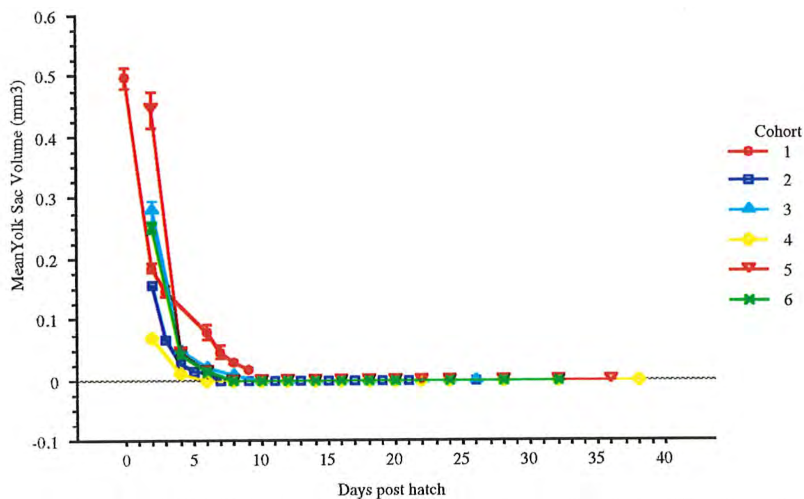

Figure 10. 1992 Yolk sac volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 10 to 28 larvae.

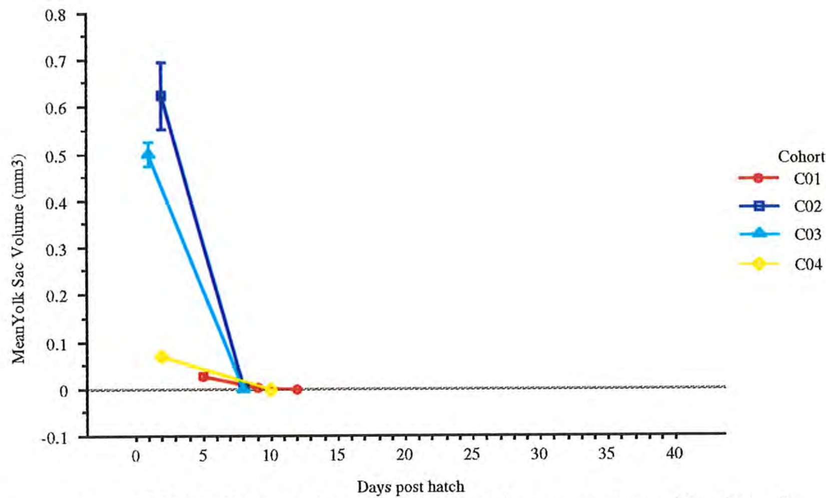

Figure 11. 1993 Yolk sac volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

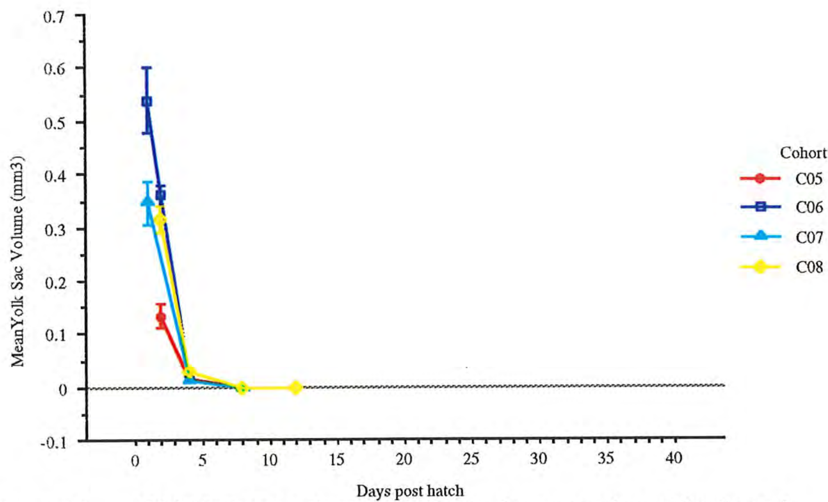

Figure 12. 1993 Yolk sac volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

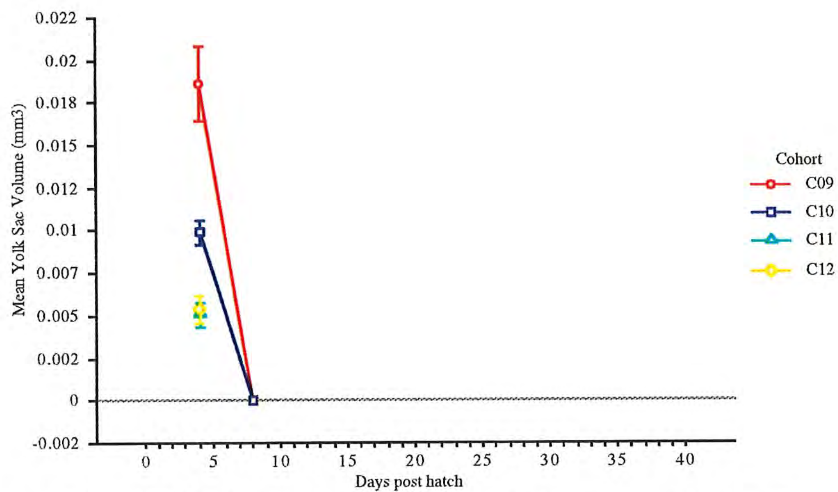

Figure 13. 1993 Yolk sac volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

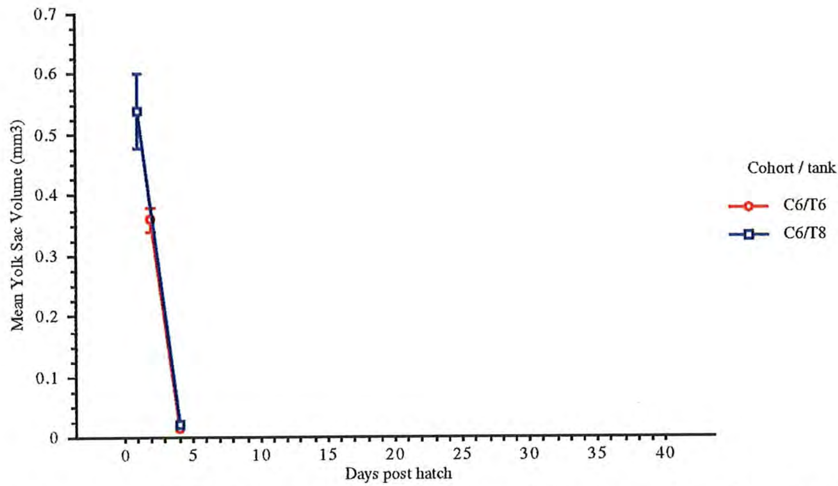

Figure 14 1993 Yolk sac volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

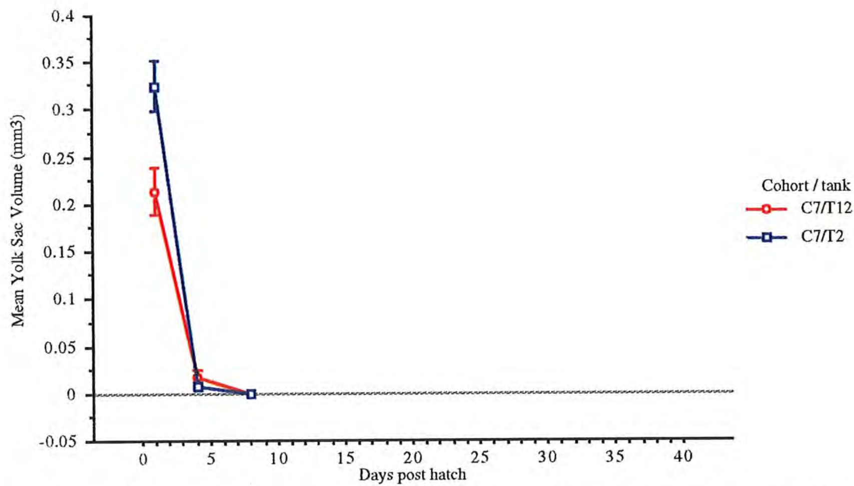

Figure 15. 1993 Yolk sac volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

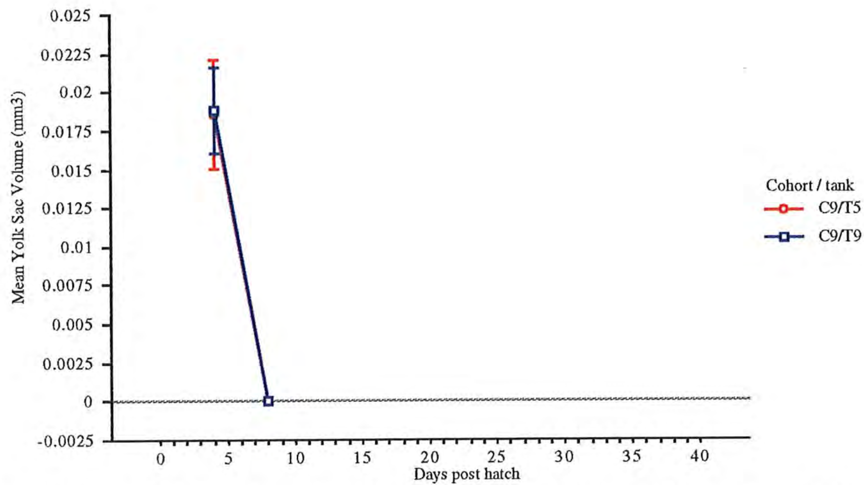

Figure 16. 1993 Yolk sac volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

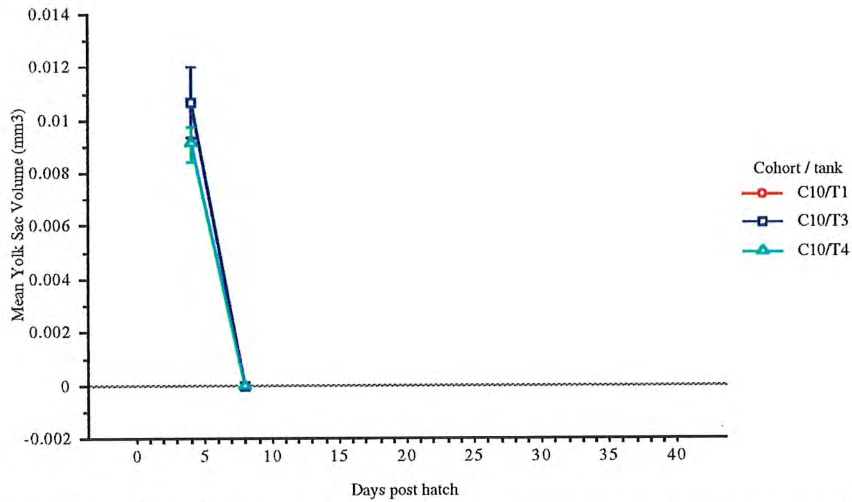

Figure 17. 1993 Yolk sac volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

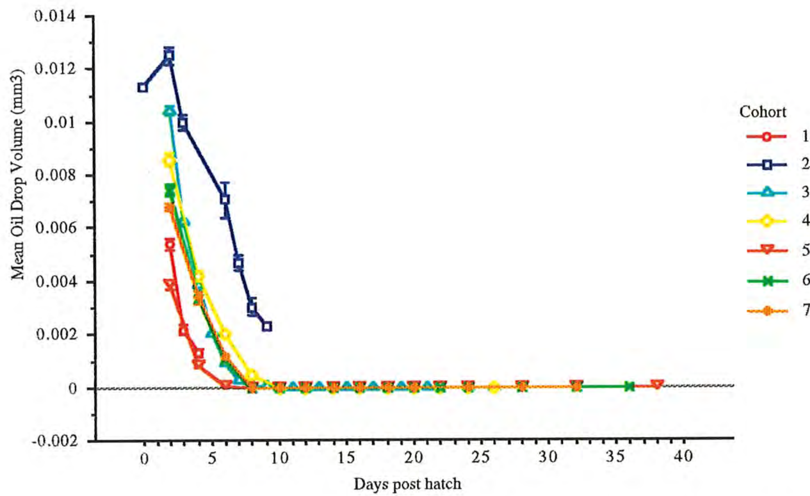

Figure 18. 1992 Oil drop volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 10 to 28 larvae.

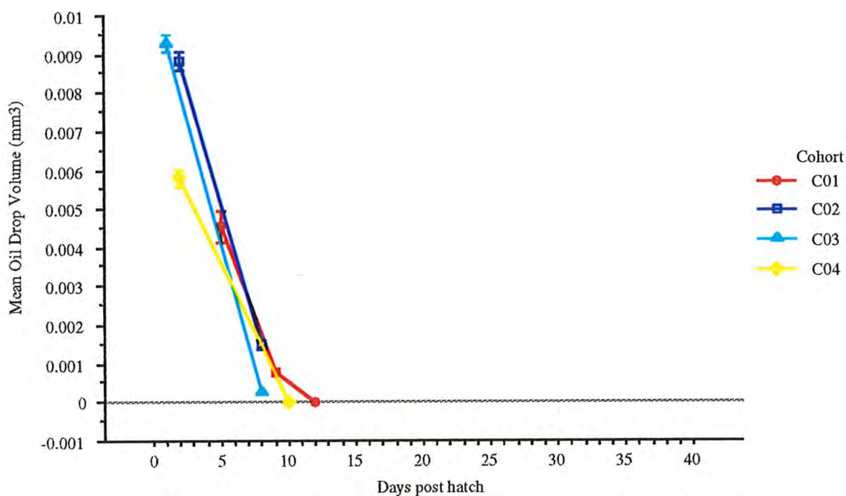

Figure 19. 1993 Oil drop volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

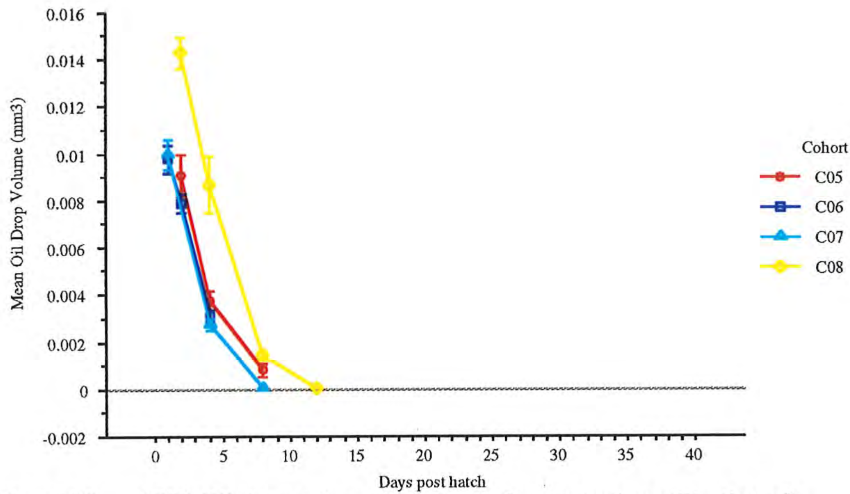

Figure 20. 1993 Oil drop volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

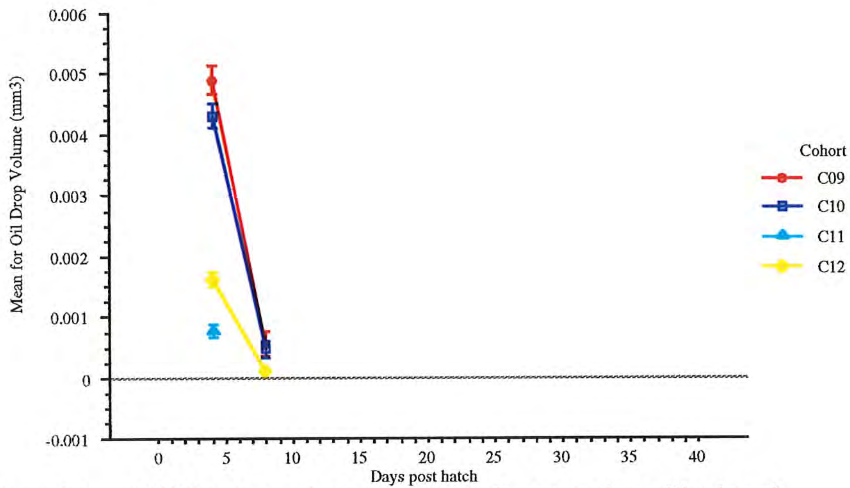

Figure 21. 1993 Oil drop volume for cohorts demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

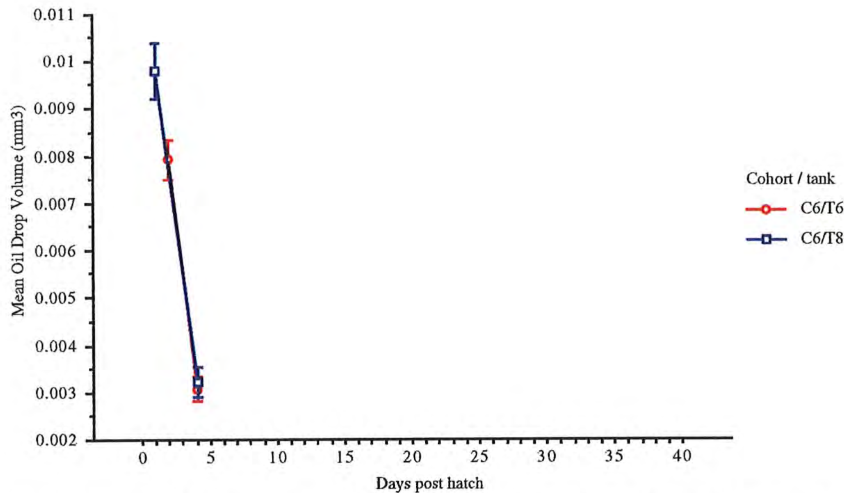

Figure 22. 1993 Oil drop volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

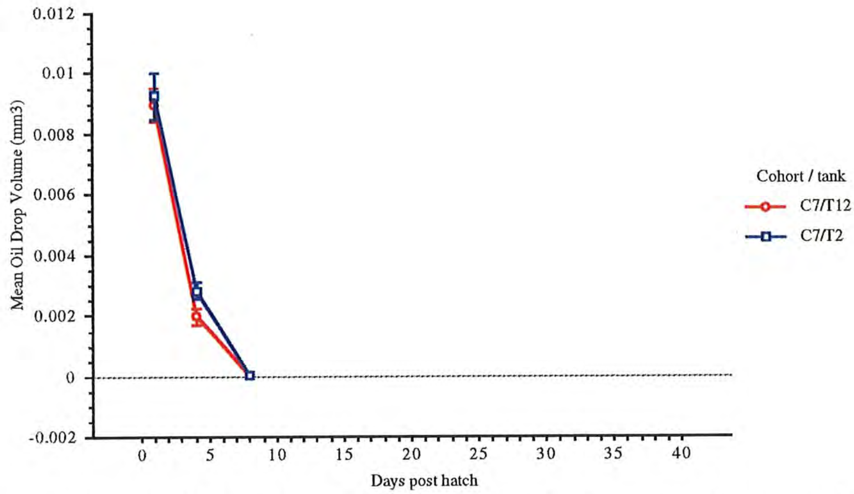

Figure 23. 1993 Oil drop volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

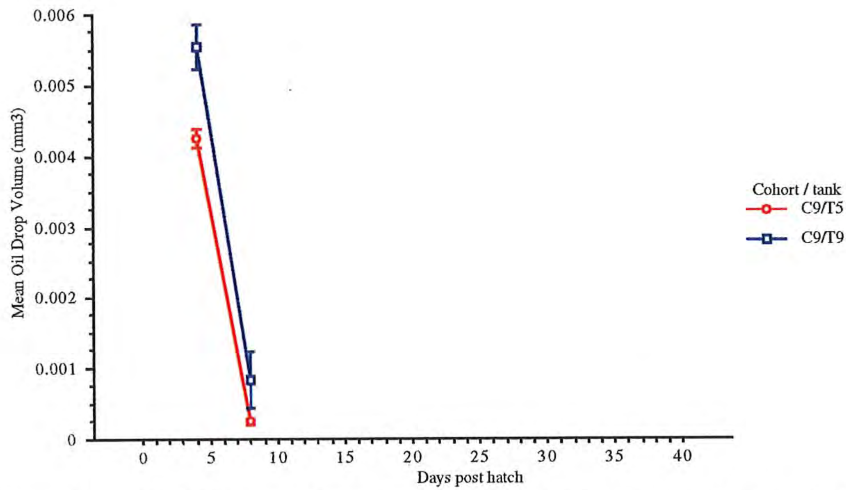

Figure 24. 1993 Oil drop volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

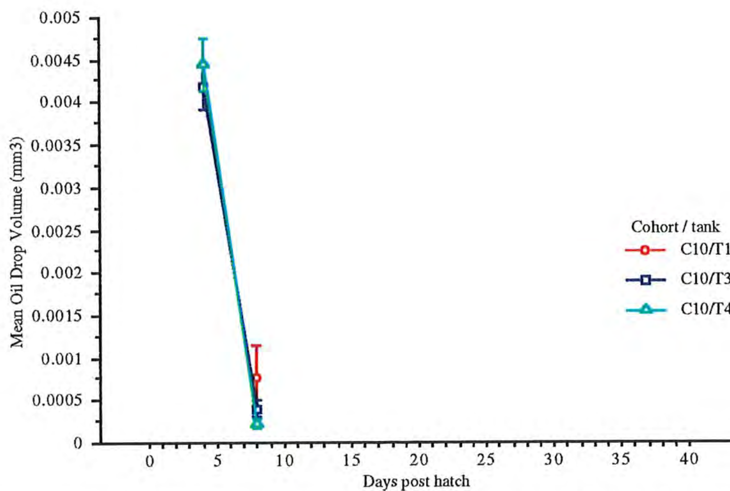

Figure 25. 1993 Oil drop volume for tanks containing larvae from the same cohorts, demonstrating utilisation of endogenous nutrition supplies. Each data point represents the Mean ± SE of 4 to 10 larvae.

Table 3. Details of 1993 cohorts and culture systems

Cohort	Date of hatch	Tank	Volume(L)	Maximum survival (days)
1	19.9.93	3	1000	38
2	21.9.93	4	1000	24
3	22.9.93	5	1000	24
4	25.9.93	2	1000	14
5	2.10.93	7	1000	24
6	3.10.93	6	4000	30
6	3.10.93	8	1000	32
7	7.10.93	2	1000	20
7	7.10.93	12	1000	17
8	26.10.93	10	1000	17
9	27.10.93	5	1000	25
9	27.10.93	9	1000	25
10	29.10.93	1	1000	22
10	29.10.93	3	1000	16
10	29.10.93	4	1000	25
11	6.11.94	7	1000	18
12	15.11.93	10	1000	8

Standard lengths for 1993 cohorts are presented in Figures 3 to 5, while standard lengths for tanks containing larvae of the same cohort are presented in Figures 6 to 9. Large standard errors for means of older larvae may be due to a small sample size; where less than 200 larvae survive in a tank, only two to three larvae were sampled

Larvae from 1992 and 1993 demonstrated similar growth and survival, and generally reached approximately 6mm by Day 25, however, significant mortalities occurred from this period onwards.

5.1.2 Yolk Sac Volume and Oil Drop Volume

Yolk sac volumes for 1992 and 1993 cohorts and tanks are presented in Figures 10 to 17. Generally, yolk sac volumes of larvae from the 1993 season were larger than those of larvae from the 1992 season, however, total depletion of the yolk sac occurred by Day 10 in both years. The volume of the yolk sac was not related to the growth and survival of larvae from each cohort in either 1992 or 1993.

Oil drop volumes for 1992 and 1993 cohorts and tanks are presented in Figures 18 to 25. Generally, volumes were greater in larvae from the 1993 season, while oil drop depletion occurred earlier in larvae from the 1992 season than in larvae from the 1993 season. No pattern between oil drop volume and survival was observed.

5.1.3 Percentage of Swimbladder Inflation

The percentages (%) of complete swimbladder inflation for each season are presented in Figures 26 and 27. During the 1992 season, cohorts 6 and 7

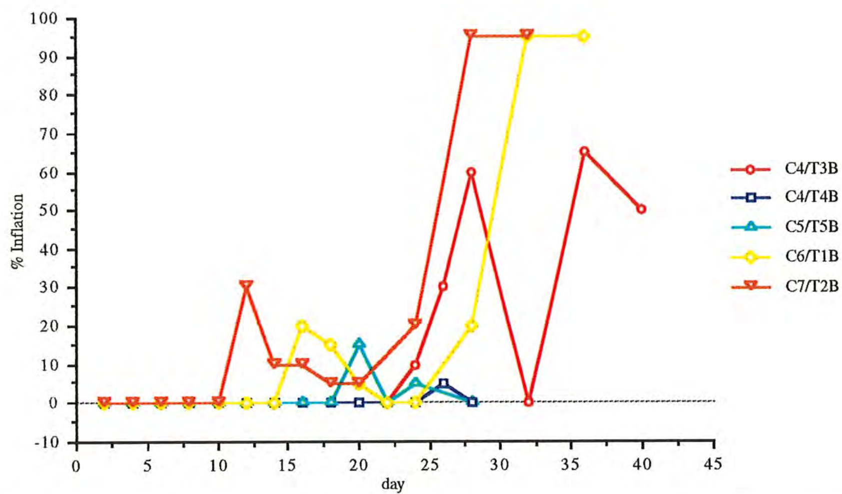

Figure 26. % Complete swimbladder inflation for cohorts (C) and tanks (T) in the 1992 rearing season. Each data point represents the incidence of inflation observed in 10 to 28 larvae.

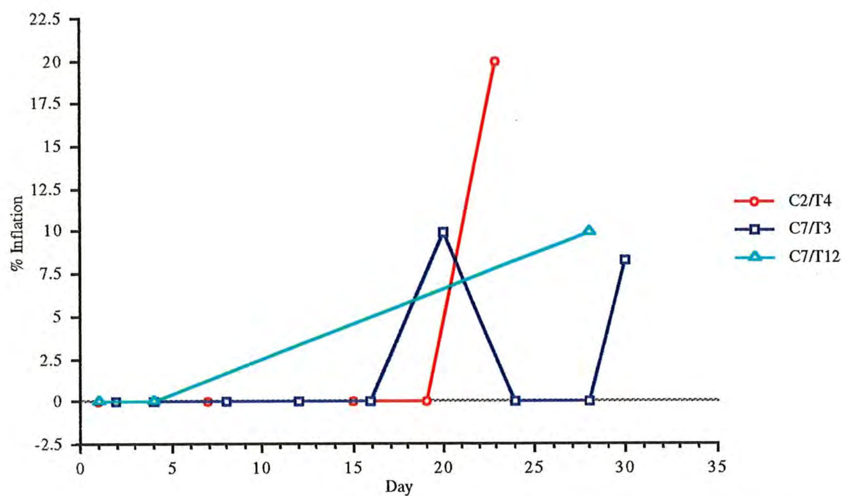

Figure 27. % Complete swimbladder inflation for cohorts (C) and tanks (T) in the 1993 rearing season. Each data point represents the incidence of inflation observed in 4 to 10 larvae.

demonstrated complete inflation in 95 % of larvae, while in the 1993 season, the maximum was 20% complete inflation in cohort 2. The variability of observed inflation in larvae from both seasons may be due to the difficulty experienced in viewing the swimbladder in older larvae, where it was either highly refractile or often obscured by chromatophores or muscle.

5.2 Histological studies

5.2.1 Swimbladder structure

Examination of several adult striped trumpeter revealed that the swimbladder is physoclistous (i.e. not connected by an open pneumatic duct to the gut) (Moyle and Cech, 1988). The swimbladder was located ventral to the kidney and dorsal to the gut. Histology of the swimbladder revealed structure similar to that of the intestine: serosa, tunica fibrosa, muscularis and epithelium, with a well developed vascular system supplying the organ.

In early larvae (Day 3 - 5), the swimbladder *anlage* was seen to "bud off" from the dorsal surface of the intestine. Prior to initial inflation, the anlage appears as a solid mass of columnar cells, with little or no lumen visible. An initial (1°) inflation occurred, where a bubble of air in the swimbladder lumen was often visible macroscopically. The appearance of the swimbladder epithelium changed irreversibly after expansion, when most of the epithelium flattens and becomes squamous (Plate 1). However, a small crescent of semi-columnar epithelium remains in the anterior section and comprises the secretory cells of the gas gland. Clear vacuoles observed in the cytoplasm of the gas gland cells may represent the generation of gas bubbles within the cells. The pneumatic duct leaves the gut via the dorsal surface of the junction of the oesophagus and the intestine (see Plate 2). The pneumatic duct then travels caudally, before bending dorsally to enter the posterior end of the swimbladder. The lumen of the pneumatic duct joins with the lumen of the swimbladder. While the pneumatic duct was found in larvae with non-inflated swimbladders at 30 days, it was not seen in larvae with completely inflated swimbladders, suggesting that inflation triggers the resorption / degeneration of the pneumatic duct. Some fish displayed a partially dilated duct when inflation began, which may have indicated the passage of the bubble of swallowed air.

In larvae which failed to inflate their swimbladder, the epithelium lining the lumen remained columnar, however, it was highly folded and often filled the entire swimbladder, obstructing the lumen (Plate 3). The folding appeared due to the

proliferation of the underlying vascular system, which pushed up into the base of the epithelium to form false villi with an underlying "*lamina propria*" -like structure.

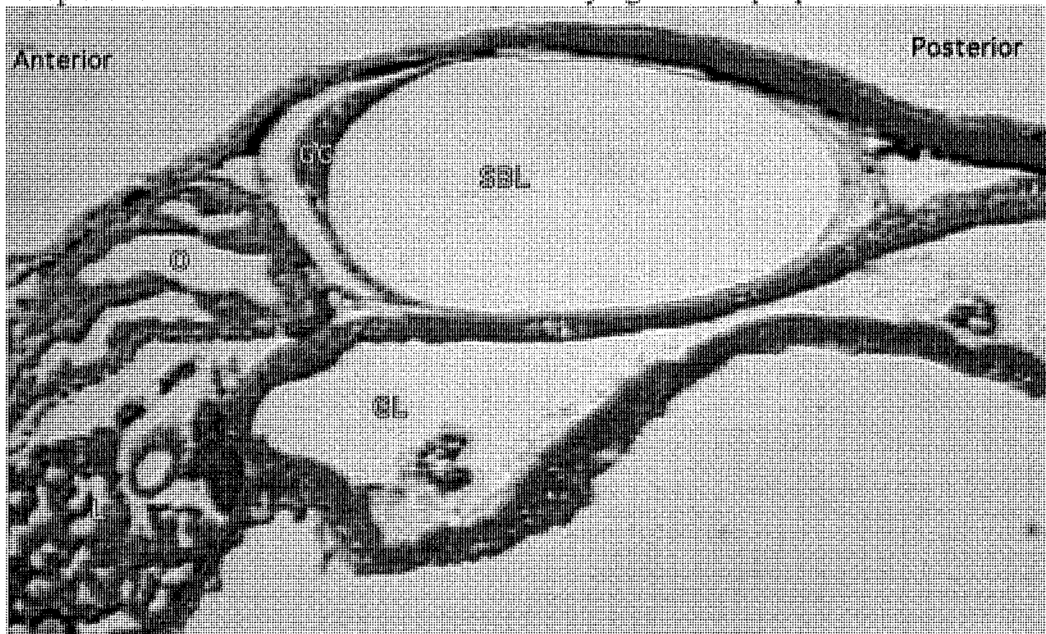

Plate 1. Detail of swimbladder displaying complete inflation. The ellipsoid shape and squamous epithelium are characteristic of a normal, fully inflated swimbladder. (GG, Gas gland epithelium; GL, Gut lumen; L, Liver; O, Oesophagus; SBL, Swimbladder lumen)

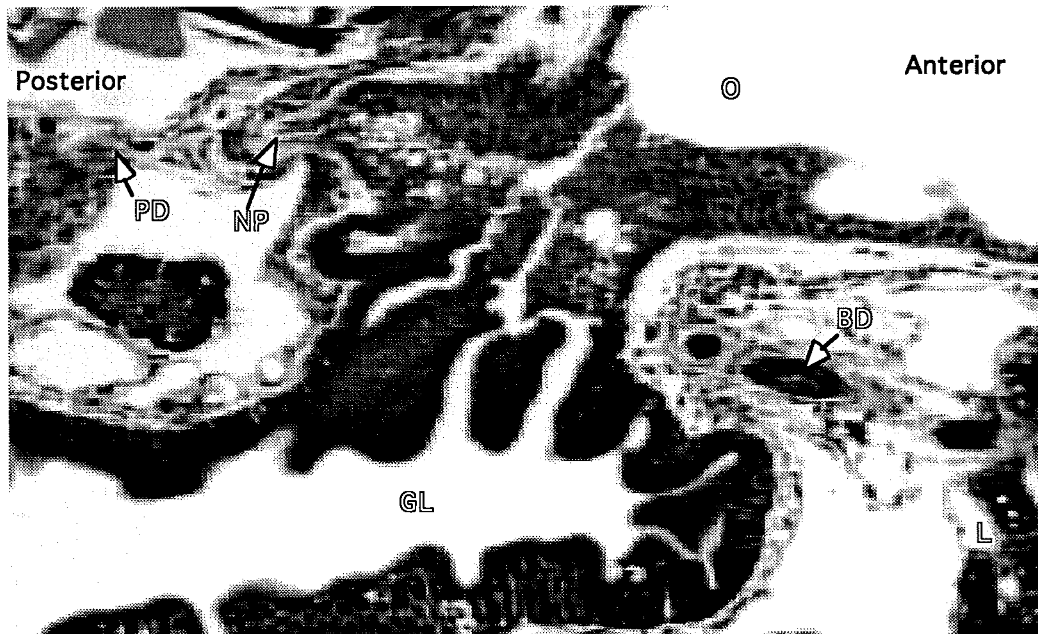

Plate 2. Location of the entry of the pneumatic duct into the digestive tract. The lumen of the pneumatic duct enters the dorsal surface of the digestive tract at the junction of the oesophagus and the intestine, directly opposite the lumen of the bile duct from the gall bladder and liver. (BD, Bile duct; GL, Gut lumen; L, Liver; O, Oesophagus; PD, Pneumatic duct; NP, neck of pneumatic duct)

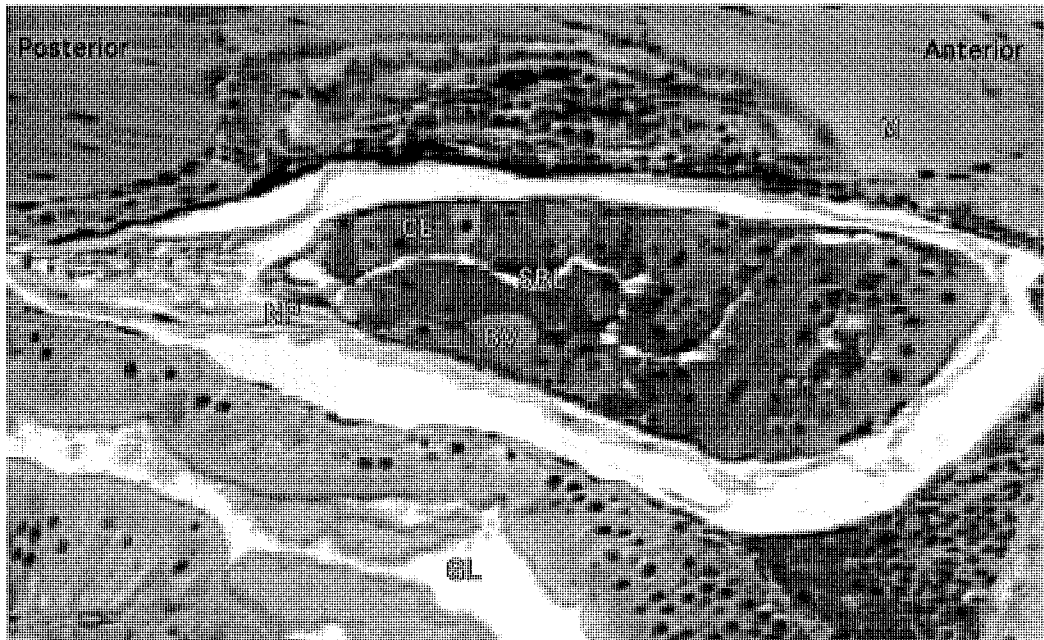

Plate 3. Non-Inflated swimbladder displaying typical, thick, folded columnar epithelium, with underlying blood vessels and a small lumen. (BV, Blood vessel; CE, Columnar epithelium; GL, Gut lumen; SBL, Swimbladder lumen; NP, neck of pneumatic duct; M, Muscle)

The pneumatic duct of fish with uninflated swimbladders was found to persist to Day 32 and may possibly remain longer, however, on Day 24 in some larvae, signs of degeneration of the duct were seen.

5.2.2 Timing of swimbladder inflation

Examination of sagittal sections of larvae revealed that primary (1°) swimbladder inflation occurred before first feeding (Day 6 in 1992; Day 8 in 1993). In larvae from the 1992 season, various stages of inflation were seen during Days 7 to 10, however, after Day 10, swimbladders were either inflated or uninflated with folded epithelium (see Table 4).

Similar observations were made with larvae from the 1993 rearing season (see Table 5) and bacteria were observed in the lumen of uninflated swimbladders in some larvae. Swimbladder inflation was observed to occur during a 4 - 5 day period, but at a later day post hatch, due to the lower incubation temperature. After this time, even though the larvae had access to the water surface and the pneumatic duct remained open, inflation did not occur and the *rete mirabilis* proliferated, causing the columnar epithelium to fold and fill the bladder lumen.

Table 4. 1992 Histological stages observed in larval swimbladders

Days Post Hatch	Swimbladder Stage	Pneumatic duct
2	anlage	
3	anlage	
4	anlage	
5	anlage	open
6	1°, anlage	
7	1°, partial 2°	open
8	partial 1° & partial 2°	open
9	1° inflation, partial 2°, non inflated, folded	
10	partial 2°, non inflated, folded	
11	non inflated, folded	
12	non inflated, folded	open
13	non inflated, folded	open
14	non inflated, folded	
15	non inflated, folded	open
16	non inflated, folded	open
17	non inflated, folded	open
18	non inflated, folded; inflated, collapsed	open
19	non inflated, folded	
21	non inflated, folded	
24	non inflated, folded	degenerating

Table 5. 1993 Histological stages observed in larval swimbladders

Days Post Hatch	Swimbladder Stage	Pneumatic duct
10	1°	open
11	1°	none visible
12	1°	open
13	2°	no duct
	non-inflated, folded	open
14	2°	duct degenerating
15	non-inflated, folded	open
17	non-inflated, folded	open
18	non-inflated, folded	open
19	2°	no duct
	non-inflated, folded	open
20	non-inflated, folded	no duct, open
21	1°	open still connected to gut.
	non-inflated, folded	
22	2°	no duct
	non-inflated, folded	open
23	non-inflated, folded	open
24	non-inflated, folded	open
30	2°	no duct
	non-inflated, folded	open

5.2.3 Stomach development

The presence of a stomach was not detected in larvae reared at Taroona during the 1992 and 1993 seasons. Mr. W. Hutchinson of UTAS, Launceston, supplied a small number of juveniles cultured in his project for evaluation. In these samples, the stomach was seen to appear between Days 50 and 60 post hatch (S.L. 11-12 mm). However, the culture conditions under which his fish were reared are unknown. It may be that the appearance of the stomach is not related to age, but to size (standard length) of the animal. Evaluation of striped trumpeter larvae and juveniles from the 1994 and subsequent rearing seasons is required to determine if this is so.

5.3 Histochemical studies

5.3.1 Enzyme activities

The larvae from the 1992 trials were preserved in fixative containing methanol (a stabiliser in commercially available formalin), which was found to inhibit enzyme activity and was thus unsuitable for use in enzyme studies.

Larvae from the 1993 rearing season were preserved in methanol-free fixative and gave variable results at all ages for all the stains trialled. Pooled results for all cohorts are presented in Table 6, as all cohorts appeared to display similar enzyme activity. The location of the stain reaction is also detailed in Table 6. When no reaction could be detected in striped trumpeter samples, methods were checked using fresh fixed tissue from greenback flounder (*Rhombosolea tapirina*) and banded morwong (*Cheilodactylus spectabilis*). Results of these trials are presented in Tables 7 and 8.

While the strength of stain reaction products was variable, reactions were detected for all enzymes and substances except Lipase, Maltase and Lipids. Acid Phosphatase demonstrated the strongest staining reaction in the yolk sac of the banded morwong. The variability in the stain reaction was noted between individuals of the same age, irrespective of cohort or tank.

Table 6. Enzyme and substance stain reactions for striped trumpeter larvae

Stain & age of larvae (days post hatch)	Intestinal Mucosa[a] Foregut	Midgut	Hindgut	Other tissues stained positively[b]
Acid Phosphatase (ACP)				
0-1	0	0	0	0
2 to 8	0	0-2	0	YS 0-4
11 to 32	0	0	0	0
Alkaline Phosphatase (ALP)				
1 to 12	0	0	0	0
16 to 21	0-2	0	0-3	0
22 to 32	0-1	0	0	0, P 0-1, K 2,
Aminopeptidase-M (AMP-M)				
1 to 8	0	0	0	0
11 to 16	0-3	3	3	0
17 to 32	0-2	0-2	0-2	0
Lipase				
0 to 32	0	0	0	0
Lipids				
0 to 32	0	0	0	0
Maltase				
0 to 32	0	0	0	0
Non-Specific Esterase (NSE)				
1 to 13	0	0	0	0
16 to 32	0-1	0-3	0-1	0
PAS (Glycogen)				
2 to 4	0	0-3	0	YS 0-3
7 to 10	0-3	0-3	0-3	YS 0-4, L 2-4
11 to 32	0-3	0-3	0-2	L 0-4
Trypsin				
2 to 11	0-2	0-2	0-2	0
12 to 1 6	0-3	0-3	0-3	L 0-1, P 0-3
17 to 32	0-2	0-2	0-2	0

a Staining reaction strengths 0 = Negative, 1 = weak variable , 2= weak, 3 = moderate, 4 = strong.
b Other tissues stained positively : L, Liver; P, Pancreas; K, Kidney; YS, Yolk Sac.

Table 7. Enzyme and substance stain reactions for Greenback Flounder larvae.

Stain & age of larvae (days post hatch)	Intestinal mucosa[a] Foregut	Midgut	Hindgut	Other Tissues Stained positively[b]
Acid Phosphatase (ACP)				
2 to 6	0	0	0	YS 0 - 3
8 to 34	0	0	0	L 0 - 1
Alkaline Phosphatase (ALP)				
2 to 6	0	0 - 2	0	0
8 to 10	0 - 3	0 - 2	0 - 2	K 0 - 3
12 to 34	0 - 4	0 - 4	0 - 4	K 0 - 4, L 0 - 3
Aminopeptidase-M (AMP-M)				
2 to 6	0	0	0	0
8 to 34	0 - 4	2 - 4	0 - 4	K 0 - 1
Maltase				
2 to 14	0	0	0	0
34	0 - 3	0 - 3	0	0
Lipase				
0 to 34	0	0	0	0
Lipids				
0 to 34	0	0	0	0
Non-Specific Esterase (NSE)				
2 to 10	0 - 4	0 - 4	0 - 4	0
12 to 34	0 - 4	0 - 4	0 - 4	P 0 - 3, L 0 - 1
PAS (Glycogen)				
8	0	0	0	L 0 - 1
10 to 34	0 - 3	0 - 3	0 - 2	L 0 - 4
Trypsin				
2 to 6	0	0	0	0
8 to 34	0 - 4	0 - 4	0 - 4	0

a Staining reaction strengths 0 = Negative, 1 = weak variable , 2= weak, 3 = moderate, 4 = strong.
b Other positively stained tissues: L= Liver, P = Pancreas, K = Kidney, YS = Yolk Sac.

Table 8. Enzyme and substance stain reactions for Banded Morwong Larvae

Stain & age of larvae (days post hatch)	Intestinal mucosa[a] Foregut	Midgut	Hindgut	Other tissues stained positively[b]
Acid Phosphatase (ACP)				
1 to 9	0	0	0	YS 2 - 4
Alkaline Phosphatase (ALP)				
0 to 11	0 - 4	0 - 4	0 - 4	L 0 - 2, K 0 - 4, YS 0 - 2
Aminopeptidase-M (AMP-M)				
3 to 5	0 - 1	0 - 1	0	0
Non-Specific Esterase (NSE)				
3 to 11	0 - 3	0 - 4	0 -	0
PAS (Glycogen)				
3 to 5	0 - 3	0 - 3	0 - 3	L 0 - 3, YS 0 - 2
Trypsin				
3 to 5	0 - 2	0 - 2	0	0

a Staining reaction strengths 0 = Negative; 1 = weak variable; 2= weak, 3 = moderate; 4 = strong.
b Other tissues positively stained : L= Liver; P = Pancreas; K = Kidney; YS = Yolk Sac.

5.3.2 Nutritional studies

Methods for these studies were developed using 1992 larvae, however, little replication was possible and the differences between diets could not be tested for significance. Enterocyte heights of larvae from the 1993 rearing season were tested from each cohort and tank at Days 12, 16, and 20 post hatch. Mean enterocyte heights for cohorts and tanks, as well as the results of statistical analyses are presented in Table 9. Significant differences were detected between the mean enterocyte heights at the P (<0.05) level for cohorts 7 and 10, run in tanks 12, and 1, 3 and 4 respectively.

Cohort 7 demonstrated a significant decrease between Days 12 and 20 (Day 16 was not tested) in tank 12. Differences between tanks were not tested due to a lack of samples at different Days in other tanks. The decrease in condition reflected an earlier slowing in growth, as shown in Figure 4. While no significant differences were detected in cohort 9, cohort 10 demonstrated significant differences between both Days and tanks. Although tank 3 demonstrated the greatest enterocyte height on Day 12, this was not reflected in the growth curves, as larvae in this tank were generally smaller and grew more slowly than those in the other two tanks. However, tank 1 demonstrated a significant increase in enterocyte height between Days 12 and 16, which was reflected in the rapid growth curve; Day 20 was not analysed. Tank 4 also demonstrated a significant increase in enterocyte heights between Days 12 and 16, however, the increase was negligible between Days 16 and 20 and reflects an earlier slowing down in the rate of growth.

There appears to be a lag between the rate of change of larval growth and the rate of change of enterocyte height, when Table 9 and Figures 3 to 9 are viewed together. However, the relationship was not consistent, which may have been due to the use of separate samples for the S.L. measurements and enterocyte height measurements. The changes in enterocyte heights demonstrated in cohorts 1, 2, 3, 5, and 6 were not significant.

Table 9. Mean enterocyte heights (μm) for cohorts and tanks in 1993 larvae and the level of significance of differences between days and tanks

Cohort	Tank	Day	Mean enterocyte ht (μm).	Level of significant difference between days (P<0.05)
1	3	12	22.04	Not significant
1	3	16	19.95	Not significant
1	3	20	19.79	Not significant
2	4	12	20.07	Not significant
2	4	16	20.52	Not significant
2	4	20	21.12	Not significant
3	5	16	18.91	Not significant
3	5	20	23.25	Not significant
5	7	12	18.89	Not significant
5	7	16	15.07	Not significant
5	7	20	18.98	Not significant
6	6, 8	12	16.25	Not significant
6	6, 8	16	18.46	Not significant
6	6, 8	20	16.59	Not significant
7	2, 12	12	18.45	days, P=0.028
7	2, 12	20	12.09	days, P=0.028
9	5, 9	12	17.42	Not significant
9	5, 9	16	16.69	Not significant
9	5, 9	20	21.21	Not significant
10	1	12	16.23	days, P=0.028; tanks, P=0.009
10	1	16	19.87	days, P=0.028; tanks, P=0.009
10	3	12	22.61	days, P=0.028; tanks, P=0.009
10	4	12	15.96	days, P=0.028; tanks, P=0.009
10	4	16	21.50	days, P=0.028; tanks, P=0.009
10	4	20	21.32	days, P=0.028; tanks, P=0.009

5.4 Abnormalities / diseases

Larvae from two tanks in the 1992 rearing trials were observed to have abnormal epithelial layers (Barry Munday, personal communication), both internally and externally. "Blisters" were in the external epidermis were extensive and separation of the epidermis from the dermis was observed (see Plate 4). Plate 5 presents normal appearance of the dermis and epidermis of a larva of similar age. The origin of the abnormality was of unknown aetiology, as no bacteria, fungi or parasites were observed in histological sections and TEM studies failed to reveal the presence of viral particles.

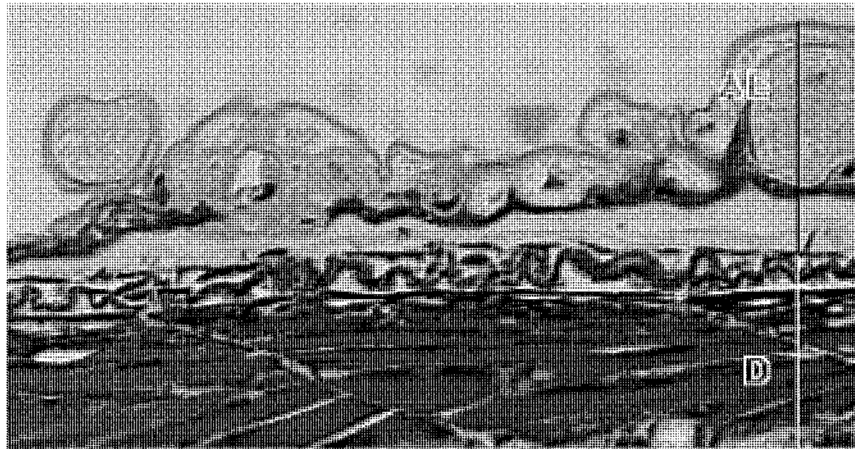

Plate 4. Abnormal epidermis of larva from 1992 rearing season (age, 19 days post hatch). Note the delamination of the epidermis (AE) from the dermis (D)

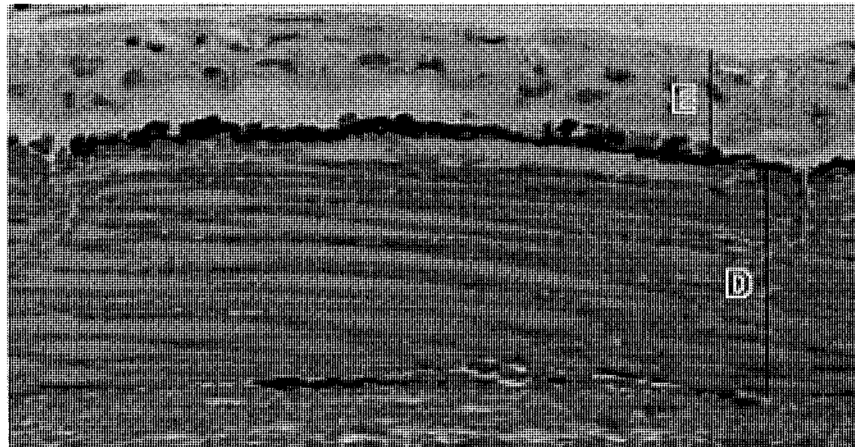

Plate 5. Normal epidermis and dermis of larva from 1994 season (age, 21 days post hatch). No abnormalities of the epidermis, or delamination of epidermis from dermis is visible.

During morphological assessment, larvae from both seasons were observed to have jaw and tail abnormalities, as well as a calculus in the urinary bladder (See Tables 11 and 12). Further study is required to determine the cause and significance of both these features.

Table 11. Percentages of abnormalities seen during the morphometric assessment of larvae from the 1992 season (n is the number of larvae observed).

Cohort	Tank	Day	n	% Jaw	% Tail	% Calculi
3	2A	14	20	15	None noted early during the season	None noted during the season
3	2A	15	20	30		
3	2A	16	17	18		
3	2A	17	25	4		
3	2A	18	27	19		
3	2A	20	21	19		
3	2A	23	26	38		
3	2B	14	20	20		
3	2B	15	20	25		
3	2B	16	27	11		
3	2B	18	23	17		
3	2B	20	29	7		
3	2B	23	25	36		
3	4A	13	20	15		
3	4A	14	28	7		
3	4A	16	20	15		
3	4A	17	22	5		
3	4A	18	25	16		
3	4A	20	23	26		
3	4A	23	25	28		
3	4B	14	21	14		
3	4B	15	20	35		
3	4B	16	28	7		
3	4B	17	26	15		
3	4B	18	22	18		
3	4B	20	28	14		
3	4B	23	25	28		
3	5A	15	20	20		
3	5A	16	21	19		
3	5A	17	21	29		
3	5A	18	18	11		
3	5A	20	26	31		
3	5A	23	25	20		
3	5B	15	20	20		
3	5B	16	28	4		
3	5B	18	27	33		
3	5B	20	23	30		
3	5B	23	26	15		
3	6A	15	20	5		
3	6A	16	20	10		
3	6A	17	24	13		
3	6A	18	25	24		
3	6A	20	20	15		
3	6A	23	25	24		
4	4B	10	18	67		
4	4B	12	20	35		
5	3B	20	20	5		
5	3B	38	18	6		
5	5B	8	20	25		
5	5B	22	21		5	
6	1B	12	20	5		
6	1B	18	20	5		
6	1B	32	20	75		
6	1B	36	20	50		
7	2B	8	20	5		
7	2B	10	20	40		
7	2B	12	20	15		
7	2B	14	20	5		
7	2B	18	20	10		
7	2B	20	20	10		
7	2B	28	20	5		
7	2B	32	20	45		

Table 12. Percentages of abnormalities seen during the morphometric assessment of larvae from the 1993 season (n is the number of larvae observed).

Cohort	Tank	Day	n	% Jaw	% Tail	% Calculi
1	3	12	10	10		
1	2 (from 3)	38	10			10
2	4	7	10			10
3	5	11	10	10		10
3	5	15	10			10
4	9	10	10			20
4	9	14	10	10		
5	7	8	10	60		
5	7	20	10	10		
5	7	24	5	20		
6	6	16	10	10		10
6	6	24	10			10
6	6	30	2			50
6	8	8	10	10		
6	8	24	10	10		
6	8	32	9	33		
7	2	4	10		10	
7	2	8	10	10		
7	2	12	10		10	10
7	2	14	10		10	
7	9	16	7			14
7	11	28	10	20	20	
7	12	12	10		10	
7	12	32	8		13	
8	10	4	10		10	
8	10	8	10		10	
8	10	16	10			10
9	5	12	10			20
9	5	14	10		10	20
9	5	18	5			20
9	5	24	6			16
9	9	8	10	20		
9	9	12	10	10		20
9	9	16	10			20
9	9	20	9	11		11
10	1	14	10			10
10	1	16	5			20
10	1	20	5			20
10	3	4	10		10	
10	3	12	10		10	10
10	3	14	10	10		10
10	4	12	10		10	
10	4	16	10	10		40
10	4	24	7			14
11	7	8	7	29		
11	7	16	6			16
11	7	18	7			14

6 Discussion.

Morphological studies were used to monitor the growth and development of larvae from both the 1992 and 1993 rearing seasons. Mean standard lengths plots were used during the seasons to monitor the performance of larval cultures by tanks and cohorts. Similarities in the survival and growth of larvae from both seasons (approximately day 40 and 7.4 to 7.7mm S.L.) suggests that the culture conditions and diets in use each season were not meeting the larval requirements, or that in each season, the quality of some egg batches was less than optimal, or a combination of the above. The appearance of features (e.g. swim bladder, stomach) may be dependent on either size, and hence the temperature at which larviculture is carried out, or age or both.

Several factors influencing the failure of larval swimbladder inflation have been reported: lack of access to the water-air interface due to turbulence, oil film or

obstruction (Chatain and Merea, 1989, Doroshev and Cornacchia, 1979; Hoss and Phonlor, 1984), incorrect photoperiod or light intensity (Battaglene and Talbot, 1993), high surface tension preventing larvae from gulping air (Searle, unpublished data).

The swimbladder structure of the larval and adult striped trumpeter has been described for the first time. The adult form lacks a pneumatic duct, while the larvae possess a pneumatic duct for a short period, which indicates that larvae require access to the water air interface for primary inflation of the swimbladder to occur. Degeneration of the pneumatic duct of larvae with non-inflated swimbladders was seen from Day 24, however, the duct was still present at Day 32 in other larvae and may persist longer (Grizzle and Curd, 1978). A pneumatic duct was not found in larvae with a completely inflated and expanded swimbladder, indicating that full inflation triggers the rapid atrophication of the duct.

Differences in the percentages of larvae demonstrating complete inflation varied between cohorts and tanks in both seasons. The relatively low incidence of inflation in both trials may be due to the build up of an oil film on the water surface from the live feeds preventing larvae from gulping a bubble of air for initial inflation of the swimbladder, or difficulty in observing the swimbladder through the pigmentation and muscle layers. In future trials, the determination of percentage swimbladder inflation by histology would be more accurate.

In the 1992 season, skimming to clean debris and the oil film from the water surface commenced at Day 8 after hatch, causing significant mortalities in all tanks within 24 hours. However, in cohorts 4, 5, 6 and 7, the previous method of skimming was not employed; instead, aeration with fine bubbles provided an area free of surface film and which did not damage the larvae, resulting in good inflation in some tanks. The design of the skimmers used in the 1993 season was altered and skimming commenced in selected tanks on Day 4 and other tanks were not skimmed. Surface skimming in all previous seasons was highly variable between skimmers and generally not totally effective in reducing surface films. In the 1993 season, inflation was achieved in cohorts 2 (tank 4) and 7 (tanks 3 and 12); of these, tanks 4 and 12 were not skimmed while skimming commenced on Day 15 in tank 3. Percent inflation in the 1992 season was greater than in the 1993 season. The maximum levels achieved were: 95% in cohorts 6 and 7 during 1992, while only 20% was achieved in cohort 2 in 1993, which was comparable to the 1991 season in which the general percent inflation was 25%, however, more than 60% inflation was

achieved in one tank (Searle, data not presented). The results were better than those of the 1989 season in which no inflation was detected (Ruwald *et al.*, 1989).

Histology of the uninflated swimbladder in larval striped trumpeter is similar to that described for the logperch (*Percina caprodes*), (Grizzle and Curd, 1978), striped bass (*Morone saxatilis*) (Bennett *et al.*, 1987; Bulak and Heidinger, 1980; Doroshev et al., 1981), sea bream (*Sparus auratus*) (Paperna, 1978). Chatain (1994) reported that sea bream weighing 7 - 54 g which had failed to initially inflate and expand their swimbladders during the larval stage, were capable of a secondary inflation of the swimbladder. The development of the swimbladder, its mode and timing of initial inflation in the striped trumpeter appears similar to that of the sea bream (Yamashita, 1982). Whether striped trumpeters with non-inflated swimbladders are capable of late secondary inflation has not been determined, as until the 1994 larval rearing season, few juvenile striped trumpeter have been produced. Further studies of striped trumpeter juveniles during growout trials in subsequent rearing seasons at the DPI&F facility at Taroona will be required to determine if the species possesses this ability. Improvements in skimmer design, tank hydrodynamics (aeration) and water quality (especially dissolved gases), as well as the use of low light levels will be used in an attempt to solve the low swimbladder inflation rate and associated problems (i.e. low levels of energy stores).

Some of the mortalities in 1992 were probably due to the increased stresses caused by the abnormal epithelium. Larvae have a large surface area relative to volume and in early larvae (Tytler, *et al.*, 1993). Early larvae lack gills and the uptake of O_2 and CO_2 occurs via the skin (Blaxter, 1969), while the gut of larval herring, plaice and cod has been found to be active in osmoregulation (Tytler and Blaxter, 1988). It is likely that larval striped trumpeter function similarly to other larval marine teleosts and impairment of the internal and external surfaces of the animal would have deleterious effects on homeostasis and respiration, increasing the energy required to maintain homeostasis and cause an energy depletion for larvae which had been compromised by poor nutrition, sub-optimal rearing conditions or were from low quality egg batches.

The occurrence of urinary calculi and jaw abnormalities was noted in some larvae from samples during morphological assessment. Separate samples of larvae were processed for histological and histochemical study and correlation of histology with the morphology was not possible.

The differences in yolk sac volumes between larvae from the 1992 and 1993 seasons may be due to differences in the source and condition of broodstock from each year, however, it is difficult to make any conclusions on this point, because broodstock were sourced from the east and west coasts of Tasmania and during the 1993 season, fish from the 1992 season held in seacages were also used. Eggs from different broodstock were often pooled to obtain sufficient numbers for trials and the detailed analysis of the differences noted was not possible. Condition of the broodstock would be affected by factors such as: nutrition, duration of holding, stresses of handling and holding.

The detection of ALP, AMP-M, NSE and trypsin indicates that larvae are capable of breaking down food to macromolecules, absorbing and digesting the molecules intracellularly. AMP-M and trypsin are both involved in the breakdown of proteins to macromolecules for absorption. The presence of intracellular as well as extracellular stain product for trypsin in the enterocytes of the hindgut indicates that this is the site of absorption of protein macromolecules, supporting the hypothesis of Pedersen and Hjelmeland (1988) that larvae do not excrete enzymes, but rather retain them by absorption or resorption. The presence of NSE indicates that larvae are able to absorb lipid macromolecules for intracellular digestion. The lipids are first emulsified by bile secreted from the gall bladder, broken down by NSE, before absorption and digestion.

The variability in the stain strength for digestive enzymes in larval striped trumpeter, both within and between daily samples for all cohorts, may have been due to differences in the production of enzymes by individuals as well as individual differences in feeding at the time of sampling. The contribution of digestive enzymes by live feed to the digestive processes of the larval striped trumpeter has not been determined, however, Munilla-Moran *et al.* (1990) report that 89-94% of the total esterase activity and 43-60% of protease activity in the gut of cultured larval turbot was derived from exogenous sources.

Glycogen deposits, of variable strength, were detected in the hepatocytes of the liver of all three species investigated. In addition, PAS positive substances were found in the basal area of enterocytes. The variability of liver deposits may have been an indication of excess energy requirements of fish with non-inflated swimbladders caused by constant swimming to maintain position in the water column, or it may reflect a diet providing insufficient energy for the animals needs.

The lack of stain reactions for lipase in striped trumpeter and greenback flounder may be normal for larval marine teleosts, as Cousin *et al.* (1987) found no lipolytic activity in larval turbot until the onset of metamorphosis. The detection of maltase activity in flounder at Day 34, but not in striped trumpeter at Day 32 suggests that this enzyme also appears after the onset of metamorphosis. Studies of juvenile striped trumpeters will be required to determine the mode of utilization of lipid and carbohydrate nutrients.

The lack of stain reactions for lipids may be due to the resin processing itself. Glycol methacrylate is an extractive substance, despite its water solubility. The relatively large oil droplets of the yolk sac larvae were probably not well infiltrated and may have fallen out of the sections during cutting, while small lipid droplets within tissues may have been extracted by the infiltration medium even though processing times were kept short (Cope and Williams, 1969; Gerrits *et al.*, 1987).

Enterocyte heights appeared to reflect a slowing down in growth or a loss of condition (negative growth) several days after it was demonstrated in the standard length plot, however, the effect was not constant and in some tanks was not demonstrated. This variation was probably due to the use of separate samples for morphological and histological assessment. A more accurate evaluation would be ensured if the one sample were used for both methods of assessment, but this may severely compromise the histological and histochemical evaluation of samples.

Significant findings from this research which have assisted in the refinement of larval culture techniques for striped trumpeter include:

1. Determination of low swimbladder inflation rates indicating that this should be a primary focus for future research.

2. Identification of the method of swimbladder inflation and the determination of the timing of the inflation "window", which has allowed significant refinement of methods employed to promote inflation (i.e. the earlier onset of surface skimming).

3. Determination of low energy reserves and inadequate proliferation of enterocytes, suggesting inappropriate nutrition and / or an energy deficit (perhaps caused by other factors such as the lack of a functional swimbladder, inappropriate rearing temperature or inappropriate prey size).

4. Identification of larval deformities with suggestion of probable causes. (i.e. incorrect incubation temperature, temperature shock, inappropriate nutrition and gas supersaturation.

While much of the first year of the project was spent in the evaluation and development of reagents and methods, the second season saw the application of methods in the assessment of material from the larval rearing trials. The project has made several valuable contributions in the area of basic biology of larval striped trumpeter. The knowledge gained by the project has resulted in the modification of larviculture techniques and equipment, resulting in the successful rearing of a significant number of larval striped trumpeter beyond metamorphosis during the 1994 rearing season. The techniques developed in 1994 were repeatable and with further diet and photoperiod manipulations, improvements in survival were achieved.

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APPENDICES

APPENDIX 1

SECTION 1 - PROJECT TITLE

Histological, Histochemical and Morphological Development of Striped Trumpeter (*Latris lineata*) Larvae and the Effect of Larval Diet.

SECTION 2 - KEYWORDS

Larval, development, diet, finfish.

SECTION 3 - OBJECTIVES

1. Characterise the histological development of larval/juvenile striped trumpeter under controlled culture conditions noting abnormalities and the effect of dietary manipulation.

2. Characterise the histochemical development of larval/juvenile striped trumpeter under controlled culture conditions noting abnormalities and the effect of dietary manipulation.

3. Characterise morphological development of larval/juvenile striped trumpeter under controlled culture conditions noting abnormalities and the effect of dietary manipulation.

4. Determine the "boosted" nutritional profile of rotifers and *Artemia* which are used as larval feeds.

5. Identify the nutritional problems encountered during the larval rearing of striped trumpeter and evaluate dietary solutions which would allow mass culture of this species.

SECTION 4 - JUSTIFICATION

The Tasmanian sea-cage salmonid industry generates more than $40 million P.A. Around half of the production is exported and the industry contributes significantly to the state economy while employing more than 400 people in rural areas.

The salmonid industry worldwide is experiencing major marketing problems due to an oversupply of product (Needham, 1989). Every salmonid producing country of note has adopted a strategy of new species development as one method of ensuring that their fin fish aquaculture industry maintains its viability into the future. These new species are white fleshed fish. It has been demonstrated that the world will experience a shortage in the supply of white fish in the near future. This shortfall in supply will be caused by declining wild catches in the face of steadily increasing demand. European market research predicts that the UK market for fresh fish and seafood will expand by 26% in the next year with a total value of approximately AUD $1,277m (Urch, 1991). Associated with the "white fish gap" will be steadily increasing prices (Gutting 1990). Finfish aquaculture provides the best long term solution to this problem (Larkin 1990).

Aquaculture currently accounts for 10 per cent of world fish, crustacean and seaweed consumption. The OECD estimates that this will rise to 50 per cent by the next decade (McKinnon et al., 1989). Presently within the EEC the aquaculture production of seabass and seabream is equal to the fisheries catch. By 1995 it is predicted that aquaculture will produce more than double the fisheries catch (Harache, 1991). This assumes that the fisheries catch is stable, not declining as is the case.

The opportunities for aquaculture in the 1990's are great and Tasmania is already a leading aquacultural centre. Mariculture industries based on salmonid and oyster farming have been established by innovative R & D programs. There is large potential for the development of a high-value white fleshed finfish mariculture industry in Tasmania and other states including South Australia and New South Wales.

There is therefore a clear need to undertake developmental research in order to evaluate both the biological and economic feasibility of the mariculture of the Striped Trumpeter and other native marine finfish. The development of native marine finfish for aquaculture is currently an active area of research worldwide. Two main factors are

responsible for the belief that finfish aquaculture will be a viable and sustainable industry of the future:

1. The wild fishery resource is depleted and catch effort is increasing.

In the case of some species the effect is felt by the consumer as an increase in price. The price of most premium table fish in Australia is now critically close to the point where aquaculture can produce fish, make a profit and compete with the wild fishery in terms of quality. Overseas (notably Europe) viable finfish culture industries based mainly on seabream and seabass will soon produce more than the wild fishery. In terms of the quality and continuity of supply, the cultured fish product has little competition.

Other fishery stocks have been fished beyond sustainable limits and controls ranging from severe limits of catch to total closure of the fishery have been imposed. With species such as these, aquaculture provides the only method for future supply. Where premium white fleshed table fisheries have been severely depleted there has been an associated increase in the price of the fish correlated to the the consumer demand for the species. This has created further opportunity for finfish aquaculture.

2. Consumer demand is steadily increasing for high quality white fleshed fish products.

Consumer demand is driven in Australia mainly by the health promoting properties of fish. Between 1984 and 1990 fish consumption in Australia has increased dramatically by 25.4% per capita. The most significant trend with respect to the type of fish preferred was demonstrated by a massive 117% increase in the consumption of Australian white fleshed fish. The per capita consumption of crustacea and molluscs over the same period has shown a gradual decrease (Skurray and Saee, 1991). Perhaps more importantly for Australian producers are the demographic trends in our Asian neighbouring countries. The population of most S. E. Asian countries is large by any comparison with Australia. Traditionally fish is eaten as both a staple part of the diet and reserved for special occasions. Fish consumption, per capita, is high and the population of most S. E. Asian countries is growing rapidly. The economies of S. E. Asian countries are in a state of rapid growth and the percentage of the population with a high disposable income is also increasing quickly (NZ Export News, 1991). Much of

this wealth is spent on food and high quality seafood (especially live fish) is at a premium.

There is increasing impetus both within Australia and overseas for the development of alternative finfish species for aquaculture. Most aquaculture centres of the world are engaged in research and development of one or a number of potential species. Some examples are listed below:

<u>Arctic Charr, *(Salvelinus alpinus)*, Norway</u>
Wallace and Kolbeinshavn,1988; Wallace et al. 1988.

<u>European Sea Bass, *(Dicentrarchus labrax)*,</u> French West Indies.
Devauchelle et al., 1986: Barnabe and Le Coz, 1987.

<u>Turbot, *(Scophthalmus maximus)*,</u> France.
Devauchelle et al., 1986; Benavente and Gatesoupe 1988; Devauchelle et al., 1988.

<u>Halibut, *(Hippoglossus hippoglossus)*,</u> Norway.
Bolla and Holmefjord, 1988.

<u>Dover Sole, *(Solea solea)*,</u> Great Britain.
Devauchelle et al., 1986; Dendrinos and Thorpe, 1987.

<u>European Sea Bass, *(Dicentrarchus labrax)*,</u> France.
Hidalgo and Alliot, 1988; Chatain and Dewavrin, 1989.

<u>Cod, *(Gadus morhua)*,</u> Norway.
Jobling, 1988; Lie and Lambertsen, 1988.

<u>Grouper ,*(Epinephelus malabaricus)*,</u> Malaysia.
Leong and Wong, 1988.

<u>Tambaqui, *(Colossoma macropomum)*,</u> Brazil.
Merola and Souza, 1988.

<u>Gilthead Seabream, *(Sparus aurata)*,</u> Israel and France.
Porter et al., 1987; Chatain, B. and Ounais-Guschemann, N.,1990.

Seabream, *(Acanthopagrus cuvieri)*, Kuwait.
Rasheed, 1989.

Red Sea Bream, *Chrysophrys major)*, Japan.
Smith and Hataya, 1982.

Snapper, *(Pagrus [Chrysophrys] auratus)*, New Zealand and Australia.
Hayden, 1988; Pankhurst and Pankhurst,1989. Bell et al. 1991.

Barramundi, *(Lates calcarifer)*, Australia.
Pearson, 1987; Garrett et al., 1987; Garret and Rasmussen, 1987; Hogan et al.., 1987; Stewart et al., 1987; Russell et al., 1987; Rodgers and Barlow, 1987; MacKinnon et al., 1987; Hogan, 1988.

Mahimahi, *(Coryphaena hippurus)*, Australia.
Southern Sea Farms Ltd., 1987.

With this knowledge the Marine Farming Branch has embarked on a program to develop standardised and reliable culture methods for a range of native marine finfish. Rather than "re-invent the wheel" the technology and basic methodology has been accessed through co-operative research with European aquaculturists. The co-operative partners are the French marine research organisation, IFREMER and the Tasmanian Department of Primary Industry. IFREMER has been involved with the development of marine finfish for aquaculture for more than ten years and is responsible for the success of the seabass and seabream culture industries of Europe (Searle, 1991). The Senior Researcher (DPI, Finfish Aquaculture) has worked in France with IFREMER scientists at Palavas and Brest for five weeks in early 1991. Contacts have been made and the technology assessed. This technology is now being refined for Australian conditions and species. In July 1991 a Finfish Development Workshop (funded by DITAC) was held in Tasmania with the cooperation of IFREMER. This 4-day workshop was organised by the Finfish Development Program (TAS DPI) and attended by more than 30 key researchers and aquaculture producers from around Australia.

To date two Tasmanian species (Striped Trumpeter, *Latris lineata* and Black Bream, *Acanthopagrus butcheri*) have been studied and considerable progress has been made towards the culture of these fish species. The results of some preliminary studies have

been included with this application. The major stumbling block in the culture of marine finfish is larval rearing. During this period (which usually lasts 1-2 months) the fish are very delicate and have very specific dietry and physical requirements. Although these dietary requirements are not completey understood at this stage a methodology has been devised which allows a quick and reliable assessment of any particular species for aquaculture based on the larval characteristics. The methodology also enables the determination of crucial aspects of larval development (in many cases in a predictive manner) and allows manipulations of diet and physical factors to be tested quickly with a high degree of confidence. With the technology and specific research techniques which have been devised over the last three years for marine finfish development we must now work through a number of native marine species, assess their potential and solve the culture problems encountered with the most promising species.

As early as 1986 both industry and the Marine Farming Branch of D.P.I. acknowledged that the striped trumpeter had excellent aquacultural potential. Some preliminary trials were undertaken by the Department in conjunction with industry. Wild caught fish were held in sea cages in a number of areas of S.E. Tasmania. The fish were found to be well suited to cage aquaculture experiencing few of the disease problems of salmon and trout. The fish were also highly tolerant of high summer water temperatures, one of the key problems facing salmonid farmers in Tasmania.

The Finfish Development Program devised techniques for brood fish capture and conditioning and achieved successful fertilization, incubation hatching and larval rearing to day 45 post hatch in 1989 (Ruwald et al., 1991). In 1990 larval rearing was achieved to day 60 but with poor survival and a low percentage of properly inflated swim bladders. During the 1991 rearing season techniques were devised that allowed high rates of swim bladder inflation (> 60%) and a nutritional inadequacy was identified. Although the life cycle has not been closed as yet, IFREMER researchers are confident that the research is on track and close to solving the larval culture problems.

The proposed research is aimed at an area vital to the understanding of fisheries. Aquaculture and wild fisheries sciences understand very little detail of the complex processes involved during the larval development of marine finfish. The studies of Knyazeva, L.M. et al., 1984; Fukuhara. O., 1984; Connes, R. and Benhalima, K., 1984; Pederson, B.H. et al., 1987; Koven, W. M., 1989; Hung, S.S.O., 1989; Kjoersvik, E. and Opstad, I., 1989; Kjoersvik, E. et al., 1989; Wallace, A. M. et al., 1989; Kjoersvik, E. et al., 1989; Zhao et al., 1989; Kjoersvik, E. et al., 1991; and Boulhic, M. and Gābaudan, J., 1992; represent some of the more notable research in

this area of larval developmental histology and histochemistry under controlled culture conditions over the last ten years. Most of these studies have been performed in the Northern hemisphere and the most applicable and thorough research has been made using the Norwegian cod, *Gadus morhua* and the Dover sole, *Solea solea*.

There is a great need to understand the larval development of native Australian species for both aquacultural and fisheries management purposes. Standardised techniques have been developed to study the histological, histochemical and morphological changes that are occurring in larval fish. Within Australia there now exists both the facilities and expertise to adopt and refine these techniques to study particular species of interest. The proposed study involving striped trumpeter would provide a basis for understanding the larval development of many Australian native species and could lead to the early mass production of juveniles of this species for aquaculture or reseeding.

The Finfish Development Program is directed in research and development by Government / Industry Steering Committee with 50% industry representation. Apart from providing a high level of commercial expertise in production and marketing this level of industry involvement ensures that the direction of research is highly applied and is directly related to establishment and support of new industry. The committee is comprised of:

Dr. Simon Stanley (Chairperson)	Assistant Director (Marine Farming) D.P.I.
Mr. Owen Carrington-Smith	Marketing Manager, AQUATAS Ltd.
Mr. Richard Doedens	Managing Director, NORTAS Ltd.
Mr. Andrew Febey	Policy Adviser, TAS Dept. of Premier & Cabinet
Mr. Pheroze Jungalwalla	General Manager, SALTAS.
Mr. Jonathan Scott	General Manager (Development) TAS Development Authority.
Mr. Lance Searle	Senior Research Officer, Aquaculture, D.P.I.E.
Mr. Peter Shelley	Managing Director, TASSAL Ltd.

In conjunction with Austrade and TASSAL the Marine Farming Branch exported a consignment of more than 100 kg of Striped Trumpeter to Japan in early 1990. The fish were appraised in both Osaka and Tokyo at an Australian seafood promotion. The product received excellent reviews. It is primarily suited to the preparation of sashimi and premium prices are paid for fish with the correct blend of taste, texture, oil content and colour. More than 10 major Japanese fish importers were interested in obtaining the product and the Yamaha Motor company is presently negotiating an agreement with TASSAL for the supply of wild caught fish. At least three other Tasmanian companies are presently exporting or intend to export striped trumpeter to Japan in the near future.

Austrade, after discussions with the Yamaha Motor Company and other Japanese importers have indicated that the fish could achieve at least $15-20/kg on the Japanese market (compared to salmon at $12-14/kg)(AUD=Y114)¥.* They stated that:

"this fish has the potential to be the most expensive finfish from Australia (apart from Bluefin Tuna) sold in Japan. It should be more expensive even than the Atlantic Salmon."

The Striped Trumpeter is an excellent white fleshed fish which will allow the diversification of the seacage aquaculture industry in Tasmania. It is fair to state that striped trumpeter would rank very highly among new finfish species presently under development for aquaculture worldwide. The product will not compete directly with salmonids in the market place and it is imperative that the development program achieve the commercial realisation of the results in a timely manner to fully exploit this Tasmanian product and ensure the survival and growth of an industry that could be worth in excess of $30 million based on striped trumpeter aquaculture alone.

The benefits to the aquaculture industry that would flow from research into the closing of the life cycle of the striped trumpeter include:

* Other independant assessments have suggested that the fish may attain up to AUD $30/kg.

1. Diversification of the established sea cage aquaculture industry in Tasmania. This would:

   a. Reduce the impact on the industry of falling world salmon prices.
   b. Reduce the risk of industry collapse due to salmonid hatchery failure or salmonid disease.
   c. Better utilize the infrastructure of the salmonid industry.
   d. Provide an alternative native finfish for aquaculture which is more tolerant than salmonids to the local seasonal conditions.
   e. Extend the geographical range of finfish aquaculture within Tasmania.
   f. Boost employment, especially in rural areas.

2. Establishment of aquaculture industry througout Australia. This would:

   a. Create employment in rural areas of Australia
   b. Boost employment in the processing, transport and related service industries
   c. Increase export earnings for Australia.

3. Increasing the profile of aquaculture produced food products in the market place. This would:

   a. Boost prices due to the continuity of supply of high quality product.
   b. Allow the development of a wide range of value added products.
   c. Encourage the development of alternative overseas markets.
   d. Allow the development of live airfreight methods to capitalize on the rewards available by satisfying the demands of a lucrative marketplace.

4. Produce information on broodstock handling, spawning, incubation, larval nutrition, larval rearing, growout and disease control which in many cases would be directly applicable to other finfish development programs in Australia.

Performance criteria which might be used to judge the success or otherwise of the proposed research and development project are listed below.

1. Establishment of standardised and refined techniques for larval evaluation based on histological, histochemical and morphological criteria.

2. Establishment of relationships between rotifer and *Artemia* boosting methods, nutritional profiles in the live prey organisms and the histological, histochemical and morphological development of larval striped trumpeter.

3. Identification of the specific nutritional deficiency or set of deficiencies in larval striped trumpeter.

4. Correction of this deficiency with the subsequent pilot and commercial scale hatchery production of striped trumpeter.

SECTION 5 - PROPOSAL IN DETAIL

a. Plan of Operation

(i) Method of Procedure:

The Finfish Development program will routinely breed the striped trumpeter during mid September - mid November. These larval rearing trials will concentrate on diet manipulation through enrichment and will be based on combinations of boosting rates with nutrients such as freeze dried fish protein, choline chloride, D-L Methionine, vitamin premix, Vitamin D, Vitamin C, Vitamin E, A niacin and highly unsaturated fatty acids. All trials will be performed in 400 litre tanks with larval densities from 50 - 100 larvae/litre at 18-20°C. Trials will be performed in duplicate in a closed recirculating system. Fish will be taken to 6.0 - 7.0mm to determine the effectiveness of different diets in curbing the slowing of growth and mortality at 6.0mm. The results will be interpreted by ANOVA..

With the assistance of FIRDC the program would employ two persons to perform the specialised tasks involved in the proposed research. A Research Officer with at least three years experience in the fields of histology and/or histochemistry would be

required to develop and implement the specific techniques necessary for the research. Their responsibility would also include the collation and interpretation of results in collaboration with other researchers working with the Finfish Development program. A Technical Officer with considerable experience in the same fields would be required to perform routine sampling, sample processing and preliminary analysis of results.

It will be important that this team can operate in an independent manner without the distractions involved in day to day production. The team will however have considerable backup in terms of expertise from researchers and technicians working on the finfish development program, DPIE's marine chemist, other DPIE histology technicians, and staff at the Key Centre for Aquaculture in Launceston.

During these two months of rearing studies at least four experimental batches of three treatments will be trialled. Daily sampling over 14 day run periods will be made from each tank. These samples will include:

1. 20-40 fish daily/tank for Total Length, Standard Length, Yolk Sac Volume, Oil Droplet Volume, Swim Bladder, Urinary Calculus and Prey Number data.

2. 50 fish daily/tank for histological histochemical and morphological analyses. These fish will be fixed, embedded in wax and resin, sectioned and specifically stained in some cases for analyses. Electron microscopy (University of Tasmania) will be utilised for some morphological studies.

The skin, motile organs (skeleton, muscle), nervous system, sensory organs, gills, vascular system, digestive system, kidney and endocrine system will be characterised under each rearing condition from a consideration of the histological, histochemical and morphological features.

Presently striped trumpeter have been held under altered photoperiod conditions for three months. These fish have been programmed to spawn out of season (December rather than Sept - Oct.) Recent gonad microsampling suggests that these fish are cued to the photoperiod and it is likely that these fish will spawn during December. In addition to this a range of slow release hormone (LHRHa/Methyltestosterone) [Lee, C.S. et al., 1986 a; Lee, C. S. et al., 1986 b; Marte, C. L. et al., 1987] implant trials have commenced. These will no doubt allow year round spawning of striped trumpeter.

With year round spawning, a two person team comprising a Research Officer and a Technician will be more than fully employed with histological, histochemical and morphological analyses of larvae. In the first year, spawning may be limited to one per six month period. During the first rearing period basic analyses only will be performed. Larvae of other species will be available to the team between striped trumpeter spawnings to allow the development and refinement of specific techniques (i.e. fixation Allen and Gendre solutions, wax and resin embedding suitable for larval fish, sectioning thickness, hematoxylin-eosin staining, Masson trichrome staining, periodic acid Schiff supplemented by amyloglcosidase digestion control for glycogen and other staining proceedures as appropriate).

By the beginning of the second 12 month period standardised and refined techniques will allow detailed study of larval development and the effects of nutrition or other factors. The third year of study will be crucial. By this stage the nutritional deficiency should be identified and dietary manipulation should allow the production of small quantities of juvenile fish. A range of dietary manipulations will be required to pinpoint the specific nutrient profile needed for normal and efficient larval development.

In conjunction with all larval analyses the nutritional profile of the boosted live prey organisms will be required to allow the larval characterisation to be related to the specific boosting method. Analyses for proteins, vitamins and highly unsaturated fatty acids (HUFA's) will be made for rotifers and *Artemia* used in each experimental run. These analyses will be performed by the National Key Centre for Aquaculture on a user pay basis.

(ii) Facilities Available

Department of Primary Industry and Energy
Division of Sea Fisheries
Marine Research Laboratories
HOBART

- 2 well equipped histology laboratories
- 1 well equipped wet laboratory

- Sea water supply 360*l* /min continuous

- Available filtered to 80 microns
  1 micron (UV sterilized)
  0.2 micron

- Larval rearing/weaning hatchery comprised of;

  10 x 1000 litre or 10 x 400 litre cylindro-conical tanks
  A 100% recirculation system with temperature regulation to ±0.5 °C capable of running 2 temperatures simultaneously through the same biofilter. Double U V sterilisation of all circulating water. Based on IFREMER seawater systems (cost of construction $85,000) (see figure 1).

- 70% Recirculation system with 8 x 3m^3 tanks.

- 90-100% Recirculation system, 2 x 7m^3 and 2 x 4 m^3 tanks

- 4 x 1000*l* and 4 x 500*l* larval rearing tanks

- 20 x 0.5m^3 fresh seawater tanks

- 12 x 500*l* rotifer culture tanks

- A range of glass aquaria 50 - 300 litres.

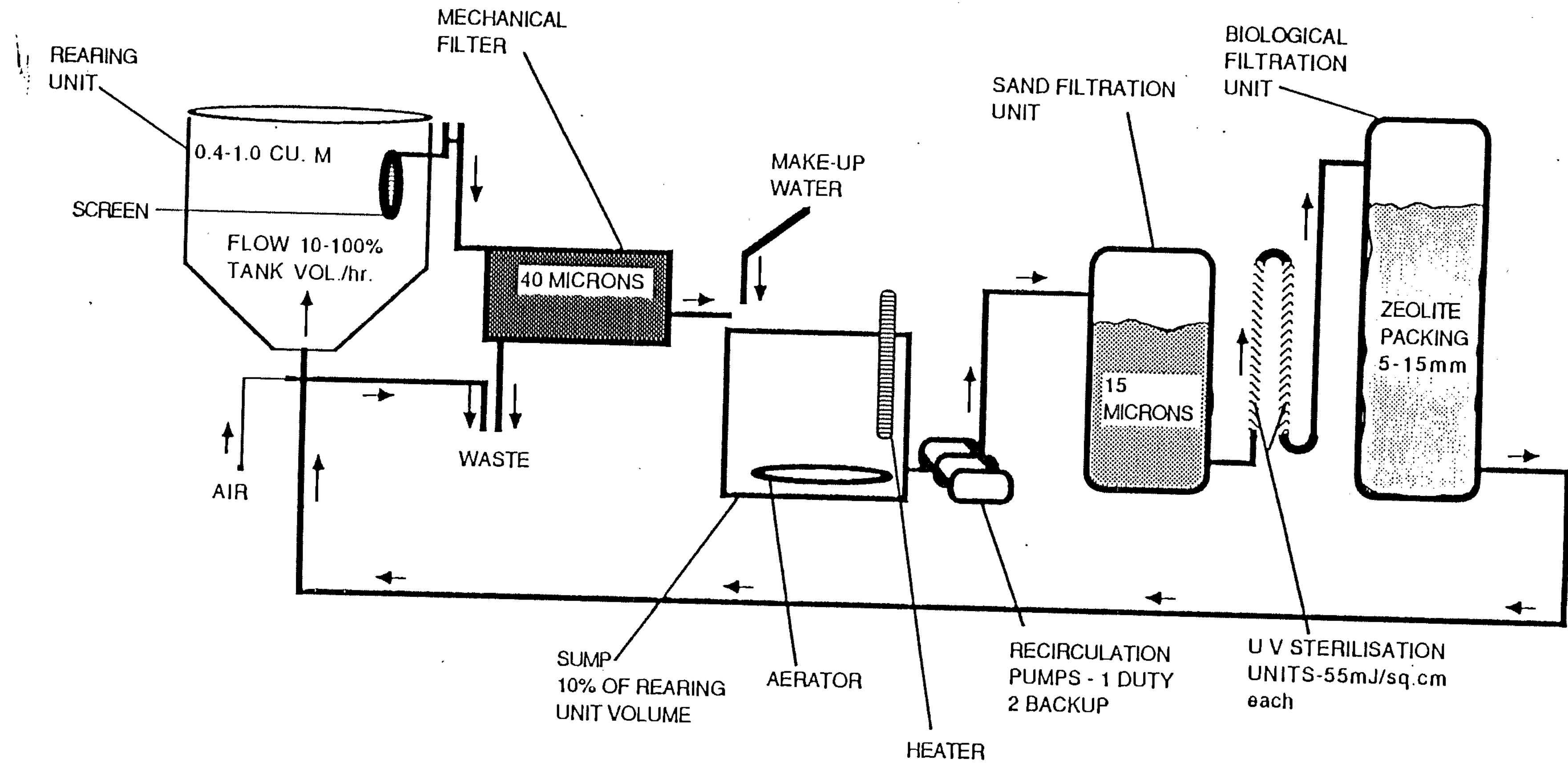
- 32 x 60*l* conical tanks

- 20 x 20*l* artemia culture bags

- Heated seawater is available to 3m^3 and 0.5m^3 tanks (up to 25°C).

- Heated sterile areas for live feed production

FIGURE 1. Configuration of Hatchery Seawater Systems



- General laboratory and wet laboratory areas are available for use. These house a wide range of analytical and diagnostic equipment to be used in the research program.

- a new seawater intake and pumping system has just been installed at a cost of $186,000 , ensuring a high quality fresh seawater supply for the program.

- The staged development (4 stages) of a research facility primarily for aquaculture research and development to be operated as a cooperative centre with TSIT, CSIRO, SALTAS and the University of Tasmania, has been planned for completion during 1995 subject . This project has an estimated cost in excess of $1.5m and could be utilized by this program in 1992-93. (A copy of the draft plan and specifications has been supplied in a previous application).

- Office space for all staff is available at the Taroona Marine Research Laboratories.

(b) Support Data:

(i) Previous Work in this or Related Fields

A summary of 1990-91 research on *Latris lineata* has been included as Attachment 2.

For three consecutive spawning seasons, striped trumpeter broodstock have successfully been held in land-based tanks and sea cages. These fish have been stripped, the eggs fertilized and viable larvae have been produced. To date larvae have been reared to day 60 (post hatch). By the stage of development reached at 6-6.5mm mortality is chronic. Histological examination of larval samples throughout these first feeding stages has revealed an improper inflation of the swim bladder with a progressive proliferation of cells within the bladder itself. A method of surface skimming (to reduce the surface film allowing larvae access to the surface to fill their bladders by gulping) has been devised and

tested with striped trumpeter larvae. Inflation was achieved in 60% of this trial batch. In addition to this very few glycogen vacuoles had developed in the livers of larvae fed either Frippak boosted rotifers, wild zooplankton or KYOWA microparticulate diets.

This indicates that early nutrition is sub-optimal in perhaps both quantity and quality. An evaluation of HUFA profiles and available vitamin E and D and digestable protein in boosted live feeds is required. Hygiene and general water quality problems encountered in earlier years have been overcome. Microbiological analysis has shown that water quality in present rearing systems is far superior to incoming fresh seawater. The preliminary results from 1991-92 larval rearing trials using a range of diets suggest a nutritional inadequacy which manifests itself at a particular developmental stage irrespective of rearing conditions. The 1991-92 spawning season was extremely short (2-3 weeks) making further nutritional trials impossible. IFREMER larval rearing expert, Dr. B. Chatain, has worked with the striped trumpeter during the 1991-92 season. Her opinion is that the striped trumpeter will be a relatively easy species of larval fish to culture as it is an excellent feeder with good initial growth. Now that the swim bladder problems have been overcome, it is simply a matter of determining the nutritional inadequacy. There is good reason to believe that experimental diets now being used for black bream and greenback flounder rearing (with high levels of soluble protein) may solve the problem.

Black bream (*Acanthopagrus butcheri*) have been studied and methods for their culture have been devised. The most significant aspect of black bream larval culture is their small size when the mouth begins to open (2.8 - 3.0mm). Available larval feeds are too large for first feeding and a modified feeding sequence was developed which has proved successful in rearing fish to metamorphosis (Day 30 -33). Trials are presently underway that will take the fish beyond this stage to weaning.

Lance Searle has been involved with finfish aquaculture for more than 8 years. As technical manager for the New Zealand Salmon company he was responsible for the construction and day to day operation of one of the world's largest chinook salmon ocean ranching operations producing 8-10 million ova/year. In addition to this he has spent a considerable amount of time developing large scale salmonid breeding systems, developed high density, long duration warmwater transport systems, carried out extensive work on the seacage grow-

out of chinook salmon. Mr. Searle was a consultant for an Auckland (NZ) company developing a live snapper transport system for the Japanese export trade.

Mr. Searle has planned a snapper (*Chrysophrys auratus*) hatchery and seacage rearing operation in the Marlborough Sounds of New Zealand for the New Zealand Salmon Co. Ltd., and experimental licences have been granted. As Senior Research Officer (Finfish Aquaculture) for the Department of Primary Industry he has been involved with disease research, seawater systems design, offshore seacage technology and the development of alternative finfish species (namely Striped Trumpeter) for aquaculture. His areas of expertise in brood holding/spawning, larval rearing, juvenile rearing, fish transport, growth performance during growout, disease research, systems design/testing and personnel management, will ensure that the research plan is carried out.

Frances Ruwald has experience in a wide range of aquaculture programs within the department. She has been involved in the striped trumpeter program from its inception and has been through four spawning and larval rearing seasons. Ms. Ruwald has assisted Sea Hatcheries (Queensland) with live larval feed production and larval rearing of Barramundi and has been involved with Grouper (*Epinephelus sp.*) culture in the South Pacific. Her areas of expertise lie in live larval feed production and the larval rearing of marine finfish.

Leigh Oates also has experience in a wide range of aquacultural programs within the department. He is a specialist in aquacultural systems design, construction, operation and maintenance. His particular contribution to the program will be in the design and operation of specific support systems. Aquacultural engineering requires an understanding of the biological requirements of a species to optimise engineering solutions within the constraints imposed by construction methods and materials. Mr. Oates has a record of highly successful innovation in this area.

Both Mr Searle and Mr Oates were responsible for the production of the working brief required for the extensive modifications to the South Australian Fisheries', West Beach Laboratory, where the S. A. Fisheries and Kinhill have been experimenting with snapper (*Pagrus [Chrysophrys] auratus*) and other marine species.

SECTION 6 - RESEARCH PRIORITY

In terms of the Corporation's 5 year Plan the research and development proposed in this paper relates to;

"Guidlines for applicants, General information", Pages 2 and 3.

"aquiring knowledge that might be used for furthering an objective of the fishing industry, including knowledge that may be of use for the purpose of improving any aspect of production....of fish."

"applying such knowledge for the purpose of attaining or furthering such an objective."

"the application of knowledge (whether or not aquired by activities referred to in the statements above) for the purpose of improving the efficiency and competitiveness of the Australian fishing industry."

"the development of equipment and techniques for culturing fish"

"the dissemination of information, and the provision of advise and assistance, to the Australian fishing industry for the purpose of encouraging the adoption of scientific and technological developments to improve its efficiency and competitiveness."

"the publication of reports....containing scientific, technical....or economic information that might be useful in improving the efficiency and competitiveness of the Australian fishing industry."

"....vocational training of persons engaged, or to be engaged, in the Australian fishing industry."

"the establishment or development of the Australian fishing industry in a particular place or for a particular purpose."

SECTION 7 -TRANSFER OF RESULTS TO INDUSTRY

The results of the proposed specific research program, "Histological, Histochemical and Morphological Development of Striped Trumpeter (*Latris lineata*) Larvae and the Effect of Larval Diet", would be transferred to industry primarily through the results obtained by the Finfish Development Program. With the success of the research program, not only will the culture of striped trumpeter be possible but efficiencies in larval rearing techniques will be indicated allowing the rapid development of industry.

The results of the proposed study program are of a basic nature and as such will have little relevance directly to industry. The main direct users of the information will include other scientists involved in fisheries disciplines related to larval development and larval rearing.

The best means for targeting this audience is through the publication of results in scientific journals such as "The Journal of Marine Biology" and "Aquaculture." In addition to this, results will be presented at relevant conferences and symposia.

In the long term, the results of research and development activities performed by the Finfish Development Program will be transferred to industry by staff training, consultancy to industry, and extension services/trouble shooting for establishing and established businesses. Workshops will be held regularly dealing with the practical applications of the results to industry fisheries.

SECTION 8 - PREDICTED COMMENCEMENT & COMPLETION DATE

Duration of Project: 3 years

COMMENCEMENT DATE	1/7/92
COMPLETION DATE	30/6/95

SECTION 9 - REQUESTED BUDGET

	1992/93	1993/94	1994/95
Salaries & Wages	72,681	72,681	72,681
Operating Expenses	25,357	25,357	25,357
Travel Expenses	0	0	0
Capital Items	3,200	0	0
TOTAL	101,238	98,038	98,038

SECTION 10 - FUNDS SOUGHT FROM OTHER SOURCES

None.

SECTION 11 - FINANCIAL CONTRIBUTION OF THE APPLICANT *

	1992/93	1993/94	1994/95
Salaries & Wages	0	0	0
Operating Expenses	27,532	27,532	27,532
Travel Expenses	4,000	4,000	4,000
Capital Items	2,100	-	-
TOTAL (D.P.I.)	33,632	31,532	31,532
TOTAL PROPOSAL	134,870	132,770	132,770

* NOTE: DPIE total financial contribution to Finfish Development (Striped Trumpeter Culture) totals $303,322 P.A.

SECTION 12 - FLOW OF BENEFITS

State	Tasmania	70%
	Other States	30%
Fishery	Aquaculture	60%
	Wild Fishery	40%

SECTION 13 - DETAILED BUDGET

ITEM	1992/93	1993/94	1994/95	Total
FTE's	2 FTE	2 FTE	2 FTE	2
SALARIES				
Salaries (S)	61,847	61,847	61,847	185,541
On Costs	9,834	9,834	9,834	29,502
Overtime	500	500	500	1,500
Allowances	500	500	500	1,500
TOTAL SALARIES	**72,681**	**72,681**	**72,681**	**218,043**
OPERATING EXPENSES				
Administration (7.5% S)	4,639	4,639	4,639	13,917
Provision of facilities (165m² @ \$90/m²)	14,850	14,850	14,850	44,550
(deprec. 0.15 A)	1,800	1,800	1,800	5,400
Transport	5,500	5,500	5,500	16,500
Consumables	7,800	7,800	7,800	23,400
Tissue processing	4,200	4,200	4,200	12,600
Usage electron microscope	1,500	1,500	1,500	4,500
Live feed analysis	12,600	12,600	12,600	37,800
TOTAL OPERATING EXPENSES	**52,889**	**52,889**	**52,889**	**158,667**
TRAVEL EXPENSES				
Travel	4,000	4,000	4,000	12,000
TOTAL TRAVEL	**4,000**	**4,000**	**4,000**	**12,000**
CAPITAL ITEMS				
Low temperature probe	2,100	-	-	2,100
MAC classic II + s/ware	3,200	-	-	3,200
TOTAL CAPITAL ITEMS	**5,300**	**0**	**0**	**5,300**
TOTAL PROGRAM	**134,870**	**132,770**	**132,770**	**400,410**

PROFORMA 'A'

FOR NEW APPLICATIONS

SECTION 8 - PREDICTED COMMENCEMENT & COMPLETION DATE

Commencement Date...1../..7.../92...

Duration of Project.......3..years......
(in years)

Completion Date..30../..6.../95...

SECTION 9 - REQUESTED BUDGET

Item	Requested 1992-93	Indicative 1993-94	Indicative 1994-95	Indicative 1995-96
Salaries & Wages	72,681	72,681	72,681	....
Operating Expenses	25,357	25,357	25,357	....
Travel Expenses	0	0	0	....
Capital Items	3,200	0	0	....
TOTAL	$101,238	$98,038	$98,038	$...............

SECTION 10 - FUNDS SOUGHT FROM OTHER SOURCES

SOURCE ....None.. $..........................

.... $..........................

.... $..........................

Continued......PROFORMA 'A'

SECTION 11- FINANCIAL CONTRIBUTION OF APPLICANT

	1992/93	1993/94	1994/95
Salaries & Wages	0	0	0
Operating Expenses	27,532	27,532	27,532
Travel Expenses	4,000	4,000	4,000
Capital Items	2,100	-	-
Total	33,632	31,532	$ 31,532

NOTE: DPIE total financial contribution to Finfish Development (Striped Trumpeter Culture) totals $303,322 P.A.

SECTION 12 - FLOW OF BENEFITS

State

Tasmania	% 70
Other States	% 30
	%

Fishery

Aquaculture	% 60
Wild Fishery	% 40
	%

SECTION 13 - PROFORMA B

SECTION 14 - ORGANIZATION

Head Responsible for Project
The Secretary, Dr. A. N. Smith

Name of Organization Department of Primary Industry, Fisheries and Energy

Address

Name of Department (if applicable)
Marine Board Building

City HOBART State TAS Postcode 7000

Telephone 002-338011 Fax 002-341335 Telex AA58352

Continued.......PROFORMA 'A'

SECTION 15 - PROJECT SUPERVISOR

Name Dr. Simon O. Stanley

Address

Department of Primary Industry, Fisheries and Energy,

Name of Department (if applicable)

GPO Box 619F

City HOBART State TAS Postcode 7001

Telephone 002-336528 Fax 002-231539 Telex

SECTION 16 - STAFF INVOLVED ON PROJECT

Research Officer (Larval Evaluation). To be appointed. Biology, Marine Biology, Biochemistry Degree or related degree. At least three years experience in histology, histochemistry or related field. Responsible for the development and implementation of the specific techniques necessary for the research and the collation and interpretation of results in collaboration with other researchers working with the Finfish Development Program.

Technical Officer (Larval Evaluation). To be appointed. Technical qualification in histology or histochemistry or degree in related field. Some experience with histological or histochemical methods. Responsible for routine sampling, sample processing and preliminary analysis of results.

SECTION 17 - ADMINISTRATIVE CONTACT

Name Ms. Wendy Dwyer-Kimber

Address Department Primary Industry, Fisheries and Energy

Name of Department (if applicable)

GPO Box 192 B

City HOBART State TAS Postcode 7001

Telephone 002-333058 Fax 002-341335 Telex AA 58333

Salaries

NEW STAFF

Name	To be appointed
Position	Research Officer - Larval evaluation
Salary/Wages	$34,752
On Costs	$5,526

Name	To be appointed
Position	Technical Officer - Larval evaluation
Salary/Wages	$27,095
On Costs	$4,308

PRESENT STAFF

Name	Mr Lance Searle
Position	Program Manager
Salary/Wages	$39,735
On Costs	$19,748

Name	Ms Frances Ruwald
Position	Research Officer - Live feed/Larval rearing
Salary/Wages	$30,575
On Costs	$15,196

Name	Mr Leigh Oates
Position	Senior Technical Officer
Salary/Wages	$26,145
On Costs	$12,994

Name	Mr Chris Pitney
Position	Technical Officer - Live feed production
Salary/Wages	$25,340
On Costs	$4,029

Name	Mr Bill Wilkinson
Position	Technical Officer - Hatchery management
Salary/Wages	$25,340
On Costs	$4,029

Note - On Costs

Permanent Staff	Super 22.5%, SAF 3%,WC 4.5%, LSL/Sick/Holidays 19.7%. TOTAL 49.7% (S)
Temporary Staff	Payroll Tax 7%, Leave Loading 1.4%, WC 4.5%, Super 3%. TOTAL 15.9% (S)

SECTION 14 - ORGANISATION

Head: Dr. A. N. Smith, Secretary
Name of Organisation: Department of Primary Industry
Address Marine Board Building
Hobart, TAS 7000.

Telephone: 002-308011 **Fax:** 002-341335 **Telex:** TASFA AA58352

SECTION 15 - PROJECT SUPERVISOR

Name Dr. Simon O. Stanley
Address Department of Primary Industry,
Division of Sea Fisheries,
GPO Box 619F,
HOBART TAS 7001

Telephone: 002-306528 **Fax:** 002-231539 **Telex:** TASFA AA58352

SECTION 16 - STAFF

New Staff

Research Officer. (Larval Evaluation). To be appointed. Biology, Marine Biology or Biochemistry or related Degree. At least three years experience in histology, histochemistry or related field. Responsible for the development and implementation of the specific techniques necessary for the research and the collation and interpretation of results in collaboration with other researchers working with the Finfish Development Program.

Technical Officer. (Larval Evaluation). To be appointed. Technical qualification in histology or histochemistry or degree in related field. Some experience with histological or histochemical methods. Responsible for routine sampling, sample processing and preliminary analysis of results.

Present Staff

Mr. Lance D. Searle. BSc. (Hons) Canterbury, NZ. Program manager. Overall program management and day to day management of brood holding/spawning, juvenile rearing, fish transport, growth performance during growout, disease identification/treatment and systems design/testing.

Ms. Frances Ruwald. BSc. Canberra C.A.; Diploma in Aquaculture, University of Tasmania. Supervision and management of larval feeding, larval rearing algal and live larval feed production.

Mr. Leigh Oates. Trade certificate - Engineering. Numerous Aquaculture course qualifications/ 10 years fisheries technical experience. Senior Technical Officer. Design, construction, operation and maintenance of systems related to the project. Day to day supervision of technical staff working on larval rearing and growout projects.

Mr Chris Pitney and Mr Bill Wilkinson. Technical Officers with qualifications and experience in relevant areas of fisheries and aquaculture . Responsible for live feed production and hatchery management.

Research Officer (Broodstock management). To be appointed. Aquaculture or related Higher Degree. Responsible for the procurement, feeding, conditioning, GSI monitoring, induction, spawning and out of season maturation of broodstock.

SECTION 16 - ADMINISTRATIVE CONTACT

Administrative Contact: Ms Wendy Dwyer-Kimber
Department Primary Industry,
GPO Box 192 B,
HOBART TAS 7001

Phone: 002 303058 **Fax No:** 002 341335 **Telex:** AA 58333

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ATTACHMENT 1

FINANCIAL INFORMATION

(i) Industry Contribution

During the course of this program no direct industry funding will be provided. Industry will however provide assistance with the feeding and management of fish held on seacage farms in net pens. In the case of AQUATAS P/L and the HUON ATLANTIC SALMON CO. LTD, staff will feed the fish and monitor the stock on a daily basis including mortality dives. In addition to this, assistance will be provided during weight checks and net changes. In terms of the monetary value of this labour contribution, it is estimated that on average at least one hour will be required on each farm per day to perform these duties with a casual labour rate of $13.34/hr. This equates to an overall industry contribution of approximately $10,000 P.A.

(ii) Justification of Information in

Salaries

$34,752 - Research Officer - Larval Evaluation.
Required for the development and implementation of the specific histological and histochemical techniques. Necessary to characterise the larval development of striped trumpeter. Responsible also for the collation and interpretation of results under different feed regimes in collaboration with other researchers working with the Finfish Development Program. This person must be a specialist in the field of histology and histochemistry. This expertise is not presently available within the program.

$27,095 - Technical Officer - Larval Evaluation.
Required for routine daily sampling, sample processing and preliminary analysis of results. Staff presently working for the Finfish Development Program do not have the time available to perform these tasks on a daily and continuous basis.

On Costs

$5,526 - Research Officer . Temporary staff 15.9% of salary (for breakdown see page 22; note - on costs).

$4,308 - Technical Officer. Temporary staff 15.9% of salary (for breakdown see page 22; note - on costs).

Operating Expenses

$4,639 - Administration

7.5% of base salaries to cover the Department's contribution in terms of clerical and administrative support to the program. To be funded by D.P.I.E.

$14,850 - Provision of Facilities

165m^2 @ $90/m^2. Includes the provision of two histology laboratories, a wet laboratory, microscope room and office space. To be funded by D.P.I.E.

$1,800 - Depreciation

Depreciation calculated at a rate of 15% pa on histology equipment valued at $12,000.

$5,500 - Transport

The cost of provision of vehicle transport as required based on the cost of running an average government vehicle P.A. To be funded by D.P.I.E.

$7,800 - Consumables

Includes the cost of glass slides, specific dyes, histochemical reagents, fixatives and other chemicals, wax, resin, sample containers and liquid nitrogen refills for cryogenic cylinder.

$4,200 - Tissue Processing

The cost of tissue processing mounting and staining for specific features best performed by the National Key Centre for Aquaculture due to specialised histological equipment. Based on 1,400 slides @ $3.00/slide.

$1,500 - Usage Electron Microscope

The cost of specialised processing for electron microscopy and equipment usage. Based on 15 hours at $100/hour.

$12,600 - Live Feed Analysis

The cost of protein, vitamin and highly unsaturated fatty acid analyses in live prey organisms such as rotifers and *Artemia*. The cost is based on the analyses of 42 treatments with three individual profiles within each treatment. Each nutritional profile i.e. protein, vitamin or HUFA will cost on average $100 including storage and processing.

Travel Expenses

$4,000 - Overnight Allowances

The cost of overnight and meal allowances for two persons to spend 20 nights each in Launceston at the Key Centre for Aquaculture for sample delivery, processing and results analysis. To be funded by D.P.I.E.

Capital Items

$3,200 - Mac Classic II

Required for data analysis and report writing. Includes the cost of wordprocessing and statistics software.

$2,100 - Low Temperature Thermometer

Required for use with cryogenic cylinder used for sample storage especially live prey. For use for protein, vitamin and HUFA analysis in liquid nitrogen to -170°C. To be funded by D.P.I.E.

(iv) **Commercial Assessment**

To date there has been a transfer of technology and "know-how" from IFREMER to the Finfish Development Program. Although there are no patents or disclosure agreements relating to this information, it should be regarded as commercially sensitive.

In the present financial year the applicant (the Tasmanian Department of Primary Industry) contributed more than $240,000 directly to this program. With consideration of the provision of facilities, office accommodation, administration, vehicle supply and running cost, the applicant's contribution was in excess of $300,000.

The Fishing Industry Research and Development Council has not previously funded this program.

No funds have been sought for any related program.

ATTACHMENT 2

APPENDIX 2

ONTOGENY OF DIGESTIVE ENZYMES IN LARVAL FISH - A SUMMARY OF THE LITERATURE

The digestive organs of many fish larvae are similar to those of stomachless fish. Many possess an undifferentiated digestive tract at hatching and rely on exogenous food to supply some of the enzymes required to digest and absorb food until metamorphosis, when the stomach and other organs become functional (Buddington, 1985; Dabrowski and Culver, 1991; Watanabe and Sawada, 1985). The pattern of digestive enzyme development and activity is closely related to the different stages of growth and the onset of activity may be programmed genetically, independent of fish mass. At metamorphosis, larval teleosts undergo a transition from larval morphology and digestive processes to those of the adult and assume adult feeding habits and diet (Buddington, 1985).

The alkaline proteolytic enzymes trypsin and chymotrypsin are usually found in the intestine during the early larval stages when fish lack morphological and functional stomachs. The activity of these alkaline proteases decreases as the stomach epithelia differentiates and the pH drops, while pepsin activity increases (Walford and Lam, 1993). Munilla-Moran *et al.* (1990) estimated that between 89-94% of the total esterase activity and 43-60% of protease activity in the gut of cultured turbot larvae at Day 3 was derived from the live diets. The size and quality of the live food may be paramount for larval survival, as the prey size may be inadequate to stretch the oesophagus or intestine and stimulate the release of digestive enzymes (Hjelmeland *et al.*, 1988) or as Walford and Lam (1993) suggested, that mechanical damage by the pharyngeal teeth is necessary to allow the rapid autolysis of rotifers in the intestine of larval sea bass.

ACID PHOSPHATASE

Cousin *et al.* (1987) found ACP activity to be widely distributed, but less well localised. Immediately post hatch, strong activity was detected surrounding the yolk sac of larval turbot, but this disappeared after yolk resorption. Activity in the nervous tissue, exocrine pancreas, liver spleen, kidney, heart, gills, retina and the fibrous layer covering the swimbladder increased with age. At Day 3, slight activity

was found in the intestine and stomach, increasing in strength through the larval and juvenile stages. Activity was localised in the apex of the epitheliocytes of the stomach, pyloric caeca and the intestine.

In juveniles, weak activity was seen in the pharynx and oesophagus, as well as the bile and hepatic duct, but the gall bladder was negative. Weak activity was detected in rotifers (*Brachionus plicatilis*) ingested by the fish, but not *Artemia* (*Artemia salina*). Activity was detected in fasting larvae, but was weaker than in comparable fed larvae.

Mitchell *et al.* (1986) found ACP activity in larval walleyes to be located in the ciliated border of the enterocytes. Activity was weak on Day 1 but strengthened with age and was found in the kidney and liver also.

Zhuraleva *et al.* (1988) found ACP activity in larval roach to be weak but increased as fish grew. Activity was localised in the brush border of the enterocytes.

ALKALINE PHOSPHATASE

Alkaline phosphatases catalyse the degradation of mono-esters of orthophosphoric acid to form an alcohol and a phosphate ion. They are active around a pH of 10, however the optimum pH varies. Intracellular ALP is associated with the plasma membrane and possibly membrane transport. Human tissues with high ALP activity are proximate convoluted tubules of kidney, intestinal mucosa, placenta and bone, while ALP is found also in serum and bile. In fish larvae, ALP is considered to be an indication of the degree of differentiation of enterocytes and is used to monitor development in the instestinal villi (Stroband *et al.*, 1979).

Zhuraleva *et al.*(1988) assessed the activity of ALP in juvenile roach (*R. rutilus*). Weak activity was found in the enterocytes, however, activity strengthened with growth. In larger fish, activity was localised in the fimbrate border of cylindrical epithelium of the intestine. Occasionally, activity was detected in the capillaries underlying the mucosa sheath of the intestine.

Segner *et al.* (1989) found ALP activity to be localised in the brush border of the enterocytes of larval coregonids (*C. lavaretus*). Peak activity was detected in the anterior intestine, although, at the very beginning of the intestine, activity was weak, increasing to the mid intestine and decreasing towards the rectum, in which no activity was detected.

Cousin *et al.* (1987) found activity in various tissues of embryos, larvae and juvenile turbot (*Scophthalmus maximus*). In larvae, activity was found in the middle and posterior regions of the digestive tract, in nervous system and developing retina. At Day 2, activity was seen in the digestive tube, while at Day 3 it was localised in the apex of the epitheliocytes. No activity was detected in the epithelium of the oesophagus, stomach or the rectal area. Activity intensified from Day 1 in the pancreas, kidney, heart, gills, pseudobranch and spleen, as did the diffuse activity in the hepatocytes. Weak activity was found in the bile and hepatic duct, while in the pyloric caecae, activity was similar to that seen in the intestinal epithelium. In larvae from fasting trials, activity began to weaken from Day 7 and at Day 9 was no longer localised in the apex of the epitheliocytes, but was diffused throughout the cytoplasm.

Stroband *et al.* (1979) found ALP activity in larval and juvenile grass carp to be localised in the enterocytes on the first segment of the intestine. Strongest activity was associated with membranes of microvilli and pinocytic invaginations, while some activity was detected in the enterocytes of the third segment.

Mitchell *et al.* (1986) found that larval walleyes appeared to have a well developed gut at Day 2. ALP activity was detected in the ciliated border of the enterocytes and activity increased with age. Other tissues with ALP activity were kidney, liver, connective tissue of the head, as well as the eye capsule.

AMINOPEPTIDASE-M (AMP-M)

Aminopeptidases are exopeptidases, hydrolysing terminal peptide bonds to form amino acids and/or smaller peptides (Kachmar and Moss, 1976) and are found both intra- and extracellularly (Lojda *et al.*, 1979). They split amino acids from the amino end of peptide chains. Aminopeptidase-M (AMP-M) is particle (microsomal) bound or membrane bound (Lojda *et al.* 1979) and is found in the brush border of enterocytes and the proximal tubules of the kidney (Cousin *et al.*, 1987; Lojda *et al.*, 1979).

Rösch and Segner (1990) found diet to affect the staining intensity of AMP-M in the brush border of enterocytes in larval *Coregonus lavaretus*. Artificial diets with high protein content, produced a stronger enzyme response than the live food (*Artemia*) diet.

Cousin *et al.*, (1987) investigated the activity of AMP-M in larval turbot (*S. maximus* L.). They found activity in the distal region of the intestine of fed larvae on

Day 1. This corresponded to the only area of the intestinal tract with differentiated epithelium. At Day 2, activity intensified and spread to the middle region, while Day 3 larvae displayed an open lumen and activity located in the epithelium. The pattern of strong activity in the posterior intestine and weak activity in the anterior intestine was constant throughout the development of the digestive tract. At Day 20, activity of a strength similar to that of the anterior intestine was detected in the pyloric caecae. No activity was detected in the stomach.

Segner *et al.*, (1989) found AMP-M activity to be localised in the brush border and adjacent cytoplasm of enterocytes in larval coregonids. Intensity increased with distance from the mouth to the end of intestine 1 and decreased in intestine 2 in the brush border. Diet was found to affect staining intensity; larvae fed zooplankton displayed more intense staining reactions in the gut than those fed dry diets.

Clark *et al.*, (1987) found AMP-M to have an optimum pH of 8.3 and its activity was greatly increased by the addition of manganese in the Dover sole (*Solea solea* L.). Activity was found to be associated with the epithelium of the intestine and was greatest in the midgut followed by the foregut, stomach, hindgut and lastly rectum.

Verreth *et al.*, (1992) investigated aminopeptidase activity in the African catfish (*C. gariepinus*). Activity was present in the foregut and midgut, while the hindgut, anus and stomach were negative. Activity was greatest in the anterior portion of the foregut and was localised in the brush border of enterocytes. Between Days 1 and 10 of exogenous feeding, only a weak increase in activity occurred.

Ferraris *et al.*, (1987) detected AMP-M activity in the brush border of enterocytes of the milkfish (*Chanos chanos* [Forsskal]) aged 21 Days or older. Munilla-Moran and Stark (1990) found the greatest activity in the foregut of turbot larvae (*S. maximus* L.) followed by the midgut, stomach, hindgut and rectum. While the levels of activity differed between fed and unfed larvae, the pattern of activity remained constant. Mitchell *et al.* (1986) detected aminopeptidase activity in the brush border of intestinal epithelial cells in 2 day post hatch larval walleyes (*Stizostedion vitreum*).

AMYLASE

Amylases are a group of hydrolases which split carbohydrates such as glycogen and starch. Those found in animal tissues are α-amylases and attack the α-1,4 linear segments of starches in which the glucose residues are linked by linkages. It will not hydrolyse cellulose in which the glucose residues are linked by β-1,4 linkages, or

the branch point linkages in glycogen or amylopectin, which are α-1,6 linkages (Kachmar & Moss, 1976).

In the turbot (*Scophthalmus maximus* L.), Cousin *et al.* (1987) detected amylase activity in the pancreas at Day 3 and in the intestinal lumen at Day 4 (particularly in the anterior and mid region). Commencing at Day 5, activity spread into the lumen of the 3 intestinal segments and 2 pyloric caecae. Activity remained most intense in the anterior portion of the intestine. The exocrine pancreas was particularly active during the larval phase. In juveniles at Day 40, the pancreas was also high in activity, while the lumen of the intestine in fish before feeding was low. Activity 4 - 5 hours after feeding was, however, high in the lumen of the intestine and low in the pancreas. A starvation trial revealed high activity in the pancreas until death, while the intestine was strong up till Day 7, when activity began to weaken and by Day 10 was barely detectable in the anterior intestine and not found at all in the middle and posterior intestine.

Kitamikado and Tachino (1960), found amylase activity in the rainbow trout (*Oncorhynchus mykiss*) greatest in the pyloric caecae, followed by intestine, liver then stomach.

Buddington (1985) investigated the digestive secretions of the lake sturgeon (*Acipenser fulvescens*) and found three phases of amylase secretion. Prior to exogenous feeding, activity in the gut was low. Initiation of feeding resulted in increased activity in the alimentary canal, with the highest levels during larval feeding. Activity in the liver was also highest during larval feeding. After metamorphosis amylase secretion decreased and remained low.

Yamane (1973) found weak amylase activity in the epithelial surface of the pharynx of carp which may have been due to contamination of the surface during processing of the tissues. However, the oesophagus showed no activity, while the intestinal bulb displayed weak activity on some surfaces. Exocrine pancreas tissue was positive while the surrounding liver was negative. It appeared that the intracellular localisation of staining corresponded with the location of zymogen granules in the exocrine pancreas.

LIPASE

Lipase is produced in the secretion granules of the exocrine pancreas and catalyses the hydrolysis of long chain fatty acids and glycerol. The enzyme can only act on emulsions of water insoluble substrates. Fatty acids (triglycerides) are hydrolysed to

produce diacylglycerol and other fatty acids. Lauric, palmitic, stearic and oleic acids are some of the fatty acids which are substrates and triglycerides are broken down more rapidly than di- and mono-glycerides.

Rösch and Segner (1990) found that lipids were removed from the food bolus in the anterior portion of the intestine of older *C. lavaretus* fry, while in younger fish, absorption occurred in the middle regions of the intestine. Absorption was greatest at the tips of mucosal folds. Lipid droplets were found in the supra-nuclear region of enterocytes in larvae 12 and 22 Days old. Larvae fed zooplankton displayed a marked difference in lipid absorption on Day 29; fat droplets filled the apical half of enterocytes, however, by Day 36, the pattern of lipid droplets was similar to that of 12 and 22 Day old larvae. Temporary storage of lipids occurs in vacuoles in the enterocyte cytoplasm, from whence it is transferred via the basolateral membranes to the submucosal space. This type of storage was observed to be greater in larvae fed dry diets compared to those fed zooplankton, which may be due to the digestibility of the lipid component of the dry diet.

MALTASE

Maltase is a disaccharidase which acts in the intestine to split maltose to form glucose (Kachmar and Moss, 1976). It has an optimal pH of 5.5 to 6.5 and is localised in the brush border of the enterocytes in the small intestine and cells of the proximal tubules of the kidney.

Segner *et al.* (1989) found weak activity confined to the brush border of the middle and posterior part of the foregut in larval coregonids. No activity was detected in the midgut or the hindgut. The type of diet was found to affect maltase activity. Larvae fed dry food diets had greater activity than those fed live zooplankton. These findings were confirmed in a later study by Rösch and Segner (1990). Larvae in the starvation trial had no detectable maltase activity. Kawai and Ikeda (1971) found that dietary starch content affected the maltase activity in carp (*Cyprinus carpio*). At 5 days after the start of the trial, fish fed diets with high starch (60 and 90%) display a marked increase in maltase activity compared to the fish on low starch (10 and 40%) diets. However, by Day 69, the highest activities occurred in fish receiving the diets with medium starch (40 and 60%) content. After Day 69, all fish were fed the diet with 60% starch and all groups developed similar activities, suggesting that carp can adapt to dietary alterations rapidly. Further experiments on the effect of carbohydrate on maltase activity found that maltase activity was greater when the substrate was lactose, followed by starch then sucrose and maltose.

NON-SPECIFIC ESTERASE (NSE)

Esterases as a group have low substrate specificity. They hydrolyse esters to alcohols and carboxylic acids. The variation in chain length of both the acyl (acid) and alkyl (alcohol) portions of esters may be marked (Kachmar and Moss, 1976). Non specific esterases (NSE) are ubiquitous in plant and animal tissues and are located in the endoplasmic reticulum and lysosomes (Lojda *et al.*, 1979).

Cousin *et al.*, (1987) found diffuse, weak NSE activity in the larvae of the turbot (*S. maximus*). At Day 3, activity was localised mainly in the anterior intestine, however the lumen of the oesophagus and early stomach were negative and weak activity was detected in the thin muscular layer of the oesophagus and proximal stomach. The exocrine pancreas displayed weak NSE activity also. Activity increased during the larval period in the anterior and middle intestine while the activity in the posterior intestine remained negligible. The *tunica muscularis* of the stomach also displayed increased activity. In juvenile turbot, activity was evenly distributed in the intestinal epithelium, including the posterior intestine as well as the pyloric caecae. Increased activity was present in the *tunica muscularis* of the oesophagus and cardiac stomach but was not found in the *tunica muscularis* of the fundic and pyloric stomach or the intestine. Activity in the pancreas also increased in juveniles. In fasting larvae between Day 7 and 10, NSE activity decreased in the pancreas and epithelium of the anterior and middle intestine. The posterior intestine was negative, while the activity found in the tunica muscularis of the oesophagus was present until Day 10.

Segner *et al.* (1989) investigated the activity of digestive enzymes, including NSE, in larval *Coregonus lavaretus* L. NSE activity was found in the cytoplasm, but not the brush border of the enterocytes. In samples with strong activity, the whole cytoplasm stained, while in those with weak activity, the staining reaction was localised in the apical cytoplasm. Enzyme activity was strongest in the mid intestine, but weaker in the anterior sections of the intestine, while the anterior rectum showed little activity and the posterior rectum was negative. In 34 Day old juveniles, both the glandular cells of the stomach and the epithelium displayed activity, however, there appeared to be no overall increase in non specific esterase activity with larval age to Day 34.

Kitamikado and Tachino (1960) investigated the NSE activity in rainbow trout larvae. Activity was detected in all digestive organs, however, liver and pancreas had the strongest activity. Esterase activity increased with increase in body weight up to 100 g, after which it slowly decreased.

Zhuraleva *et al.* (1988) investigated the enzymes of the digestive tract of the roach *Rutilus rutilus caspicus* during early development. Low activity was found in cylindrical epithelial cells of the intestine in larvae up to 34 mg body weight. In juveniles (80 mg), activity was moderate and found in fimbrate cylindrical epithelium of the intestine. In juveniles weighing 134 mg, NSE activity was high and was located in the apical portion of the brush border of the enterocytes. Activity was greatest overall in the mid section of the intestine.

Hirji and Courtney (1983) investigated the NSE activity in the perch (*Perca fluviatilis* L.) and found the greatest activity in the pancreas. The activity in the stomach, liver, pyloric caecae, upper and mid intestine was moderate, while that in the lower intestine was low and in the rectum negligible. The activity in the stomach was in the chief cells and the surface epithelium, where it was localised in the supranuclear cytoplasm, not in the distal cytoplasm. In the chief cells, activity was strong in the basal cytoplasm, while the distal cytoplasm bordering the lumen of the gastric gland displayed weak activity. In the pyloric caecae, upper and middle intestine, activity was localised in the columnar cells, particularly in the supranuclear cytoplasm, but was absent from the brush border area while some areas of activity were observed in the submucosa. Activity was greater in the columnar cells lining the crypts than in the cells at the apices of the intestinal folds. The activity in the rectum was greater in the columnar cells lining the apices of the folds than in the crypts and the activity in the rectal submucosa was stronger than that observed in the submucosa for the rest of the intestine. Enzyme activity in the pancreas was localised in the cells of exocrine pancreas, while in the liver, activity was localised in the hepatocytes, particularly those bordering the central canals and the blood vessels.

Verreth *et al.* (1992) found NSE activity in the African catfish (*Clarias gariepinus*) at the onset of exogenous feeding was weak in the gut, stronger in the pancreas and the kidney tubules. At Day 4, activity was detected in the glandular tissue of the stomach, but not the epithelium. By Day 5, however, the epithelium was demonstrating strong activity. Activity increased with age, with the reaction occurring in the stomach and anterior intestine, decreasing towards the anus. Activity was localised intracellularly at the apical end of the enterocytes, but absent from the brush borders.

TRYPSIN

Trypsin is an endopeptidase specific for the peptide bonds involving the carboxyl groups of arginine and lysine. The chemical bond attacked may not be a peptide bond; esters and amides are split more rapidly than peptide bonds. Trypsin is synthesised in the acinar cells of the pancreas in the form of the inactive proenzyme trypsinogen, which is stored in the zymogen granules and is secreted in to the intestine under the stimulus of either the vagal nerve or the intestinal hormone cholecystokinin - pancreozymin. In the intestinal tract, the removal of short peptide fragments by the enzyme enterokinase or by existing trypsin alters it to the active form (Kachmar and Moss 1976; Bronk, 1973). The enzyme is active in the pH range of 6 - 9.5, with an optimum activity at pH 8.0 at which its activity rapidly decreases due to autodigestion. Jany (1976) found that a peak of proteolytic activity at around pH 9 consisted of two peaks, one at 8.7 and the other at 9.2, which was thought to be trypsin and chymotrypsin.

Segner *et al.* (1989) found a strong positive reaction for trypsin in the anterior lumen of the foregut and a weaker reaction in the mid gut region. As well as food materials in the lumen, a positive reaction was obtained along the brush border of the intestinal epithelium, probably due to the binding of trypsin molecules to the glycocalyx of the enterocytes. Activity rapidly decreased with passage through the gut.

Trypsin may take several days or weeks after first feeding before its activity increases (Dabrowski and Culver, 1991). Munilla-Morán *et al.* (1990) found 3 day old turbot larval (*Scophthalmus maximus* L.) to have $210 \pm 3.2 \times 10^{-3}$ units per organism, of which they estimated that 43-60% was derived from the live food. Buddington (1985) found the trypsin concentrations in the alimentary canal of the lake sturgeon, *Acipenser fulvescens* to decrease during absorption of the yolk, remain low during larval feeding and to increase after metamorphosis. Baragi and Lovell (1986) measured the activity of digestive enzymes of larval striped bass (*Morone saxatilis*) fed different diets. Activity for all diet groups at Day 4 was approximately 60% of those at Day 32.

Lauff and Hofer (1984) studied the development of proteolytic enzymes in three fish species: *Coregonus* sp., *Oncorhynchus mykiss* and *Rutilus rutilus*. Each possessed several trypsin isoenzymes and in *O. mykiss* and *R. rutilus*, the pattern did not alter with age. However, *Coregonus* sp. displayed a change in the pattern during Days 1 - 14, when only two isozymes were detected, while on Day 14, a third isozyme was detected and the most cathodic isozyme began to decrease. The

change was correlated with the formation of the stomach. In early larvae of *Coregonus* sp., most of the trypsin activity was found to be of exogenous origin; after formation of the stomach, this decreased to levels comparable to those of the rainbow trout and roach.

Hofer and Uddin (1985) found trypsin activity of larval roach fed an artificial diet to increase in early life, while fry had lower levels which increased in adults. Roach larvae fed Artemia displayed a low level of trypsin activity. Trypsin activity decreases markedly in the hindgut of the adult fish, possibly due to absorption, as a means of "recycling" the enzyme, however, this is not the case in larvae and occurs to a lesser extent in fry.

Kawai and Ikeda (1972) studied the effects of dietary content on digestive enzymes of carp (*Cyprinus carpio*) and found that proteinase activity was greatest in fish fed the highest protein diet. However, proteinase activity tended to decrease with an increase in age until Day 69, when it was found to increase in all but the fish fed the diet lowest in protein.

Fagbenro (1990) studied the digestive enzymes of the catfish, (*Clarias isheriensis*, Sydenham 1980) and found trypsin activity to be greatest in the duodenum, followed by the ileum and absent from other parts of the digestive tract. They also found that the activity of proteases was dependent on the concentration of proteins in the diet. Uys and Hecht (1987) state that protein digestion occurred principally in the stomach and foregut in *Clarias gariepinus*, while Uys *et al.* (1987) found maximum protease activity to occur in the stomach 2.5 hours after feeding. Activity gradually decreased 4 hours after feeding probably due to the denaturation of the enzyme or its passage, with food, out of the stomach and into the intestine where it may have bound to the glycocalyx of the enterocytes.

Hjelmeland *et al.* (1988) investigated trypsin activity in larval herring (*Clupea harengus*) fed various live diets and inert polystyrene spheres. They found that activity was slightly increased by the ingestion of the polystyrene spheres, but that the ingestion of copepodites produced the greatest activity at Day 14, which suggests that not only does the live food provide enzymes, but its size and/or shape stimulates the release of the enzyme from the fishes own system. Pedersen and Hjelmeland (1988a) investigated the fate of trypsin in larval herring (*Clupea harengus*) and suggest that larvae absorb the enzyme before it can be excreted with the faeces and resecrete it during subsequent meals, thereby conserving energy and protein. Trypsin levels were found to vary with phases of feeding in larvae from

Day 13 to 34. Except in Day 13 larvae, post ingestion trypsin levels increased with respect to pre-ingestion basal levels and post digestion levels after the first feeding were markedly decreased, followed by a significant increase in levels after the second feeding. The enzyme content of the pancreas increased from Day 13 to Day 34, as did the variability in trypsinogen levels. The faecal trypsin content varied between 1.0 and 9.2 ng per fish; the higher value was obtained when larvae defaecated under stress (tank transfers). Pedersen and Hjelmeland (1988b) found that larvae starved for 1 to 7 Days could, when fed, produce increased amounts of trypsin after ingestion, while larvae starved for 8 Days when fed did not and food appeared undigested in the hindgut.

Baragi and Lovell (1986) found trypsin activity in Day 4 striped bass larvae to be at least 25% that found in Day 32 larvae irrespective of the diets tested. By Day 12, the activity was approximately 60% that found in larvae at Day 32. Walford and Lam (1993) investigated the development of the digestive tract in seabass (*Lates calcarifer*) larvae and found the trypsin-type enzyme activity to be high immediately post hatch, but by Day 8 it had decreased, before rising again on Day 17. Despite the still alkaline pH of the intestine, the trypsin-type activity decreased after Day 17 until it was not detected on Day 30. This correlated with the increased function of the stomach.

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APPENDIX 3

CSIRO Marine Laboratories

Report 94-SF1

FATTY ACID COMPOSITION OF *ARTEMIA SALINA* AND *BRACHIONUS PLICATILUS* FED ON ARTIFICIAL DIETS

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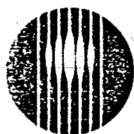

Australian Science, Australia's Future

INTRODUCTION

The fatty acid composition and total lipid of 10 samples of *Artemia salina* and 5 samples of *Brachionus plicatilus* fed different artificial diets at the Tasmanian Department of Primary Industry, Division of Sea Fisheries, were determined using gravimetry, capillary gas chromatography (GC) and capillary gas chromatography-mass spectrometry (GC-MS). The study was carried out to test the efficacy of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) boosting in these two animals used as mariculture feeds.

SUMMARY OF RESULTS

Total Lipid

The total lipid present in the *Artemia* and *Brachionus* samples is given in Table 1 along with details describing each sample.

The lipid content of the starved *Artemia* ranged from 62 to 119 mg per gram of dry weight. The lipid content of the 24 hour starved sample is higher than the 12 hour starved sample which may be attributable to the history/culturing of the different samples of *Artemia*. The *Artemia* fed the various diets had lipid contents varying from 164 to 217 mg per gram of dry weight, which were higher than the starved animals, except for the sample fed Frippak where the amounts were comparable.

The *Brachionus* fed the different diets all had lipid contents above that of the starved sample, and were also variable, ranging from 87 to 147 mg per gram of dry weight. The lowest value for the fed animals again was for those fed Frippak.

Fatty Acid Composition

The fatty compositions (as % of total fatty acids) are given in Table 2.

Boosting of EPA and DHA in Artemia

There was no DHA (docosahexaenoic acid, 22:6(n-6)) present in the starved *Artemia* but EPA (eicosapentaenoic acid, 20:5(n-3)) ranged from about 2% to 8% of the total fatty acids. The proportion of EPA are quite different in the two zero hour starved samples, numbers 1 (AF1:3),

6.5%, and 4 (SS00A), 1.9%, suggesting a different culturing/growth history of these two samples of *Artemia.*

All diets boosted levels of EPA and DHA in *Artemia* from those present in the zero hour starved animals of sample number 4 (SS00A), with Frippak being the least effective (boosted EPA by 0.9% and DHA by 0.4% only). Compared to the other starved *Artemia* samples (numbers 1, 2 and 3) Petsalis/PVM and Frippak appear to have only boosted DHA, not EPA as the percentage of this fatty acid were higher in these starved samples. However, as the animals on the Petsalis/PVM diet have a higher lipid content, the actual amounts of EPA in the animals on this diet are slightly higher than in these starved animals.

Artemia fed SuperSelco gave the highest boosting of EPA and DHA, reaching 17.4% EPA and 11.6% DHA after 24 hours. After 48 hours fed SuperSelco, EPA levels had risen to 21.4%, but there was only a slight increase in DHA (11.7%). The amount of lipid also increased only slightly, suggesting that 24 hour boosting may be sufficient. ProteinSelco produced the second highest boosting of EPA and DHA, followed by Nutripak.

Boosting of EPA and DHA in Brachionus

The unfed *Brachionus* sample contained a trace amount (0.1%) of DHA and 5.8% EPA. All diets enhanced the amounts of these two fatty acids with SuperSelco having the best boosting effect of both EPA and DHA, followed by Petsalis. The Frippak-fed animals may appear to have the next highest degree of boosting of EPA on a percentage basis (10.1%) but the Petsalis-fed sample (6.5% EPA) contains more lipid so the actual amounts of EPA present are more than for the Frippak-fed animals.

The lipid content of the Frippak-fed animals is so low that even though the Nutripak-fed animals have less EPA and DHA on a percentage basis, the amounts present of each are more than for Frippak. Therefore, of all the *Brachionus* diets, Frippak produced the lowest boosting of EPA and DHA.

Other fatty acids

The amounts of the polyunsaturated fatty acid (PUFA) 18:3(n-3) present in the starved *Artemia* also show a difference between the two zero hour starved samples, number 1 (AF1:3) had 20.7% while

number 4 (SS00A) had 34.9%, the highest proportion found of this fatty acid. This trend is the opposite to the difference in EPA levels. The *Artemia* fed Frippak, which produced the lowest boosting of EPA and DHA, had the second highest amount of 18:3(n-3), while those fed SuperSelco (producing the highest boosting of EPA and DHA) had lower amounts of 18:3(n-3). In contrast, 18:3(n-3) was not a major constituent in the *Brachionus* samples (0.9% to1.7%).

CONCLUSIONS

The SuperSelco diet produced the highest, and Frippak the lowest boosting of EPA and DHA in both species. A rating of the diets in order from those producing highest to lowest boosting of these two PUFAs is SuperSelco, ProteinSelco, Nutripak, Petsalis/PVM then Frippak in *Artemia*; and SuperSelco, Petsalis, Nutripak then Frippak in *Brachionus*. There was quite a difference in the amounts of EPA, DHA and other fatty acids in the two zero hour starved *Artemia* samples, presumably due to the different histories of the two samples.

MATERIALS AND METHODS

Samples

Frozen samples of *Artemia* (10) and *Brachionus* (5) were supplied in test tubes and were freeze-dried immediately before extraction.

Lipid Extraction

Lipids were extracted using a modification of the method of Bligh and Dyer (1959). The lipid extracts were stored at -20°C under nitrogen prior to fatty acid analysis. The amount of lipid present (under nitrogen) was determined by weighing using a Mettler AE163 digital balance.

Fatty Acid Analysis

The lipid extracts were transesterified with a methanolic hydrochloric acid solution to form fatty acid methyl esters (FAME). The FAME fractions were dissolved in chloroform to which a known amount of C_{23} FAME was added as an internal standard and were stored under nitrogen at -20 °C until analysis.

Analysis by gas chromatography (GC). The FAME samples were analysed with a Hewlett-Packard 4890 gas chromatograph equipped with an FID and cooled OCI-3 on-column injector and a polar 70% cyanopropyl siloxane (BP-X70) fused-silica capillary column (50 m x 0.25 mm i.d.) (SGE, Australia). Samples were injected at 45°C and after 2 minutes the oven temperature was raised to 120°C at 30°C per minute and then to 240°C at 3°C per minute. The final temperature was maintained for 10 minutes. Hydrogen was used as the carrier gas. The detector temperature was 260°C. Peak areas were quantified with DAPA software on an IBM-compatible computer.

Analysis by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses FAME fractions were performed as necessary with an HP 5890 GC and 5790 MSD fitted with a direct capillary inlet. The polar column, injector and chromatography conditions were the same as those described above with the exception that helium was used as the carrier gas. Electron impact mass spectra were acquired and processed with an HP 59970A Computer Workstation. Typical MSD operating conditions were: electron multiplier 2000 volts; transfer line 310 °C; electron impact energy of 70 eV; 0.8 scans per second; mass range 40-600 dalton.

Fatty Acid Nomenclature

Fatty acids are designated as X:Y(n-z) where "X" is the number of carbon atoms (as it is with the C_X notation), "Y" is the number of double-bonds and "z" is the position of the ultimate double-bond from the terminal methyl group. The prefix *i* indicates an *iso* methyl branch and *a* an *anteiso* methyl branch. FAME are fatty acid methyl esters, while PUFA is an abbreviation for polyunsaturated fatty acids. Docosahexaenoic acid, 22:6(n-3) is abbreviated to DHA and eicosapentaenoic acid, 20:5(n-3) to EPA. Double bonds in PUFA are separated by a methylene group unless designated NMI (non-methylene interrupted).

Reference

Bligh, E. G. & Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can J. Biochem Physiol.* **37**:911-917

Table 1. Sample codes, diets, total lipid and other details

Sample number	Sample code used by Sea Fisheries	Species	Diet	Duration (hours)	Temperature (°C)	Total Lipid (mg/g dry weight)
1	AF1:3	*Artemia*	nil	0	27	106
2	AF2:3	"	nil	12	20.5	62.4
3	AF3:3	"	nil	24	20.5	86.9
4	SS00A	"	nil	0	27	119
5	SS24A	"	SuperSelco	24	27	164
6	SS48A	"	SuperSelco	48	27	171
7	OLPP	"	Petsalis/PVM	?	27	200
8	OLPS	"	ProteinSelco	?	27	185
9	OLNP	"	Nutripak	?	27	217
10	OLFP	"	Frippak	?	27	106
11	RO:OA	*Brachionus*	nil	0	20	66.7
12	RPetA	"	Petsalis	6	20	137
13	RSSA	"	SuperSelco	6	20	115
14	RNPA1/RFPA	"	Frippak	4	20	86.6
15	RNPA2	"	Nutripak	2	20	147

Table 2. Fatty acid composition (% of total fatty acids) of *Artemia* (1-10) and *Brachionus* (11-15) fed different diets.

SF1 sample number	1	2	3	4	5	6	7	8
Sea Fisheries code	AF1:3	AF2:3	AF3:3	SS00A	SS24A	SS48A	OLPP	OLPS
Diet	nil 0h	nil 24h	nil 48h	nil	SuperSelco	SuperSelco	Petsalis/PVM	ProteinSelco
Saturated Fatty Acids								
14:0	0.1	1.1	0.8	0.5	0.4	0.4	1.5	1.6
16:0	11.5	11.1	10.9	9.7	6.5	5.9	10.8	12.9
18:0	4.7	5.4	5.9	6.0	5.1	4.8	5.9	5.4
Monoenoic fatty acids								
16:1(n-7)	6.7	6.3	6.0	2.1	1.4	1.3	3.3	1.8
17:1(n-8)	1.5	1.5	1.4	0.6	0.4	0.4	0.5	0.4
18:1(n-9)	20.2	18.6	18.8	15.7	15.4	15.6	17.6	14.1
18:1(n-7)	8.8	9.7	10.9	5.5	5.0	5.0	4.9	3.3
20:1(n-9)	0.5	0.5	0.6	0.6	1.2	1.3	4.5	3.1
22:1(n-11?)	-	-	-	-	0.3	0.3	2.7	4.0
22:1(n-9)	-	-	-	-	0.1	0.1	0.3	0.4
Polyenoic Fatty Acids								
18:2(n-9 or NMI)	0.6	0.6	0.5	1.3	0.5	0.4	0.8	0.6
18:2	-	-	-	-	-	-	-	-
18:2(n-6)	5.4	5.1	5.0	5.0	6.7	7.3	3.5	4.9
18:3(n-6)	1.0	1.0	0.9	1.1	0.5	0.3	0.6	0.4
18:3(n-3)	20.7	19.9	18.8	34.9	15.4	12.2	19.7	15.4
18:4(n-3)	3.8	3.5	3.2	7.6	3.3	2.5	4.4	3.5
20:3(n-3)	0.8	0.8	0.8	2.1	1.1	0.8	1.2	1.0
20:4(n-6)	1.7	1.8	2.0	0.5	1.3	1.6	0.6	0.7
20:4(n-3)	0.8	0.8	0.8	1.4	1.4	1.4	1.3	1.2
20:5(n-3)	6.5	7.3	7.7	1.9	17.4	21.4	5.5	9.8
21:5(n-3)	-	-	-	-	0.3	0.3	0.2	0.6
22:5(n-6)	-	-	-	-	0.3	0.4	0.1	0.3
22:5(n-3)	-	-	-	-	1.5	1.8	0.8	1.8
22:6(n-3)	-	-	-	-	11.6	11.7	6.5	9.8
other*	4.7	5.0	5.0	3.5	2.9	2.8	2.8	3.0

*includes fatty acids which are less than 1% of total in all samples such as 15:0, *a*15:0, *i*15:0, 17:0, 20:0, 22:0, 14:1, 18:2, 18:3, 20:2(n-6) and 20:3(n-6) where present.
NMI = non-methylene interrupted.

Table 2 cont. Fatty acid composition (% of total fatty acids) of *Artemia* (1-10) and *Brachionus* 11-15) fed different diets.

SF1 sample number	9	10	11	12	13	14	15
Sea Fisheries code	OLNP	OLFP	RO:OA	RPetA	RSSA	RNPA1	RNPA2
Diet	Nutripak	Frippak	nil	Petsalis	SuperSelco	Frippak	Nutripak
Saturated Fatty Acids							
14:0	2.7	0.7	2.3	3.6	1.4	3.6	5.7
16:0	10.1	10.2	7.0	10.9	4.9	9.6	11.5
18:0	4.1	6.9	4.4	5.0	3.7	4.7	2.8
Monoenoic Fatty Acids							
16:1(n-7)	3.9	2.3	25.3	14.1	10.4	19.2	11.3
17:1(n-8)	0.5	0.6	0.7	0.6	0.5	0.6	0.5
18:1(n-9)	21.4	15.9	24.8	20.5	15.7	18.6	20.2
18:1(n-7)	4.3	5.6	6.4	5.2	4.3	5.1	3.4
20:1(n-9)	4.0	0.6	3.8	6.4	3.1	2.8	7.3
22:1(n-11?)	4.0	-	0.3	3.2	0.7	0.3	9.2
22:1(n-9)	0.3	-	1.3	0.8	0.8	0.9	-
Polyenoic Fatty Acids							
18:2(n-9 or NMI)	0.5	1.2	-	-	-	-	-
18:2	-	-	2.0	0.7	0.8	1.2	0.6
18:2(n-6)	4.8	4.8	3.9	2.9	6.8	4.8	3.9
18:3(n-6)	0.5	1.0	0.1	0.2	0.2	0.3	0.2
18:3(n-3)	15.6	32.2	0.9	1.3	1.4	1.2	1.7
18:4(n-3)	4.1	6.8	0.3	2.2	1.6	1.9	2.2
20:3(n-3)	0.9	2.1	0.1	0.1	0.2	0.1	0.1
20:4(n-6)	0.5	0.6	2.2	1.0	1.7	2.2	0.9
20:4(n-3)	1.0	1.4	0.5	0.9	1.3	1.0	0.6
20:5(n-3)	8.7	2.9	5.7	8.2	16.5	10.1	7.9
21:5(n-3)	0.2	-	-	0.3	0.5	-	0.3
22:5(n-6)	0.1	0.1	-	0.1	0.6	-	0.1
22:5(n-3)	0.7	0.1	1.1	1.2	2.7	1.7	1.0
22:6(n-3)	4.1	0.4	0.1	6.5	15.8	3.5	4.4
other*	3.0	3.6	6.8	4.1	4.4	6.5	4.1

*includes fatty acids which are less than 1% of total in all samples such as 15:0, *a*15:0, *i*15:0, 17:0, 20:0, 22:0, 14:1, 18:2, 18:3, 20:2(n-6) and 20:3(n-6) where present.

NMI = non-methylene interrupted.