Larval development and metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*): influences of nutritional, environmental and physiological factors.

A thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

This work has been composed entirely by my own investigation. Except where specifically acknowledged, work in this Thesis has been conducted independently and has neither been accepted nor is being submitted for any other degree.

Candidate (signature)

Supervisor (signature)

Acknowledgements

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Abstract

The present work reflects investigations performed, in the frame of a Ph.D. thesis, on the larval development and metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*). The emphasis was placed on the influence of nutritional, environmental and physiological factors.

Three important periods have been identified during the larval development of Atlantic halibut; namely, the pre-metamorphic stage (up to 35 days post first-feeding (PFF)), the metamorphic climax (from day 35 to 45-50 PFF) and the post-metamorphic stage. The pre-metamorphic stage is characterised morphologically by a change of larval body shape with a widening of the body. The period of metamorphic climax is initiated by a surge in thyroid hormone synthesis followed by the differentiation of the stomach and the accumulation of lipid in the liver. Meanwhile, larvae fed enriched-*Artemia* insted of copepods, showed a delayed appearance of morphological changes associated with the shift from pelagic to demersal life-style. Their ability to digest and accumulate lipids was also delayed until the time at which the stomach became functional. Associated with this, the recruitment of rods into the retina was depleted as shown by a larval rod/cone ratio of 1.32 compared to 2.53 for the copepod-fed larvae. The ultrastructure of the outer segments of the retinal photoreceptors was characterised by the poor stacking of the membranes; and the retinal pigment epithelium revealed signs of lipid deficiencies such as an increase in appearance of phagosomes and the absence of lipid droplets.

The use of *Artemia* HUFA-rich enrichments helped to improve the larval performances, especially in terms of growth and stress resistance. However, the occurrence of pseudoalbinism remained widespread. The modifications of the light environment used to rear the larvae could not be linked with an improvement in larval pigmentation. Nevertheless, the use of blue light (475nm) improves the survival and the food intake during part of the pre-metamorphic stage. The present results support the importance of HUFA in the diet of larval fish. However, it is suggested that the larvae are unable to fully digest *Artemia* at a time when their digestion relies solely on the pancreatic enzymes. It was observed that the exoskeleton of *Artemia* was not fully digested by larvae whose stomach was not functional. Consequently, the hypothesis under which only the food accessible via the anus and mouth of the *Artemia* was digestible, was raised, therefore questioning the adequacy of long term enrichments.

Overall, the present study strengthened existing knowledge of the physiological, environmental and morphological parameters affected and affecting the halibut larval metamorphosis. The most prominent experimental evidence further emphasised the nutritional importance of HUFA especially for the development of the retina and presumably the whole nervous system.

Keywords: halibut, larval development, live food, digestibility, retina, thyroid, light regimen, PUFA.

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Chapter 1. General Introduction

The life cycle of fish, from the beginning to the end of their development, consists of five periods according to Balon (1985): embryo, larva, juvenile, adult and senescence. A sixth stage is often mentioned in the literature and widely used by fish farmers. Indeed, the embryonic phase is divided into the egg stage and the yolksac stage. Balon (1985) argues that hatching is rarely a developmental threshold as throughout the embryos rely on endogenous food supply. Beside the biological differences between these two developmental stages, the rearing techniques required to maintain eggs or yolksac larvae are quite different, especially for halibut (Harboe *et al.*, 1994b). Therefore, and to avoid confusion, the terms first-feeding larvae or exogenous feeding will be used hereafter to refer to Balon's larval stage.

It is to the larval stage of development of halibut that this study was devoted. In their effort to successfully control the rearing of Atlantic halibut, farmers as well as scientists have faced a series of problems over the past fifteen years. The attention was first focused on embryos (Riis-Vestergaard, 1982; Bolla and Holmefjord, 1988; Bergh *et al.*, 1989). Attempts to rear first-feeding larvae soon became the main bottleneck (Blaxter *et al.*, 1983). The succession of problems associated with the design of rearing system for eggs and larvae highlighted the need for more knowledge to be acquired on developmental biology of halibut (Pittman *et al.*, 1989; Pittman *et al.*, 1990; Kjørsvik and Reiersen, 1992). As knowledge was acquired and the production potential of halibut as a major cold water species became obvious, at least one major problem remained.

Some authors showed the adequacy of wild plankton and in particular marine copepods as a good first-feeding prey organism (Skjolddal *et al.*, 1990; Næss *et* al., 1995; McEvoy *et al.*, in press). In the mean time, the intensification of the culture techniques and the use of *Artemia* appeared to be linked to incomplete metamorphosis and malpigmentation as in other flatfish (Seikai *et al.*, 1987c; Sanchez and Miranda, 1997). It is in that frame of research that this thesis was initiated and in the light of new findings (Murray *et al.*, 1994a; van der Meeren, 1995; Gulbrandsen, 1996; Shields *et al.*, 1997) that it progressed.

The use of a calanoid copepod, *Eurytemora velox*, as prey organism, was combined with the application of the best environmental conditions adapted as a result of years of experience at the SFIA Marine Farming Unit in Ardtoe (Argyll, Scotland). Indeed, the improvement of larval rearing requires suitable environmental, nutritional and physiological conditions. It can be expected that larvae are adapted to the environment in which they develop in the wild. Despite the rather poor success of campaigns of capture destined to collect data on wild animals (Haug, 1990), it is well established that larval halibut are pelagic. By comparison to other fish of other genus for which more information is available, a number of assumptions could be made as to the diet and development of larval halibut. As a result, the above mentioned rearing conditions were considered as mimicking natural conditions. Larvae reared under these conditions were considered to provide a positive control.

Further knowledge on the nutritional and physiological aspects of the metamorphosis were gained based on these larvae. In order to apply these findings to the current

rearing procedures, larvae from identical batches were fed exclusively on enriched-*Artemia*. Each aspect studied such as the ontogeny of the digestive tract, the importance of thyroid hormones during the metamorphosis and the development of the retina, were compared according to the nutritional status of the larvae. It allowed a better understanding of halibut larval development and metamorphosis and the impact of the diets on their regulation.

As a result, the main objectives of this thesis were as follows:

1) to describe the development and metamorphosis of halibut larvae and to assess the effects of the nutritional quality of two different prey organisms on their morphology and digestive ontogeny;

2) to assess the importance of thyroid hormones on the larval metamorphosis and development;

3) to describe the retina of metamorphosed larvae and to assess the effects of nutritional quality of prey organisms on its structure and functions:

4) to test the impact of commercially available lipid sources used as enrichments for *Artemia* on the development and metamorphosis of the larvae;

and,

5) to test the impact of different light environments on the development and the metamorphosis of the larvae.

The experiments designed to fulfil these objectives are presented hereafter in Chapters numbered from 3 to 8. Each Chapter deals with one objective and includes a brief introduction, a specific materials and methods if required, a presentation of the results and a discussion. The materials and methods common to more than one experiment are combined in a single Chapter (Chapter 2). Finally, a conclusion assesses the progress made in completing each objective and highlights the main findings and their implications (Chapter 9).

Chapter 2. General Materials and Methods

2.1. Standard procedure used for larval production

2.1.1. *Artemia* culture

2.1.1.1. Background information

Artemia and rotifers are the most widely used live feed organisms in larviculture of marine finfish around the world (Sorgeloos and Léger, 1992). In halibut larval culture, *Artemia* have received most of the attention. The large size of the larvae at first-feeding allows them to ingest brine shrimp nauplii straight away. Furthermore, when exogenous feeding has been well established, larvae can be fed on moulted *Artemia* (Næss *et al.*, 1995; Shields *et al.*, 1997). *Artemia* are collected in the wild as cysts and commercialised as such. They are commonly decapsulated, a process designed to ease hatching and to clear the cysts of potentially pathogenic bacteria, and partially ongrown in hatcheries filling the demand for live prey.

2.1.1.2. Decapsulation

2.1.1.2.1. Materials

* *Artemia*: EG cysts from the Great Salt Lakes (Utah, USA) commercialised by INVE Aquaculture NV (Baasrode, Belgium).
* decapsulation solution: 15% hypochlorite solution (^-OCl) and a sodium hydroxide solution (NaOH) used at a rate of 0,15g of NaOH per 1g of cysts.
* filter: piece of 25cm diameter pipe with one end sealed by a 64µm mesh.
* safety wear: disposable laboratory gloves and breathing mask.
* 5 L glass beaker

2.1.1.2.2. Methods

Distilled water was added in the glass beaker at a rate of 14 l kg^{-1} of dry cysts. The hypochlorite solution, 3.5 l kg^{-1} of dry cysts, was then added. The pH of the decapsulation solution was stabilised by the addition of the sodium hydroxyde solution. This procedure avoids over-oxidation. Thorough mixing was performed using a glass rod until the colour of the cysts changed from brown to orange indicating that they had been oxidised. The solution was then transferred to the large filter and rinsed thoroughly with seawater until the effluent was perfectly clear. The cysts were then rinsed using distilled water and kept in a small volume of distilled water (just enough to keep the cysts submerged) at 4° C until further use.

2.1.1.3. Hatching

2.1.1.3.1. Materials

* incubator: 80 l cylindrico-conical semi-transparent tank with a central stand pipe.
* water: UV sterilised and filtered (5μm) sea water heated to 27° C.
* aeration: perforated round pipe (5mm in diameter) surrounding the bottom of the stand pipe, producing fine air bubbles rising evenly over the whole tank.
* lighting: two PAR 38 tungsten floodlights (Osram Concentra, 80 watt), one placed directly on top of the incubator and one positioned to the side of the conical section of the incubator, near the bottom.

2.1.1.3.2. Methods

Up to 300 ml of cysts kept in distilled water at 4° C were hatched at any one time. The cysts were added to the heated water of the incubator. The aeration was increased until a thorough mix of the *Artemia* in the incubator was assured. A precise value of the volume of air used in relation to the volume of the incubator can not be provided as the

aeration was adapted to every batch. Only the light placed above the incubator was lit. The cysts were allowed 18 h to hatch. The top light was then switched off and the second one was lit. All other sources of light in the room were also switched off. The hatched nauplii, positively phototactic, were allowed to swim to the light for 5-10 min. They were removed using a siphon, leaving in the incubator the undecapsulated cysts that had sunk to the bottom and the empty shells that had floated to the surface. The nauplii were rinsed first in sea water and then in distilled water as a precaution against pathogenic bacteria. Despite the osmotic shock and the temperature shock associated with this procedure, preliminary experiments revealed that the nauplii survival rate was high (>90%).

2.1.1.4. Ongrowing

2.1.1.4.1. Materials

* tanks: three 80 l cylindrico-conical tanks and 10 smaller 2 to 5 l containers of similar shape placed in two water baths.
* water: the water was UV sterilised and filtered (5 μm); in the case of the large tanks it was heated to 27° C within the tank while for the smaller containers, only the water of the water bath in which they were placed was heated.
* filters: 20 cm diameter pipe with one end sealed by a 100 μm mesh.
* measuring cylinders: 50, 250, 500, and 1000 ml graduated cylinders.
* *Artemia* counter: 1 ml automatic pipette with a glass pipette used as a tip.

2.1.1.4.2. Methods

The rinsed nauplii were transferred from the filter into a measuring cylinder of appropriate size. Water was added to adjust the volume to the closest round number to ease calculation. An air stone was immersed in the cylinder to assure an even

distribution of the nauplii. Then 3 to 5 samples of 1 ml were taken using an automatic pipette also referred to as '*Artemia* counter'. Each sample was used to estimate the density of nauplii by dropping the counter's content slowly in front of a light. Individual nauplii could be counted as they passed through the thin section of the glass pipette. The average number of nauplii per ml in each sample was calculated and used to estimate the overall number of nauplii hatched. The same technique was also used to estimate ongrown *Artemia* number. The nauplii were then transferred to the incubators in appropriate numbers to reach an average of 200 nauplii ml^{-1}; this culture density was adopted following preliminary experiments, which revealed that it was associated with the best *Artemia* growth. The rinsing and counting procedure described above for the nauplii was repeated on a daily basis when the *Artemia* were ongrown for more than 18 h. It is important to note that the distilled water rinsing was introduced as a result of preliminary experiments which showed that such a procedure was efficient in decreasing the bacterial load associated with the *Artemia*.

2.1.1.5. Enrichments

2.1.1.5.1. Materials

* Enrichments:

- a microparticulate product solely based on marine ingredients, having a protein content of 75% in dry matter, referred to as SSF microfeed, and provided by the Norwegian Herring Oil and Meal Industry Research Institute (Norsildmel, Fyllingsdalen, Norway).

- a microparticulate product prepared from a marine protist (*Schizochytrium*) and referred to as Algamac 2000™ (Aquafauna Biomarine Inc., USA).

- a marine fish oil referred to as Herring Oil and provided by the Norwegian Herring Oil and Meal Industry Research Institute (Norsildmel, Fyllingsdalen, Norway).

- an oil emulsion, Super Selco™ produced by INVE Aquaculture NV (Baasrode, Belgium).

- a PUFA-rich oil extracted from the eye orbit of tuna (referred to as Tuna Orbital Oil) provided by the NERC Unit of Aquatic Biochemistry of the University of Stirling and emulsified with 12% (w/w) soya lecithin.

* Mixer:

- 1 l standard kitchen blender.

- vortex mixer.

2.1.1.5.2. Methods

The quantity of enrichment was determined by the number of *Artemia* to be fed. Based on culture densities of 200 000 *Artemia* l^{-1}, it was established, following a series of preliminary experiments, that 0.5 to 0.6 g l^{-1} was the most appropriate dilution for all the enrichments in order to maximise growth and survival. Indeed, an homogenous population of *Artemia* showing low mortality rates was seen as ideal to secure the larval feeding. Prior to their addition to the culture tanks, the enrichments were dissolved in a small volume of water (300 ml g^{-1}). In the case of the microparticulate enrichments, they were added directly into the blender, mixed for 2 min and added to the *Artemia* culture tanks. The oil emulsions were first weighed in a 20ml test tube. A few ml of distilled water were added and the solution was mixed thoroughly for 1 min using a vortex mixer. This solution was then decanted in the blender and mixed for 2 min with the required volume of water. The resulting solution was separated in two aliquots, one

was added to the *Artemia* culture tank and the other stored at 4° C. The second aliquot was added to the culture half way through the 18h enrichment period.

2.1.2. Copepod culture

2.1.2.1. Background information

As part of a project financed by the Commission of the European Communities, Directorate General XIV (AIR3 - CT94 – 2094), a large 150 m^3 tank was set up in Ardtoe in order to improve the productivity of cultures of a calanoid copepod, *Eurytemora velox*. The tank was seeded with all the copepod stages remaining from the previous small-scale experiments (for details on the role and production of copepods, see Naess, 1997 and Shields, 1997).

2.1.2.2. Materials

* tank: 150 m^3 round outdoor tank in a concrete base.
* aeration: single air stone positioned in the centre of the tank.
* water temperature: ambient temperature, no additional heating installed and no water renewal.
* algae population: during the spring approximately 200 l of algae (*Nannochloris atomus*) were added and allowed to bloom freely (no nutrient added).
* copepod collector: air lift system filtering 2 l min^{-1} of tank water through a 64 µm mesh adding up to 2% of the total volume per day.
* copepod separator: series of filters of increasing mesh size (64 µm, 200 µm and 400 µm).

2.1.2.3. Methods

While the copepod and the algae population was allowed to bloom during the spring, periodic samples were taken using the copepod collector. These samples were passed through the series of filters forming the separator to quantify the different size groups: the nauplii and copepodites (between 64 and 200 μm), the copepodites and juveniles (between 200 and 400 μm) and the adults (above 400 μm). A count was performed on each fraction before replacing them in the culture tank. A minimum of 15000 copepods of the intermediate size population was obtained per sample by late spring-early summer at the time when the first larval rearing experiment was initiated. This fraction was thereafter used as live feed for the larvae.

2.1.3. Production of first-feeding larvae

2.1.3.1. Background information

The experiments were conducted at SeaFish Aquaculture's Marine Farming Unit at Ardtoe (Argyll, Scotland). The following section briefly summarises the procedure adopted for the production of first-feeding larvae. As the present work did not deal with the culture of halibut prior to or after the exogenous feeding stage, only the general characteristics of the culture techniques are mentioned (for complementary information see Haug, 1990; Pittman, 1996; Millard and FitzGerald, 1997 and Shields *et al.*, 1997).

2.1.3.2. Methods

* Eggs (see figure 2.1):

- holding facilities: black cylindro-conical tanks of 80 to 300 l with slow upstreaming water ($\cong 2\ l\ min^{-1}$).

- temperature regime: 6° C.

- light regime: total darkness except for brief daily checks.

- timing: the incubation starts 16h after fertilisation. At this stage, 8 cells can be seen. Criteria relating to their symmetry are being used to select batches of good quality (Nick Brown 1997, personal communication). Hatching occurs approximately 16 days later (85 day-degrees).

* Yolksac larvae:

- holding facilities: black cylindro-conical tanks or silos of 500 to 3500 l with slow upstreaming water ($\cong 2\ l\ min^{-1}$).

- temperature regime: 6° C.

- light regime: total darkness.

- timing: after 190 day-degrees post hatch at 6°C, the temperature in the yolksac tanks was raised by 1°C per day until the water temperature reached 10°C. At this stage (approximately 225 day-degrees post hatch) the larvae are removed from the upper water layers of the tanks using 2 l plastic beakers.

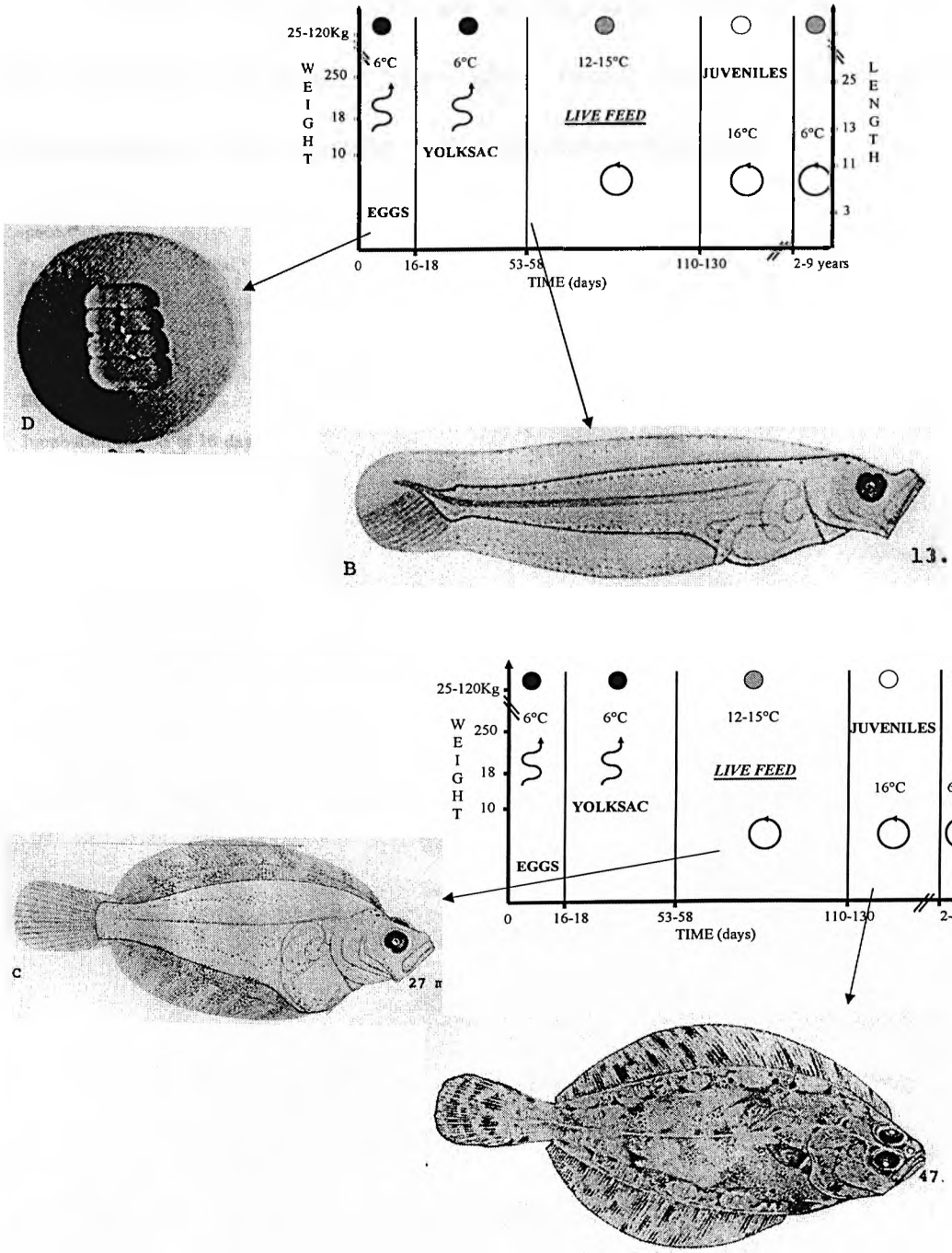
* First feeding larvae:

- holding facilities: 1000 l black circular tanks with a circular flow of $250ml^{-1}$ started on the fifth day post first-feeding (PFF).

- temperature regime: daily increase of 1°C from 6 to 10° C followed by a slow increase up to 13-14° C.

- light regime: light of maximum 1500 lux (50 lux at transfer) at the water surface and green microalgae added to the water (turbidity < 4 NTU).

Figure 2.2: summary figure presenting the different developmental stages of halibut larvae. The circles on top of the charts refer to the light regime (from total darkness at the egg stage to daylight at the larval stage and to dim light at the adult stage). The arrows schematise the water flow (from upwelling to circular flow). The drawings of egg, larvae and juvenile have been copied from an unidentified publication of the Cummunications Directorate of the Canadian Department of Fisheries and Oceans.



- timing: after transfer from the yolksac incubators, the larvae are kept for 10 to 15 days in these conditions to select the feeding individuals. Larvae are then transferred to experimental facilities.

- feeding regime: mix of *Artemia* nauplii and 18 h enriched *Artemia* fed 3 times daily to satiation at the onset of first-feeding. Dietary regimes following the initiation of experiment may vary according to the experimental objectives.

2.2. Comparison between copepod and *Artemia*-fed larvae

2.2.1. Aim

Two larval rearing experiments were conducted in order to compare the effects of enriched *Artemia* versus a marine copepod (*Eurytemora velox*) as live prey for halibut larvae. Both experiments were initiated with larvae described as established feeders (see section 2.1.3.2.) and lasted until the end of metamorphosis.

2.2.2. Experiment 1

2.2.2.1. Experimental design

2.2.2.1.1. Materials

* tanks: 2 black circular 1000 l tanks with a central stand pipe.
* water: constant temperature (11 ± 2° C), salinity (34 ± 1.8 ppt), and pH (7.5 ± 1.1).
* temperature control: the incoming water was chilled and the room was equipped with an air chiller.
* lighting: dimmable PAR 38 tungsten floodlight (Osram Concentra, 80 watt), place above each tank providing a maximum of 1200 lux on the water surface.
* aeration: single air stone placed in the centre of the tank.
* turbidity: 10 to 20 l of algae (*Nannochloris atomus*) added daily (maximum final turbidity = 3 NTU) until a majority of the larvae became demersal.
* anaesthetic: 3-aminobenzoic acid ethyl ester methane sulphonate (MS222) (Sigma Chemical Co., Poole, England).

2.2.2.1.2. Methods

Using 2 l plastic beakers, approximately 900 larvae aged 21 days post first-feeding (21 days PFF) were scooped out of the first feeding tank and randomly distributed between the two experimental tanks. Both tanks were treated identically in terms of water quality, lighting, and sampling.

The larvae were fed 3 times daily to satiation. One group was fed with 18h SSF-enriched *Artemia* exclusively and the other with *Eurytemora velox* and 18 h SSF-enriched *Artemia*. The exact number of *Artemia* or copepods given per larva per day was not calculated. Indeed, this task was not part not of the hatchery standard practise for larval production. However from previous experiments it was estimated as varying from 300 to 2500 *Artemia* larva^{-1} day^{-1} or 120 to 500 copepod larva^{-1} day^{-1} between the onset and the end of the experiment. The size of the rations was determined by the ability of the larvae to eat all the prey between the morning and the afternoon feed and by visual observation of their guts. If the larvae had emptied the tanks of all its prey and if some displayed empty guts, the rations were increased. The evening feed consisted solely of enriched-*Artemia* for both groups as the supply of copepods (minimum 15000 copepod day^{-1}) was not sufficient to feed the larvae to satiation. The *Artemia* supplement varied in size accordingly to fulfil this requirement.

2.2.2.2. Sampling schedule

The larvae to be sampled were randomly selected from each tank using a 2 l plastic beaker and killed with an overdose of 3-aminobenzoic acid ethyl ester methane sulphonate (MS222). Sampling started on day 21 PFF. Five groups of 5 larvae were taken from each tank. Four of these groups were destined for growth measurements and

dry weight measurements. The fifth group of 5 larvae was destined for growth measurements, pigmentation characterisation and histology (see sections 2.5 and 2.6 for details on these techniques). This procedure was repeated on a weekly basis (days 21, 28, 35, 42, 49, 56, 63 and 70 PFF).

2.2.3. Experiment 2

B. Gara and Dr. R.J. Shields at Ardtoe carried out a further diet comparison the following year. This experiment was a collaborative effort, as samples were generated for histological, retinal structural and ultrastructural observations, as well as lipid analysis (performed by Dr. J.G. Bell and Dr. L.A. MacEvoy). My personal involvement was restricted to the sampling, the histological and the retinal observations.

The experiment was conducted in replicated 100 l circular tanks (3 tanks per treatment) using 44 fish per tank at 16 days PFF. The larvae were either fed *Eurytemora velox* copepods or *Artemia* enriched with Super Selco (morning feed) or Algamac 2000 (afternoon feed). The experiment lasted until day 69 PFF with samples collected on three occasions; after day 35, 49 and 63 PFF. Out of the 10 larvae sampled on each date, 2 x 3 larvae were used for lipid analysis and the 4 remaining larvae were used for the histological observations.

More details about this experiment are available in Shields *et al.*, 1997, and the lipid extraction and analysis methods are presented in section 2.6.

2.3. Morphological measurements

2.3.1. Larval growth

The following measurements were used to assess the growth of the larvae throughout development: weight, length and condition factor.

2.3.1.1. Weight

2.3.1.1.1. Materials

* microbalance accurate to 0.0001 g (Sartorius H110).

* freeze dryer (Edwards Micro Modulyo, Edwards High Vacuum International, Sussex, UK).

2.3.1.1.2. Method

* wet weight (also referred to as blotted weight): the larvae were removed gently from the anaesthetic solution with a pair of forceps, dipped in fresh water to remove the salt and blotted on both side with tissue paper to remove all surface water. They were then transferred into a weighing basket previously used to tare the microbalance and then weighed.

* dry weight: if the average wet weight of larvae exceeded 200 mg they were kept separate otherwise they were pooled in groups of 5. Individual larva or groups of 5 larvae were transferred from the anaesthetic solution into pre-weighed plastic vials. The cap of each vial was perforated and they were placed in a previously cooled freeze dryer for up to 48 h. Once the freeze-drying was completed each vial was weighed using a microbalance to determine the larval dry weight.

* Specific Growth Rate (SGR): the SGR is a measure of growth expressing the daily weight gain during a specific number of feeding days. It eases the comparison of growth measurements between fish of different ages or having been reared under different conditions. It is calculated using the dry weight values as follows:

$$SGR = (\ln W_T - \ln W_0) / T \times 100$$

where, T is the number of feeding days, W_T is the weight at time T and W_0 is the initial weight.

2.3.1.2. Length

2.3.1.2.1. Materials

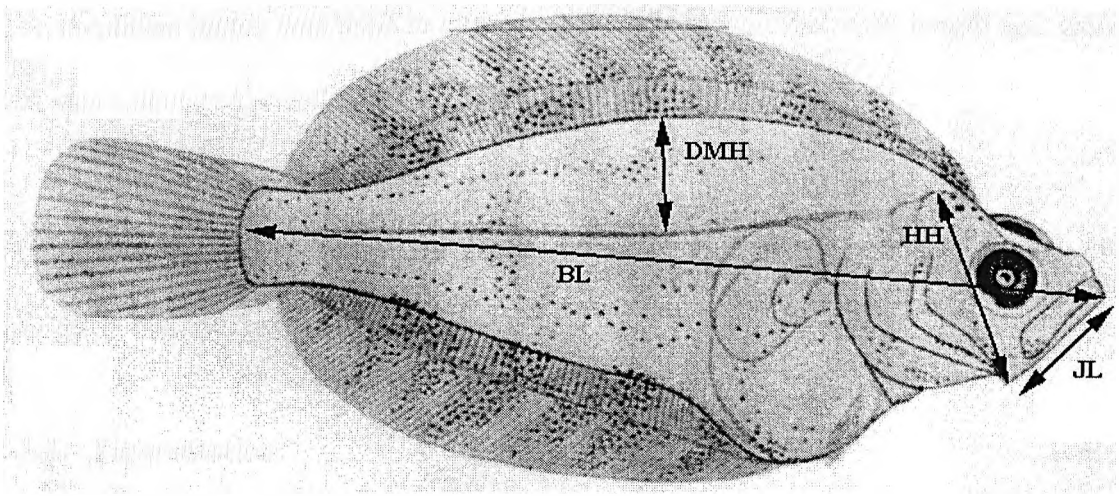
* light microscope (Olympus SZ60)
* microscopic objective equipped with an eyepiece: the graticule allowed measurements to be made in arbitrary units. The conversion from this unit to mm was calculated and applied using the following formula:

length in mm = number of units / (4.1881 x magnification + 1.0051)

2.3.1.2.2. Methods

Anaesthetised larvae were transferred to a cold room where the microscope was placed. The room temperature was set not to exceed the water temperature of the tank from which the larvae were sampled. Several measurements of the larvae were taken. They included body length, or standard length, head height, lower jaw length (referred to as jaw length) and dorsal muscular block height (referred to as dorsal myotome height). The measurements are represented in Figure 2.2. The body length was taken from the tip of the lower jaw to the tip of the bone structure forming the caudal complex. The head height was taken from the tip of the lower jaw to the top of the cranial bone

Figure 2.2: schematic representation of the growth measurements: DMH stands for Dorsal Myotome Height, BL stands for Body Length, JL stands for Jaw Length and HH stands for Head Height (drawing copied from an unidentified publication of the Cummunications Directorate of the Canadian Department of Fisheries and Oceans).



encapsulating the brain. The dorsal myotome height was taken from the backbone to the dorsal fin margin edge above the anus.

2.3.1.3. Condition factor

The condition factor was used to expressed the relationship between length and weight and was calculated as follows:

$$\text{Condition factor (K)} = 100 * W / L^3$$

where, W is the wet weight of the fish in grams (or milligrams), and L is the length of the fish in centimetres (or millimetres).

2.3.2. Pigmentation

2.3.2.1. Pigmentation categories

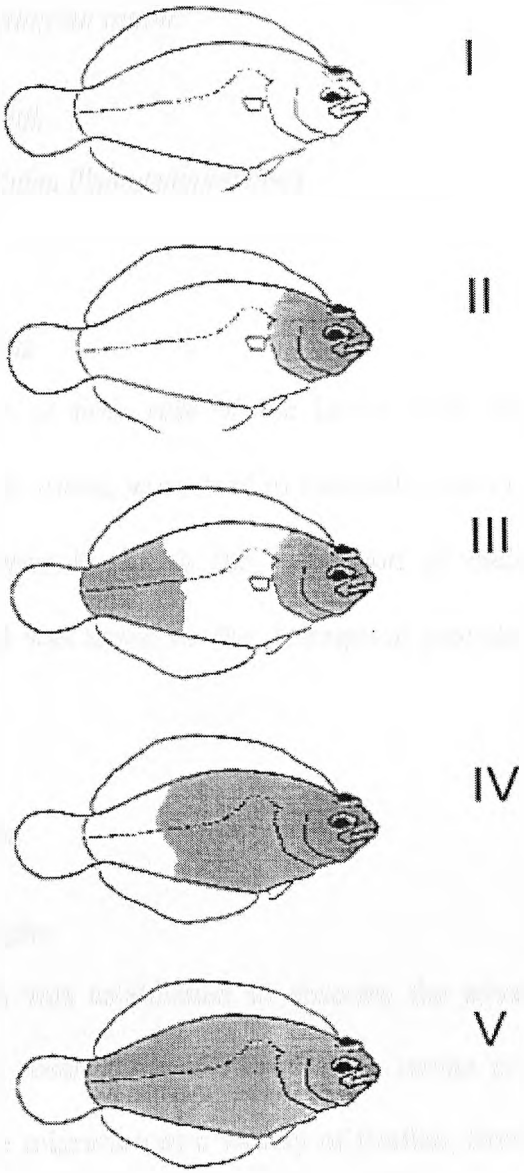
2.3.2.1.1. Principle

This method consisted of a visual assessment of the blind side and ocular side pigmentation of the larvae aiming at differentiating between normal pigmentation and pseudo-albinism or ambicoloration. Rather than giving an approximate value or index on the percentage of pigmentation of the larvae (Nicolaides and Woodall, 1962; Fukusho *et al.*, 1987; Dickey-Collas, 1993), a series of categories were defined based on those established for the Japanese flounder (Seikai, 1980; 1985a).

2.3.2.1.2. Methods

Based on visual observations of first-feeding larvae and juveniles produced at Ardtoe, a limited number of common pigmentation abnormalities were identified. These abnormalities were classified into 5 categories numbered 1 to 5 (Figure 2.3). Category 1 referred to a total lack of pigment, category 5 to a perfect pigmentation pattern whilst

Figure 2.3: schematic representation of the pigmentation categories, applicable for both sides of the larvae (1 = lack of pigment; V = normal pigmentation).



categories 2, 3 and 4 referred to intermediate patterns. This assessment system was used to describe both the blind and the ocular side of the metamorphosed larvae sampled.

2.3.2.2. Melanocytes count

2.3.2.2.1. Material

* light microscope (Zeiss Photomicroscope)

* manual counter

2.3.2.2.2. Method

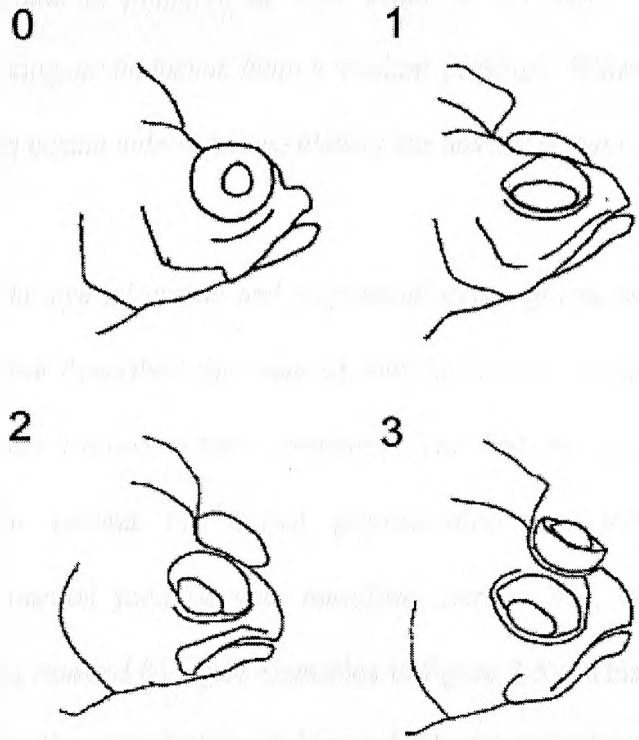
Photographic records of both side of the larvae were taken. These pictures, once projected on a 17 inch screen, were used to manually count (using a manual counter) the number of melanocytes in which the dispersion of melanin was apparent. Such identification method was based on the description provided for Japanese flounder by Suzuki, 1994.

2.3.3. Eye migration

2.3.3.1.1. Principle

A qualitative system was established to describe the advancement of eye migration based on the visual observation of first-feeding larvae produced at Ardtoe. In his description of the eye migration of a variety of flatfish, Brewster (1987) referred to the position of the eye in relation to the dorsal fin. Consequently, 4 categories were defined and numbered from 0 to 3 (Figure 2.4). Category 0 referred to larvae for which both eyes were symmetrically placed on both sides of the head. Category 1 referred to the appearance of the left eye on the right side of the head. Category 2 referred to the left eye reaching the level of the dorsal fin on its way to the right side. And finally category 3 referred to the left eye having passed the level of the dorsal fin and appearing

Figure 2.4: schematic representation of the eye migration categories (0 = symmetrical disposition of the eyes; 3 = complete eye migration).



next to the right eye on the ocular side of the larvae.

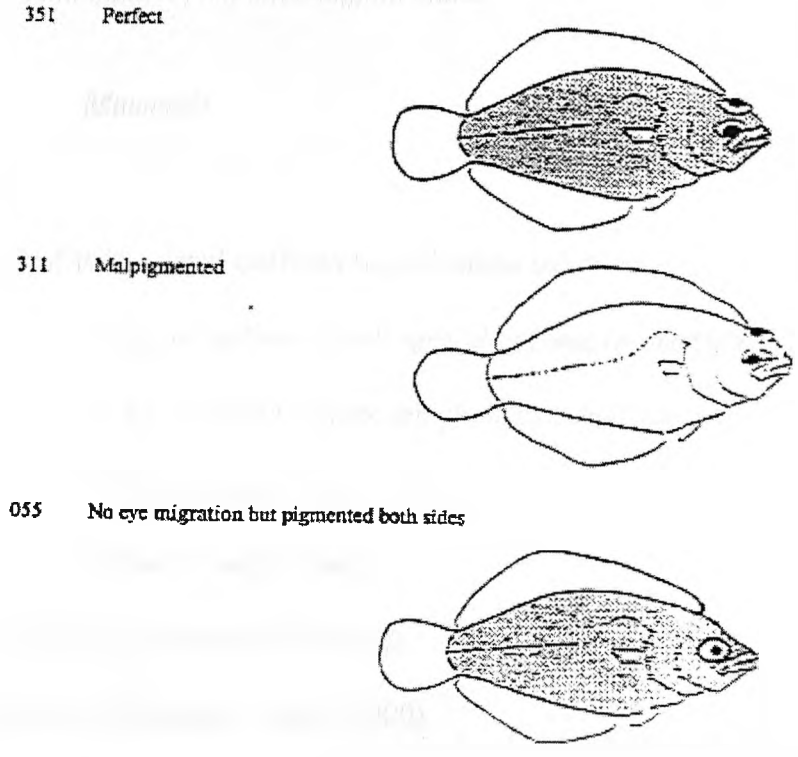
2.3.3.1.2. Methods

The larvae were transferred from the anaesthetic solution to a Petri dish using forceps. They were laid as flat as possible on their blind or left side. The categories were identified from looking at the larvae from a vertical position. When in doubt, the larvae were turned on their ocular side to assess clearly the advancement of the eye migration.

A combination of the eye migration and pigmentation categories was used to determine further categories that described the state of metamorphosis of the larvae as a whole. These categories were formed of three numbers. The first one represented the level of eye migration, the second the dorsal pigmentation and the third the ventral pigmentation. A normal juvenile was therefore marked 351, while a pelagic mal-pigmented larva was marked 011 (see examples in figure 2.5). This technique was seen as practical tool for the description of larvae both for experimental and production purposes. Indeed, several farmers, members of the British Halibut Association, adopted this technique during the life span of this thesis.

Although the left eye, if looking at the larvae from tail to head, was seen migrating, in some cases the right eye migrated. Attempts were made to relate this abnormality to the rearing parameters recorded but this was unsuccessful. As the migration of the right eye was recorded in less than 5% of the larvae, it was not taken into account in the following study.

Figure 2.5: examples of metamorphosis categories based on a combination of the eye migration and pigmentation assessment.



2.4. Histological preparations

2.4.1. Preparation of the histological slides

2.4.1.1. Materials

* fixative:

-1 l of 10% neutral buffered formal saline solution:

- 4.0g. of sodium dihydrogen phosphate (monohydrate)
- 6.5g. of sodium hydrogen phosphate (anhydrous)
- 100ml. formaldehyde
- 900ml. distilled water

- Bouin Picro Formol (BDH Ltd)

* tissue processor (Shandon Citadel 2000)

* embedder (Jung Histoembedder)

* decalcifier (RDC Rapid Decalcifier –1 l, CellPath, UK)

* cold plate (Raymond A Lamb)

* microtome (Reichter-Jung Biocut 2035)

* water bath (Raymond A Lamb)

* hot plate (Raymond A Lamb)

2.4.1.2. Methods

The larvae collected for histological examination were placed in 10% neutral buffered formal saline solution for at least 24 h (6 h when in Bouin's solution) for fixation. Once removed from the fixative, the larvae were placed in cassettes with one larva in each.

The cassettes were allocated a case number using a pencil (ink would have been removed by solvents during the processing) and left in a bowl of water to avoid drying.

Larvae were embedded in paraffin wax following dehydration in a graded series of alcohol and cleared in chloroform (see Table 2.1). Following embedding, the wax was solidified on a cold plate and the larva was held in position. The larvae were oriented to provide longitudinal sections.

The surface layer of wax had to first be removed to expose the complete surface of the specimen. This was carried out on the microtome using a used blade. The rate of advancement of the block towards the knife was determined manually at this stage.

Before sections could be obtained, it was necessary to surface decalcify the blocks. In whole larval samples, the bone or cartilaginous structures were too hard and affected the cutting; the decalcification softened those tissues. This was carried out by placing blocks face down in a vessel containing a layer of decalcifying solution for approximately 1h. Then the blocks were cooled using a cold plate prior to sectioning.

Specimens were clamped into the block holder that was automatically advanced by 5μm per rotation. When a 'ribbon' was obtained it was removed and floated on a water bath. The best section(s) was(were) selected and picked up on a clean glass slide. When possible, all the sections were collected. Indeed, due to the variety in density between tissues (bones, organs, cartilage, etc.) serial sections were very difficult to obtain. The

Table 2.1: schedule of the tissue processing.

50% Methylated Spirit	1 h
80% Methylated Spirit	2 h
100% Methylated Spirit	2 h
100% Methylated Spirit	2 h
100% Methylated Spirit	2 h
100% Alcohol	2 h
100% Alcohol	2 h
Chloroform	2 h
Chloroform	1 h
Molten wax	1 h
Molten wax	2 h
Molten wax	2 h

selection of the sections did not follow a rigorous procedure therefore restricting the possibilities for 3 dimensional representation of tissues.

The case number was marked on the slide using a diamond pencil and the slide placed face up on a hot plate. Racked slides were then dried in an oven at 60° C for at least one hour before staining.

2.4.2. Staining techniques

2.4.2.1. Haematoxylin and Eosin (H&E)

This is the most common staining technique in histology. Haematoxylin stains acid structures purple. For example, nuclei and rough endoplasmic reticulum (RER) are strongly stained by the haematoxylin due to their high content of nucleic acids. On the other hand, eosin stains the alkaline structures in red or pink. As the cells' cytoplasm is full of protein, it appears stained in pink (Mayer, 1903). The different steps of the staining process are described in Table 2.2.

2.4.2.2. Masson's trichrome

This technique is most commonly used to stain the connective tissue. It includes three stains. The nuclei and the other basophilic structures appear blue, the collagen green and the cytoplasm as well as the muscles bright red (Masson, 1929). The different steps of the staining process are described in Table 2.3 and the preparations of some of the reagents are presented in Table 2.4.

Table 2.2: Procedure for Haematoxylin and Eosin (H&E) stain

Xylene	5 min
Alcohol I	2 min
Methylated Spirits	1 1/2 min
Running tap water	wash
Haematoxylin	5 min
Running tap water	wash
Acid Alcohol	3 quick dips
Running tap water	wash
Scott's tap water	½ min
Check staining microscopically at this stage	
Running tap water	wash well
Eosin	5 min
Running tap water	quick wash
Methylated spirits	30 sec
Alcohol II	2 min
Alcohol I	1 1/2 min
Xylene	5 min
Xylene	until coverslipped

Table 2.3: Procedure for Masson's trichrome stain

Xylene	5 min
Alcohol I	2 min
Methylated Spirits	1 1/2 min
Running tap water	wash
Celestine blue	5 min
Running tap water	wash
Haematoxylin	5 min
Running tap water	wash well
Acid alcohol	4 quick dips
Running tap water	wash well
Distilled water	rinse
Cytoplasmic stain in 1% Acetic acid (1:10)	60 min
Distilled water	rinse
1% Phosphosmolibdic acid	1-2 min*
* until collagen is decoloured, and muscle and fibres are red	
Distilled water	rinse
Counterstain	2-3 min
1% Acetic acid	wash well
Blot	
Alcohol II	2 min
Alcohol I	1 1/2 min
Xylene	5 min
Xylene	until coverslipped

2.4.2.3. Periodic acid Schiff (PAS)

Materials stained purple by PAS are said to be PAS positive. They include glycogen, mucin produced by the mucous cells of the digestive tract, the basal membrane and the brush border of the intestine (MacManus, 1946). The different steps of the staining process are described in Table 2.5.

2.4.2.4. Modified aldehyde fuchsin

The aim of the modified aldehyde fuchsin staining technique is to differentiate the various cells of the endocrine pancreas, *i.e.* the insulin secreting cells, the glucagon secreting cells and the somatostatin secreting cells. The aim here was to identify pancreatic tissues rather than their specific cells. The different steps of the staining process are described in Table 2.6.

Table 2.4: Detailed description of the preparation of some of the reagents used for Masson's trichrome stain.

Cytoplasmic stain	
1% Ponceau de Xylidine in 1% Acetic acid	2 parts
1% Acid Fuchsin in 1% Acetic acid	1 part
Counterstain	
2% light green in 1% Acetic acid	
Celestine blue	
Celestine blue B	0.5 g
Ferric Ammonium Sulphate	6 g
Glycerol	14 ml
Distilled water	100 ml

Table 2.5: Procedure for Periodic Acid Schiff (PAS) stain

Xylene	5 min
Alcohol I	2 min
Methylated Spirits	$1^{1/2}$ min
Running tap water	wash
1% Aqueous periodic acid	10 min
Running tap water	5 min
Distilled water	rinse
Schiff	20 min
Running tap water	wash
Haematoxylin	5 min
Running tap water	wash
Acid Alcohol	4 quick dips
Scott's tap water solution	1 min
Methylated spirits	rinse
Tartrazine	5 min
Blot on paper	
Methylated spirits	30 sec
Alcohol II	2 min
Alcohol I	$1^{1/2}$ min
Xylene	5 min
Xylene	until coverslipped

Table 2.6: List of the different steps of the staining technique known as modified aldehyde fuchsin.

Xylene	5 min
Alcohol I	2 min
Methylated Spirits	$1^{1/2}$ min
Running tap water	wash
Lugol's iodine	10 min
Running tap water	wash
2.5% sodium thiosulphate	up to 5 min
Running tap water	wash well
70% ethanol	wash
Aldehyde-fuchsin stain	15-30 min
95% ethanol	wash
Running tap water	wash
Celestine blue	5 min
Running tap water	wash
Haematoxylin	5 min
Running tap water	wash well
Acid alcohol	4 quick dips
Running tap water	wash well
Distilled water	rinse
Orange G - Light green	45 sec
0.2% Acetic acid	rinse briefly
95% ethanol	rinse briefly
Alcohol II	2 min
Alcohol I	$1^{1/2}$ min
Xylene	5 min
Xylene	until coverslipped

2.5. Stress resistance

2.5.1. Aim

Tests designed to determine the physiological conditions of fish are all based on the same principle and can be termed secondary stress tests (Schreck, 1990). A secondary stress test consists of subjecting fish to an additional standardised stress and measuring physiological variables that are part of the general adaptive response (GAS). Weaker fish will have less energy available to maintain homeostasis and so will either die or show exaggerated GAS response.

Based on this principle and following several preliminary experiments, a high salinity (65 ppt) test similar to that described by Dhert *et al.* (1990) for Asian seabass was established. This test was performed on larvae collected from experiments 3 and 4.

2.5.2. Materials

* tank: 4 l white rectangular container filled with 3.5 l of 65 ppt salt water.
* aeration: single air stone placed in a corner of the container.
* temperature: identical to that of the experimental tanks from which the larvae were sampled.
* lighting: also identical to that of the experimental tanks from which the larvae were sampled.

2.5.3. Methods

Groups of 10 larvae sampled at the end of experiment 3 and 4 were transferred (using a small hand net) to small containers filled with the 65 ppt salt water at a given time. Every hour, they were assessed for mortalities. Fish that were swimming were not disturbed, however static fish were slightly pushed aside using a sharp-ended forceps. If no reaction followed this first stimulation, the fish were gently picked in the tail. Once again, if no reaction followed the fish was picked harder and eventually counted as dead. The experiment lasted until the death of the last fish. In addition, a stress index was calculated for each group of larvae. The index represented the added cumulative mortality over a period of 12 h.

2.6. Lipid extraction and analysis

2.6.1. Materials

* chloroform: methanol solution (2:1 v/v)

* gas-liquid chromatography (Carlo Erba Vega, Thermo Separation Products, Stone, UK)

* capillary column (CP Wax 52CB, Chrompak Ltd., London, UK)

2.6.2. Methods

Total lipids were extracted from frozen samples of *Artemia*, copepods and larval tissue samples by homogenising in 10 volumes of chloroform: methanol solution using a glass/teflon homogeniser. The method of Folch *et al.* (1957) was used to prepare and measure the total lipid gravimetrically. The method of Christie (1982) was used for the preparation of fatty acid methyl ester. They were obtained by acid catalysed transesterification of total lipids. Extraction and purification of fatty acid methyl esters was performed as described by Ghioni *et al.* (1996). They were separated and quantified by gas-liquid chromatography using a 30m x 0,32mm capillary column. Hydrogen was used as carrier gas and temperature programming was from 50°C to 150°C at 40°C min^{-1} and then to 230°C at 2.0°C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980).

These analysis were performed, as part of a collaboration, by Dr. J.G. Bell and Dr. L.A. McEvoy at the NERC Unit of Aquatic Biochemistry of the University of Stirling, UK.

2.7. Statistical analysis

All the basic statistical analyses were performed using the data analysis tool of the software Microsoft Excel version 5.0a (Microsoft Corporation, USA). They included Student's t-test, analysis of variance with one (ANOVA 1) and two factors (ANOVA 2). When the results of the ANOVA revealed significant differences, a comparison of the means was performed using Tukey's method. For this purpose, a statistical package (Minitab 2.0) was used. Those results are generally presented in a matrix-like table. In the cells at the intersection of two parameters, the significance of the differences between the treatments appears. Differences were considered significant when $p \leq 0.05$ and were marked 'S'. When differences were not significant ($p > 0.05$) they were marked 'NS'.

All sets of data were tested for normality, and values available in percentage were transformed (arcsin transformation) prior to any statistical test.

Chapter 3. Morphological parameters characterising metamorphosing larvae

3.1. Introduction

The sequence of events characterising the development of fish from different species may vary greatly with different phases being distinguished between the embryo and the adult. For species presenting a clear larval stage, the larval metamorphosis represent a major threshold (Youson, 1988). Morphologically, metamorphosis is often associated with three processes: (1) a transformation of larval tissues into their adult form, (2) a regression of larval structures, and (3) the development of new adult tissues and functions (Youson, 1988). In farming conditions, this period of development often remains the main bottleneck.

Two lines of approach have appeared in the struggle to improve the culture of larval Atlantic halibut. Firstly, the culture of larvae using wild plankton and mimicking the natural conditions was intended to define and study the main parameters and constraints affecting the larval quality and performance while initiating commercial production. Indeed, despite the lack of comparison with larvae captured in the wild, a definition of metamorphosis had to be refined. Secondly, the culture of larvae in intensive systems relying on *Artemia* as the sole prey organism was intended to identify the husbandry problems associated with halibut in order to adapt and improve existing methods designed for other species. In both scenarios, the final aim was to confirm the Atlantic halibut as a promising species for the future of cold water aquaculture.

The *Artemia*-based approach adopted at Ardtoe revealed both advantages and limitations. The advantages relate to the availability and the ease of culture of *Artemia*, the disadvantages relate mostly to their poor nutritional quality in terms of their long

chain poly-unsaturated fatty acid (PUFA) content. Therefore a comparison with the copepod-based approach seemed beneficial in providing a 'control' in the present attempt to describe and clarify larval metamorphosis. For this purpose, a few morphological parameters have been identified. The most important, to which all experimental results refer, is growth or size. The others relate to the morphology and the developmental particularity of flatfish, including eye migration and pigmentation.

3.1.1. Larval growth

Three feed rations can be identified in relation to growth. They are described as minimum, ensuring only the maintenance needs of the species considered; maximum, corresponding to the assimilation capacity of the fish; and optimum, leading to the best food utilisation in terms of growth. At this stage of development of halibut culture such an approach is not possible. At the larval stage, nutritional deficiencies, developmental abnormalities and high mortalities remain of such importance that the optimisation of feed ration is not a major priority, although some experiments have been conducted on the matter (van der Meeren, 1995). However, prior to the study of other larval developmental aspects, a better description of the growth characteristic seems necessary. Indeed, larvae showing depleted growth rates or abnormal morphological characteristics would not provide an ideal base for further research.

For this purpose, the identification of growth parameters is important. The reshaping of the body of flatfish occurring during metamorphosis implies that parameters other than body length must be considered. In the present study, the variations of body width were characterised by the measure of dorsal myotome height. This parameter was preferred to the more commonly used myotome height as the variability between measurements

was reduced due to the easier observation of the muscular block on the dorsal side. The change in shape of the cranium occurring during the metamorphosis (see Brewster, 1987 for details) was characterised by the measurements of the head height and lower jaw length. The body length was also examined in relation to the body weight expressed as a condition factor. Such a factor has been used to characterise the metamorphic stage of lampreys (Potter *et al.*, 1980; Beamish and Thomas, 1984).

3.1.2. Larval pigmentation and the importance of melanin

Asymmetric pigmentation is a common feature in most pelagic fish which often display a darker colour on their dorsal side and a lighter colour on their ventral side. Such characteristics also apply to demersal fish such as flatfish in which the blind side lacks pigments (Seikai and Matsumoto, 1994). Therefore, a more precise description and understanding of larval pigmentation seemed important in this morphological study.

Different cells produce different types of pigments in fish. They are known as melanocytes, xanthocytes and iridocytes. The melanocytes produce melanin, the most common pigment. Xanthocytes produce a variety of brightly coloured pigments, and iridocytes produce a bright yellow-white pigment (Seikai and Matsumoto, 1994). The relations and interactions between these pigment cells have been studied. The iridocytes, found on both sides of plaice skin were reported as more numerous than melanocytes in large animals (Roberts *et al.*, 1971). They are also the only pigments in the skin of unpigmented fish suggesting that iridocytes have a separate origin from the melanocytes (Roberts *et al.*, 1973). On the contrary, studies on flounder pigmentation have led to the conclusion that the three types of pigment cells are regulated in a similar fashion (Seikai *et al.*, 1987a; Seikai and Matsumoto, 1994).

Melanocytes are dermal cells originating during the embryonic life from precursor cells migrating to the developing epidermis from the neural crest. They contain organelles called melanosomes where the production of the melanin takes place. When undifferentiated, melanocytes are termed melanoblasts, but when differentiated in the dermis they are often referred to as melanophores (Grønås *et al.*, 1993).

A membrane glycoprotein, tyrosinase, is the principle active enzyme in melanin production (Gilchrest *et al.*, 1996). It catalyses tyrosine hydroxylation which is the first reaction in the metabolic pathway leading to the polymerisation of the black pigment called melanin. These chemical reactions are facilitated by natural light and promoted by superoxide radicals (Gilchrest *et al.*, 1996).

With the exception of neural cells, which produce neuromelanins, melanocytes are the only cells producing melanin. The melanin produced in membrane-bound structures, called premelanosomes, accumulates through oxidation of tyrosine to form the melanosomes. These can be phagocytosed by other dermal cells, the keratocytes, in a process called cytocrine secretion as some of the cytoplasm surrounding the melanosomes is also phagocytosed (see review by Grønås *et al.*, 1993). The aggregation of the melanosomes is believed to be influenced by nerves whose activation depends on the recognition of a stimulus by the eye (Fernando and Grove, 1974; Kanazawa, 1993). Hormonal regulation also occurs and will be discussed in more detail in the chapter dealing with the role of the thyroid hormones (see chapter 6). Furthermore, the production of melanin is influenced directly and indirectly (via the

keratinocytes of the skin) by light (see review by Gilchrest *et al.*, 1996). Hence, melanin inhibits the formation of reactive chemicals by photic oxidation (Grønås *et al.*, 1993).

The role of melanin is not limited to the protection against photic damage done by absorbing short wavelengths. It also protects against photic damage by neutralising oxidative moieties generated by photic exposure or other agents. The main source of these reagents is the oxidative metabolism. In higher vertebrates there is a complex anti-oxidant system of cell defence involving enzymes such as catalase and peroxidases (Jacobson, 1996). As a result, in mammals, melanin is almost exclusively found in the skin.

In most fish, melanin is found throughout the internal organs and most specially in the abdominal linings. By contrast to the melanin present in the skin, this melanin does not prevent photic damage but acts as an anti-oxidant defence mechanism. This system implies that fish are less protected against oxidative damage than higher vertebrates.

Another distinctive feature of fish when compared to higher vertebrates also involves the melanin. It has been suggested that melanin plays a role in the immune system of fish although its function has not yet been clearly defined (Grønås *et al.*, 1993). The immune system in fish rests heavily on its primary components, the macrophages. These cells use oxidative and peroxidative agents to kill intruding microorganisms (Kemenade *et al.*, 1994). In order to avoid self-destruction they must be protected against their own oxidative agents. The existence of pigment-macrophages and the

evidence of melanin containing macrophages present in the epidermis (Roberts *et al.*, 1971) suggest that fish may depend on melanin for protection (Grønås *et al.*, 1993).

The establishment of proper pigmentation by the production of melanin at the larval stage appears as a most important parameter for the survival of the individuals at later stages. As detailed above, the lack, or limited amounts of melanin could sensitise the juveniles to a number of environmental and internal factors associated with the oxidative metabolism and the immune system.

3.1.3. Eye migration

Early studies on flatfish used the observation of the eye migration to define metamorphosis (Richardson and Joseph, 1973). Although, such an approach seems appealing, because of its simplicity, the need for more general characteristics of the metamorphosis remains. However, eye migration takes places during flatfish larval development and can be problematic in culture conditions, notably in halibut (Shields and Bell, 1995). In order to assess the occurrence of eye migration during the development and until the juvenile stage, categories were identified. They refer to the position of the migrating eye in relation to the dorsal fin (see section 2.3.3 for details).

3.1.4. Objectives

The present investigation aimed firstly at describing the morphological parameters affected during larval metamorphosis, and secondly at acquiring a better understanding of the impact of the nutritional quality of the live prey organisms on these parameters.

3.2. Materials and Methods

The larvae and the experiments referred to in this Chapter were those described in section 2.2 of the General Materials and Methods. Similarly, the techniques used to rear the *Artemia* and copepods are described in section 2.1. As these larvae were also used to provide materials for histology, the experimental design has already been described in section 2.3. Briefly, the experiments consisted of a comparison between two groups of first-feeding larvae fed either copepods and enriched-*Artemia* or enriched-*Artemia* only. During experiment 1, weekly samples were taken to characterise the morphological parameters affected during the larval development and metamorphosis. The parameters identified and described in section 2.3 were body length, dorsal myotome height, jaw length, head height, SGR, wet and dry weight, condition factor, and melanocyte counts. During experiment 2, samples were only taken on 3 occasions, after 35, 49 and 63 days PFF.

3.3. Results

For experiment 1, the different growth measurements taken from day 21 PFF to day 70 PFF on larvae fed enriched-*Artemia* only or copepods and enriched-*Artemia* are presented in Figures 3.1 to 3.4. The relation between these measurements and the larval age was characterised by the best trendlines calculated for each set of data using the software Excel 5.0™ statistical package (Table 3.1). The head height, the lower jaw length, and the body length measurements all increased according to a linear relation with time ($y = ax + b$). For the dorsal myotome height, the relation was logarithmic ($y = a \ln(x) + b$). In all cases the coefficient of correlation was above 0.9 except for the equation expressing the variation of body length and dorsal myotome height against the age PFF of larvae fed *Artemia*. From these coefficients it was clear that all measurements considered increased with time. These results were obtained from samples ($n = 5$) collected during experiment 1. During the second experiment (experiment 2), a smaller number of samples ($n = 4$) and fewer sampling dates did not provide sufficient data to plot graphs and calculate trendlines. Therefore the data is summarised and expressed as percentage of growth between sampling dates (see Table 3.2). The difference in the relation of the growth parameters and the larval age revealed a slow increase in body length until day 35 PFF, associated with a fast increase in myotome height resulting in a great variation of body shape. Between day 35 PFF and day 49 PFF, the increase in both measurements was comparable. The last period, after day 50 PFF, was characterised by a fast increase of the body length. These results combined with the daily observation of the larvae allowed the distinction of three stages

Table 3.1: summary table showing the equation of the trendlines characterising the evolution of larval growth measurements with time during experiment 1 (n = 5).

	Body Length	Dorsal Myotome Height	Head Height	Jaw Length
Artemia	$y = 0.1114x + 11.13$ $r^2 = 0.8594$	$y = 1.3577 \ln(x) - 3.5604$ $r^2 = 0.8165$	$y = 0.0606x + 1.516$ $r^2 = 0.9552$	$y = 0.0286x + 0.9322$ $r^2 = 0.9322$
Copepods	$y = 0.1434x + 10.499$ $r^2 = 0.9552$	$y = 1.5672 \ln(x) - 4.1443$ $r^2 = 0.9152$	$y = 0.0746x + 1.1584$ $r^2 = 0.9908$	$y = 0.0332x + 1.2404$ $r^2 = 0.9758$

*Figure 3.1: average body length (error bars indicate the standard deviation) of first-feeding larvae fed enriched-*Artemia *and copepods (dotted line) or enriched-*Artemia *only (plain line) (n = 5).*

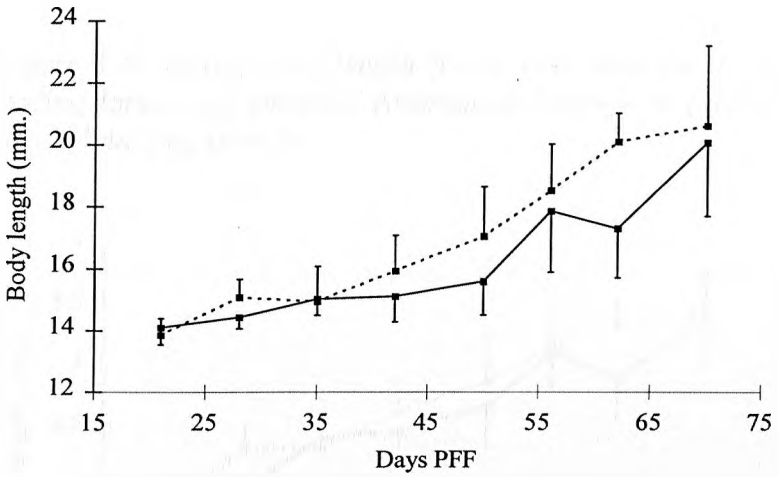


*Figure 3.2: average dorsal myotome height (error bars indicate the standard deviation) of first-feeding larvae fed enriched-*Artemia *and copepods (dotted line) or enriched-*Artemia *only (plain line) (n = 5).*

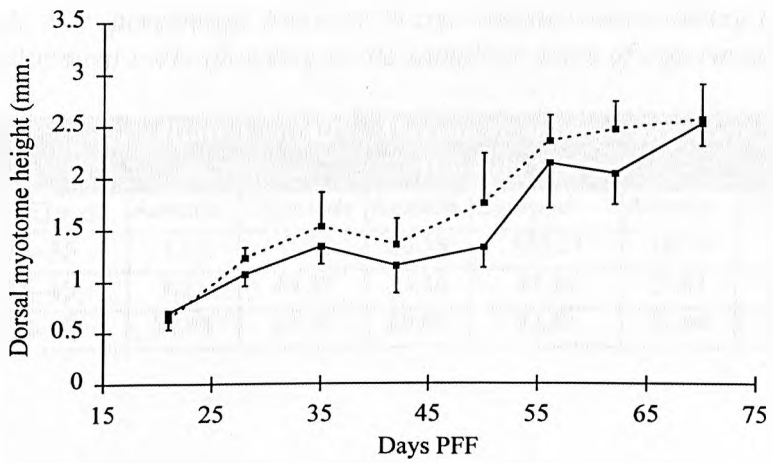


*Figure 3.3: average head height (error bars indicate the standard deviation) of first-feeding larvae fed enriched-Artemia and copepods (dotted line) or enriched-*Artemia *only (plain line) (n = 5).*

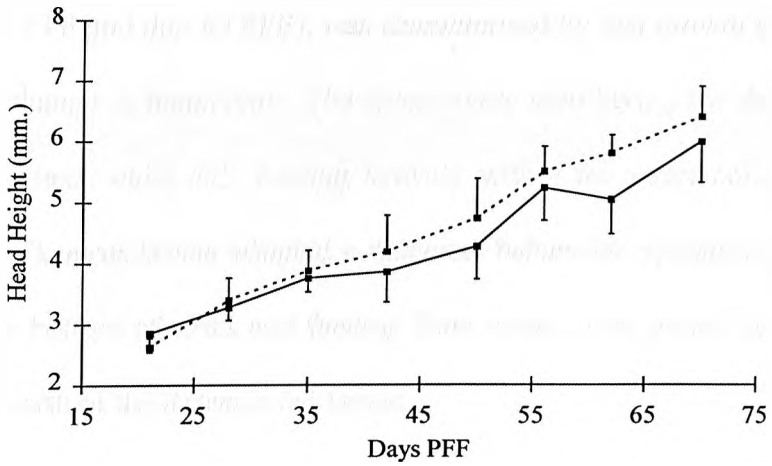


*Figure 3.4: average jaw length (error bars indicate the standard deviation) of first-feeding larvae fed enriched-*Artemia *and copepods (dotted line) or enriched-*Artemia *only (plain line) (n = 5).*

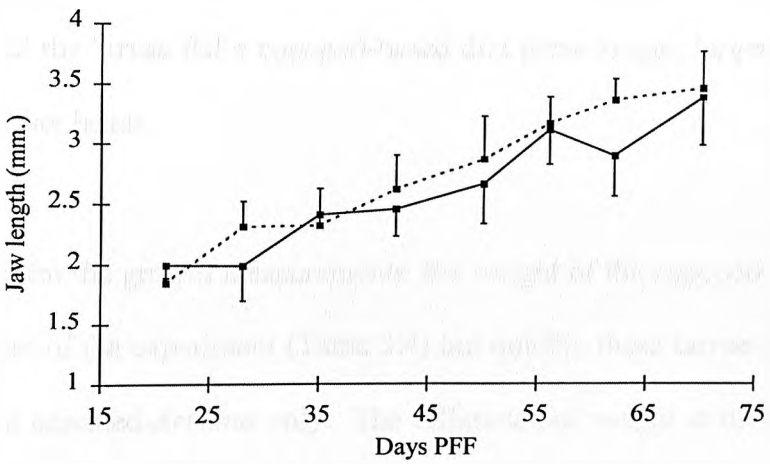


Table 3.2: percentage increase in size measurements during three periods of the larval development corresponding to the sampling dates of experiment 2 (n = 4).

	Body Length (%)		Dorsal Myotome Height (%)		Jaw Length (%)		Head Height (%)	
Age (Days)	Artemia	*Copepods*	Artemia	*Copepods*	Artemia	*Copepods*	Artemia	*Copepods*
16-35	4.53	*9.66*	80.18	*132.22*	19.19	*23.76*	27.56	*42.03*
36-49	9.51	*16.22*	23.36	*25.36*	17.41	*26.27*	24.46	*30.54*
50-63	22.63	*15.14*	60.60	*33.84*	22.04	*15.77*	31.90	*26.41*

in the larval development. The first stage (up to day 35 PFF), was characterised by a widening of the body while the larvae remain pelagic. The second stage (between day 35 PFF and day 49 PFF), was characterised by fast growth both in length and width and a change in behaviour. The larvae were seen laying for short period on the bottom of the tank while still feeding actively within the water column. Finally (after day 50 PFF), most larvae adopted a demersal behaviour, spending most of the time laying on the bottom of tanks and feeding from there. This period corresponded to an improved growth of the *Artemia*-fed larvae.

In terms of the impact of the prey organisms on the growth, the factor 'diet' was shown as being significant or highly significant for all the measurements considered (Table 3.3). This information, combined with the growth figures presented earlier, highlights that the larvae fed a copepod-based diet grew longer, larger, developed longer jaw and higher heads.

As for the growth measurements, the weight of the copepod-fed larvae was lower at the start of the experiment (Table 3.4) but quickly these larvae appeared heavier than those fed enriched-*Artemia* only. The difference of weight at the start of the experiment can only be attributed to the result of the random distribution of the larvae amongst the two treatments one week before the first supplementation of copepods. The faster growth of the copepod-fed larvae was also reflected by the SGR during the first period. The condition factor also increased during the experimental period suggesting a proportionally greater gain of weight in comparison to the gain in length. Indeed, the statistical analysis (Anova 2 with replication) of the dry weight measurements revealed

Table 3.3: summary table of the effects of time and diet on the growth measurements of Artemia*-fed larvae and copepod-fed larvae (experiment 1). HS = highly significant ($p < 0.01$); S = significant ($0.05 > p > 0.01$); and ns = non significant ($p > 0.05$).*

Factor	Body Length	Dorsal Myotome Height	Jaw Length	Head Height
Time	HS	HS	HS	HS
Diet	S	HS	S	HS
Interaction	ns	ns	ns	ns

Table 3.4: mean dry weight ± standard deviation based on 4 pooled samples of 5 larvae collected at the indicated days PFF during experiment 1. The SGR and condition factors shown were calculated from the same data set.

	Dry Weight (mg)		SGR		Condition Factor	
Days PFF	Artemia	*Copepods*	Artemia	*Copepods*	Artemia	*Copepods*
21	2.5 ± 0.1	*2.2 ± 0.1*			0.09	*0.08*
35	3.9 ± 0.6	*5.7 ± 1.2*	9.88	*13.78*	0.12	*0.16*
49	9.9 ± 2.0	*14.9 ± 1.1*	4.77	*6.65*	0.23	*0.26*

highly significant changes. In addition to the effect of the two selected factors, time and diet, their interaction was also highly significant demonstrating that the weight advantage of the copepod-fed over the *Artemia*-fed larvae increased with time. By contrast, the growth performances of the larval group fed exclusively on *Artemia* in experiment 2 (Table 3.5) were better than those of the group which also received copepods. However, once again and despite the random distribution of the larvae between treatment, there was a weight difference at the start of the sampling period. Nevertheless, the weight differences appeared to result from improved performances of the *Artemia*-fed larvae rather than a slower growth of the copepod-fed ones. Other significant differences also appeared. Indeed, the percentage of larvae perfectly metamorphosed was much greater, 39.7 ± 1.9 compared to 3.5 ± 3.1, within those that had received a supplementation of copepods. Similarly, this group did not include any albino larva and the percentage of larvae adequately pigmented almost reached 90% (89.2 ± 5.4).

The melanocytes, in which the melanin was visibly dispersed, covering the dorsal but also the ventral sides of the larvae were counted until 49 days post first feeding in experiment 1. Further counts on older larvae were rendered impossible under light microscopy due to the increased numbers of melanocytes and the associated difficulty in identifying individual cells. A sharp increase in numbers was observed after 42 days post first feeding. While the dorsal side of the copepod-fed larvae averaged the highest number of melanocytes containing expressed pigments and their ventral side the lowest, the *Artemia*-fed larvae showed an intermediate increase in melanocytes covering both sides of their bodies (Figure 3.5 and Table 3.6). At that stage, there was no

Table 3.5: growth, survival, pigmentation and eye migration parameters in larvae fed enriched Artemia or Eurytemora velox copepods from day 16 to 63 PFF during experiment 2. These measurements were collected by B. Gara and R. Shields.

	Artemia	*Copepods*
Start wet weight (mg)	39.5 ± 2.4*	*24.7 ± 2.3*
Final wet weight (mg)	363.8 ± 25.7*	*249.6 ± 8.3*
Survival (%)	44.7 ± 9.5*	*66.4 ± 2.3*
SGR	4.2 ± 0.1	*4.1 ± 0.1*
Eye migration index	2.1 ± 0.1	*2.3 ± 0.1*
% perfect metamorphosis	3.5 ± 3.1*	*39.7 ± 1.9*
% albinos	32.5 ± 7.5*	*0*
% perfect pigmentation	28.9 ± 6.6*	*89.2 ± 5.4*
% incomplete metamorphosis	53.6 ± 9.9*h	*7.9 ± 3.4*

** values which are significantly different (P < 0.05).*

*Figure 3.5: counts of melanocytes in which the pigments were expressed of developing larvae fed enriched-*Artemia *only or enriched-*Artemia *supplemented with copepods during experiment 1. The melanocytes were counted on the body only; fins were excluded.* Artemia O. *stands for the ocular side of* Artemia*-fed larvae and Copepod Bl. stands for the blind side of copepod-fed larvae (n = 5).*

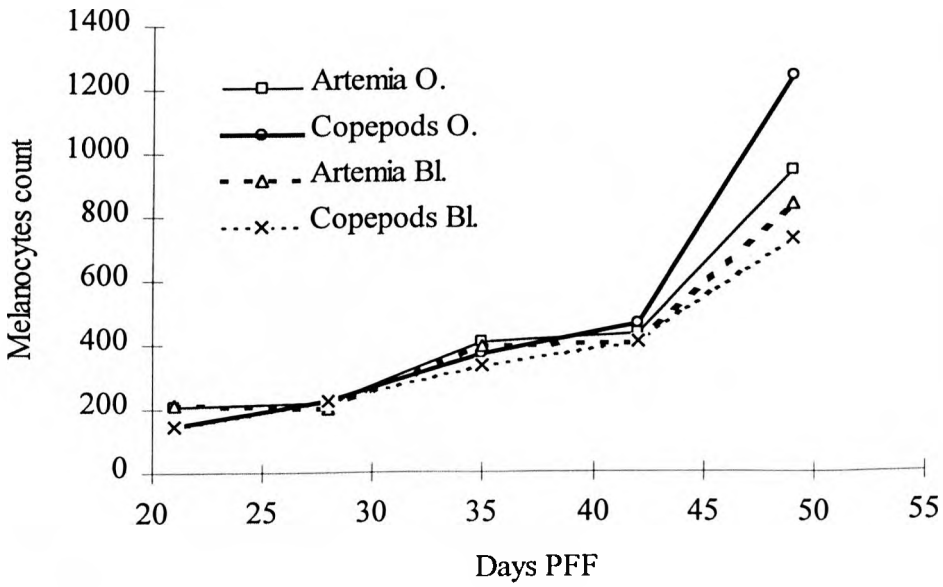


Table 3.6: mean ± standard deviation of counts of melanocytes in which the pigments were expressed on the ocular and blind side of first-feeding larvae aged between 21 and 49 days PFF (experiment 1) (n = 5).

	Ocular side		Blind Side	
Days PFF	*Copepod*	Artemia	*Copepod*	Artemia
21	*146 ± 43*	208 ± 51	*146 ± 56*	213 ± 54
28	*222 ± 4*	215 ± 37	*221 ± 9*	201 ± 33
35	*370 ± 47*	405 ± 125	*331 ± 50*	393 ± 116
42	*460 ± 59*	433 ± 93	*408 ± 47*	402 ± 93
49	*1239 ± 337*	990 ± 463	*726 ± 246*	838 ± 407

statistical difference between the groups. In experiment 2, a melanocyte count was not performed. The pigmentation of the larvae was characterised using categories as described earlier (see section 2.3.2). This method revealed that the appearance of perfectly pigmented juveniles was significantly higher when feeding copepods. In terms of eye migration, the calculation of the eye index in experiment 2 did not highlight any differences between the larval groups.

3.4. Discussion

The growth measurements characterising halibut larvae fed either enriched-*Artemia* supplemented with copepods (referred to as the copepod-fed larvae) or enriched-*Artemia* only (referred to as the *Artemia*-fed larvae) from day 20 to day 70 PFF allowed the differentiation of three developmental periods associated with the transition from a pelagic to a demersal life style and with metamorphosis. The first period (until day 35 PFF) could, in reference to the Japanese flounder (Seikai *et al.*, 1987b), be named the pre-metamorphic stage. It was characterised by a fast weight gain, with a SGR of almost 14 for the copepod-fed larvae and nearly 10 for their *Artemia*-fed counterparts. Such growth rates were higher than had been previously observed at Ardtoe, but lower than recent Norwegian observations (McEvoy *et al.*, in press). During the same period the condition factor also increased revealing an important gain of weight per unit of length. By contrast, in other species such as lampreys, the condition factor of larvae showed an increase only during the pre-metamorphic phase highlighting the difference in body shape between the two species (Potter *et al.*, 1978). Indeed, in halibut while the body length only increased by less than 10% during this period, the dorsal myotome height more than doubled. A similar but lower rate of growth was seen for the *Artemia*-fed larvae. These observations were confirmed by the linear changes of body length with time by contrast with the logarithmic changes of dorsal myotome height.

That period was also characterised by the highest increase in terms of percentage of the height of the head. In combination with the great changes of myotome height, these modifications of the larvae's body shape reflected what could be described as the onset

of the metamorphosis. Likewise, different authors (Brewster, 1987; Wagemans *et al.*, 1998) observed a change in cranial bone structure from the start of the eye migration in their study of flatfish cranial development during metamorphosis.

During the following two weeks (from day 36 to 49 PFF), the growth in terms of percentage body length increase progressed more quickly, reaching 16% for the copepod and 9.5% for the *Artemia*-fed larvae. As a result, the difference in size between the two groups widened. However, the overall shape of the larvae had changed from that of pelagic fish more towards that of a flatfish. The ratio between dorsal myotome height and length had dropped from 1/21.8 to 1/9.4 in the copepod fed group, and from 1/20.4 to 1/10.5 in the *Artemia* fed group. In the meantime, the number of melanocytes on the dorsal side increased exponentially. This period also saw a change in larval behaviour with the larvae spending more time on the bottom of the tanks. According to the stages described for the Japanese flounder (Seikai *et al.*, 1987b), one could talk of this as the metamorphic climax.

The third period, from day 50 to 70 PFF, was characterised by a good performance of the *Artemia*-fed larvae. Indeed, they reduced most of their weight and length deficit. It appeared as if they had undergone similar morphological changes as the control group with a slight delay in time. As a detailed count of the number of copepod fed to the larvae was not performed, it could be suggested that the group of larvae which received a supplement of copepod were under-fed, hence the better performances of the larvae fed enriched-*Artemia* only. However, this hypothesis does not apply as the copepod-based diet was supplemented by enriched-*Artemia* in order to avoid such deficiencies.

Therefore, and despite some limitations in relation to the experimental design, these observations are to be accounted for. Beside, similar observations have been reported by Næss *et al.* (1995) in a study aimed at improving the growth of halibut larvae by combining *Artemia* and wild zooplankton during first feeding. If considering the copepod-fed larvae only, one could refer to this period as the post-metamorphic stage. In the second experiment (experiment 2), only final measurements were available and they revealed identical growth rates irrespective of the diet. Based on experiment 1, it could be argued that despite these identical growth rates (see table 3.4), the *Artemia*-fed larvae may have had a delayed growth until the metamorphic climax. Such observations could be related to the pigmentation abnormalities and poor eye migration. Indeed, growth, and length in particular, has been suggested as the main parameter affecting eye migration (Policansky, 1982; B. Gara, personal communication). The identical larval size at the end of the metamorphosis could have accounted for the similar eye migration indices in both groups. On the other hand, the appearance of adult type melanocytes seemed to be associated with the timing of the metamorphic climax and the nutritional quality of the live prey (Estevez and Kanazawa, 1996). The experimental evidence obtained during these experiments are sufficiently clear to support many conclusions. However, it can be argued that a delayed climax would affect pigmentation. Whether it could explain the occurrence of impaired pigmentation in the larval group fed exclusively on enriched-*Artemia* would have to be confirmed using a more rigorous dietary comparison. Nevertheless, the availability of copepods is likely to remain problematic in the near future jeopardising such investigations.

As the use of copepods appeared to be beneficial in promoting growth and development, it is arguable that experiment 1 may have started too late (21 days PFF), especially as the larvae were fed exclusively on *Artemia* before the onset of the experiment. However, the size of halibut larvae first fed at comparable age and in comparable conditions has been reported to average between 11.5 mm to 12.4 mm in body length (Blaxter *et al.*, 1983; Haug, 1990; Kjørsvik and Reiersen, 1992, Lein and Holmefjord, 1992). From previous unpublished experiments and according to the results of the present research the growth during the end of the yolk resorbtion was very limited, as reported by Skjolddal *et al.* (1990). Indeed, at the beginning of the sampling period (21 days PFF) the larvae measured only slightly more than 14mm in length and were very thin (dorsal myotome height of less than 1mm). Such measurements are in agreement with observations reported by Pittman *et al.* (1990). Besides Næss *et al.* (1995) indicated that the use of zooplankton during the first 19 days post first-feeding was not required, similarly as Seikai *et al* (1987b) reported that copepod were not required for larval flounder until day 9 to 15. At the time of that experiment, it could be assumed that the larvae were in comparable conditions and had similar growth rates to larvae used for experimentation elsewhere. However, by comparison to the higher growth rates observed in subsequent studies, it cannot be said that the larvae did not show a reduced growth at the onset of the experiment. The interpretation of the present results must therefore be considered in their context, and the timing of the developmental stages would have to be reviewed to apply to the improved situation at the time of writing. In view of these considerations, it seems recommended that any future dietary investigations undertaken at the SFIA Marine Farming Unit at Ardtoe should be

initiated as close as possible to the time of transfer of the larvae from the yolksac incubators.

In terms of pigmentation, the results on Atlantic halibut differed from those in flounder. The main pigmentation problem associated with *Artemia* was not albinism but ambicoloration. However, the use of copepods was also associated with improved pigmentation. In flounder, the lack of skin differentiation resulted in juveniles having only few larval melanocytes on both sides, in halibut, it seemed as if the lack of skin differentiation resulted in juveniles having a complete pigmentation of the skin on both sides. However, a histological study of the skin would be required to identify the type of melanocytes present in ambicolored fish.

In flounder, it was shown that the use of rotifers as prey organisms during the first two thirds of the development was sufficient to establish normal pigmentation patterns. They could not be reversed by feeding *Artemia* although some strains of *Artemia* induced better performances than others (Seikai *et al.*, 1987c). In the mean time larvae fed *Artemia* at an earlier stage developed pseudo-albinism (Seikai, 1985a) adding to the hypothesis that pigmentation relates more to the timing of the metamorphic climax and the nutritional quality of the diet rather than to eye migration. Furthermore, the pigments present on both sides of larval flounder at the pre-metamorphic stage were confined to one type of large melanophores. During metamorphosis, a second smaller type of melanophore accounted for the pigmentation on the ocular side. In malpigmented larvae, only the large melanophores could be identified. It is believed that the feeding of *Artemia* and the consequent malpigmentation interfered with the

differentiation of the skin, with only the larval type of melanophore being produced (Seikai *et al.*, 1987a; Suzuki, 1994). This effect on the differentiation of the skin is not believed to be related to an inhibition factor in *Artemia,* as the tyrosinase activity of cell extracts was not inhibited in presence of *Artemia* (Seikai *et al.*, 1987a).

As mentioned earlier, the larvae fed copepods appeared to have grown faster than their *Artemia*-fed counterparts during the pre-metamorphic and the metamorphic stages. Similar findings have been reported for turbot (Sanchez and Miranda, 1997). In flounder, although copepods resolved the pigmentation problem, the larvae fed *Artemia* grew larger and showed a better survival (Seikai, 1985b). Japanese literature reported by Seikai (1985b) reveals that the proximate analysis, mineral and amino acid composition of *Artemia* differs only slightly from that of copepods, whilst the main difference in composition between the species resides in their fatty acid content. Similar to copepods, the use of red sea bream eggs also reduced flounder pigmentation problems. Although, Fukusho *et al.* (1987) concluded that the beneficial effect of the eggs originated in a trace substance contained in the eggs, it has been reported that eggs of marine species are very rich in n-3 polyunsaturated fatty acids (PUFA) (Falk-Petersen *et al.*, 1986; Planas *et al.*, 1993). The use of enriched *Artemia* as a larval diet for flatfish later confirmed the importance of the PUFA content in improving pigmentation (Dickey-Collas, 1993; Reitan *et al.*, 1994, Næss *et al.*, 1995). Similarly, the use of vitamin A enriched *Artemia* has been recognised as a potential source of improvement of the malpigmentation although it is also associated with bone deformities and poor larval growth (Miki *et al.*, 1990; Takeuchi *et al.*, 1995). Bearing in mind the discrepancies in results between halibut and flounder, the benefit associated with

copepods as larval feed remained, suggesting that the research findings for flounder may be applicable for halibut as described in Chapters 5 and 7.

In Japanese flounder, the sensitive period during which the provision of wild zooplankton was most effective in solving the pigmentation problem was identified as being during the pre-metamorphic stages. These stages are characterised by the initiation of the asymmetric changes occurring during larval development (Seikai *et al.*, 1987b). Using a similar classification system, a pre-metamorphic stage in halibut larvae has been identified as lasting from the onset of exogenous feeding until days 35-40 PFF (average body length = 16mm). During the first 20 days PFF, Næss *et al.* (1995) suggested that there was no beneficial effect in feeding copepods instead of *Artemia*. By comparison with the Japanese flounder, it could suggest that the sensitive period of development of Atlantic halibut larvae, during which copepods are beneficial, could be restricted to the period between 20 and 35 days PFF. As mentioned earlier, such comments have to be taken with care and should only provide support for an hypothesis to be tested further in experiment specially designed for that purpose. Indeed, detailed comparison would have to include a variety of other parameters such as body size and precise developmental stages.

In addition further research would be required, especially concerning the mechanism involved with skin differentiation in halibut. In the meantime, improvements of the nutritional quality of *Artemia* could be achieved based on the findings for the Japanese flounder. The use of high HUFA enrichments appears as a logical step for the future, keeping in mind that an approach based solely on the dietary PUFA might be restrictive.

Indeed the lack of pigmentation has been also associated to very low levels of riboflavin (vitamin B2) in both skin and muscles of flounder (Nakamura and Iida, 1986). Other components such as zinc and iron have also been reported to be associated with the ocular side of the skin (Nakano *et al.*, 1992). They all have been shown to have some role in the enzymatic process leading to the melanin production. A general nutritional approach to pigmentation seems required and further studies on the other aspects of metamorphosis should be envisaged to broaden the scope of research and understanding. As a result, the following section deals with the ontogeny of the digestive system and the ability of the larvae to digest copepods and *Artemia* while the nutritional value of *Artemia* in terms of PUFA is presented later (Chapter 5 and Chapter 7).

Chapter 4. Ontogeny of the digestive system

4.1. Introduction

The study of the morphological characteristics of metamorphosing halibut larvae revealed an important discrepancy between larvae fed a variety of diets (Gara *et al.*, in press). Indeed, the use of wild planktonic invertebrates dominated by *Eurytemora velox* in comparison to *Artemia* revealed the importance of the prey organisms on the completion of the metamorphosis (Næss, 1997; Shields *et al.*, 1997). Such differences emphasise the importance of nutrition at the first-feeding stage of larval development. Larval fish are capable of digesting live prey at the time of first-feeding but the assimilation of artificial food remains problematic as shown by the failure of early weaning experiments (N. Brown, personal communication, 1997). The differentiation of the digestive tract, and more particularly of the stomach and pyloric appendages, are the main features lacking in larvae. Consequently, the successful rearing of larval fish and the formulation of adequate diets will depend in part on the understanding of their digestive tracts and functions (Specker and Bengtson, 1995).

In adult halibut, as in other vertebrates, the alimentary canal is modified from the mouth to the anus to perform the different aspects of digestion. Similarly, its organisation from the oesophagus onwards consists of four concentric layers (Hibiya, 1982; Wheater and Burkitt, 1988; Gartner and Hiatt, 1994; Ross *et al.*, 1995). Each section contains from the lumen outwards:

- a mucosa assuring a secretory role. The mucosa is itself subdivided into three layers, an absorptive and secretory epithelium, a *lamina propria* composed of connective tissue containing glands and vessels, and a *muscularis mucosae* responsible for the mobility of the mucosa.

- a submucosa formed of connective tissue containing the blood vessels, lymphatic vessels and nerves, which physically support the mucosa.

- a *muscularis externa* composed generally of an inner circular and an outer longitudinal muscle layer responsible for the peristaltic contractions.

- and a *serosa* or *adventitia.*

Despite the good general knowledge of the digestive tract of vertebrates, there are specificity associated with the dietary regime and the developmental stage of each species. For halibut, the digestive ontogeny during the embryonic stage has been described (Pittman *et al.*, 1990; Kjørsvik and Reiersen,1992). Similarly, information is available on specific sections of the adult digestive tract (Murray *et al.*, 1994a,b). However, little is known of the digestive ontogeny of first-feeding larvae especially in relation to the prey organisms.

This chapter focuses on the organogenesis of the larvae with most of the emphasis placed on the development of the digestive tract and the timing of functionality. For this purpose, the larvae sampled in experiments 1 and 2 (see section 2.2) provided histological material for the following study. Special features of the digestive tract's development associated with the use of *Artemia* as prey organism are investigated and compared to the features described in larvae fed enriched-*Artemia* and a wild plankton dominated by a marine copepod, *Eurytemora velox.*

4.2. Materials and Methods

The materials and methods used for the experiments described hereafter in Chapter 4 have been already described in the Chapter on General Materials and Methods (see section 2.4). Briefly they consist mainly of the description of histological techniques used for the staining of tissues samples. The staining techniques used for the present experiments and throughout the thesis were the following: haematoxylin and eosin (H&E), Masson's trichrome, periodic acid Schiff (PAS) and modified aldehyde fuchsin.

In the following section, the photomicrographs have all been obtained from larvae fed a copepod-based diet supplemented with enriched-*Artemia* unless otherwise specified. Each photmicrograph, as each comment, are based on the observation of at least three larvae showing similar characteristics. The comparisons are based on larvae of the same age (expressed in days PFF) rather than size. All the larvae were sampled from either experiment 1 or experiment 2.

4.3. Results

4.3.1. The digestive tract

4.3.1.1. The oesophagus

The oesophagus of the young halibut larvae (up to 30 days PFF) was only partially organised according to the general scheme characterising the digestive tract. Indeed, the mucosa was present but within that mucosa, only the epithelium could be identified. The loose connective tissue bordering the epithelium was not recognised as the mucosal *lamina propria* but as the submucosa. The mucosal muscular layer, the *muscularis mucosa*, normally separating the lamina propria from the submucosa was not clearly observed. The connective tissue was therefore referred to as the *propria-submucosa*.

Within the first few weeks of exogenous feeding the larval mucosa was folded longitudinally forming long cone shaped elongation into the oesophagus' lumen. The epithelial cells, the mucous cells and the *propria-submucosa* (Plate 4.1a) constituted the folds.

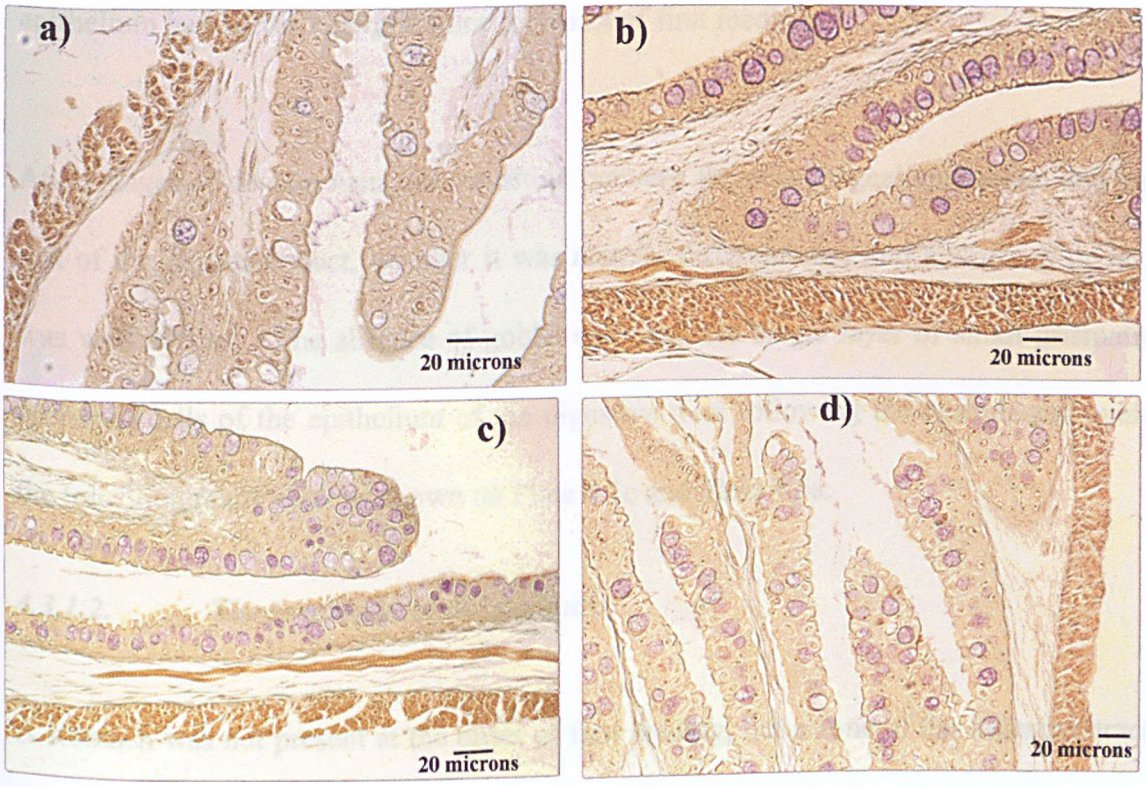
The epithelium of the mucosa was mainly composed of epithelial cells of the cuboidal type. Several layers of cuboidal cells were observed on the tip of the mucosal folds, however, the rest of the epithelium was mostly composed of bi-layer and classified as pseudostratified. Among those cells, goblet cells were distributed all along the oesophagus' mucosa. The goblet cells were present in relatively small numbers, 1 in 18

to 35 epithelial cells being a goblet cell. Their mucus was positively stained with PAS and with Modified Aldehyde Fuchsin. These large mucous vacuoles formed the bulk of these cells, which were larger than other epithelial cells. In longitudinal sections of the epithelium, their nuclei appeared confined to the basal side of the cells, directly adjacent to the *propria-submucosa*.

During the metamorphic climax (between day 35 and 50 PFF), a muscle layer orientated longitudinally was observed in the *propria-submucosa* suggesting either a complete differentiation of the mucosa or of the *muscularis externa*. Indeed, if that muscle layer belonged to the mucosa, it was then possible to identify its sublayers, *i.e.* the epithelium, the connective tissue forming the *lamina propria* and the *muscularis mucosa*. While if the muscle block belonged to the *muscularis externa*, it would represent the second muscle block often observed within that layer. However, a definite identification remained difficult as on one hand connective tissue present on both sides of the muscle layer suggested that it marked the limit between the *lamina propria* and the submucosa. But on the other hand, the muscle layer could not be observed all along the oesophagus. And furthermore, none of the vessels or glands characterising the oesophagus submucosa could be identified on the outer side of the muscle (Plate 4.1b).

In the meantime, the relative importance of the goblet cells in the epithelial layer had increased to account for most of the epithelium's cells. The level of folding of the mucosa had also increased. The original cone like elongation had turned into heavily branched folds (Plate 4.1c).

Plate 4.1: photomicrographs of longitudinal sections of the mucosa, submucosa and muscularis externa of the oesophagus of larvae aged: a) 28 days PFF (magnification: x500; stain: Modified Aldehyde Fuchsin); b and c) 49 days PFF (magnification: x400; stain: Modified Aldehyde Fuchsin) and d) 63 days PFF (magnification: x312.5; stain: Modified Aldehyde Fuchsin).



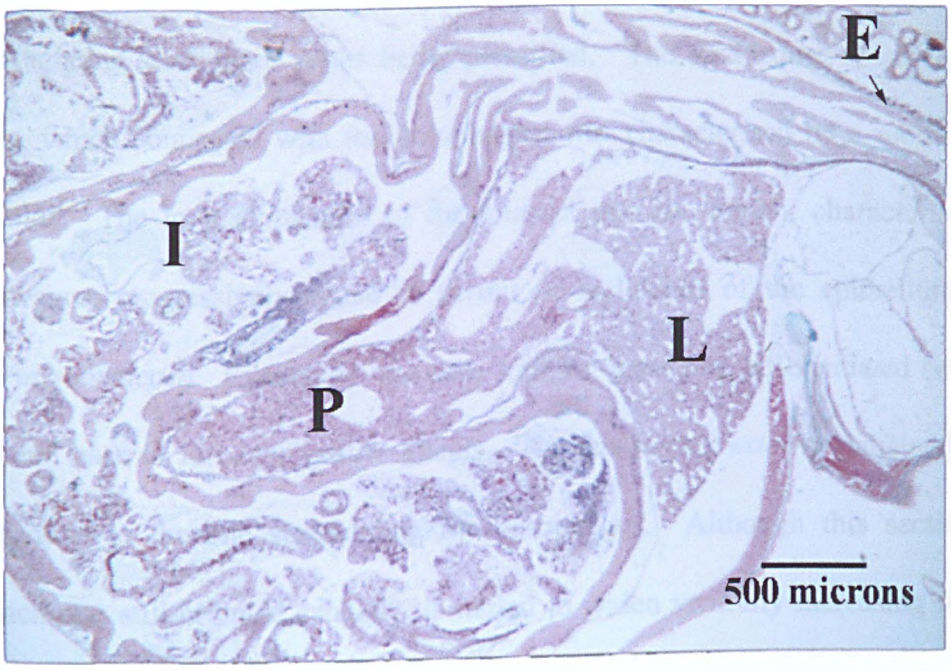
By the end of the first-feeding period (from 50 to 80 days PFF), the connective tissue between the epithelium and the *muscularis externa* was further separated by a longitudinal muscle layer (Plate 4.1c). However, its identification remained debatable. The goblet cells still accounted for most of the epithelial cells and the width of this epithelium had hardly changed since the onset of first feeding.

At all stages of development the transition between the oesophageal epithelium and the rest of the digestive tract, whether it was already differentiated into a stomach or not, was well defined. The absence of goblet cells and the single layer of small prismatic epithelial cells of the epithelium of the digestive tract following the oesophagus made the transition really clear as shown on Plate 4.1c and Plate 4.5a.

4.3.1.2. The formation of the stomach

A stomach was not present at the onset of first feeding, but a zone of the digestive tract from which the stomach will differentiate could be identified. Indeed, the intestine or midgut did not start before a constriction that will later correspond to the pyloric caecae (Plate 4.2 and 4.5d). At low magnification, this area appeared heavily folded compared to the intestine. It was orientated caudally in the prolongation of the oesophagus. Its caudal part bent towards the anus and formed a constriction. One could not yet talk of a sphincter as the *muscularis externa* surrounding it was not any wider than along the rest of the digestive tract. At higher magnification, the mucosa was observed to be composed only of a simple lining epithelium underlain by a thin and loose *propria-submucosa*. Only circular muscle blocks were identified in the *muscularis externa*. The epithelial cells were of the columnar type, each with its round nucleus confined to the

Plate 4.2: photomicrograph of a longitudinal section into the abdominal cavity of a larva aged 21 days PFF (magnification: x50; stain: Masson's trichrome) showing most of the digestive tract from the oesophagus (E) to the rectum. I stand for intestine; P stands for pancreas and L stands for liver.



basal side of the cells. Close observation showed microvilli at the apical end of the cells (Plate 4.3).

Between 35 and 50 days PFF, during the metamorphic climax, the stomach began differentiating with three zones being identified. The first zone, usually called the cardia, which connected with the oesophagus, was characterised by a modification of the folds. The long elongation of the mucosa into the lumen, characteristic of the oesophagus, progressively ended in favour of inclusions of the epithelium into the *propria-submucosa*. Eventually, the cells forming these pits differentiated between the neck and base to form the gastric glands. The *muscularis externa* remained thin and comparable to the one surrounding the oesophagus. Although this section of the stomach was still oriented caudally, it started to widen ventrally increasing the size of the lumen.

In the main body of the stomach, also called the fundus or fundic region, these inclusions were numerous in the epithelium and the differentiation of the gastric glands was well under way. The cells lining the base of the gastric pits were of a cuboidal type rather than columnar as the surface epithelial cells. In addition, microvilli could not be identified on their apical side as shown by a PAS stain (Plate 4.4 and Plate 4.5c). Indeed, the coloration disappeared in the cells lining the future glands. However, their cytoplasm was still dense and their nuclei were still large and positioned in the centre of the cells, suggesting that the newly formed gastric glands were not functional at this stage. Furthermore, the various cells forming the gastric epithelium of other fish could not be identified at this stage (Ezeasor, 1981).

Plate 4.3: photomicrograph of a longitudinal section into the mucosa of the 'stomach' of a larva aged 28 days PFF (stain: Masson's trichrome).

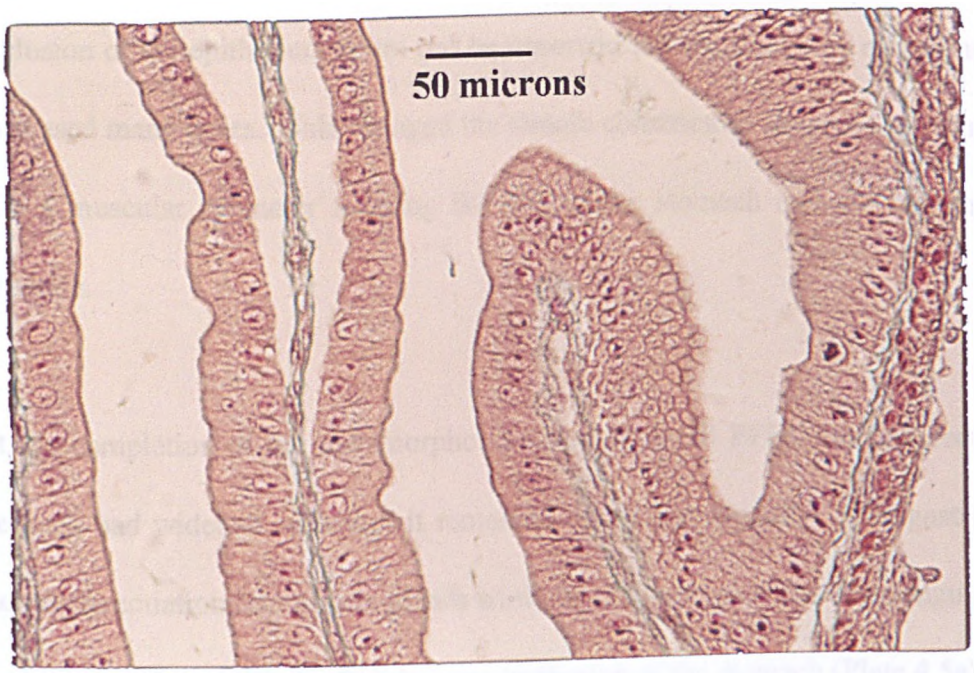
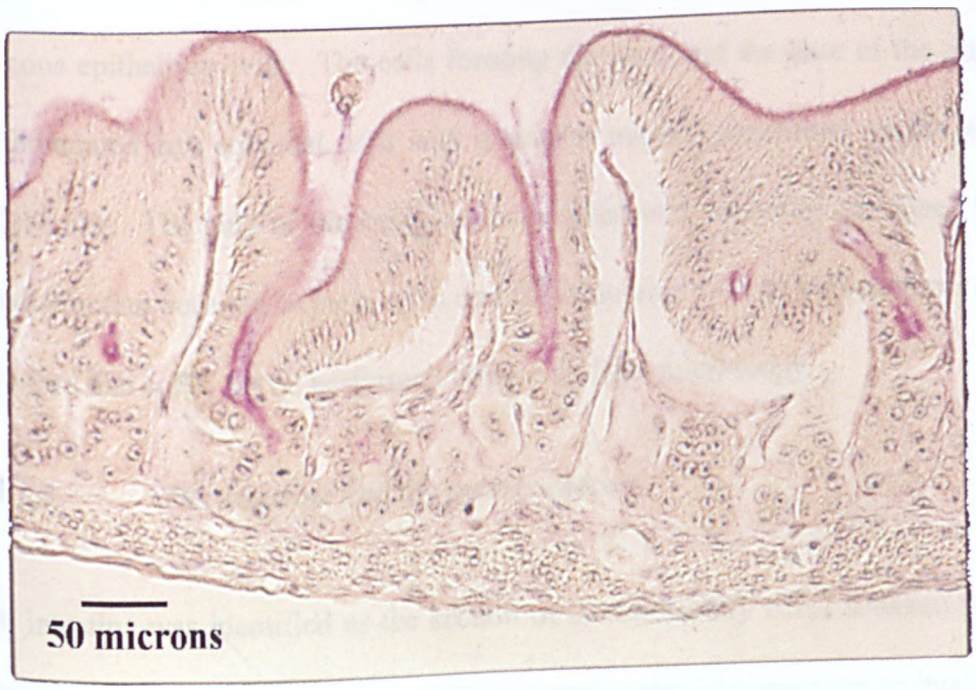


Plate 4.4: photomicrograph of a longitudinal section of the stomach mucosa of a larva aged 44 days PFF. The PAS stain showed the differentiation of the epithelial cells in the gastric pits.



The third section of the stomach, commonly called the pylorus or pyloric region, saw mainly a differentiation of the *muscularis externa* rather than of the mucosa. Indeed, the inclusion of the epithelium could not be observed but the thickness of the muscle layer increased many times. This changed the simple constriction observed in younger larvae into a muscular sphincter marking the end of the stomach and the beginning of the intestine.

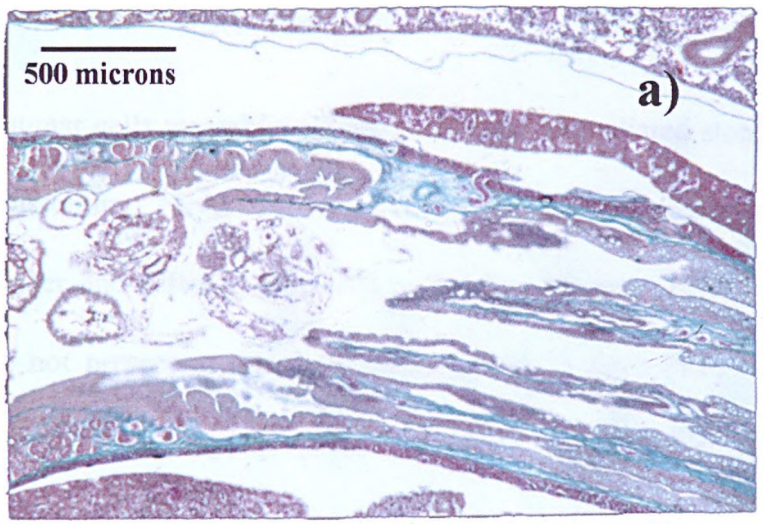
At the completion of the metamorphosis (days 50 to 80 PFF), the first section of the stomach had widened, although it remained orientated caudally. The gastric pits and their differentiation into gastric glands could be observed near the oesophagus. Besides, the long folds were restricted to the very beginning of the stomach (Plate 4.5a).

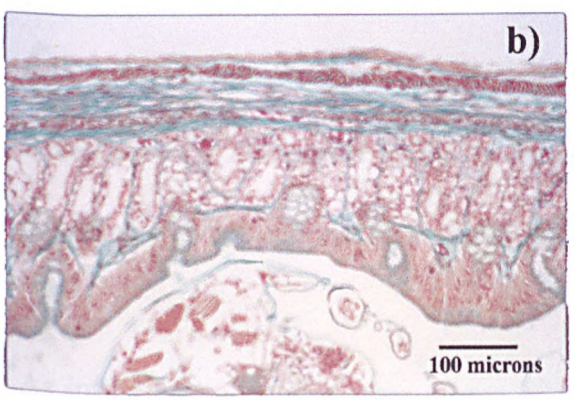
The tubular gastric glands were of adult type and appeared fully differentiated (Plates 4.5b and 4.5c). They formed elongated canals underneath the single layer of columnar mucous epithelium cells. The cells forming the neck and the base of the glands were differentiated into cuboidal cells with a smaller nucleus condensed on the side of the cells' walls. The bulk of the cytoplasm was filled with secretory granules. However, the distinction between oxyntic cells, and the endocrine cells of various types described for other fish could not be performed using only light microscopy.

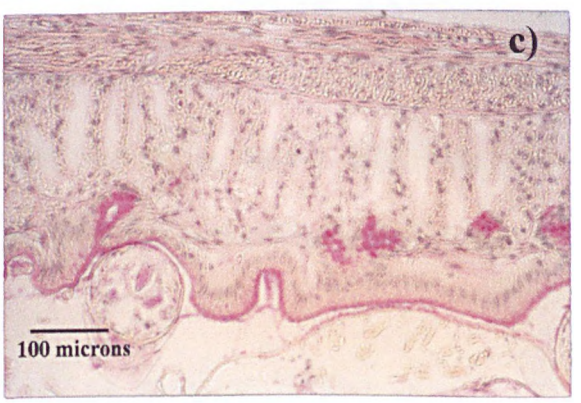
4.3.1.3. The intestine and the pyloric caecae

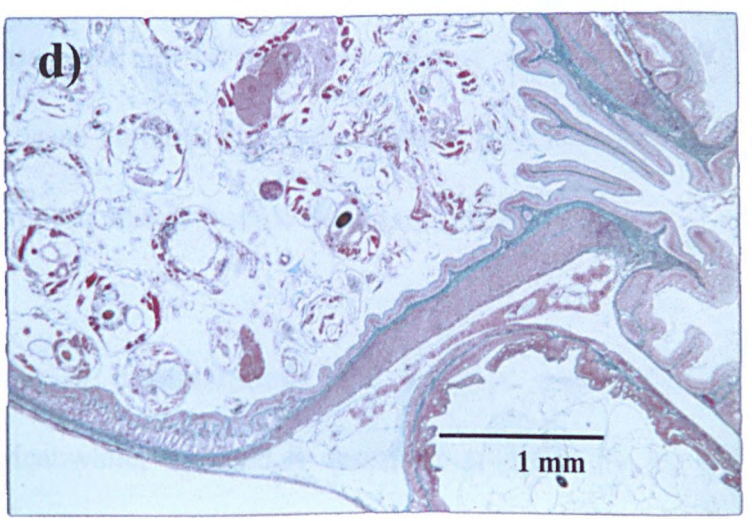
The intestine was identified as the section of the alimentary canal between the pyloric sphincter and the ileo-rectal valve. As mentioned earlier, it was observed that at the

Plate 4.5: photomicrographs of longitudinal sections into the stomach of a metamorphosed larva (age 63 days PFF) showing its three regions. a) cardia of the stomach connecting with the oesophagus (magnification: x80; stain: Masson's trichrome); b and c) differentiated gastric glands in the stomach's mucosa (magnification: x312.5; stain: Masson's trichrome and PAS); d) pyloric sphincter (magnification: x60; stain: Masson's trichrome).









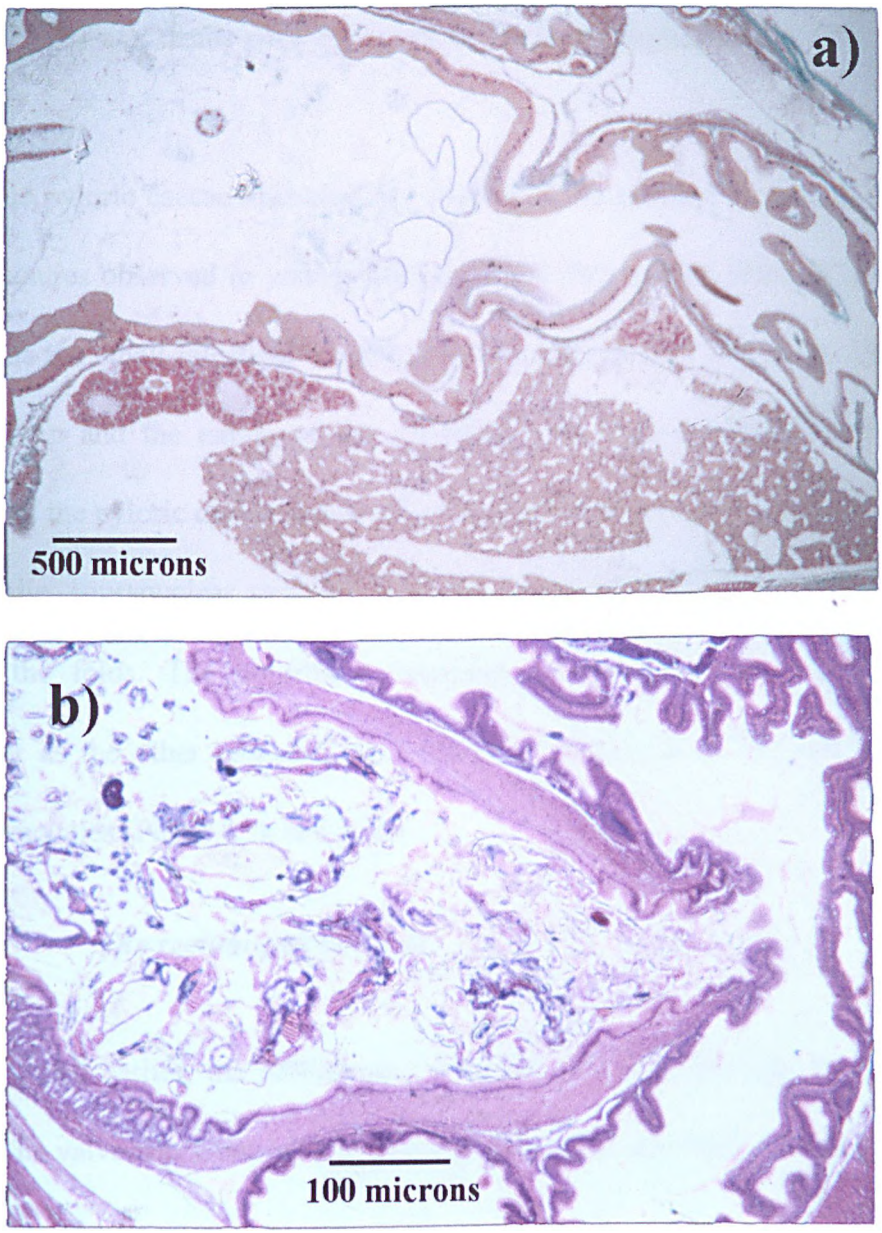
onset of first feeding the pyloric sphincter was only composed of a constriction of the digestive tract mucosa. Similarly, at that stage, the ileorectal valve appeared as a simple fold of the intestinal mucosa. In between these two features, the intestine formed a single loop and accounted for at least two thirds of the length of the alimentary canal.

Mainly columnar cells resembling those of the undifferentiated stomach composed the intestinal epithelium. Nevertheless, the microvilli on the apical surface of these cells appeared longer and a few goblet cells could also be seen (Plate 4.7a). The pyloric caecae were not present at this early stage (up to 35 days PFF) but the area of the intestine directly following the pyloric constriction was wider forming a 'storage pocket' in this tube-like digestive tract (Plate 4.6a). This area was described as such following our observations that it contained most of the food items at this stage of development. A detailed study of the transit time of the digesta and of the site of digestion was not undertaken so these comments have to be considered cautiously, etc.

As in the oesophagus, a clear demarcation between the *lamina propria* of the mucosa and the submucosa could not be identified. Indeed, there was only a small layer of loose connective tissue between the epithelium and the *muscularis externa*. Only one layer of circular smooth muscle formed the *muscularis externa*.

During the course of metamorphosis, the intestine moved forward as stomach differentiated. Meanwhile, the pocket described as following the pyloric sphincter expanded forming the pyloric caecae. Although, the longitudinal sections rarely showed the formation of more than two caecae forming, dissection of whole larvae and juveniles

Plate 4.6: photomicrographs of longitudinal sections into the intestine of developing larvae showing the formation of the pyloric caecae. a) undifferentiated stomach and intestinal 'pocket' of a larva age 21 days PFF (magnification: x64; stain: Masson's trichrome); b) pyloric sphincter and pyloric caecae of a metamorphosed larva aged 63 days PFF (magnification: x312.5; stain: H&E).



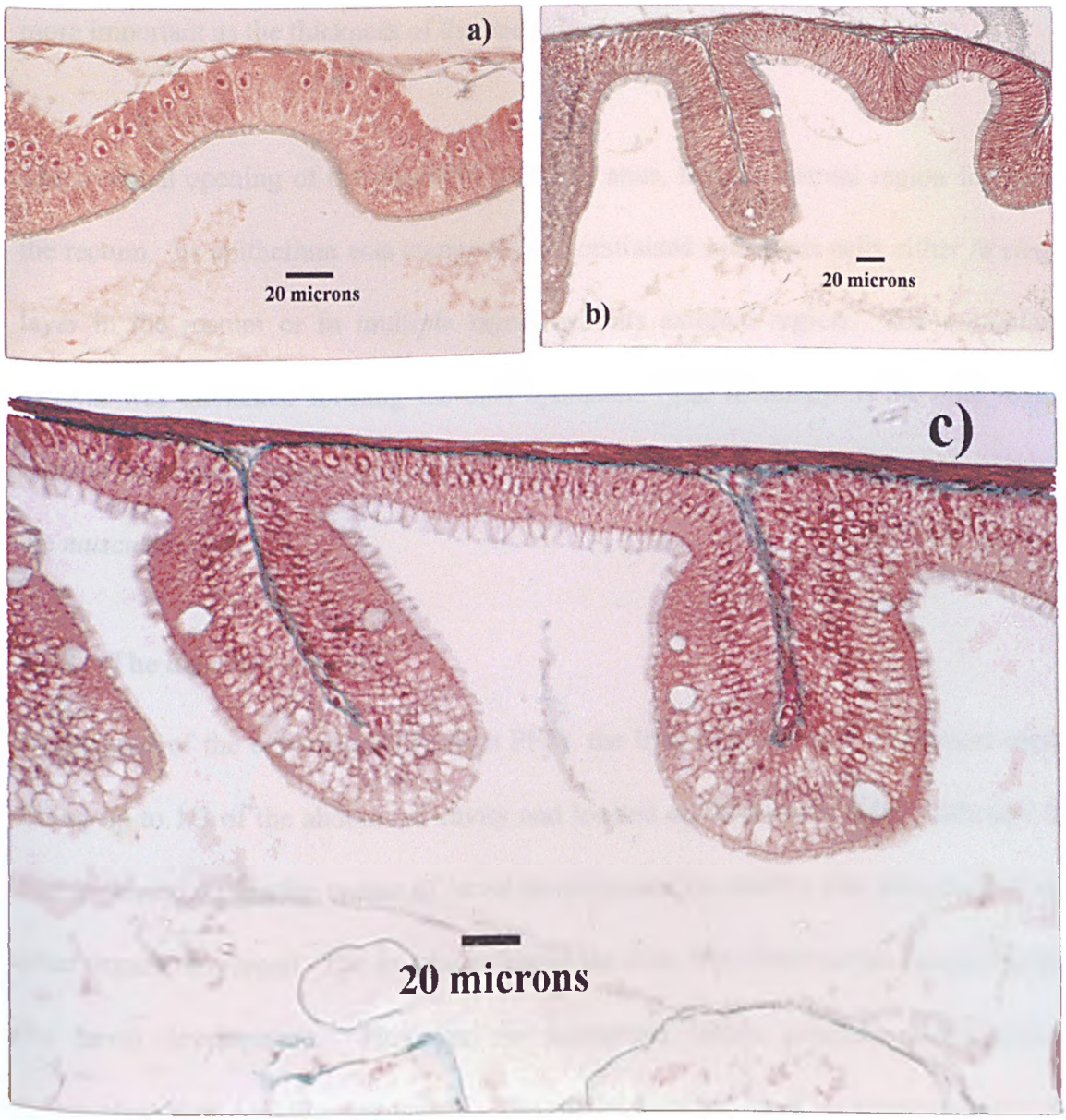
revealed the presence of four caecae (exceptionally five). The caecae formed from expansions of the intestine growing in the opposite direction to the movement of the digestive chyme (Plate 4.6b). All the caecae were located around the pyloric sphincter and developed at a steady pace during the entire metamorphosis.

When the pyloric caecae appeared, the folding of the mucosa increased and the wave-like structures observed in younger larvae turned into conical folds. These folds were not yet as the small-intestinal mucosa of mammals (Gartner and Hiatt, 1994) but their organisation and the nature of the epithelial cells were similar to those villi. The mucosa of the pyloric caecae did not show any difference from its intestinal counterpart. Meanwhile, supranuclear vacuoles appeared mostly in the epithelial cells forming the top of the folds. The absorptive enterocytes containing these vacuoles appeared columnar as the other epithelial cells with the apical side of the cell full of one or several vacuoles (Plates 4.7a and 4.7b).

4.3.1.4. The rectum and the anus

As mentioned earlier, the rectum was separated from the intestine by the ileo-rectal valve. The valve was composed of an expansion of the intestinal coating into its lumen. Soon after the beginning of exogenous feeding, the valve partially obstructed the intestine while by the end of metamorphosis it was large enough to close the lumen completely.

Plate 4.7: photomicrographs of longitudinal sections into the intestinal mucosa showing the appearance of vacuoles in the absorptive cells of copepod fed larvae. a) intestinal mucosa of a larva aged 28 days PFF showing the simple columnar epithelium (stain: Masson's trichrome); b and c) intestinal mucosa of metamorphosed larvae aged 63 days PFF having been fed (b) exclusively on Artemia and (c) on copepods and Artemia (stain: Masson's trichrome).



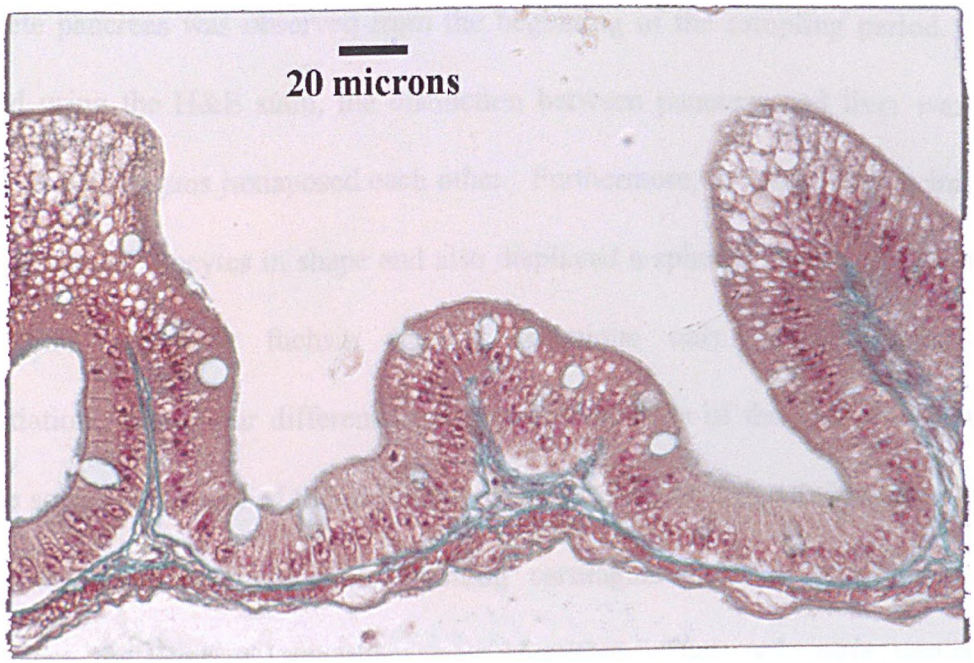
The organisation of the rectal epithelium appeared similar to that of the intestine. However, the appearance of vacuoles amongst the cells forming the top of the fold could already be seen in large numbers sometimes during the end of the metamorphic climax (days 40 to 50 PFF). Furthermore, the folding of the mucosa was progressively more important as the thickness of the *muscularis externa* increased (Plate 4.8).

The terminal opening of the digestive tract, the anus, had an internal region following the rectum. Its epithelium was composed of keratinised squamous cells either in single layer in the rectum or in multiple layers near its external region. The *muscularis externa* was thickened forming the anal sphincter. The histology of the anal region showed very little changes during the larval development except for the thickening of the *muscularis externa*.

4.3.2. The digestive organs

At the start of the experiment (21 days PFF), the liver was the most prominent organ filling up to 1/3 of the abdominal cavity and located on its anterior side. Although its size increased during the course of larval development its relative size decreased as the other organs developed. The lobular nature of the liver was observed unchanged during the larval development. However, no hexagonal lobule structure characterising mammalian liver could be recognised. The spatial distribution of the parenchymal cells (hepatocytes) was less structured forming a relatively loose organ. Nevertheless, the hepatocytes had a characteristic rounded polygonal shape and contained a clear spherical nucleus. Large quantities of lipids were seen in their cytoplasm according to the age and the diet of the larvae (see details in section 4.3.3.) (these observations were confirmed in the study by Shields *et al.*, in press).

Plate 4.8: photomicrograph of a longitudinal section into the rectal mucosa of a larva aged 49 days PFF fed a copepod-based diet. It shows the numerous vacuoles in the absorptive cells of the epithelial cells forming the top of the folds (magnification: x400; stain: Masson's trichrome).

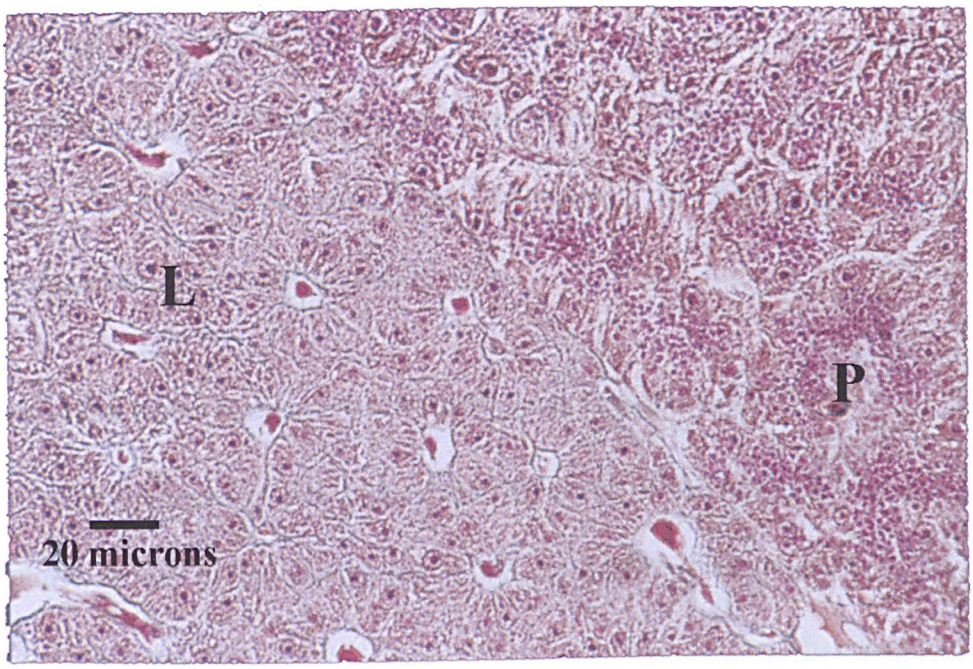


The pattern of ramification and distribution of the sinusoids, the portal vein, the bile duct and the hepatic artery could not be identified although all these canicules were singled out on larvae sampled at all ages of development.

A discrete pancreas was observed from the beginning of the sampling period. When observed using the H&E stain, the distinction between pancreas and liver was made difficult as both organs juxtaposed each other. Furthermore, the pancreatic acinar cells resembled the hepatocytes in shape and also displayed a spherical nucleus. The use of the modified aldehyde fuchsin staining technique only partially achieved a differentiation. No colour differentiation between the cells of the endocrine pancreas could be seen irrespective of the modification done to the technique. However, colour differentiation was achieved when staining cartilaginous tissue or mucous cells. Nevertheless, the islets of Langerhans were identified. Their cells were smaller and more rounded than those of the exocrine pancreas. The islets were perforated by numerous capillaries and formed circular lobes surrounded by exocrine tissue. A maximum of three islets have been identified per pancreas. Meanwhile, the acinar cells were unexpectedly identified using the Masson's trichrome stain which stained heavily the zymogen granules in purple, therefore distinguishing those from hepatocytes and confirming the functionality of the exocrine pancreas. The granules could be observed in large numbers at the apical end of the cells (Plate 4.9).

Unlike the liver, the structure of the pancreas changed during the development although its cellular organisation remained identical throughout. At the early stages of larval development (up to 30 days PFF), the pancreas was a discrete organ of about half the

Plate 4.9: photomicrograph of a section into the liver (L) and pancreas (P) of a 35 days PFF larvae showing the distinction between hepatocytes and pancreatic acinar cells. The zymogen granules of the acinar cells are stained in purple. (magnification: x400; stain: Masson's trichrome)



size of the liver lying next to it under the intestinal loop. As the stomach started to form and to push the intestine further, lobes formed in the pancreas. Progressively, pancreatic tissues could be identified around the pyloric sphincter and along the pyloric caecae. By the end of the metamorphosis a single pancreatic organ could not be singled out. Pancreatic tissue could be seen along the intestine and along the hepatic vein (Figure 4.1). These observations suggested that the pancreas of adult halibut might be a scattered organ as in other teleost species.

4.3.3. Digestive features associated with the prey organisms

The major developmental events such as the formation of the stomach, the appearance of the pyloric caecae or the elongation of the ileo-rectal valve occurred at the same stages of development in all larvae irrespective of their diet. Closer observations on the other hand showed differences occurring with time between the copepod-fed larvae and the *Artemia*-fed larvae. The main variations were seen in the liver and along the intestinal and rectal mucosa.

4.3.3.1. The liver

As the larvae from the copepod and *Artemia*-fed groups initially received *Artemia*, their hepatocytes appeared identical at the beginning of the sampling period (days 21 PFF). During the pre-metamorphic stage (up to 35 days PFF) the large glycogen field of the copepod-fed hepatocytes, visualised by PAS stain, decreased. Simultaneously, there was an accumulation of lipid droplets progressively filling most of the cells' cytoplasm. The nuclei were pushed towards the basal side of the cell, next to the sinusoids as seen on a 49 days PFF larva (Plate 4.10). Meanwhile, little change could be seen in the hepatocytes of *Artemia*-fed larvae.

Figure 4.1: schematic representation of the digestive tract and its extramural glands during the larval development. The pancreas is represented in black and the liver in grey. The number 1 stands for the oesophagus; 2 for the stomach; 3 for the intestine and 4 for the rectum. The direction of movement of the digestive chyme is symbolised by the arrows.

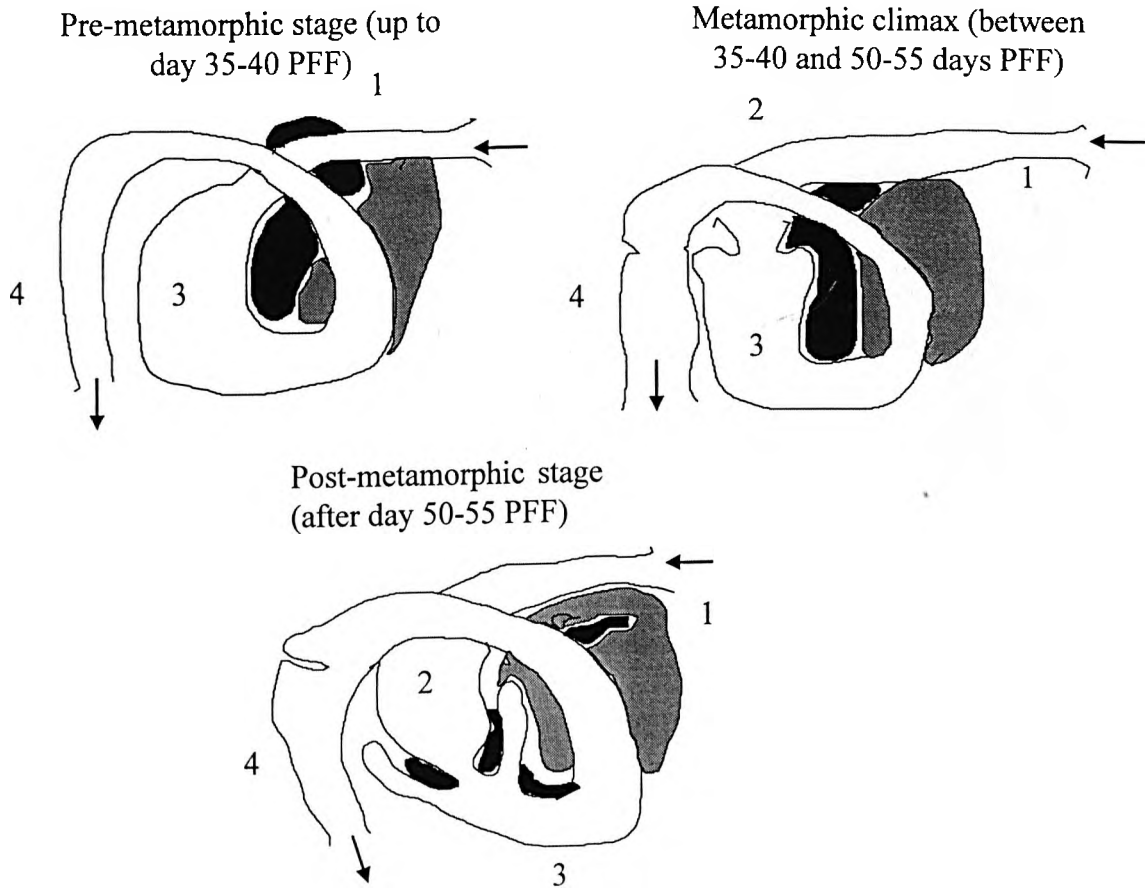
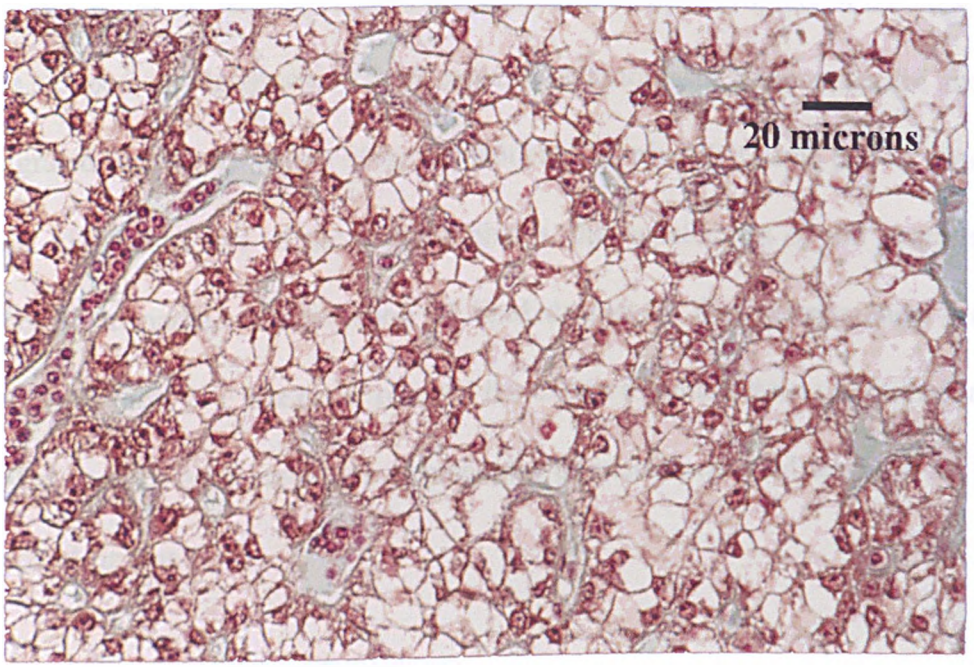


Plate 4.10: photomicrograph of a section into the liver of a 49 days PFF larva fed a copepod-based diet showing the accumulation of lipid and the displacement of the cells nuclei (magnification: x400; stain: Masson's trichrome).



Their nuclei remained in the centre of the cells and no accumulation of lipid droplets was observed. From there onwards, the accumulation of lipid droplets in the copepod-fed larvae stopped and decreased in most cases. The cytoplasm became denser, and the nuclei migrated back to the centre of the hepatocytes. During that same period, from 40 to 45 days PFF to the end of the metamorphosis, the hepatocytes of the *Artemia*-fed larvae showed a marked accumulation of lipid droplets. This phenomenon peaked around 60 to 65 days PFF but never reached the extent to which it was observed in the copepod-fed group. As a result, the hepatocytes of metamorphosed larvae from both groups looked similar.

4.3.3.2. The digestive epithelium

Typically, the absorptive cells of the intestinal and rectal mucosa were columnar cells showing a large round nucleus on their basal side. Two types of supranuclear inclusions were identified in these cells. The first type was described as lipid vacuoles due to the lack of coloration. Indeed, lipids are known to be extracted in the chloroform and alcoholic solvents used during the processing of samples. The second type of inclusions was described as protein inclusions resulting from the pinocytosis of macromolecules by the enterocytes. These inclusions contrary to the former were coloured by all the stains used.

The intestinal mucosa of the *Artemia*-fed larvae showed very little variation during the course of metamorphosis. No lipid vacuoles nor protein inclusions were noted. Meanwhile, the intestinal epithelium of the copepod-fed larvae showed great variation.

Progressively, the cells lining the top of the mucosal fold displayed increasing numbers of small lipid vacuoles. It was only at the post-metamorphic stage (55 to 60 days PFF and later) that these small vacuoles appeared to be combined into larger vacuoles which filled most of the cell and pushed the nuclei to the basal end of the cells (Plate 4.7c).

In the rectal epithelial cells, inclusions or vacuoles appeared in both groups of larvae (Plate 4.8). The *Artemia*-fed larvae showed small inclusions from the beginning of the sampling period. They appeared darkly-stained and positioned in the apical side of the enterocytes' cytoplasm and were identified as proteinic inclusions. Later, by days 40-50 PFF, some vacuoles could also be seen, especially in cells lining the tip of the folds. Finally, by the end of the metamorphosis, the apical side of the enterocytes appeared very lightly stained and sprinkled with dark inclusions. The appearance of the rectal epithelium of the copepod-fed larvae was similar; inclusions were visible from the beginning to the end of the sampling period. However, a number of vacuoles were also observed in the enterocytes from day 21 PFF, and increasing in number and size during the following 25 days.

4.4. Discussion

From hatching to adulthood, the development of the fish alimentary canal changes from a straight, undifferentiated gut to a complex and segmented digestive tract (Govoni *et al.*, 1986). At the time of first exogenous feeding, it is partially differentiated except for the stomach that is lacking (Hjelmeland *et al.*, 1993a,b; Pedersen, 1993). The present study confirmed similar observations performed on halibut (Pittman *et al.*, 1989).

In most teleosts, with the exception of a few herbivorous species (Anderson, 1986; Loewe and Eckmann, 1988), the muscular layer surrounding the mucosa of the oesophagus is thin with its main function relating to the peristaltic contraction easing the circulation of the digesta. Such contractions have been reported for halibut larva reaching the end of their yolk-sac resorbtion (Pittman *et al.*, 1990).

Besides its mechanical role, the oesophagus also has a secretory role. Its production of mucus lubricates the lumen, thereby easing the transport of the digesta (Ross *et al.*, 1995). This function, assumed by cilia until the opening of the mouth (Morrison *et al.*, 1997), was present at the time of first-feeding in halibut and was further developed through the larval period as shown by the increase in the number of goblet cells. Unlike the goblet cells identified in the intestinal mucosa, these showed a negative reaction to the PAS stain but a positive one to the modified aldehyde fuchsin stain suggesting the presence of different mucopolysaccharides (Boulhic and Gabaudan, 1992; Tibbetts, 1997).

A third role for the oesophagus has been suggested. Its posterior section is believed to have a pre-gastric digestive function (Murray *et al.*, 1994b). This hypothesis is based on the complex histochemistry of the mucus, the mucosal folding and the secretory activity. None of the observations made in this study are in disagreement with these findings, but the evidence is too scarce to confirm such a suggestion.

The epithelium of the transition zone between the oesophagus and the stomach of halibut larvae was a simple prismatic epithelium, as in larval sole (Boulhic and Gabaudan, 1992). The portion of the digestive tract of the pre-metamorphic larvae (up to 35 days PFF) located between this transition zone and the pyloric constriction differentiated into a stomach. Meanwhile, the pyloric caecae differentiated from the anterior part of the intestine as in cod larvae (Pedersen and Falk-Petersen, 1992).

Three regions were distinguished in the stomach including a transition zone following the oesophagus, the cardia, the glandular zone, the fundus and an aglandular region preceding the pyloric sphincter, the pylorus. These regions have been detailed intensively in mammals (Wheater and Burkitt, 1988; Gartner and Hiatt, 1994; Ross *et al.*, 1995) and in several species of fish (Hibiya, 1982; Caceci *et al.*, 1997). However, these findings for the larvae disagree with observations of the stomach of adult halibut where the pyloric aglandular region was not reported (Murray *et al.*, 1994a). It may suggest that the differentiation of the stomach and the formation of gastric glands continue after the end of the metamorphosis. Indeed, the pyloric region was only clearly identified on the ventral side of the stomach while the glandular region of the fundus almost reached the pyloric sphincter on the dorsal side.

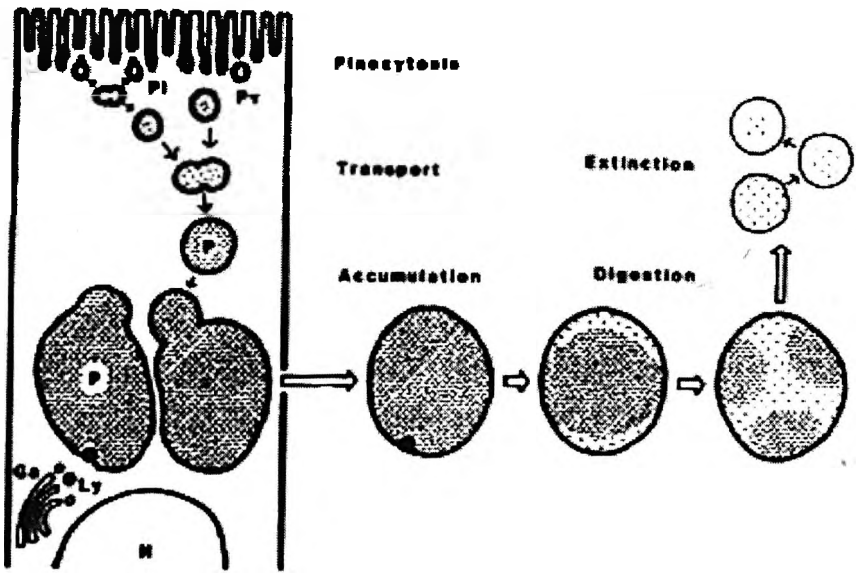
In adults, the stomach disrupts the configuration of protein by its secretion of hydrochloric acid. The action of pepsin continues this pre-digestion (Grabner and Hoffer, 1989). The complete digestion, which follows, takes place in the intestine. This extracellular digestion involves aminopeptidases located on the intestinal striated border membranes. These complete the digestion started by the luminal pancreatic enzymes (Hendriks *et al.*, 1990). Independently of the time at which the larvae gain control of digestive enzyme secretion, the stomachal protein digestion occurring in adult will not take place until the gastric glands become functional (Govoni *et al.*, 1986).

The apical striated border of the intestinal enterocytes in the halibut stained with PAS as in sole (Boulhic and Gabaudan, 1992) and flounder (Jenkins *et al.*, 1992). Although changes occurred in the size of the mucosa, its folding and absorptive activity, it has been shown in other species that the enterocytes are fully functional at hatching (Segner *et al.*, 1994). Nevertheless, the larvae seem to compensate for the lack of gastric pre-digestion by an active intracellular digestion in the rectal epithelial cells following the pinocytosis of macromolecules from the lumen (Dabrowski and Dabrowska, 1981; Govoni *et al.*, 1986; Loewe and Eckmann, 1988; Bengtson *et al.*, 1993). The histological evidence for this process is the acidophilic, supranuclear inclusion bodies of the hindgut epithelial cells. Such inclusions were, indeed, observed in the rectal epithelial cells of both the *Artemia* and the copepod-fed group during the course of development.

These inclusions disappeared when the stomach became functional and allowed for extracellular digestion. However, some reports of supranuclear bodies exist for juveniles possessing a stomach (Ezeasor, 1981) and in some cases, they have been identified as containing lipids (O'Connell, 1976). On the other hand, studies using a histochemical tracer, horseradish peroxidase (HRP), confirmed that macromolecules are pinocytosed and intracellular digestion occurs in the rectum (Watanbe *et al.*, 1985; Kurokawa and Suzuki, 1996) (Figure 4.2).

Concerning the digestion of fat, the first step consists of intraluminal digestion, which requires the presence of pancreatic lipase. These enzymes break down triglyceride into monoglyceride and free fatty acids by their action on the water-lipid interface. The second step, micellar solubilisation, is essential to the completion of triglyceride hydrolysis. The resulting water-soluble micelles transport the lipids from the emulsion to the absorptive surface. The mucosal cells' passive absorption follows after the disruption of the micelles. Once inside the cell, the fatty acids are activated by the addition of coenzyme A, forming acyl-CoA prior to re-esterification. The subsequent synthesis of triglycerides, cholesterol, phospholipids and protein forms the chylomicrons that are transported into the intracellular space. The chylomicrons then pass by the lymphatic system before entering the circulation and being delivered to the liver (see reviews by Shiau, 1981; Krogdahl, 1985; Sheridan, 1988). The liver re-packages the dietary lipids and combines them with synthesised lipids and protein to form very low density lipoproteins (VLDL) (Gnoni and Muci, 1990).

Figure 4.2: schematic drawing of the successive stages of protein absorption and intracellular digestion by the hindgut epithelial cells of fish larvae. Pi stands for pinocytotic invaginations, Pv stands for pinocytotic vesicles, P stands for protein inclusions bodies, Ga stand for Golgi apparatus, Ly stands for lysosomes and N for the nucleus. (Copied from Watanabe 1984 in Govoni et al., 1986).



The digestion of lipid in larval fish seems to depend on esterase (Munilla *et al.*, 1993) as lipase does not seem to be present until metamorphosis (Cousin *et al.*, 1987). Nevertheless, evidence of lipid digestion is apparent in fish larvae as early as 2h after the first food intake (Segner *et al.*, 1994).

Histologically, active lipid digestion is confirmed by the appearance of large lipid droplets in the mucosal epithelial cells, which are thought to be temporary storage sites (Watanabe and Sawada, 1985). In halibut larvae, as shown by the present investigation, the lipid vacuoles were supranuclear and appeared in both the intestine and rectum of copepod-fed larvae. The lack of such vacuoles in the intestine of *Artemia*-fed larvae suggests a reduced intestinal lipid digestion or absorption. In sole, the lipid vacuoles were infranuclear and appeared in the antero-median intestine while the eosinophilic inclusions were supranuclear and appeared in the rectum (Boulhic and Gabaudan, 1992). In gilthead seabream, similar observations were reported, although the lipid vacuoles were supranuclear (Sarasquete *et al.*, 1995).

Furthermore, it has been shown that the site of absorption of amino acid in the intestine moves posteriorly in relation to the dietary protein digestibility (Dabrowski and Dabrowska, 1981). In addition, several reports exist of the rapid passage of *Artemia* through the alimentary canal of fish larvae (Rosenthal and Hempel, 1970), especially when fed high rations (Werner and Blaxter, 1980). Longer retention times have been associated with the feeding and digestion of copepods (Perdersen and Hjelmeland, 1988).

The rapid autolysis of rotifers in the intestine of seabass larvae only takes place when autolytic processes have been triggered by damage inflicted to the rotifers (Walford and Lam, 1993). Since there is no acidity in the stomach to cause such damage biochemically, the pharyngeal teeth may perform this function. In the case of halibut, the appearance of teeth was noted around half way through the yolksac period (Pittman *et al.*, 1990). Nevertheless, according to the food provided to larval seabass, Walford and Lam (1993) reported a variability in the mechanical damage inflicted and consequently to the level of digestion. In the present experiment, the gut contents of larvae were examined. Intact or partially intact *Artemia* were commonly observed in the intestinal lumen while individual copepods could not be identified. However, it could not be established if these differences should be attributed to differential damages inflicted by the teeth.

Furthermore, no evidence was found of macromolecular phagocytosis in eel rectal epithelial cells of larvae fed rotifers (Kurokawa *et al.*, 1995). Two possible explanations were given by the authors: either the rectal cells had not yet acquired the ability to phagocytose or rotifers were an unsuitable prey for larval eel.

In the light of these reports, the observation that rectal rather than intestinal lipid storage was observed only at the end of metamorphosis in *Artemia*-fed larvae questions the suitability of *Artemia* as live prey for Atlantic halibut larvae. It can be suggested that *Artemia*, whether they are mechanically damaged by the larval teeth or not, are not properly digested. It is only following the differentiation of the stomach and consequent pepsin and hydrochloric acid pre-digestion that the *Artemia* lipids become available to

the larvae. Even then, the appearance of the vacuoles in the rectum suggests that the retention times are too short for the process to take place normally involving the intestinal enterocytes.

It is questionable whether the food consumption rates as standardised for halibut larvae by van der Meeren (1995) represent the optimal rations for maximal absorption. It seems that there is only a limited availability of the body nutrients of *Artemia* for the larvae. Therefore, the use of long term enrichment might also be questioned. Indeed, such enrichment procedures are designed to improve the nutritional value of the tissues of *Artemia*. Based on these observations and on the literature cited above, it is suggested that the *Artemia* are poorly digested as reflected by the presence of intact exoskeletons in the rectum. Therefore, it can be speculated that the larvae only benefit from the dietary value of the enrichment which they can access via the mouth and the anal opening of the undigested *Artemia*. In commercial terms, short-term enrichments may achieve the same goal whilst reducing the costs of live feed rearing. However, neither of these approaches would solve the more fundamental problem of the digestibility of *Artemia*.

Pittman *et al.* (1990) described the formation of the main organs associated with the digestive system as well as the formation of the alimentary canal of halibut. The liver was reported to appear after about 15 to 18 days post hatch. By the end of yolk resorption, the intestine had formed a loop and the first peristaltic contractions were observed. Furthermore, Kjørsvik and Reiersen (1992) identified distinct pancreatic cells by day 20 post-hatch. Although the cells looked very similar to the hepatocytes, small

amounts of zymogen granules could already be seen. The pancreas of first-feeding halibut larvae, despite its defined status compared to the diffuse adult organ, is of great importance for the larval digestion.

Notwithstanding the short retention time associated with the short digestive tract, first-feeding larvae differ from adults in other ways related to the lack of a functional stomach. The absence of gastric glands and therefore of pepsin and hydrochloric acid secretion implies that the protein digestion depends only on the pancreas (Hjelmeland *et al.*, 1988). Indeed, the digestion of protein and starch is made possible by the pancreas-borne enzymes present in the lumen and on the enterocytes striated border (Hjelmeland *et al.*, 1988; Youson and Cheung, 1990; Segner *et al.*, 1993b). Furthermore, strong amylolytic and proteolytic activities have been reported, at the time of first feeding, in turbot (Cousin *et al.*, 1987), catfish (Segner and Verreth, 1995), white fish (Segner *et al.*, 1993a), herring (Hjelmeland *et al.*, 1988; Perdersen *et al.*, 1987; Pedersen, 1993), striped bass (Baragi and Lovell, 1986), and halibut (Ugelstad *et al.*, 1993). The pancreatic enzymes secreted by the exocrine portion of the gland are either precursors of active enzymes such as trypsin or chymotrypsin, or are released in their active form. The mechanisms of release of these enzymes are believed to be of neural and hormonal origin in mammals. In larval fish, the position is less clear as reflected by the different observations reported in the literature. For example, Hjelmeland *et al.* (1988) concluded that the release of active substances (lipids, amino acids or polypeptides) from prey stimulate the secretion of cholecystokinin (CCK), which in turn triggers the release of pancreatic enzymes. This corresponds to the chain of event reported in adult fish (Holmgren, 1993). On the other hand, Cousin *et al.* (1987) noted the presence of

amylase and protease in the intestinal lumen of fasting turbot larvae. This suggests that at that stage the larvae are not capable of controlling the release of these enzymes.

A way of assessing the pancreatic activity or the functionality of the pancreas consist of identifying the presence of trypsin, one of its major secreted enzymes (Pedersen, 1993). This technique has often led to the assumption that the pyloric caecae were sites of trypsin activity, suggesting a digestive function in addition to their absorptive function. However, the pancreas is scattered in most fish species and pancreatic cells can often be found along the pyloric appendages (Groff and Youson, 1997). Kurokawa and Suzuki (1995) suggested that the location of tryptic activity in flounder larvae was the result of contamination, by pancreatic cells, of the pyloric samples. The authors subsequently questioned the digestive function of the pyloric caecae reported in other teleosts. Similarities exist between flounder and halibut with respect to the timing of trypsin secretion (Ugelstad *et al.*, 1993) and the presence of pancreatic cells along the intestine and pyloric appendages. The function of the pyloric caecae of larval halibut may therefore be only absorptive as suggested for flounder. Independently of its absorptive function or in conjunction with it, the pyloric caecae have also been associated with the regulation of pancreatic activity by means of hormone production (Kurokawa and Suzuki, 1995). However, little data are available to date on the exact involvement of the intestinal and pyloric mucosa in the regulation mechanisms of digestion.

Nevertheless, the liver and the pancreas have been reported as functional at the beginning of the exogenous feeding period in halibut larvae (Kjørsvik Reiersen, 1992). The clear observation of numerous zymogens in the pancreatic acinar cells confirmed

these findings. The ultrastructure of the hepatocytes has been reported to reflect the quality of the diet and its adequacy (Storch *et al.*, 1983; Segner *et al.*, 1984; Sieg, 1992; Segner *et al.*, 1993b; Abi-Ayad and Kestemont, 1994). The lipid accumulations in the hepatocytes are thought to originate from the intracisternal droplets formed by lipoprotein-like particles aggregated within the lumen of the endoplasmic reticulum (Segner *et al.*, 1994). In the present study, lipid had become the dominant energy store in the liver at the expense of the glycogen halfway through the metamorphosis of the copepod-fed larvae (Shields *et al.*, in press). These results are in agreement with the apparent large availability of lipids as seen by the temporary storage accumulated in the intestinal enterocytes. Similarly, the lack of lipid accumulation in the hepatocytes of *Artemia*-fed larvae until the end of metamorphosis also suggests a limited availability of lipid, or difficulty in digestion and absorption of *Artemia* lipids.

While the digestibility of the *Artemia* has been questioned, one important aspect of the digestive development of halibut larvae remains to be addressed. This relates to the time of weaning as the differentiation of the digestive system and more particularly the functionality of the gastric glands and the appearance of the pyloric appendages are considered as a juvenile characteristic (Boulhic and Gabaudan, 1992).

In flounder, the ability to digest artificial diet has been related to the exocrine capacity of the pancreas and mostly to the mechanisms of its regulation (Kurokawa and Suzuki, 1996). Indeed, larvae are capable of surviving on heat-killed *Artemia* while they do not seem to produce any digestive enzymes in the presence of artificial food. These observations suggest the presence of a stimulatory factor in the zooplankton that would

be required during the larval stages until all the adult-like features of the digestive system have been established (Kurokawa and Suzuki, 1996). Practically, a relation between the food ration and the trypsin secretion has been established for other species (Abi-Ayad and Kestemont, 1994; Zambonino Infante *et al.*, 1996).

Enzymatic data is not available for halibut larvae so the timing of weaning can only be assumed to be related to the functionality of the stomach. In such circumstances, the present results have shown that by the end of the metamorphosis the stomach was fully developed morphologically. However, the gastric glands were developed, if not fully differentiated by the time of metamorphic climax (between 35 to 50 days PFF). The changes in absorptive activity of the *Artemia*-fed larvae also started to occur around the same period so one could speculate that the functionality of the halibut stomach occurs around day 45 PFF (in the conditions experienced for this experiment). At this age, as reported in section 3 the larvae had shown morphological changes including a widening of the dorsal myotome height and an exponential increase of the number of melanocytes on their dorsal side. Whether the developmental stages, *i.e.* pre-metamorphic, metamorphic climax and post-metamorphic, identified when studying the digestive ontogeny and the morphological changes in body shape are linked seems probable.

Chapter 5. The larval retina

5.1. Introduction

For a fish to see an object under-water, the intensity of the light that the object reflects must be either greater or smaller than the light intensity of the background to provide a contrast (Northmore *et al.*, 1978). For the image to be informative, the eye must trap as much light as possible as the amount of information in the retinal image depends on the number of photons that form it. For the image to be clear, the density of the visual cells forming the retina must be high as details can only be resolved if their images on the retina are separated by at least one unstimulated photoreceptor (Blaxter and Jones, 1967). Furthermore, light at great depth is scarce as the intensity of daylight decreases tenfold every 75m increase in depth (Denton, 1990). Consequently, the development of the eye of a deep-sea fish such as halibut and more particularly the development of their retina is of vital importance for their ability to survive in their natural environment. In addition, the requirements for highly unsaturated fatty acids (HUFA) of the central nervous system (CNS) tissues and most particularly the retina are high (Tocher and Harvie, 1988; Gülcan *et al.*, 1993). Having shown the relative inadequacy of *Artemia* in fulfilling the larval needs in terms of lipids, the study of the larval retina appeared as a interesting area of research in connection with development, metamorphosis and larval nutrition.

5.1.1. The retina

The retina is the photosensitive layer of the eye and is constituted by three types of cells: neural cells, pigmented epithelial cells and support cells. These three types of cells, specialised in various ways form its 10 cell layers (Figure 5.1), namely, following the path of entering light;

- (1) the internal limiting membrane;

- (2) the nerve fibre layer;
- (3) the ganglion layer;
- (4) the inner plexiform or inner molecular layer;
- (5) the inner nuclear layer;
- (6) the outer plexiform or outer molecular layer;
- (7) the outer nuclear layer;
- (8) the external limiting membrane;
- (9) the photoreceptor layer;
- (10) and the pigment epithelium.

The first 8 layers are characteristic of any fully differentiated vertebrate's retina and therefore more detailed description is limited, in the following sub-sections, to the last two layers (*i.e.* layers 9 and 10).

5.1.1.1. The photoreceptors

Two types of specialised cells make up the photoreceptor layer, the cones and rods. These are bipolar cells, containing an inner and an outer segment connected by a thin band of cytoplasm (Figure 5.2). The inner segment contains a voluminous Golgi apparatus and numerous mitochondria (Wheater and Burkitt, 1988).

Figure 5.1: Schematic drawing of the layers of the retina. Copied from Ross et al., 1995.

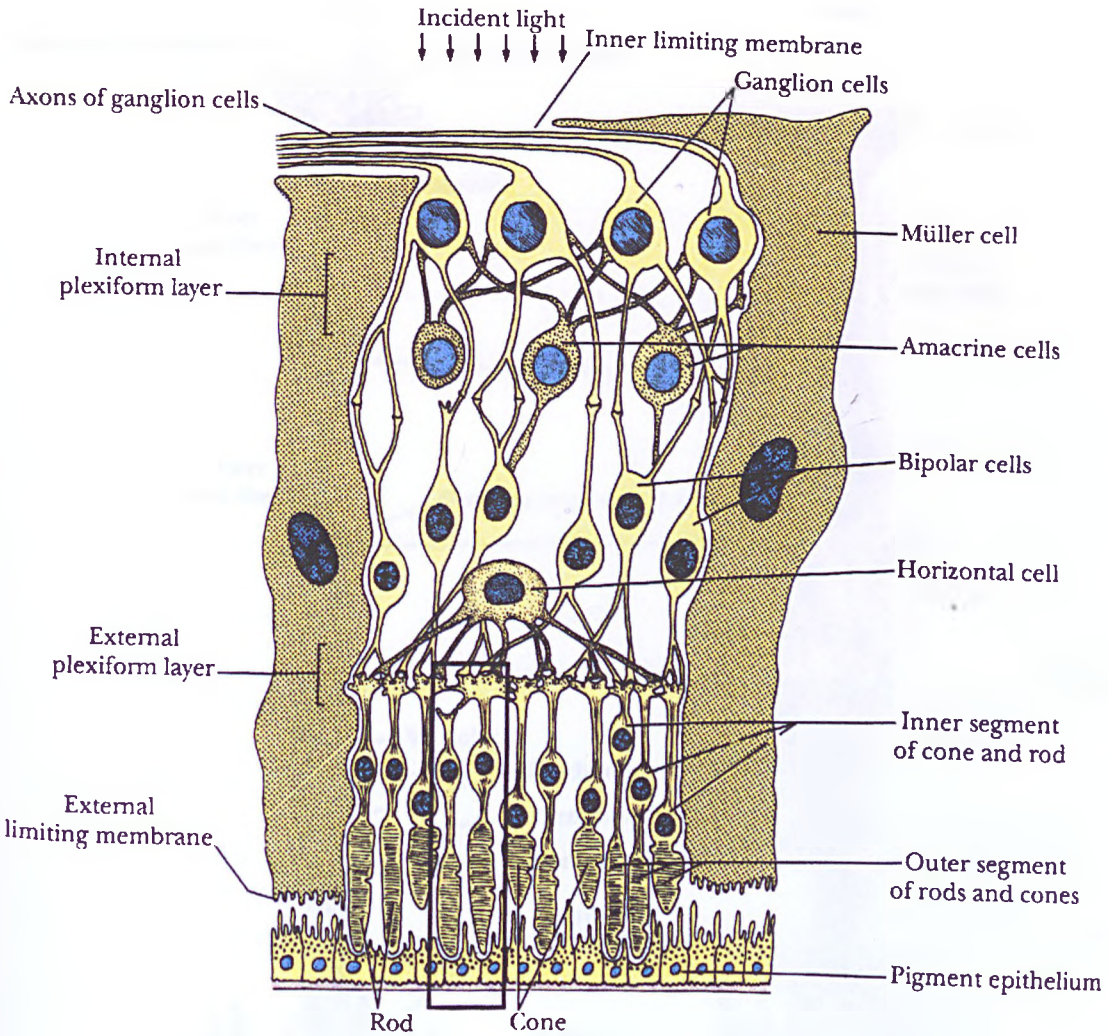
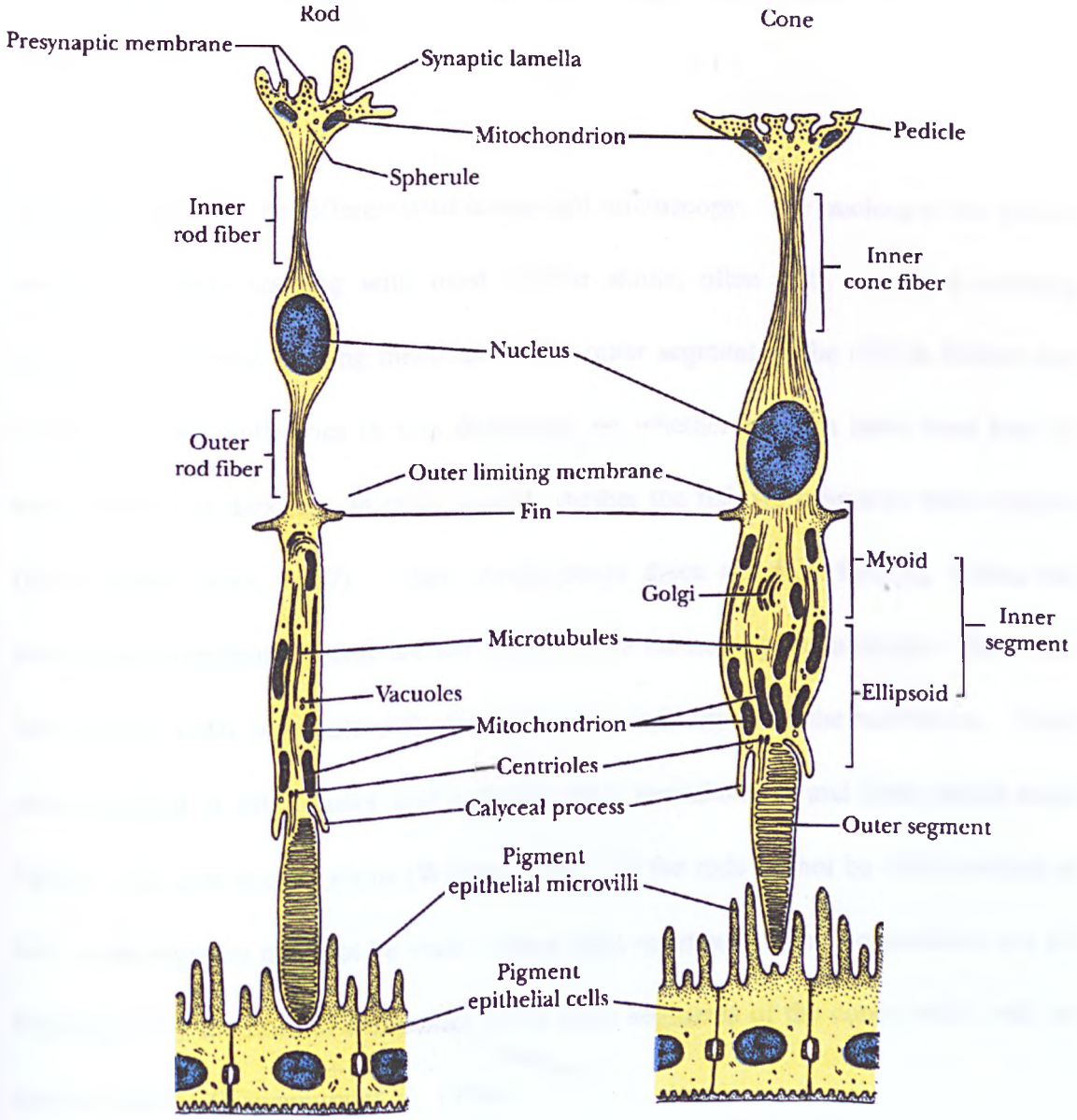


Figure 5.2: schematic diagram of the ultrastructure of rod and cone cells. Copied from Ross et al., *1995.*



The outer segment is a specialised region of cylindrical shape, containing membrane-like disks in which are incorporated all of the photosensitive visual pigments (Wolken, 1975). Their main role is to convert the light energy into neural excitation (Fernald, 1993).

Cones and rods can be differentiated using light microscopy. The nucleus of the rods is small and darkly staining with most nuclear stains, often with processes pointing towards the external limiting membrane. The outer segment of the rods is thinner and more elongated and varies in size depending on whether the fish have been kept in constant light or darkness, in other words, whether the fish were light or dark-adapted (Blaxter and Jones, 1967). Their membranous discs are free floating within the enveloping membrane as they are not connected to the outer plasmalemma. The cones have shorter outer segments with their discs in continuity with the membrane. Their inner segment is often bulky and contains large mitochondria and their nuclei stain lightly with most nucleic stains (Wagner, 1990). If the rods cannot be distinguished as their outer segment may not be visible using light microscopy, their occurrence can be highlighted by the ratio of cell nuclei to the outer segments of the cones which will be greater than 1:1 (Kawamura *et al.*, 1984a).

Cones are often distributed in a very precise spatial order forming mosaics. These patterns may vary from species to species and even during the life span of individuals. Their role has been related to a series of behavioural and visual aspects (see review by Wagner, 1990). The cones are also associated with colour vision as they usually present more than one spectral sensitivity (Kröger and Fernald, 1994). Their number and their visual pigments may vary greatly between fish species (Partridge, 1990).

During the course of development, most fish larvae start with a pure-cone retina with rods and double cones appearing around the time of metamorphosis (Sandy and Blaxter, 1980; Kawamura *et al.*, 1984b; Kitamura, 1990; Huse, 1993; Mani-Ponset *et al.*, 1993; Pankhurst, 1994; Kvenseth *et al.*, 1996). The double cones are believed to allow an increase in visual acuity and a better perception of movement. The cones contains a variety of pigments, whereas the outer segments of the rods contain only rhodopsin which is responsible for vision in the dark (Kanazawa, 1993).

It is worth noting that photoreceptors can be found outside the retina, in the pineal. Although in many respects, they remain an enigma, these are known to mediate some behavioural (Foster and Roberts, 1982) and physiological responses (Porter *et al.*, 1995). However, they were not taken into account in the present study.

5.1.1.2. The retinal pigment epithelium (RPE)

The most external retinal layer, the RPE is a monolayer of neuroepithelial cuboidal cells lying on top of the choroid's surface. They are usually mononucleate and contain a series of organelles and cytoplasmic inclusions having a functional relationship with the photoreceptors (Wagner, 1990).

The RPE cells may contain reflecting granules forming the *tapetum lucidum* which increase the light sensitivity of the eye by reflecting the light back to the photosensitive cells (Zyznar and Ali, 1975). These granules filled with crystals are randomly distributed in the long processes of the RPE engulfing the outer segment of the

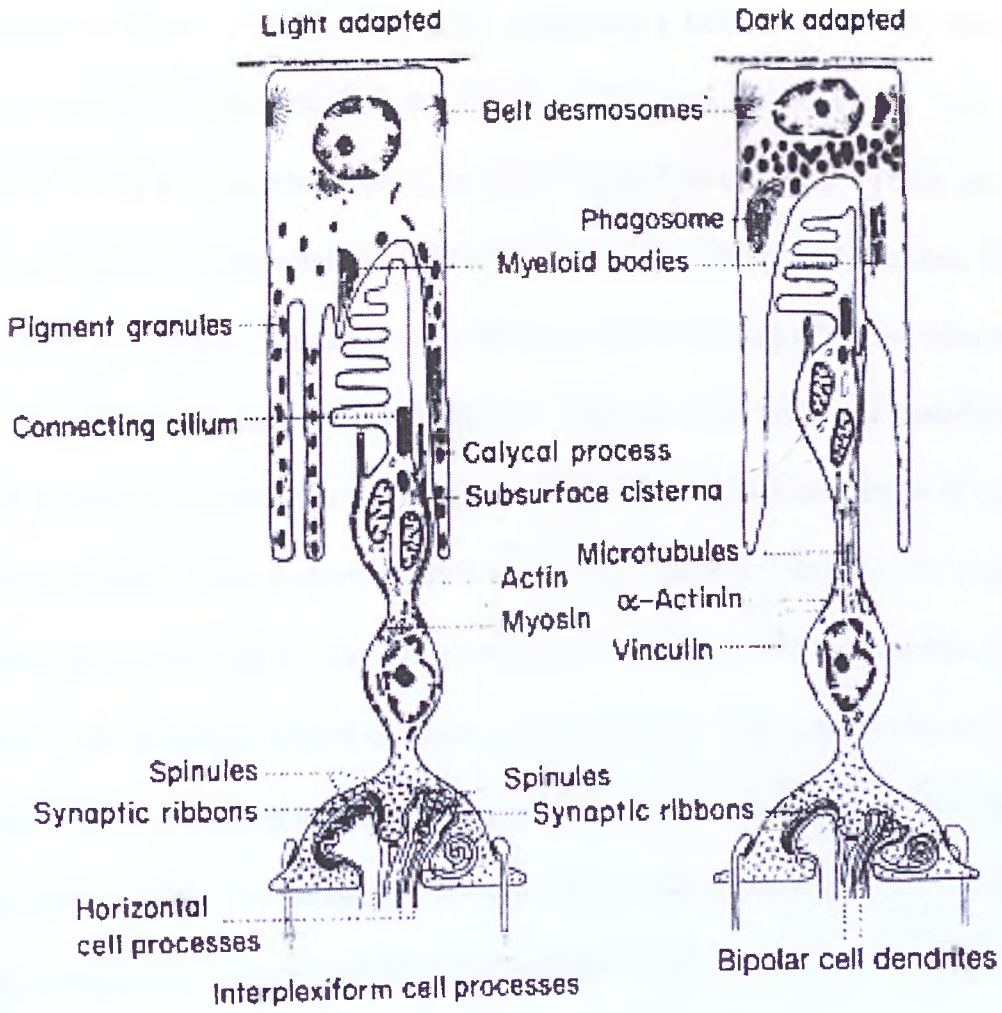
photoreceptive cells. They are absent from the base of the cell and exhibit geometrical profiles.

Although there are at least two types of structures in the retinal *tapeta,* fish with *tapeta* can usually be characterised by the fact that they exhibit eyeshine. It can represent an adaptation to dim light conditions in their natural environment such as turbid water in the case of the sauger (Ali and Anctil, 1977).

The RPE also contains spherical or rod-shaped pigment granules distributed in the apical processes under intensive light. They improve the image resolution by absorbing extra light and optically isolating individual photoreceptors, thereby reducing interference between adjacent cells. Under low light intensity, they are moved to the cell bodies of the RPE. These two states are generally described as light and dark adaptation respectively (Wagner, 1990; Nag and Sur, 1992) (Figure 5.3).

Besides acting as a light screen, the RPE participates in many processes necessary for normal photoreceptor function. The first recognised function is its participation in the renewal of the outer segment of the rods (Young and Bok, 1969). Using labelled membranes, the authors showed that the detached end of the outer segment was absorbed by RPE's inclusions called phagosomes. The phagosomes are degraded by lysosomes. This process is governed by daily cycles. The cones outer segments are renewed during the night and the rods outer segments during the day when the cells are less active (Young, 1977; Bosch *et al.*, 1993). These dual functions of the RPE and the photoreceptor appear to be regulated to some extent by cyclic adenosine mono phosphate (cAMP) which itself is regulated by neurotransmitters and neuromodulators

Figure 5.3: schematic drawing of morphological alterations in a cone and a pigment epithelial cell during light and dark adaptation. Copied from Wagner, 1990.



including thyroid stimulating hormone (TSH) and the melanocyte-stimulating hormone (MSH) (Koh and Chader, 1984). Meanwhile, melatonin was shown not to have any regulatory effects on the cAMP activity suggesting a lack of interaction between the pineal and RPE's functions (Koh and Chader, 1984; Begay *et al.*, 1994). The myeloid bodies (MBs) form another type of organelle present in the RPE. These are special forms of smooth endoplasmic reticulum (Wagner, 1990; Abran and Dickson, 1992a,b). They are presumed to be active in the conversion and recycling of retinol released from photoreceptors following photostimulation. They are also cyclically associated with lipid droplets and lysosomes suggesting a role in lipid metabolism (Baker *et al.*, 1986; Wagner, 1990). This involvement in lipid renewal was first believed to be passive but studies on the frog retina were not consistent with this hypothesis (Cai and Dickson, 1993). The authors reworded the hypothesis as follows: the requirement for building materials for outer segment membrane synthesis is increased during the light and decreased at night. The remodelled phospholipids (docosahexaenoic acid, DHA being a major component) are removed from the myeloid bodies (MBs) for transfer back to the photoreceptor cells in the light. The overall size of MBs is then reduced. In contrast, in the dark, because the requirement for building materials is low, the size of MBs increases. The authors then speculated that MBs are involved in a process that actively supplies, remodels and recycles lipids, particularly HUFA such as DHA, that are destined for the synthesis of new outer segment membranes.

The RPE is also involved in transfer of materials from the photoreceptors outer segment to the choroidal circulation. Water is pumped away from the retinal photoreceptors (Koh and Chader, 1984) and nutrient exchange occurs among the three cellular layers (Gülcan *et al.*, 1993). Finally, the RPE is believed to be involved with the regulation of

the transcription and/or translation of the pigments proteinic component, opsin (Stiemke *et al.*, 1994).

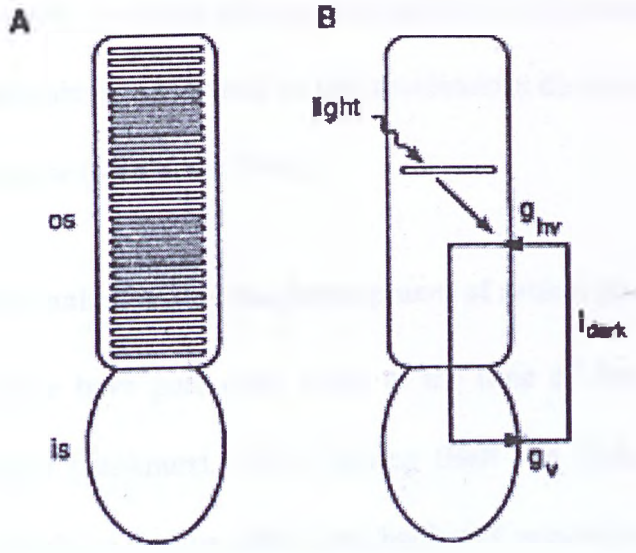
5.1.2. The visual pigment, rhodopsin

The mechanism of the visual process in the vertebrate retinal rod involves rhodopsin, which bleaches upon exposure to light, yielding retinal and its carrier protein opsin.

Retinal is a vitamin A aldehyde that is associated with the outer segment membrane; in particular to the membrane phospholipids such as phosphatidylcholine (PC), of which an abundant fatty acid is DHA. Since the role of rhodopsin is to convert the light stimulus to a chemical one, it ensures the stimulation of the central nervous system (CNS). It is believed that the visual transmission from the retina is not delivered to the CNS if the rhodopsin formation is interrupted (Kanazawa, 1993). Indeed, Brown (1994) studied and partially established the influence of membranes lipids on the photochemical function of rhodopsin. The three components of the pigment complex, namely, protein (opsin), retinal (vit. A aldehyde) and phospholipid (with high content of DHA), are therefore of vital importance to maintain any visual acuity and sensibility (Kanazawa, 1993).

As mentioned earlier the rod outer segment (ROS) is specialised in the process of phototransduction. Its membranous discs contain the photopigment rhodopsin. In frog and cattle for example, the protein content of the ROS membranes is 80+% rhodopsin (Papermaster and Dreyer, 1974). The inner segment of the photoreceptor cell is responsible for the transmission of the electric photoresponse (figure 5.4). In darkness, an electrical current flows from the voltage-gated conductance of the inner segment (g_v

Figure 5.4: (A) schematic diagram showing the structure of a vertebrate rod. (B) diagram illustrating the electrical basis of the photoresponse. Copied from Matthews, 1990.



in figure 5.4) into the rod outer segment. Absorption of light has two effects. It destroys the cellular transmitter of excitation, cyclic guanosine monophosphate (cGMP) and rhodopsin is converted to trans retinal + opsin. As a result, there is a decrease in the light-sensitive cGMP activated conductance (g_{hv} in figure 5.4), thereby reducing the circulating current. In cones, although the phototransduction mechanism remains that of the rods, rhodopsin is not located on the membranous discs but in sacs formed from the surface membrane (Matthews, 1990).

5.1.3. Nutritional effects on the development of retinal photoreceptors

Most fish larvae have pure cone retina at the time of first feeding, including New Zealand snapper (Pankhurst, 1994), herring (Bell and Dick, 1993), sole (Sandy and Blaxter, 1980), plaice (Neave, 1984), and halibut (Kvenseth *et al.*, 1993; 1996). During metamorphosis, the single cones fuse to form twin cones, and rods are recruited as a pre-adaptation to life at greater depths (Kawamura *et al.*, 1984b; Kitamura, 1990). Indeed, the decrease in the relative abundance of single cones has been shown as an adaptation to dim light environments (Ali and Anctil, 1977). Simultaneously, the amounts of phospholipids containing two molecules of DHA in the retina increase (Bell and Dick, 1993).

Indeed, the fish retina in comparison to that of terrestrial mammals is characterised by its higher content of (n-3) highly-unsaturated fatty acids (HUFA) and particularly 22:6 and 20:5 (Tocher and Harvie, 1988; Gülcan *et al.*, 1993; McEvoy *et al.*, in press). This feature was later confirmed and it was revealed that the fish retinal membranes, and particularly those of photoreceptor outer segments, contain the most highly unsaturated phospholipids of all vertebrates tissues (Bell and Tocher, 1989; Koven *et al.*, 1993; Lin

et al., 1994). The DHA appears most important as it is the major component of the phospholipids in trout retina (Bell and Tocher, 1989). Furthermore, it was suggested that DHA is actively synthesised in the RPE from a precursor originating from the shedding of photoreceptors outer segments (Wang and Anderson, 1993). It is speculated that these HUFAs are important for the regulation of localised membrane structure and fluidity in neural tissues (Tocher and Harvie, 1988).

In adult animals, any dietary deficiency in n-3 fatty acids is minimised by the transfer of DHA from the retinal pigment epithelium layer to the photoreceptor (Bazan *et al.*, 1992). In young animals however, such dietary deficiencies may impair the visual acuity as shown in baby monkeys (Neuringer *et al.*, 1984), piglets (Hrboticky *et al.*, 1991), and juvenile herring (Bell *et al.*, 1995b). In the latter, the authors even outlined a linear relationship between the recruitment of rods in the retina and the content of di 22:6(n-3) molecular species of phospholipids. These observations emphasise the importance of the dietary lipids for the development of larval fish neural tissues.

Although other retinal pathologies exist (Nasisse *et al.*, 1989), of all the eye pathologies known in fish to have a nutritional basis, only deficiencies in vitamins A (Hughes, 1985; Acott and Weleber, 1995) or C (Collins *et al.*, 1993) affect the retina. All the other pathologies result in a clouding of the lens or the cornea. While vitamin A plays a metabolic role in the formation of the visual pigment, vitamin C acts as a protective agent against photic injury.

5.1.4. Objectives

The transformation of the light stimulus into a nervous response influences and/or regulates the pituitary secretion of some hormones such as the melanocyte-stimulating hormone (Kanazawa, 1993). Any defects in the retinal function could alter the stimulus pathway and perturb the physiological status of the larvae affecting their development. Furthermore, the nutritional quality of diets and more particularly the PUFA content of diets have been associated with disfunction of the retina.

The first goal of this study was to describe the structure and ultrastructure of the retina of first-feeding halibut larvae. Secondly, the effects on the retina of a nutritionally deficient diet, *i.e. Artemia*, versus a nutritionally complete diet, *i.e.* copepods, and its functionality were investigated. In addition, eye samples were analysed for their lipid composition by Drs. J.G. Bell and L.A. McEvoy at the NERC Unit of Aquatic Biochemistry at the University of Stirling.

5.2. Materials and Methods

5.2.1. Rod and cone counts

Microscopical observations of the outer nuclear and the photoreceptor cell layers of the retina allowed the calculation of the rod:cone ratio. A zone of the retina was selected, the ventro-temporal region. All the counts were performed within that region as there may be marked variations in the distribution of photoreceptors throughout the retina (Wagner, 1990). Thirty cone outer segments were counted and, for the corresponding region of the nuclear layer, all the nuclei were counted. The number of nuclei exceeding the number of cones was used as an estimate of rods (Kawamura *et al.*, 1989).

5.2.2. Ultrastructural observations

5.2.2.1. Materials

* light microscope (Olympus CH-2)
* fixation: Karnovsky's fixative, cacodylate, 1% osmium tetroxide
* dehydration and impregnation: Acetone, Epoxy-resin (Araldite CY212), hardener (DDSA and MNA), accelerator (BDMA)
* knife maker (Reichert, Wien - Austria)
* microtome (Ultracut E - Reichert, Wien - Austria)
* grid
* staining:
    - uranyl acetate (20% solution in absolute methanol stored at 4° C)
    - lead citrate (1.33 g lead nitrate and 1.76 g sodium citrate)
* centrifuge (Sanyo - Centraur 2)

* transmission electron microscope (TEM Philips 301, The Netherlands)

5.2.2.2. Methods

The eyes of larvae were removed and placed in a drop of Karnovsky's fixative on a paraffin plate. Using razor blades, the ventro-temporal portion of the retina was dissected under a light microscope. The samples of approximately 1 mm^3 were transferred into a small vials containing Karnovsky's fixative at 4° C using a tooth pick. After 2-4 h, the fixative was pipetted off and replaced with Cacodylate Rinse at 4° C for 12 h. The samples were then fixed in 1% osmium tetroxide in a fume cupboard for 1 h.

Osmium was pipetted into a waste bottle and equal volume of vegetable oil added. Tissues were then treated with a graded series of acetone as follows:

- 60% acetone	10 min.
- 90% acetone	10 min.
- 100% acetone (2 times)	15 min.
- 100% acetone + resin 1:1	1-2 h.
- 100% acetone + resin 1:3	1-2 h.
- pure resin	2-12 h.

In order to facilitate impregnation with resin, the vials containing the tissues were placed at an angle on a rotator.

BEEM capsules were warmed in an oven (at 60° C) for 1 h prior to the embedding in order to remove any moisture, which may have been present. One drop of resin was placed in the bottom of each capsule, the specimens were then added and the capsule

filled with resin. Capsules were placed in the oven at 60° C for 48 h to complete polymerisation.

The resin was prepared in a fume cupboard wearing gloves. Warm araldite and DDSA in separate containers were placed in the oven for 30 min before mixing them in a third, warmer container. The mixture was stirred gently with a wooden spatula before adding MNA and BDMA. The resin was stirred again before being placed in a container on the Howie Resin Stirrer.

All used resin, together with plastic containers, spatulas, syringes and specimens vials were polymerised at 60° C for 48 h before disposal.

Each resin block was removed from its capsules, fixed on the microtome and trimmed using razor blades. Semi-thin sections were then taken and observed using a light microscope to localise the most appropriate portion of the block to be used for ultra-thin sections. The ultra-thin sections (90 nm) were collected, stretched out with chloroform and placed on small grids. All the sections were cut using glass blades prepared with the knife maker.

The grids were stained for 20 min in uranyl acetate, rinsed with distilled water, and stained for 7 min in lead citrate previously centrifuged for 5 min at 3000 rpm. A minimum of 2 h elapsed prior to observations of the grids.

The samples were then observed on a transmission electron microscope and electronmicrographs taken when possible. Indeed, it appeared that the impregnation

procedure should have been slightly adapted for retinal samples. Under the present protocol, the resin did not always penetrate adequately between all the photoreceptor cells. It implies that when exposed to the electron beam, the samples only resisted for a few tens of seconds before breaking. This problem only affected the observations to a limited extend but greatly limited the possibilities for taking electron-micrographs. Indeed, a long set up and exposure time was required. This procedure proved to be incompatible with a lost of the fragile sections. Therefore a limited number of micrographs could be obtain to reflect the observations performed on a much larger number of sections. The magnification used to observe each section is specified individually as it varied greatly according to the structures being observed. The electronmicrographs were processed locally following the normal procedure for black and white prints.

5.2.3. Fatty acid and lipid class analysis

As similar analyses were performed to provide lipid data on the larvae sampled for experiment 3 (see Chapter 7), the description of the method is presented in the Methods of Chapter 2 (see section 2.6).

5.3. Results

5.3.1. Recruitment of rods

Twelve larvae from experiment 2 were sampled (after 63 days PFF), 6 had been fed copepods and 6 *Artemia*. From histological observations of these larvae, nuclei and cone outer segment counts were performed. For each retina observed, three counts were taken in the ventro-temporal region. The mean of these counts and the calculated rod/cone ratios are presented in Table 5.1. These results show a highly significant ($p = 0.004$) difference in the number of rods having been recruited in the retina of larvae fed a copepod or *Artemia*-based diet. Indeed, the rod/cone ratios show that the number of rods per cone in the retina of copepod-fed larvae was almost double that of their *Artemia* fed counterparts.

As the feeding experiment (experiment 2) was not originally designed to provide material for rod/cone ratio calculation, only a limited number of counts were performed on younger larvae (days 35 and 49 PFF). Due to the lack of replication, these counts are not presented here, but it can be noted that at the onset of the experiment, all the larvae sampled displayed pure cone retinas.

Table 5.1: photoreceptor counts performed on the ventro-temporal region of the retina of larvae (aged 63 days PFF) fed a copepod or Artemia*-based diet. The number of rods was estimated from the difference between the number of cone outer segments and the number of nuclei. Each value presented in the table is the mean of triplicate counts.*

***Artemia*-fed larvae**			**Copepod-fed larvae**		
Cones	**Rods**	**Rod/cone**	**Cones**	**Rods**	**Rod/cone**
30	59	1.98	30	82	2.74
30	55	1.84	30	62	2.07
30	42	1.39	30	49	1.63
30	35	1.18	30	80	2.68
30	12	0.39	30	70	2.34
30	34	1.12	30	111	3.70
average	40	**1.32**	average	76	**2.53**
st. dev.	17	0.57	st. dev.	21	0.71

5.3.2. Ultrastructure of the photoreceptors and the retinal pigment epithelium

Common features of all the retinas observed were the pigment granules of the long processes of the retinal pigment epithelium (RPE) isolating the photoreceptor outer segments. Furthermore, myeloid bodies (MBs) were observed near the nucleus of the RPE cells in the retinas of both groups of larvae (the *Artemia*-fed and the copepod-fed group) (Plates 5.1 and 5.2). Other features observed are suggested to relate to the dietary treatment of the larvae.

The *Artemia*-fed larvae were characterised by irregular stacking of the outer segment membranes. Indeed, there were plenty of gaps in between the adjacent membranes as shown on plates 5.3 and 5.4. The retina of the larvae from this group was also characterised by the presence of numerous phagosomes in the RPE. Although, the phagosomes were present in the whole RPE cells, they were observed in larger numbers near the tip of the photoreceptors outer segments (Plates 5.3 and 5.4). At very high magnification, they were seen to contain membranes (Plate 5.5).

The retina of the copepod-fed larvae was characterised by a better stacking of the photoreceptors outer segment membranes. Although, some small gaps were seen between adjacent membranes the overall parallelism of the membranes was better than that of the photoreceptor outer segment of their *Artemia*-fed counterparts (Plate 5.6). The number of phagosomes observed near the tip of the outer segment and in the rest of the RPE cells was lower. However, the main feature characterising the retina of these larvae was the presence of numerous lipid droplets within the RPE. These were

Plate 5.1: electronmicrograph of the RPE of an larva fed enriched-*Artemia only showing its nucleus (N) and myeloid bodies (MB).*

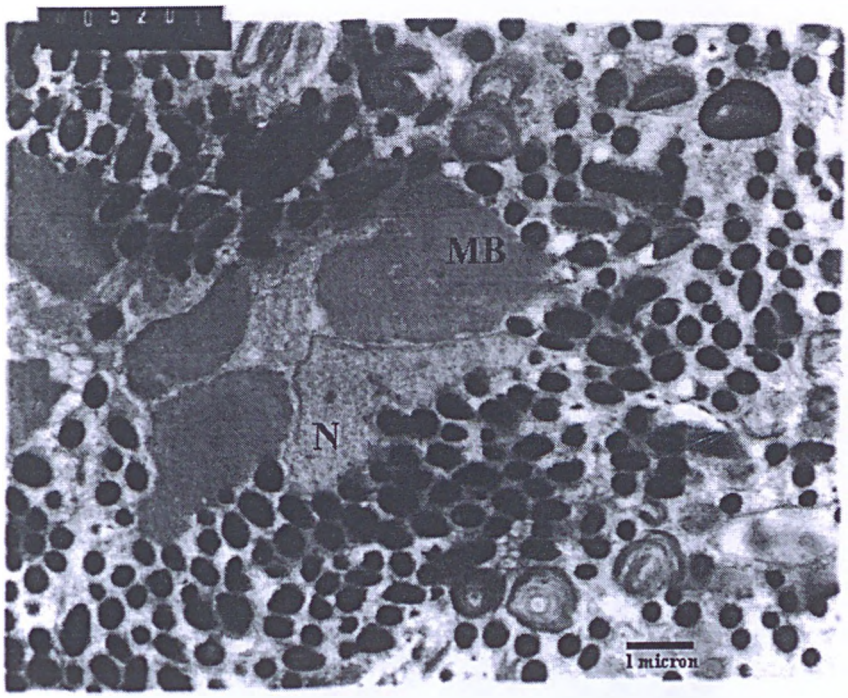


*Plate 5.2: electronmicrograph of the RPE of a larva fed a copepod-based diet supplemented with enriched-*Artemia *showing its nucleus (N), its MBs and lipid droplets (L).*

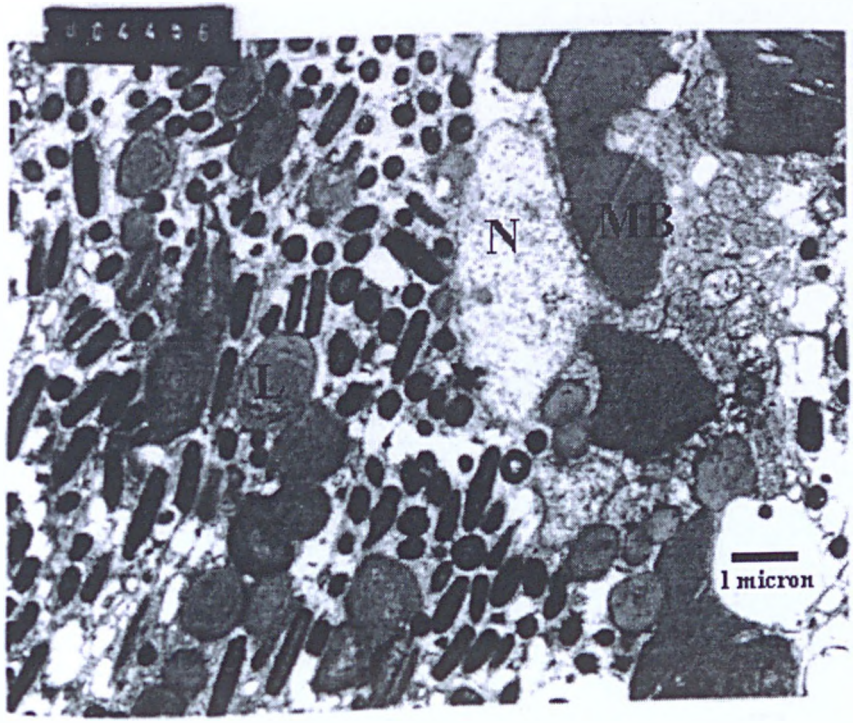


*Plate 5.3: electronmicrograph of the retina of an larva fed enriched-*Artemia *only showing its photoreceptor outer segments (OS) and the RPE's phagosomes (Ph).*

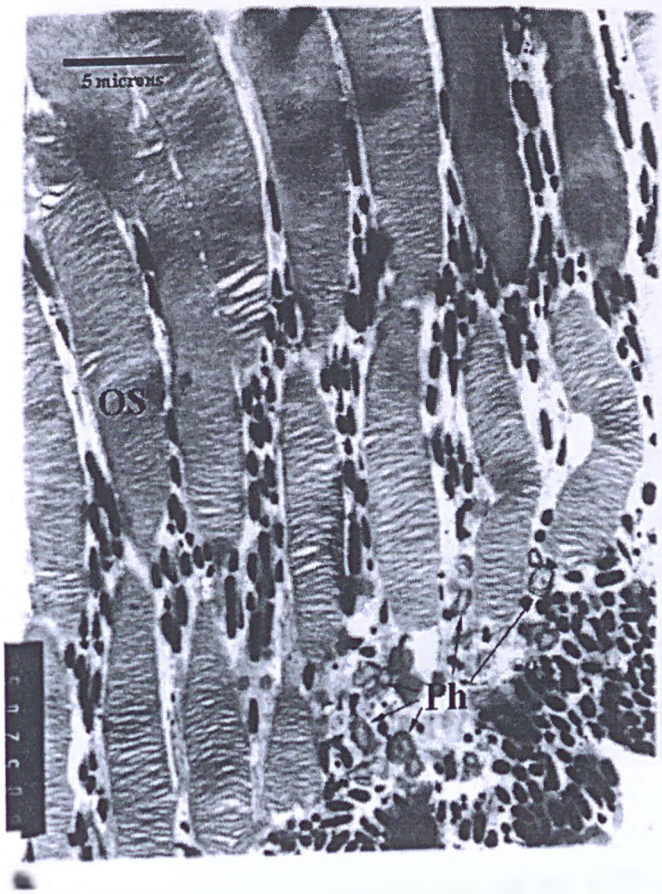


*Plate 5.4: electronmicrograph of the photoreceptor outer segments (OS) of a larva fed enriched-*Artemia *only showing the membrane stacking and the presence of phagosomes (Ph).*

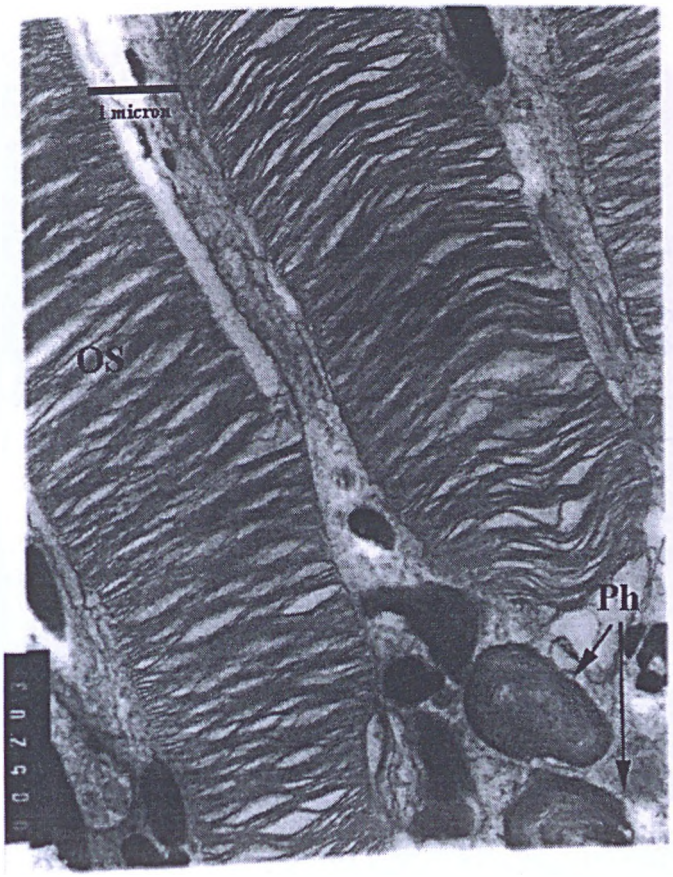


*Plate 5.5: electronmicrograph of a phagosome of the RPE of a larva fed enriched-*Artemia *only.*

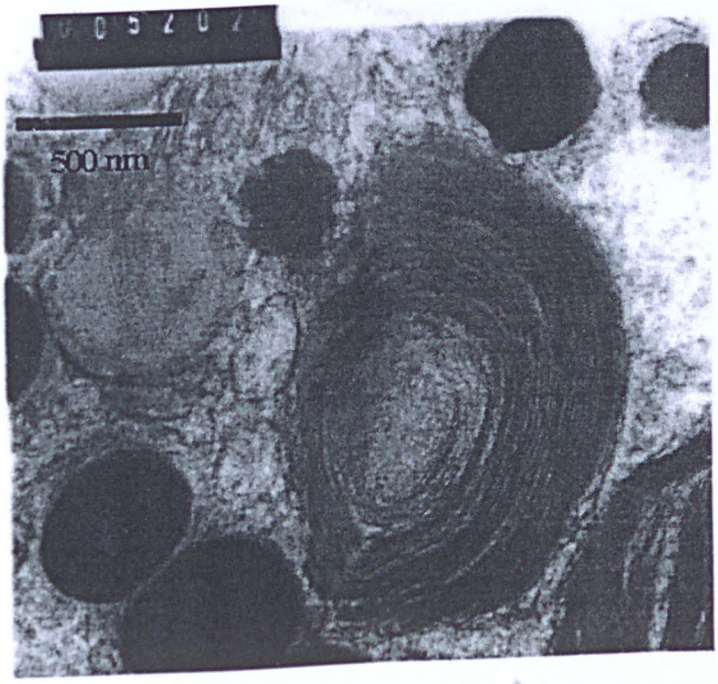
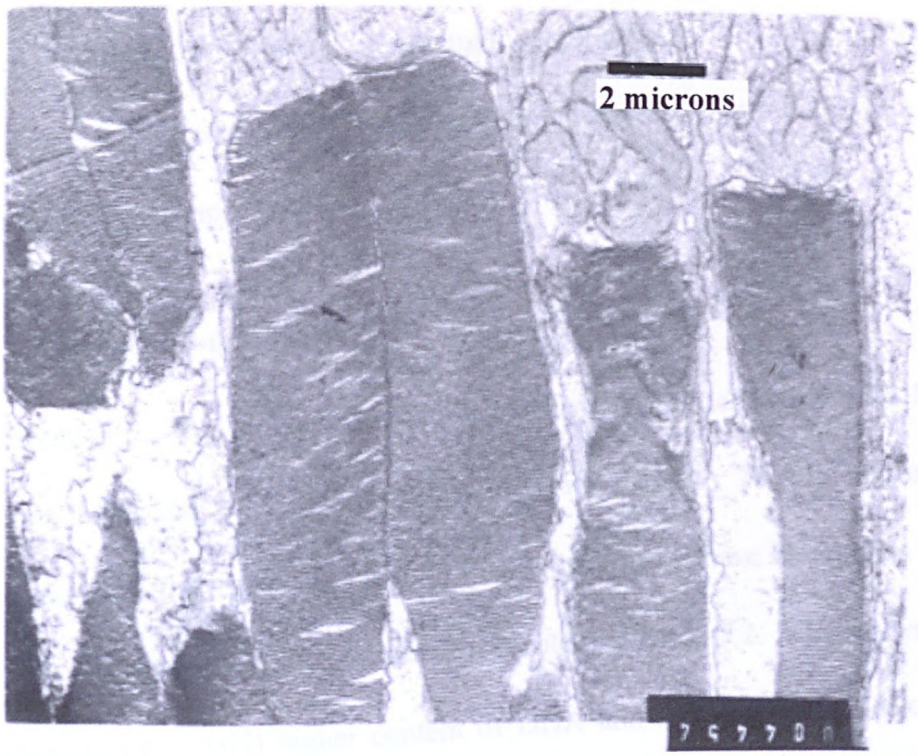


*Plate 5.6: electronmicrograph of the photoreceptor outer segments of a larva fed a copepod-based diet supplemented with enriched-*Artemia *showing the membrane stacking.*



observed equally distributed within the RPE processes, between the photoreceptors outer segment, and in the cytoplasm of the RPE cells (Plate 5.7). At magnifications lower than 13000, they appeared similar in colour to the MBs. Only their round shape allowed their distinction. When increasing the magnification, the myeloid bodies could easily be distinguished as they were filled with membranes (Plate 5.8) while the content of the lipid droplets appeared homogenous.

5.3.3. Fatty acid composition of the eyes

In experiment 2, the larvae fed *Artemia* received nauplii enriched with Super Selco (morning feed) and with Algamac 2000 (afternoon feed). The other group received copepods. The fatty acid composition of the eyes from these larvae was analysed by Drs. J.G. Bell and L.A. McEvoy and is presented in Table 5.2. The analysis showed a significantly ($P < 0.05$) higher content of DHA and a significantly ($P < 0.05$) lower content of EPA and AA in the eye of larvae fed copepods. This resulted in a DHA/EPA ratio twice as high for these larvae while there was no difference in the EPA/AA ratio. However, the larvae fed enriched-*Artemia* only showed increased levels of 22:5n-6 and 22:5n-3. These larvae also had larger amounts of short chain fatty acids such as 18:3n-3.

*Plate 5.7: electronmicrograph of the RPE of a larva fed a copepod-based diet supplemented with enriched-*Artemia *showing its MBs and the wide spread distribution of the lipid droplets (LD) surrounding the outer segment of the photoreceptors (OS).*

*Plate 5.8: electronmicrograph of a MB of a larva fed enriched-*Artemia *only showing its membranous structure.*

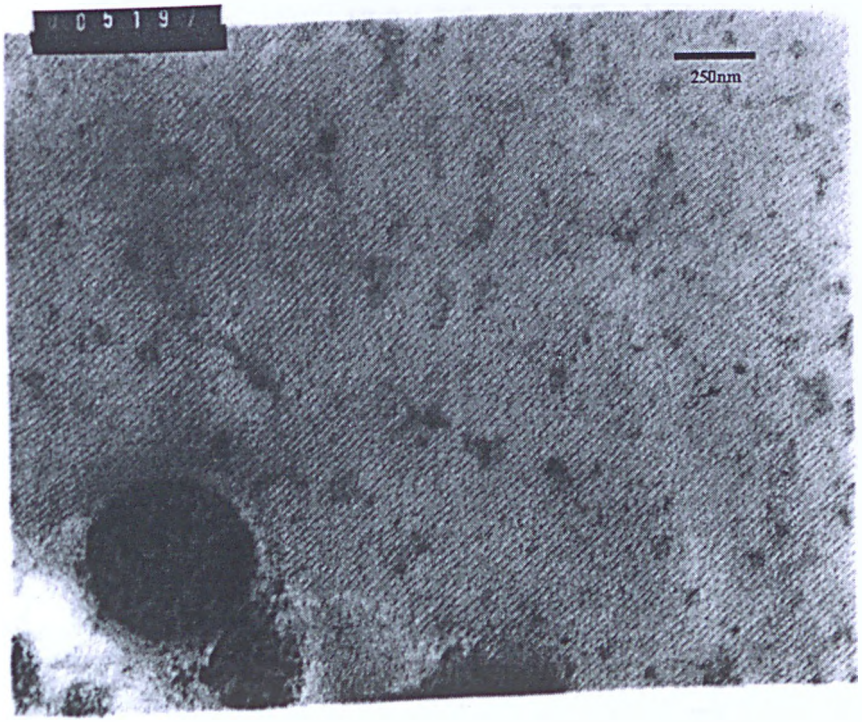


Table 5.2: fatty acid compositions of total lipid from eyes *of larvae fed either* Artemia *enriched with Super Selco and Algamac 2000 only or* Eurytemora velox *copepods supplemented by enriched-*Artemia. *Values are in weight % of total fatty acids. The analyses were performed at the NERC Unit of Aquatic Biochemistry of the University of Stirling by Drs. J.G. Bell and L. McEvoy.*

Fatty acid/ Sample	*Artemia*-fed eyes	*E. velox*-fed eyes
14:0	0.9 ± 0.1	0.8 ± 0.1
16:0	16.2 ± 1.0	18.0 ± 0.3
18:0	10.5 ± 0.4	9.8 ± 0.5
Total saturates[1]	28.0 ± 1.2	28.9 ± 0.1
16:1n-7	3.9 ± 0.3	8.2 ± 1.1
18:1n-9	12.5 ± 1.3	9.8 ± 0.6
18:1n-7	7.2 ± 0.5	4.2 ± 0.5
20:1n-9	0.6 ± 0.1	0.4 ± 0.0
24:1	0.5 ± 0.1	1.0 ± 0.1
Total monoenes[2]	24.9 ± 1.9	23.7 ± 1.8
18:2n-6	2.1 ± 0.1	1.0 ± 0.2
20:2n-6	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	3.1 ± 0.1	2.7 ± 0.1
22:5n-6	2.5 ± 0.3	0.5 ± 0.1
Total n-6[3]	8.1 ± 0.2	4.3 ± 0.1
18:3n-3	4.5 ± 0.3	0.4 ± 0.1
18:4n-3	0.3 ± 0.1	0.2 ± 0.1
20:3n-3	1.1 ± 0.1	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.2 ± 0.1
20:5n-3	9.0 ± 0.6	7.0 ± 0.4
22:5n-3	2.9 ± 0.2	1.5 0.1
22:6n-3	18.0 ± 3.5	30.9 ± 1.9
Total n-3	35.9 ± 4.2	40.1 ± 1.6
Total PUFA	44.0 ± 4.5	44.4 ± 1.4
DHA/EPA	2.0 ± 0.2	4.4 ± 0.4
EPA/AA	2.9 ± 0.1	2.7 ± 0.2

[1]includes 15:0 and 20:0. [2]included 20:1n-11, 20:1n-7, 22:1n-9 and 22-1n-11. [3]includes 18:3n-6 and 20:3n-6

5.4. Discussion

The most striking difference in the structure of the retina of metamorphosed copepod-fed larvae compared to *Artemia*-fed larvae was the increased number of rods. These results were obtained using a histological technique based on the estimation of rod numbers, by comparing the number of cone outer segments and the number of nuclei of a specific region of the retina. This technique was preferred to that described by Blaxter and Jones (1967) based on the observation of the nuclei. Indeed, the rod nuclei are smaller and stain more darkly with nuclear stains and often present processes pointing towards the external limiting membrane. However, the distinction between rod and cone nuclei remained difficult.

Despite the low light intensity of its natural environment, halibut do not develop a *tapetum* as an adaptation to dim light as shown in other species (Zyznar and Ali, 1974; Ali and Anctil, 1977; Mani-Ponset *et al.*, 1993). The main adaptive feature associated with the transition from pelagic to demersal life style in metamorphosing halibut larvae is the recruitment of rods, adding to the potentially detrimental effects associated with the use of *Artemia* as prey organisms.

In response to high light intensities there are sclerad movements of rods (*e.g.*. away from the centre of the retina); this is considered to be a light adaptation, protecting the rods from damage by bright light. Similarly, there are vitread movements of rods (*e.g.* towards the centre of the retina) when the light intensity is low, exposing a larger number of rods (Neave, 1984). As the larvae were kept under constant light in the

present work, it is assumed that the number of rods was underestimated. Further support for the suggestion that the number of rods was underestimated comes from the findings that in flounder the rods appear later in cultured than in wild larvae (Kawamura *et al.*, 1989). However, the light regime applied to the different groups was identical, so the validity of the comparison between *Artemia*-fed and copepod-fed larvae remains.

Different hypotheses could explain the differences in rod recruitment. Indeed, the reduced numbers of photoreceptors and especially the rods can be associated with prolonged photoperiod (Nasisse *et al.*, 1989; Rozanowska *et al.*, 1995). However, other symptoms of degenerative retinopathy associated with the exposure to constant light include the proliferation of pigments and the hypertrophy of the RPE. These symptoms were not observed. The present observations can therefore be attributed to differences in rod recruitment rather than retinopathy.

The depleted rod recruitment in *Artemia*-fed larvae could therefore be linked to the nutritional quality of *Artemia*. It has been shown, as summarised in the introduction (section 4.1), that the appearance of rods occurs in parallel with an increase in HUFA content of the retina (Bell and Dick, 1993). Dietary deficiencies in these fatty acids are responsible for retinal alterations and the replacement of DHA by other molecular species in the rod outer segment membranes and results in impaired vision (Bell *et al.*, 1995b). Indeed, the fatty acid composition of the eyes of copepod-fed larvae showed a significantly larger % weight of DHA than their *Artemia*-fed counterparts resulting in a DHA/EPA ratio higher by two fold. In addition, it appears that larvae fed enriched-*Artemia* only tried to compensate for the lack of DHA by incorporating 22:5n-3 and 22:5n-6 as alternative PUFA as observed in a study by Bell *et al.* (1995b). These results

suggest that feeding halibut larvae with *Artemia* does not meet the larval nutritional requirement for HUFA and most particularly DHA. This situation may be reversed provided the juvenile fish received a more appropriate feed. Indeed, it has been shown that adult teleosts continue to produce new rods (Mack and Fernald, 1995).

In the present work, electron microscopical studies have helped to understand the developmental problems associated with the use of *Artemia* as prey organisms.

The larger number of phagosomes observed in the RPE of *Artemia*-fed larvae suggest a more active uptake of outer segment membranes. Indeed, the phagosomes have long been shown to actively participate in the process of membrane renewal (Young and Bok, 1969). Under a normal photoperiod regime, with the alternation of day and night, this process is governed by the daily cycles (Young, 1977). The cone outer segments appear to be recycled during night-time, when they are less needed, while the rods outer segment membrane renewal is delayed by half of a cycle. In constant darkness, the interactions between the photoreceptor outer segments and the RPE are absent in Antarctic fish (Tokumaru-Phan and Massao-Nachi, 1992). By contrast, constant light inhibits outer segment membranes shedding in the leopard frog (Cai and Dickson, 1993). The present results seem to suggest that in larval halibut the process of renewal of outer segment membranes is not governed by light but remains cyclic irrespective of the photoperiod.

The myeloid bodies (MBs) have been described as a special form of smooth endoplasmic reticulum (SER). They are involved in the active conversion and recycling of retinal and HUFA released from the photoreceptors (Yorke and Dickson, 1984;

Wagner, 1990; Abran and Dickson, 1992b; Chen *et al.*, 1992; Braekevelt, 1993; Cai and Dickson, 1993; Dickson and Morrison, 1993; Wang and Anderson, 1993; Braekevelt *et al.*, 1996). Their membranous structure was observed in the present study (Plate 5.8). Although, accurate measurements of their size relative to the RPE cells was not performed, the MBs appeared larger in the RPE of copepod-fed larvae (Plate 5.7). If further investigations were to confirm this suggestion, it would indicate that the requirement for membrane building material was lower in this group. This suggestion is supported by the observations of lower numbers of phagosomes and by the more numerous lipid droplets. Indeed, by storing lipids, these are thought to participate in the membrane renewal process (Yorke and Dickson, 1985).

These differences in the cellular features characterising the RPE of copepod-fed and *Artemia*-fed larvae support the hypothesis stating that the rod recruitment was depleted in the latter by comparison to the former. They suggest that feeding halibut larva with *Artemia* results in a lack of HUFA which are required for the development of the retina. Indeed, the dietary HUFA, absorbed from the chorion capillaries by the RPE, passed through the interphotoreceptor matrix and are incorporated into the membranes of the photoreceptors before being actively recycled (Bazan *et al.*, 1992).

A consequence of this deficiency was highlighted by the observation of the photoreceptor outer segments. The stacking of their membranes appeared more irregular in the retina of the *Artemia*-fed group. The dark current transmitting the photoresponse across the outer segment membranes from the stimulated rhodopsin is dependent on the stability and the structure of these membranes (Matthews, 1990). The metabolic and physical properties of different molecular species of lipids forming the

membranes not only influence the membrane fluidity, but also the function and activity of proteins that are bound to them (Lin *et al.*, 1994). Despite the fact that Brown (1994) did not associate the functionality of the photosentive pigment complex and the transmission of the light stimulus to a single lipid species, this author showed that there is indeed a relation between two and that the composition of the latter affects the functionality of the former. In fact, knowing that the visual pigment, rhodopsin is an integral membrane protein (Matthews, 1990), it seems implicit that the structure of the membrane and the lipid molecular species from which it is constituted are or major importance in the visual process.

In summary, the present findings indicate that the deficiencies in HUFA associated with feeding *Artemia* may be responsible for the impaired vision and poor transmission of light to the CNS of the larval halibut. This could lead, in culture conditions, to reductions in feed intake and the disruption of the light-regulated pituitary functions such as MSH secretion (Kanazawa, 1993). Consequently, two working hypothesis were established for further research: (1) the performances of halibut larvae could be improved by increasing the HUFA content of *Artemia* (see Chapter 7), and (2) an increase in the stimulation of rhodopsin could compensate for the disrupted light-regulated pituitary functions and therefore enhance the pigmentation (see Chapter 8).

Chapter 6. Role of thyroid hormones during larval development

6.1. Introduction

The effects of thyroid hormones differ during the life of fish. Nevertheless they have been closely associated with the metamorphosis of several species (see section 6.1.2 for details). To date little data is available on their role in halibut larval development except for a recent study by Solbakken and co-authors (1997). More information on that subject would be of great interest to help understand the processes involved during the metamorphic threshold. The following sub-section aims to summarise the literature available on this issue. A review of the metabolism of these hormones is followed by a description of their role throughout the life of fish. A survey of the quantitative and qualitative techniques available to express thyroid activity is briefly described prior to the description of the objectives.

6.1.1. Thyroid hormone metabolism

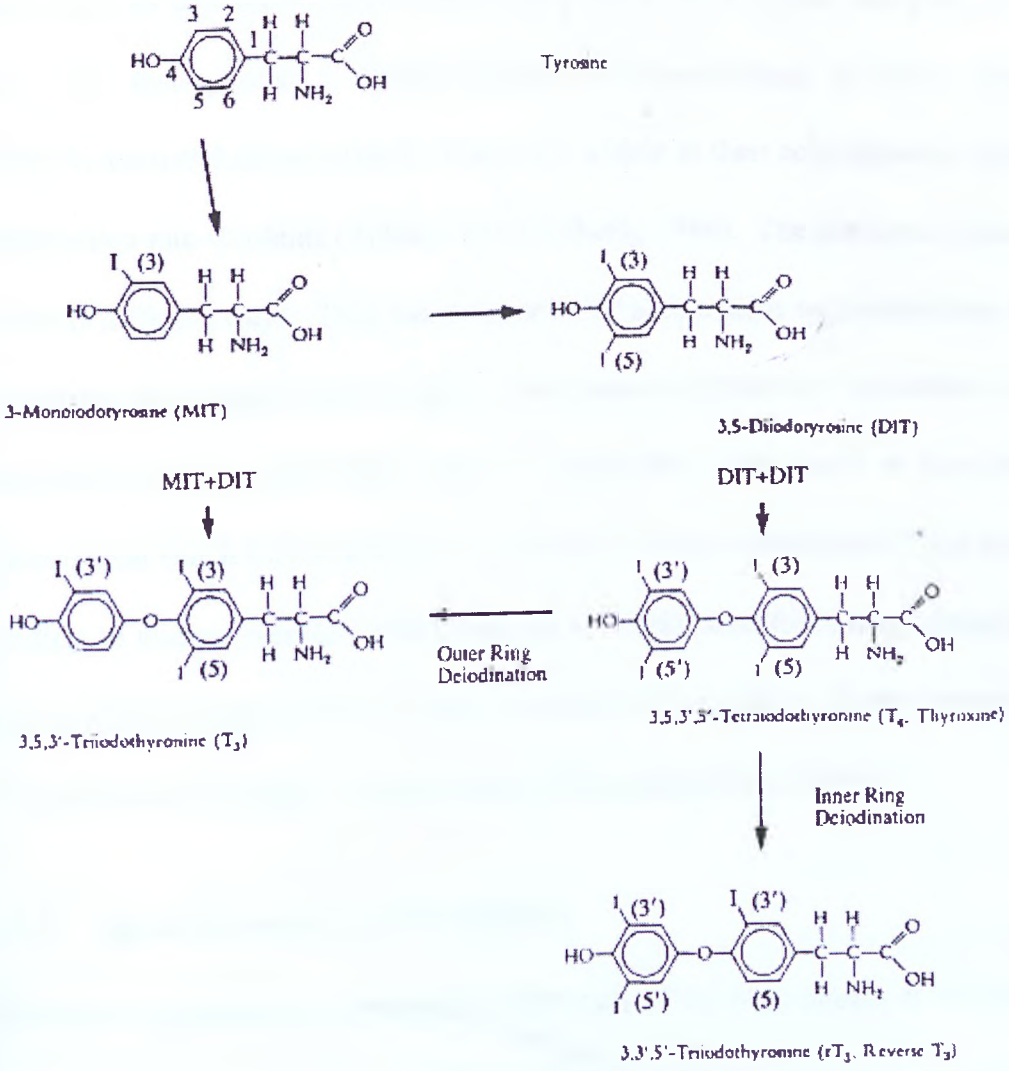
In adult fish, the effects of thyroid hormones may vary from tissue to tissue (Shambaugh III, 1986), however, some general trends can be observed. Firstly, seasonal variations of thyroid activity have been described in several species (Youngson *et al.*, 1986). Their concomitant changes with ambient temperature (Tanangonan *et al.*, 1989; Leatherland *et al.*, 1990a), photoperiod (Cyr *et al.*, 1988), pH (Fok *et al.*, 1990), salinity (Specker and Richman III, 1984; Parker and Specker, 1990), water velocity (Youngson and McLay, 1989), and rearing density (Sower and Fawcett, 1991) have been established. Nevertheless, the complexity of the interactions between these environmental and physiological factors does not allow any direct relationship with thyroid involvement to be isolated (Eales and Fletcher, 1982; Leatherland, 1982; Sukumar *et al.*, 1997).

The interactions of thyroid hormone activity in relation to nutritional factors have also been envisaged. In such studies, Eales (1988) found similarities between various vertebrates. The response to starvation of mammals, birds and salmonids showed some evidence for altered production of thyroxine (T4) at least partially explained by regulation of hepatic T4 5'monodeiodinase (Himick and Eales, 1990; Farbridge and Leatherland, 1992; Farbridge *et al.*, 1992). The level of carbohydrates in the diet and the availability of glucose determined postprandial elevation in plasma T4 in rainbow trout (Himick *et al.*, 1991).

These few examples point out to the diversity of the interactions between the thyroid hormones and the environment and emphasise the care required in monitoring and standardising rearing conditions when studying these hormones. Similar diversity of interactions overshadows the understanding of the effects of thyroid hormones with different physiological events during the fish's development.

The unit of thyroid secretion is the follicle. It produces the glycoprotein matrix on which the hormones are synthesised. The thyroglobulin, a large globular glycoprotein provides the tyrosine residues from which the active hormones, thyroxine (T4) and 3,5,3'-triiodothyronine (T3), originate (Halmi, 1986; Norris, 1997) (Figure 6.1). Thyroxine is deiodinated to form T3 under the control of various hormones including growth hormone and prolactin (de Luze and Leloup, 1984). Meanwhile, cortisol appears to have a role linked with the clearance of T3 rather than its conversion into T4 (Brown *et al.*, 1991). Monodeiodination can also take place in peripheral tissues such as the liver (Flett and Leatherland; 1989; Leatherland *et al.*, 1990c). The hypothalamic

Figure 6.1: Synthesis of thyroid hormones and precursors (copied from Norris, 1997).



control of thyroid stimulating hormone (TSH) secretion in teleosts is thought to be inhibitory rather than stimulatory as in higher vertebrates (Sage and Bromage, 1970; Leatherland, 1982; Gorbman, 1986; Sukumar *et al.*, 1997). In humans, thyroid hormones are delivered to their target organs via a set of plasma transport proteins, as they are hydrophobic. These include thyroxine-binding globulin, prealbumin (transthyretin) and albumin itself. They vary widely in their concentration, affinity and dissociation rate constants (Robbins and Edelhoch, 1986). The hormones affect tissues in many different ways. They assist transport of amino acids and electrolytes from the external environment to the cell matrix; they promote synthesis or activation of specific enzymes within the cell; they enhance intracellular events such as translation and transcription which lead to changes in cell size, function and number. They proceed by binding to nuclear receptors (Bres and Eales, 1988), therefore acting directly on the genome (Shambaugh III, 1986). They also modulate the actions of other hormones such as corticosteroids through selective receptor interactions (Lam, 1994).

6.1.2. Thyroid hormone and development

The thyroid hormones play an active role through all the main phases of the life of fish. They were first shown to be associated with the periods of fast growth and changes in the biochemical constitution of the tissues in Atlantic salmon (Hoar, 1939). Thyroxine was later shown or suggested as a regulator of the ovarian development by increasing its sensitivity to gonadotropic stimulation in goldfish (Hulburt, 1977), trout (Pickering and Christie, 1981; Cyr and Eales, 1989), and brown bullhead (Burke and Leatherland, 1983).

Although, a common mechanism of action for thyroid hormones in either the embryos or the adults has not been identified, a role for the thyroid hormones during early development is beyond doubt. In monkey, the treatment of pre-natal hypothyroidism with a thyroid hormone analogue helped prevent developmental retardation of several structures including brain, lung and skeletal system (Shambaugh III, 1986). Indeed, the T3 has been shown to play a role in osteoblastic differentiation (Fratzl-Zelman *et al.*, 1997). Similarly, in a study of the thyroid hormones in early development of teleost fish, Brown and Bern (1989) suggested a role of maternal thyroid hormones in the initial formation of the central nervous system in fish embryos.

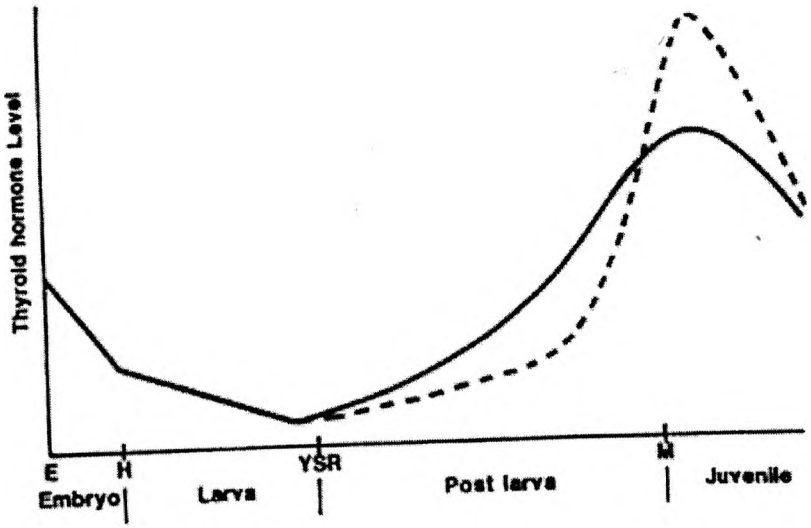
In vivo, T3 and T4 present in maternal circulation are transferred into the oocytes and subsequently into the larvae (Brown *et al.*, 1988; Brown and Bern, 1989). This allowed researchers to improve larval development following maternal (Brown *et al.*, 1989; Ayson and Lam, 1993) or embryonic thyroid hormone treatment (Hey *et al.*, 1996). In an attempt to improve the understanding of the hatching process and its regulation, Reddy and Lam (1991) conducted experiments on tilapia. Their results led to the suggestion that thyroid hormones promote the development of the embryo while inhibiting hatching. As observed in several species, T3 and T4 concentrations in the yolk decrease before hatching (Tagawa *et al.*, 1990b; de Jesus, 1994; Tay *et al.*, 1995). This decline at the end of the embryogenesis corresponds to an increase in prolactin secretion, which would overcome the inhibiting effect of T3 and T4 to stimulate hatching (Reddy and Lam, 1991).

Studies on other species showed that the levels of thyroid hormones decrease to lower values, not before, but after hatch. It suggests a transfer from the yolk to the larval circulation (Brown *et al.*, 1987; Tagawa and Hirano, 1987; Leatherland *et al.*, 1989a, Tagawa and Hirano, 1990; de Jesus *et al.*, 1991). In addition, maternal hormones are not the sole source in larvae of some species. The thyroid follicles and TSH producing cells, the thyrotrophs, seemed active from a week prior to hatching in salmon (Leatherland and Lin, 1975; Greenblatt *et al.*, 1989), one day after hatching in sea bass (Cambré *et al.*, 1990) and after 3 weeks in striped bass (Brown *et al.*, 1987). However, an embryonic control of the thyroid hormones transfer from the yolk to the plasma has been suggested (Leatherland *et al.*, 1989b; Tagawa *et al.*, 1990a). It would, according to these authors, undermine the importance of the hormone content in the yolk over required limits. Furthermore, in medaka, 90% of the hormones contained in the eggs appear not to be essential for early development (Tagawa and Hirano, 1991).

Independently of species differences in levels of thyroid hormones during embryonic life, there seems to be a surge at the end of the larval development (Figure 6.2). It was associated with the emergence of salmon larvae (Kobuke *et al.*, 1987; de Jesus and Hirano, 1992), and with the metamorphosis of flounder (Miwa and Inui, 1987; Inui *et al.*, 1989), milkfish (de Jesus, 1994) and greasy grouper larvae (Tay *et al.*, 1994).

At later stages of development, the thyroid hormones seem to be related with migratory behaviour. Indeed, there is a variation in activity of the thyroid hormones during the salmon's downstream migration (Dickhoff *et al.*, 1978; Youngson, 1989) and during the lamprey's upstream migration (Leatherland *et al.*, 1990b). However, migration is often

Figure 6.2: General pattern of change in the thyroid hormone level during development in fish. The plain line may represent either T3 or T4 for species having a gradual transformation while the dotted line may represent T3 or T4 level for species with metamorphic climax. E stands for fertilisation; H stands for hatching; YSR stands for yolksac resorbtion and M stands for metamorphosis or other marked changes in morphology, physiology, behaviour, food habit or habitat. (copied from Lam, 1994).



associated with physiological changes such as smoltification and spawning in the above mentioned examples. A clear understanding of the interactions between thyroid hormones and the physiological changes and/or the migratory behaviour has not been achieved to our knowledge although a relation between water velocity and thyroid activity has been suggested (Youngson and McLay, 1989).

6.1.3. Quantification of thyroid hormone

Initially, thyroid levels in plasma samples were assessed using methods based on protein-bound iodide. The precision of these methods was questioned by Higgs and Eales (1973) who pointed out that virtually all iodothyronines were protein-bound and that the influence of high plasma levels of iodide were not accounted for. They proposed a new, more specific, method based on saturation or competitive binding analysis, which gave lower values. This method was later improved to measure both T4 and T3 by radioimmunoassay (Brown and Eales, 1977).

Due to the low levels of hormones associated with periods of thyroid inactivity in fish, the search for more precise methods continued. The use of chromatographic methods was progressively favoured (Osborn *et al.*, 1978; Hearn and Hancock, 1979; Gordon *et al.*, 1982) due to their accuracy in detecting thyroid hormones and related metabolites. Later, combined methods using chromatographic columns for the separation of the compounds and radioimmunoassay for their extraction allowed Omeljaniuk *et al.*, (1984) to measure simultaneously T3 and T4 in trout plasma with high precision.

Today, measurements of T3 and T4 are most commonly performed using radioimmunoassay although other methods are also used (Larsen, 1986; de Jesus *et al.*, 1991; de Jesus and Hirano1992; Faustino and Power, 1997).

6.1.4. Thyroid follicles

Unlike higher vertebrates such as mammals and birds, adult cyclostome and most teleost fish do not have an organised thyroid gland. Instead, they are characterised by scattered follicles located mainly in the subpharyngeal connective tissue in proximity of the ventral aorta and its principal branches into the gills (Gorbman, 1986). The structure of the follicles is very similar in all vertebrates. They are formed by a single layer of secretory epithelium, the lumen of which contains a viscous fluid, the colloid. They have been reported to appear prior to hatching (Hoar, 1939; Wabuke-Bunoti, 1983) and their number increases during the pre-metamorphic stages (Miwa and Inui, 1987).

The epithelial cells forming the follicle vary in size depending on the intensity of the stimulus from the TSH. When active, they turn from squamous to tall columnar cells (Halmi, 1986). In the meantime, the colloid which contains the thyroglobulin and from which the active hormones are synthesised is used up (Norris, 1997). This correlation between the activity of the follicles and their histological appearance has enabled some authors to use cellular clues to reflect the thyroid activity (Miwa and Inui, 1987; Nishioka *et al.*, 1987). Similarly observations of the histology of the pituitary and most particularly of the TSH producing cells allows similar conclusions to be drawn concerning thyroid activity (Narayan *et al.*, 1985; Miwa and Inui, 1987).

6.1.5. Objectives

The aims of this preliminary study were firstly to determine whether a surge of thyroid hormones is present during halibut metamorphosis and secondly to establish whether the use of *Artemia* as prey organisms affected thyroid activity.

6.2. Materials and Methods

6.2.1. Background information

The role of the thyroid gland and hormones was investigated in two ways: firstly, thyroid hormone levels in the head and body regions of larval halibut collected from a commercial fish farm (Otter Ferry Salmon Ltd.) were measured by radioimmunoassay. Secondly, thyroid structure was observed in histological sections of larvae collected from experiment 1 and 2 and assessments made of secretory activity.

6.2.2. Larval collection

First feeding larvae, which had been reared on copepods, were sampled from a commercial halibut hatchery, Otter Ferry Salmon Ltd. Duplicate groups of larvae were collected 14, 28, 35 and 42 days PFF from a single batch. The larvae were removed from a rearing tank using a small plastic beaker, transferred directly to pre-weighed and pre-labelled tubes and placed in a portable container filled with liquid nitrogen. The samples were transferred later to a -70° C freezer. All larvae were sampled at the same time of the day (between 13:00 and 14:00) to minimise the effect of daily variations of hormone content (Osborn *et al.*, 1978; Youngson *et al.*, 1986).

The samples were transported in dry ice to the University of Algarve (Faro, Portugal) for assay. They were replaced in a -75° C freezer immediately on arrival. Prior to extraction the larvae were decapitated and the heads and body kept separately. The samples were then weighed using a microbalance and pooled in order to produce 100 mg samples.

6.2.3. Radioimmunoassay

6.2.3.1. Principle

The principle of radioimmunoassay (Odell and Daughaday, 1971) is based on competition for a fixed number of antibody binding sites between a set quantity of added radioactive antigen and a variable quantity of non-radioactive antigen in the samples.

6.2.3.2. Materials

* centrifuge (Heraeus - Varifuge 20RS)
* gamma counter (Pharmacia - Wallac 1470 - Wizard Automatic Gamma Counter)
* vortex mixer
* T3 label (Dupont - Triiodothyronine I-125 - cat. n° NEX-110)
* T4 label (Dupont - Thyroxine I-125 - cat. n° NEX-111)
* T3 and T4 buffer: 0.1M Tris buffer.
* T3 standard buffer: 0.07M barbitol (pH 8.6) + 0.1% BAS/NaN_3 (BSA stands for bovine serum albumin and NaN_3 stands for sodium azide).
* T4 standard buffer: 0.1M Tris + 0.1% BSA/NaN_3.
* solution G: secondary antibody diluted to 1/25 in PBS (pH 7.4) and mixed with 1 volume of 0.1M EDTA (pH 7.8).
* PEG solution: 20% solution of polyethylene glycol.

6.2.3.3. Methods

6.2.3.3.1. Extraction of T3 and T4

Frozen samples were homogenised in 1 volume of ice cold 100% ethanol. The homogenisation was carried out on ice to avoid heating up the samples. The

homogenate was centrifuged at 3000 rpm. for 10 min at 4° C and the supernatant decanted into a clean labelled tube and stored on ice. The pellet was resuspended in 0.5 volume of ice cold 100% ethanol and re-extracted The homogenate was centrifuged at 3000 rpm for 10 min at 4° C, the supernatant removed and combined with the previous supernatant.

The combined supernatants were dried by lyophilisation overnight and reconstituted in 1 volume of 0.1 M Tris buffer. The samples were then frozen (-75° C) until assayed.

6.2.3.3.2. T3 and T4 radioimmunoassay

Each assay required a standard curve. Small vials suitable for radioactive counters were used. They were labelled and filled as presented in Table 6.1 for T3 and Table 6.2 for T4. The samples were treated exactly as the standard except that they were diluted in different buffers (see section 6.2.3.2.). The primary antibody, specific to T3, was diluted to 1/7000 while that of T4 was diluted to 1/3000. The labelled solutions were also diluted (to 1/2000 for T3 and 1/3000 for T4) to give radioactive counts of between 8 and 10 000 cpm. The above listed reagents were added in the given order, mixed thoroughly and incubated overnight at 4° C.

After the incubation, the solution G was added. The vials were mixed thoroughly and the PEG solution was added.

6.2.3.3.3. Separation

The free and bound antigen were separated as follows. Once the secondary antibody and PEG were added, the samples and standards except for the 'total count' tube were centrifuged for 60 min at 3000 rpm at 4° C. The supernatant discarded and each tube

Table 6.1: Detailed procedure used for the T3 radioimmunoassay, listing all the reagents, their order of addition and their volume of addition. All volume are given in µl and it is important to note that the sum of the standards or the samples and the buffer always adds up to 200µl.

Tube label		Standard	Buffer	1° Anti-body	Labelled T3		Sol. G	PEG
1	Total Count	/////	/////	/////	100		/////	/////
2	Non Specific	/////	200	/////	100		200	500
3	Total Binding	/////	100	100	100	I	200	500
4	0.1µg/ml	50	50	100	100	N	200	500
5	0.1µg/ml	25	75	100	100	C	200	500
6	0.1µg/ml	10	90	100	100	U	200	500
7	0.1µg/ml	5	95	100	100	B	200	500
8	0.01µg/ml	50	50	100	100	A	200	500
9	0.01µg/ml	25	75	100	100	T	200	500
10	0.01µg/ml	10	90	100	100	I	200	500
11	0.01µg/ml	5	95	100	100	O	200	500
12	0.001µg/ml	50	50	100	100	N	200	500
13	0.001µg/ml	25	75	100	100		200	500
14	0.001µg/ml	10	90	100	100		200	500
15	0.001µg/ml	5	95	100	100		200	500
16	samples	X	100-X	100	100		200	500

Table 6.2: Detailed procedure used for the T4 radioimmunoassay. The standard curve varies slightly for that of T3.

Tube label		Standard	Buffer	1° Anti-body	Labelled T4		Sol. G	PEG
1	Total Count	/////	/////	/////	100		/////	/////
2	Non Specific	/////	200	/////	100		200	500
3	Total Binding	/////	100	100	100	I	200	500
4	1µg/ml	50	50	100	100	N	200	500
5	1µg/ml	25	75	100	100	C	200	500
6	1µg/ml	10	90	100	100	U	200	500
7	1µg/ml	5	95	100	100	B	200	500
8	0.1µg/ml	50	50	100	100	A	200	500
9	0.1µg/ml	25	75	100	100	T	200	500
10	0.1µg/ml	10	90	100	100	I	200	500
11	0.1µg/ml	5	95	100	100	O	200	500
12	0.01µg/ml	50	50	100	100	N	200	500
13	0.01µg/ml	25	75	100	100		200	500
14	0.01µg/ml	5	95	100	100		200	500
15	0.001µg/ml	25	75	100	100		200	500
16	0.001µg/ml	10	90	100	100		200	500
17	samples	X	100-X	100	100		200	500

paper dried and placed in a gamma counter. The radioactivity of all the tubes, including the 'total count' was then counted.

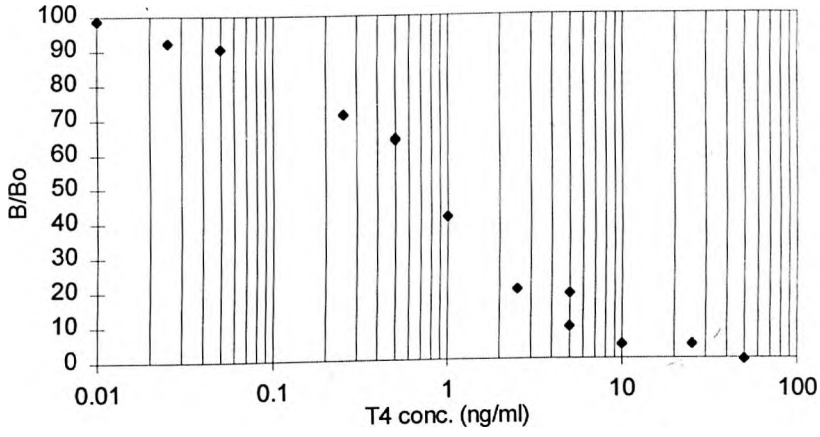
6.2.3.3.4. Calculations

The results obtained in cpm (counts per minute) were converted to the percentage of relative binding (%B/Bo) using the following formula:

$$\%B/Bo = \frac{\text{Sample Counts - Non Specific Counts}}{\text{Total Binding Counts - Non Specific Counts}} \times 100$$

The results were plotted against the standard curve calculated for each assay (see Figure 6.3 for example of standard curve). Both ends of the standard curve deviated from linearity. Those values were not taken into account when calculating the intercept line used to determine the hormone concentration in the samples from the relative binding percentages. For all the samples, only dilutions, which gave relative binding percentage corresponding to the linear section of the standard curve, were used.

Figure 6.3: Example of a T4 standard curve for a radioimmunoassay. The x-axis is presented using a logarithmic scale. B/Bo stands for the percentage of the total binding.



6.3. Results

6.3.1. Radioimmunoassay

The analysis performed on developing larvae fed a copepod-based diet (n = 4) revealed changing levels of T3 and T4 with time. At the start of the sampling period, 14 days PFF, the levels of T3 in the head and body regions were similar, respectively 13.1 ± 3.0 and 14.7 ± 5.5 ng gBW^{-1} (Figure 6.4). The levels of T4 in the head and body regions were also similar although two folds higher than those of T3, respectively 28.8 ± 12.4 and 27.3 ± 5.0 ng gBW^{-1}. The standard deviation was high, especially in the head region (Figure 6.5). By day 28 PFF, only the concentrations of T3 in the head region remained similar (14.4 ± 2.0 ng gBW^{-1}), while the levels of T3 in the body region and both measurements of T4 dropped by two folds reaching 7.7 ± 3.1, 13.0 ± 3.2 and 11.5 ± 3.9 ng gBW^{-1} respectively.

The following sampling date, 35 days PFF, was characterised by a significant ($p<0.05$) surge in both T4 measurements and in T3 levels in the head region. Despite a very large variability as shown by the standard deviation, the mean concentrations of both T3 and T4 reached about 60 ng gBW^{-1} in the head region (respectively, 59.1 ± 48.0 and 61.7 ± 52.3 ng gBW^{-1}). The surge in T3 in the body region was of lowest amplitude with a 3 folds increase in comparison to a 4 fold increase for the other measurements. Meanwhile, the level of T4 in the body region continued to decrease to its lowest level of 8.4 ± 1.1 ng gBW^{-1}.

Figure 6.4: mean concentrations (± standard deviation) of T3 measured in the head and body regions of developing larvae fed a copepod-based diet. The results are expressed as ng of hormone per gram of larval body weight. (n = 4)

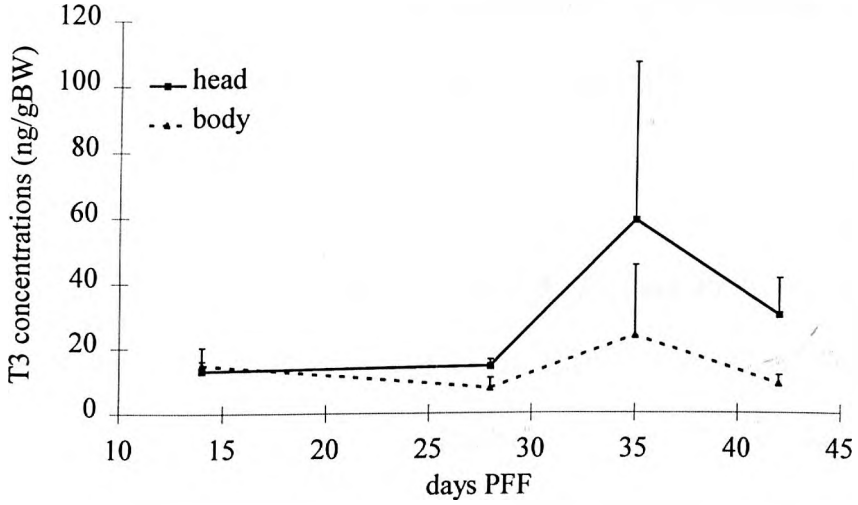
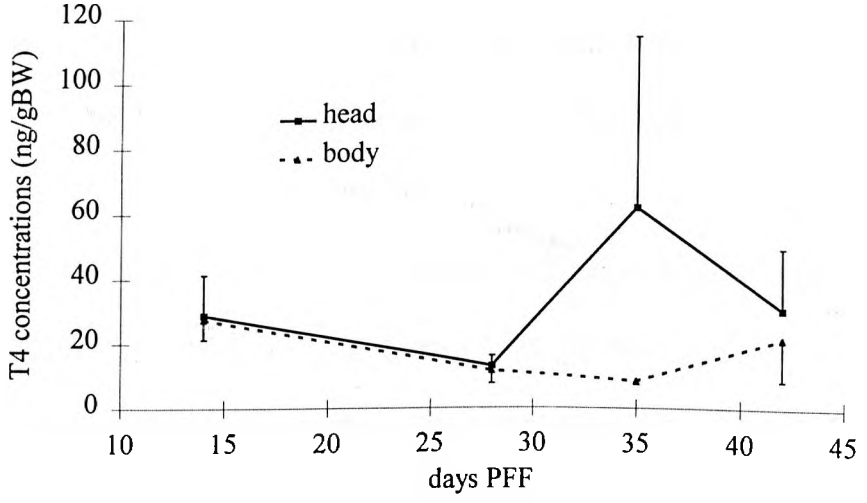


Figure 6.5: mean concentrations (± standard deviation) of T4 measured in the head and body regions of developing larvae fed a copepod-based diet. The results are expressed as ng of hormone per gram of larval body weight. (n = 4)



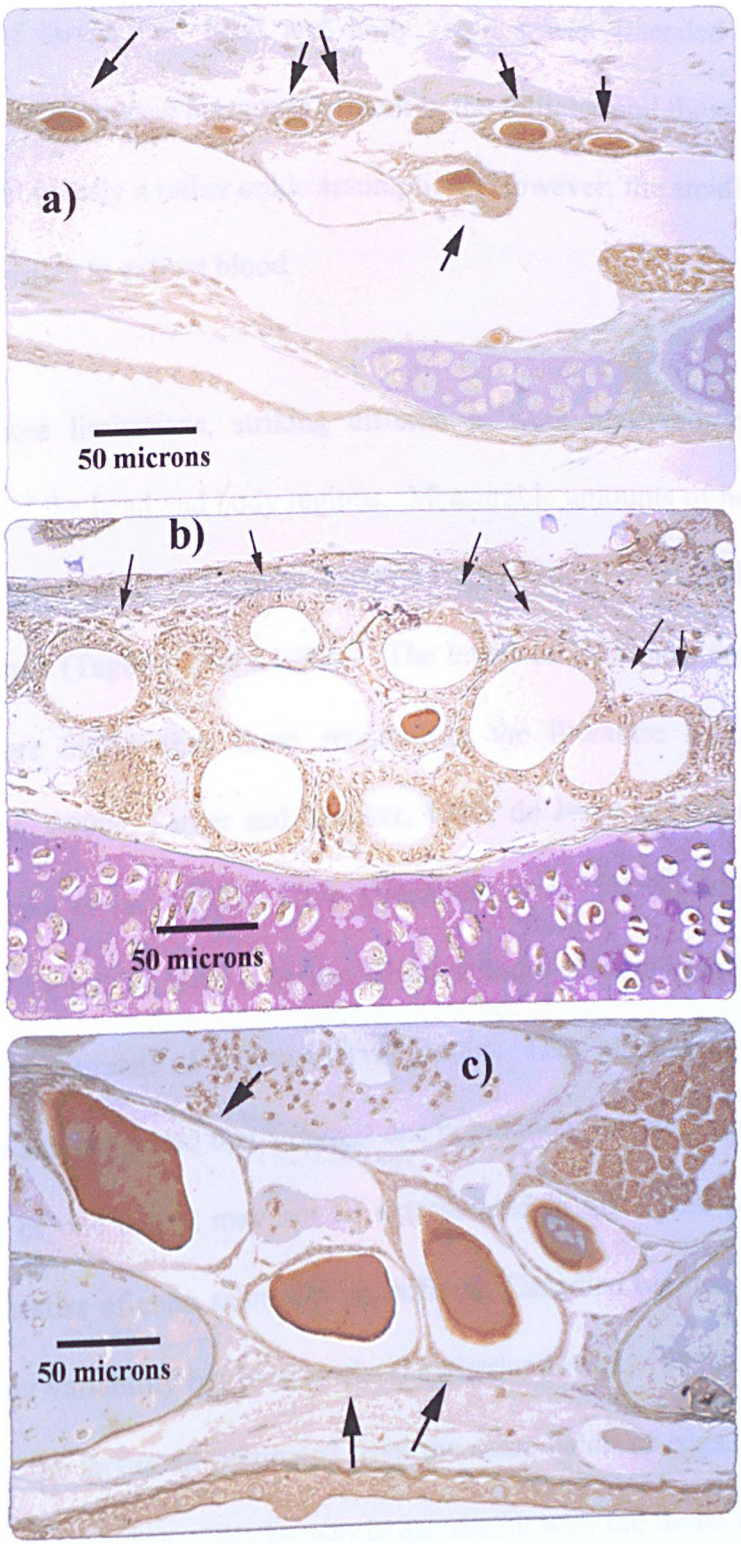
By the last sampling date, 45 days PFF, the levels of T3 and T4 in the head region had decreased to a similar value (30.1 ± 11.4 and 30.2 ± 18.9 ng gBW^{-1} respectively) although the standard variation was more consequent for the T4 measurements. In the mean, the level of T3 in the body region also decreased while the level of T4 in the body region increased almost 3 fold to 22.2 ± 12.7 ng gBW^{-1}.

6.3.2. Follicle structure

At the beginning of the sampling period (21 days PFF), the thyroid follicles were observed on transverse sections of the gill arches. The follicles were scattered along the arches in small groups. Their epithelial cells were of the columnar type in both the copepod and the *Artemia*-fed groups. Colloid was present in all the follicles observed and filled most of the follicular lumen (Plate 6.1a). During the following 15 to 20 days (up to day 35 to 42 PFF), the shape of the follicular cells only changed slightly with an increase in the epithelial cell height. However, the amount of colloid in the lumen decreased (Plate 6.1b) and many of the follicles did not contain any colloid 30 days PFF. Similar changes were seen in larvae on both *Artemia* and copepod diets.

By day 42 to 49 PFF, the epithelial cells forming the follicles had changed from columnar to squamous. Furthermore, colloid was visible in the majority of follicles observed and filled the centre of the lumen (Plate 6.1c). During the following days and to the end of the metamorphosis, variability between follicles of individual larvae appeared. Although, most follicles had their lumen full of colloid some displayed columnar cells while others had squamous ones.

*Plate 6.1: photomicrographs of thyroid follicles (arrows) (magnification: x312.5; stain: Modified Aldehyde Fuchsin). a) group of follicles from a 21 days PFF larva fed enriched-*Artemia. *b) group of follicles from a 35 days PFF larva fed enriched-*Artemia. *c) group of follicles from a 49 days PFF larva fed a copepod-based diet.*



6.4. Discussion

The dissection of larvae into head and body regions was intended to allow the distinction between the thyroid hormones present in the follicles and those present in the circulation. It is obviously a rather crude assumption. However, the small size of larval fish made it impossible to extract blood.

Regardless of these limitations, striking differences were observed in the thyroid hormone content of the head and body regions. Measurable amounts of hormones were present in all samples unlike in flounder where T3 only becomes detectable during the metamorphic climax (Tagawa *et al.*, 1990a). The levels of hormones measured in the present work were higher than those reported in the literature for other species (Leatherland *et al.*, 1990a; Parker and Specker, 1990; de Jesus and Hirano, 1992; de Jesus, 1994; Tanaka *et al.*, 1995). Only Greenblatt *et al.* (1989) found such high concentrations of T3 and T4 level in larvae. These authors observed high variability in their hormone measurements of different larval groups. They attributed this variability to the broodstock and suggested that amongst one population, variation may be so great that examination of one cohort may not be extrapolated to the whole population. A study of various stocks of coho salmon from different Canadian regions confirmed the possible significant variability between stocks (Leatherland *et al.*, 1989b). However, in the present study, the larvae all originated from the same batch of eggs. Furthermore, the histological structure of the follicles was in agreement with the tissue levels in terms of the timing of the hormonal peak. Variability between larval stocks cannot be excluded due to the lack of replication but the occurrence of a surge in thyroid

hormones around the time of metamorphic climax is clearly shown. Furthermore, the high levels could be attributed to the fact that contrary to other experiments referred to, the larvae were not analysed as a whole. As the head samples revealed the highest concentrations of both T3 and T4 it may be that the concentration in the follicles was indeed higher than in the circulation. However, in comparison to other species, even the levels encountered in the body region were high suggesting that the metamorphic surge of thyroid hormone in larval halibut may be of higher magnitude than in related species such as the Japanese flounder.

The peak was particularly evident when measuring the hormonal concentrations in the head region. It suggested an intense follicular activity. Indeed, the follicles of larvae sampled during the metamorphic climax (sampled between days 35 and 42 PFF) showed all the appropriate signs: the epithelial cells were columnar, reflecting the cellular activity, and the colloid area was small or absent, suggesting that the thyroglobin reserves had been used for the secretion of active hormones. Furthermore, the columnar shape of the cells of younger larvae and the presence of large colloid, suggested that the activity of the follicle was initiated very soon after the onset of exogenous feeding.

The rapid decrease in hormone secretion by the follicle was evident after 42 days PFF from both the quantitative determinations and the histological observations. Such patterns of thyroid secretion have been associated with the metamorphic climax in several other species (Miwa and Inui, 1987; Tanangonan *et al.*, 1989; Tay *et al.*, 1994). It can therefore be assumed that thyroid hormones play an active role in the

metamorphosis of halibut larvae. Such a role has recently been emphasised by the findings of Solbakken *et al.* (1997) who improved the rate of successful metamorphosis and more particularly the pigmentation and the eye migration of halibut larvae using thyroid treatments. These authors used T4 in their treatment as T4 was the most available hormone. Similarly, thyroxine was the hormone showing a surge after TSH treatment of flounder larvae, although T3 is reportedly the active hormone and T4 a pro-hormone (Inui *et al.*, 1989). The consequent improvement of the flounder larval metamorphosis was therefore related to the thyroxine secretion rather than to T3 which remained undetectable (Inui *et al.*, 1989). The authors suggested that the fish follicles only secrete T4 and that T3 originates solely from peripheral tissue conversion. However, in a later study also on flounder, it was shown that T3 was more potent than T4 in inducing the dorsal fin-ray resorbtion which is an important feature of larval flounder metamorphosis (de Jesus *et al.*, 1990). According to our observations, T3 and T4 are both synthesised in the follicles probably reflecting a follicular conversion. More detailed studies of the interactions between the hormones and their receptors will be required to clarify the specific action of the two hormones. Experiments on these aspects of thyroid metabolism have been initiated on flounder (Inui *et al.*, 1995) and seabass (D. Power, personal communication, 1997).

Regardless of which of the two hormones is the most active, their involvement with halibut metamorphosis seems clear. Beside, thyroid hormones have been linked to a series of developmental changes in other species, features which also characterise halibut metamorphosis. In red seabream, T4 treatment induced enhanced pigmentation and behavioural changes associated with the change of habitat from surface to benthic

layer (Hirata *et al.*, 1989). In goldfish, similar treatment accelerated larval differentiation, growth and scale formation. After 8 days, the body of T4 treated fish was covered with scales and the number of melanophores on the scales were significantly greater. (Reddy and Lam, 1992). Similarly, combined treatment of Pacific threadfin with T3 and cortisol resulted in an accelerated onset of pigmentation (Brown and Kim, 1995).

Another important change occurring during the metamorphosis was characterised by the increase in myotome height (see Chapter 3). In flounder, such changes have been correlated with biochemical modifications occurring in the muscles during the change from larva to adult. The regulation of these changes has been associated with the thyroid hormones and particularly thyroxine (Yamano *et al.*, 1991; Inui *et al.*, 1995). The difference in swimming ability among all T3-treated larvae suggests that the development of the neuro-muscular system may be influenced directly or indirectly by thyroid hormones (Brown and Kim, 1995).

In terms of nutrient absorption, larval fish metamorphosis is associated with the differentiation of the digestive tract (see Chapter 4 for review). Furthermore ion and/or nutrient transport in the gut depends on energy-requiring transport functions which themselves depend on functional mitochondria. The early observation of functional mitochondria following T3 and cortisol treatment led to the suggestion that these hormones accelerated intestinal differentiation (Brown and Kim, 1995). Consequently, nutrient uptake could be facilitated as was also suggested by the findings of Tanaka *et al.* (1995) when treating larval fish with T4. Indeed, these authors observed an increase

vacuolisation of the gut mucosal epithelium suggesting an enhanced pinocytotic activity. Similar observations have also been reported in poultry (Krogdahl, 1985).

In addition, thyroid hormone seems to interact with other pituitary hormones. They appear to play a permissive role on growth due to the promoting effect of the growth hormone (GH). When food is restricted, thyroid hormone levels are reduced which would lower the effectiveness of GH in enhancing growth. As a result a larger portion of the nutrient available would be directed away from growth allowing a better maintenance of other metabolic activities (Farbridge *et al.*, 1992).

A difference between the larvae fed a copepod-based diet and those fed an enriched-*Artemia* diet could not be established based on the observations of the follicles. It suggests that the surge in thyroid activity in both groups occurred at the same time. However, the development of the *Artemia*-fed group, which involved slower growth, poor eye migration, occurrence of malpigmentation and reduced myotome height, seems to be under the control of thyroid hormones in other species (Reddy and Lam, 1992; de Jesus, 1994; Lam, 1994; Tay *et al.*, 1995; Hey *et al.*, 1996). In addition, thyroid treatment of *Artemia*-fed larvae has proved successful in improving halibut metamorphosis (Solbakken *et al.*, 1997). Further research would be required to establish the link between the type of prey organisms and the thyroid metabolism and activity in halibut larvae. In addition, the high variability experienced between samples tend to suggest that the timing of the samples taken were too far apart. It may reflect a difference in the rate of development of the larvae. In further studies, it would be recommended to sample a larger number of larvae and more frequently. If would also

appear wise to link the development of the follicles, the synthesis of thyroid hormones not only with the age and the diet of the larvae but also with their size. Despite the fact that the thyroid hormone levels are calculated as concentrations, thereby eliminating the variation due to larval size, it does not distinguish between the variety in development and growth rates between larval samples. As the hormonal peak is assumed to be of a short duration in time, such variety can result in high variabilities as experienced in the present investigations. The inclusion of size and developmental stages as new factors of comparison would account for this problem and ease any conclusion drawn from such studies.

Chapter 7. Effects of dietary lipids on the metamorphosing larvae

7.1. Introduction

The findings presented earlier (see Chapter 4) showed that *Artemia* may not be able to provide the necessary dietary lipid, especially HUFA (Navarro *et al.*, 1993; Estevez and Kanazawa, 1996; McEvoy *et al.*, in press), to the developing larvae due to the apparent larval inability to digest them. In addition, *Artemia* have been shown to retroconvert DHA to EPA thereby reducing the effectiveness of DHA-rich enrichments (Navarro *et al.*, 1997). They also provide fatty acid mostly in the form of triglycerides which are less digestible to the larvae by comparison with phospholipids. As a result, it was suggested that the use of HUFA-rich commercially available enrichments may match the larval requirements by improving the dietary value of *Artemia*. An experiment was designed based on this hypothesis.

7.1.1. Digestion of nutritional fatty acids

Lipids are generally classified in two categories, the neutral lipids, mainly triglycerides, and the polar lipids, mainly phospholipids. The triglycerides are glyceryl esters with three, mostly different, fatty acids. In the phospholipids, the 'head' of the molecule is highly polar as one of the three fatty acids has been replaced by phosphoric acid itself bound to alcohol (Lehninger, 1985). The structure of the fatty acids forming the 'tail' of the phospholipids determines to a large extent the physiological properties of phospholipids in cell membranes. Furthermore, the enzyme-substrate interactions in lipid and fatty acid metabolism are dependent on weak hydrophobic interactions. They infer relatively low specificity of incorporation to fatty acyl groups. Consequently, the structure or the species of dietary lipids will have a great influence on the cellular metabolism of lipids as the rate of incorporation of some fatty acids will correspond to

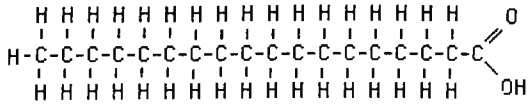
their abundance in the diet. By contrast, the essential amino acids have cellular functions that are mostly independent of the composition of dietary proteins (Sargent *et al.*, 1993).

The main criteria in fatty acids' structure are the length of the carbon chain and the nature of the bonds between the carbon atoms. The length of the chain of fatty acids incorporated in phospholipids varies mainly from 14 to 22 carbon atoms, with the longer molecules having a higher melting point. When the carbon atoms of the chain are all linked by single bonds, the fatty acid is described as saturated while the number and the position of double bonds will determine the degree of unsaturation of the fatty acid and its family group. Fatty acids having double bonds linking the third carbon of the chain starting from the methyl end will be said to belong to the n-3 series. Similarly if the first double bond links the sixth or the ninth carbon atom, one will be speaking of the n-6 and n-9 series respectively (Steffens, 1989; Guggenbühl, 1997) (see Figure 7.1).

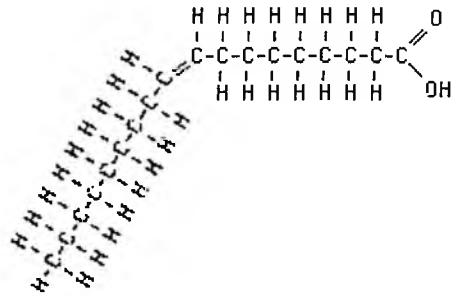
Independently of their structure, the triglycerides and the phospholipids, present in the diet, are digested following a similar sequence of events.

Bile salts from the liver emulsify the lipids to form micelles, where they are partially digested into glycerol and fatty acids by pancreas-borne lipases. The resulting mixed micelles are absorbed by the intestinal mucosa where the triglycerides and the phospholipids are reconstituted and incorporated into lipoproteins. The intestine produces the lipoproteins of the lowest densities, the chylomicrons. Once released into the intestinal lymph, they migrate to the circulatory system where they are available to

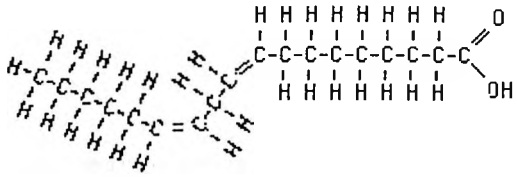
Figure 7.1: Examples illustrating the fatty acid classes and nomenclature



Saturated fatty acid :
e.g. 18 :0, stearic acid



Monounsaturated fatty acid :
e.g. 18 :1 n-9, oleic acid



Polyunsaturated fatty acid (PUFA) :
e.g. 18:2 n-6, linoleic acid

other tissues, principally adipose tissues and the liver (Norum *et al.*, 1983) (Figure 7.2). The proportion of each component, *e.g.* triglycerides, phospholipids, cholesterol and protein, in the lipoproteins affects their density and is used in this classification as shown in Figure 7.3.

This metabolic pathway is of great importance in many species of fish as, by contrast to mammals, they utilise lipids as their main source of energy in preference to carbohydrates (Black and Skinner, 1987). It results in plasma levels of lipids and cholesterol being higher by several fold in comparison to those of humans (Babin and Vernier, 1989; Luizi *et al.*, 1997). In larval fish, with their limited digestive capacities, phospholipids provide a better source of lipid than triglycerides as the polar lipids are more easily emulsified and consequently more rapidly hydrolysed and assimilated (Sargent *et al.*, 1993).

7.1.2. Fatty acids in the marine environment

During starvation, the important decrease in neutral rather then polar lipids, suggest that triglycerides are the major energy source and therefore are primarily catabolised. In contrast, the polar lipids are conserved since they play an important role in the production of cellular membranes (Koven *et al.*, 1989; Navarro and Sargent, 1992).

Amongst the fatty acid composition of polar lipids, a lot of research on fish nutrition has been focused on docosahexaenoic acid [DHA, 22:6(n-3)] and eicosapentaenoic acid [EPA, 20:5(n-3)] and more recently on arachidonic acid [AA, 20:4(n-6)] (Castell *et al.*, 1994; Bell *et al.*,1995a; Bell and Sargent, 1996; Zheng *et al.*, 1996; Sargent *et al.*, 1997b). They appear to be the most important essential fatty acids (EFA), with possibly

Figure 7.2: Transport and fate of major lipid substrates and metabolites (LPL = lipoprotein lipase; TG = triglycerides; FFA = free fatty acids). From Mayes (1988).

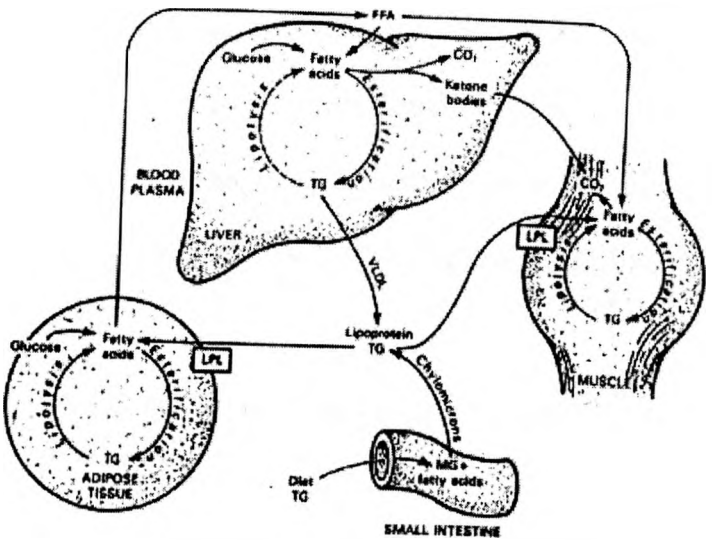
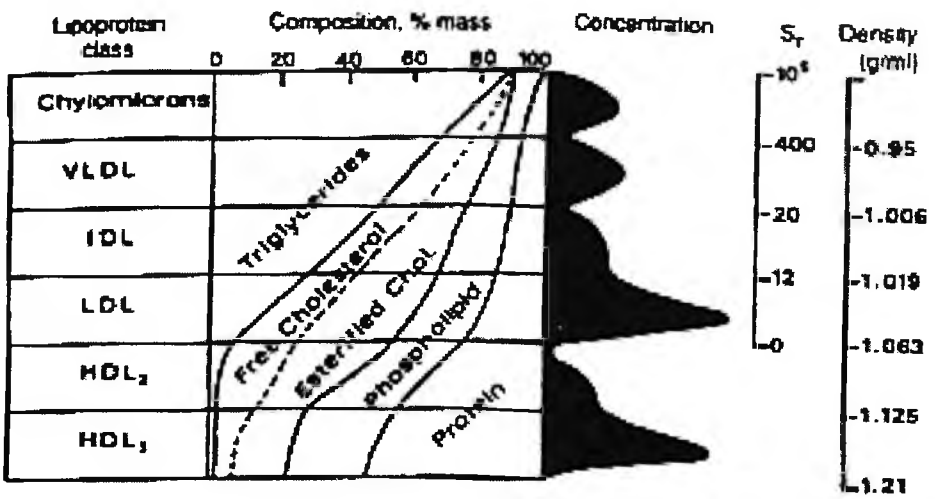


Figure 7.3: The classification, composition and ultracentrifugation properties of the lipoproteins (S_F = Sverdberg flotation units). From Mackness and Durrington, 1992.

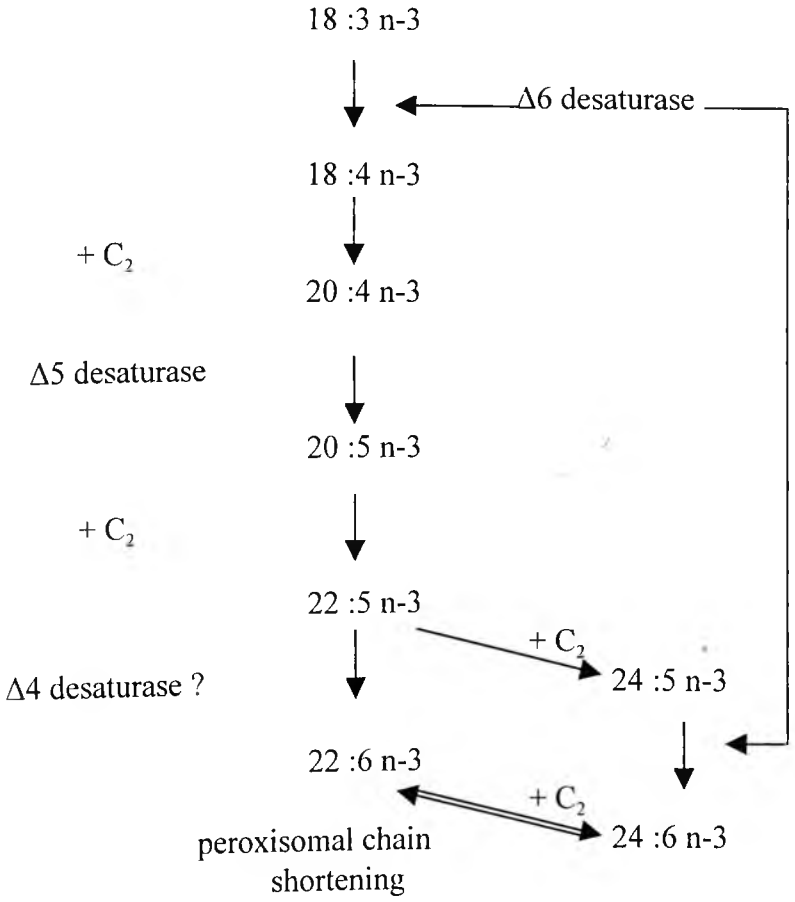


DHA being more important (Watanabe *et al.,* 1989a) since the rate of conversion from EPA to DHA of marine species is very limited (Watanabe, 1991; Sargent *et al.*, 1997b). Freshwater fish are capable of converting polyunsaturated fatty acids with 18 carbon atoms (C18 PUFA) of both the (n-3) and (n-6) series [18:2 (n-6), 18:3 (n-3) to 20:4 (n-6), 22:5 (n-6), 20:5 (n-3), 22:6 (n-3)] (see Figure 7.4). By contrast, marine fish are not capable of these conversions due to a deficiency or impairment of one or more enzymes in the pathway of desaturation and elongation (Sargent *et al.,* 1993; 1997).

EPA, DHA and AA have been shown to improve notably survival, growth, pigmentation, development, etc., of many fish species such as gilthead seabream (Koven *et al.,* 1990; 1992), carp (Radünz-Neto *et al.,* 1994), turbot (Bell *et al.,* 1994; Reitan *et al.*, 1994; Bell *et al.*, 1995a), flounder (Estevez and Kanazawa, 1996), halibut (Shields *et al.*, in press) and several others (Tuncer *et al.,* 1993); an exception being plaice (Dickey-Collas and Geffen, 1992), which appear to have low requirements for EPA and DHA.

However, it has been found that rather than the absolute value of each EFA, the ratio between them was of major importance. For example, the brain and neural tissue in fish have higher ratios of DHA/EPA than other tissues (Koven *et al.,* 1993; MacEvoy *et al.*, in press). Evidence suggests that DHA-containing molecular species of phosphatidylethanolamine (PE) provide a particularly fluid membrane conformation which allows maximal intra-membrane movement of associated enzymes and proteins. On the other hand, AA along with EPA are precursors of physiological mediators known as eicosanoids (prostaglandins and leukotrienes) (Johnston *et al.*, 1983; Steffens,

Figure 7.4: Pathways of elongation and desaturation of 18:3 n-3 as identified in rainbow trout (Buzzi et al., *1997).*



1989; Watanabe *et al.*, 1989a; Bell *et al.*, 1994; Bell *et al.*, 1995a). However, AA is the most effective precursor implying that a high EPA/AA ratio can reduce the production of prostaglandins (Sargent *et al.*, 1997b).

7.1.3. Fatty acids and the nervous system

By the time of exogenous feeding, most fish larvae are of such small sizes that they are usually recognised as the smallest vertebrates of the animal kingdom. From those early stages to the completion of metamorphosis and the juvenile stage, they develop and differentiate their essential organs and systems. Amongst the systems undergoing a rapid rate of development is the central nervous system, particularly the brain and the retina. Those tissues are characterised by highly specialised cellular membranes where a balance between rigidity and flexibility is required to ensure very rapid protein conformation changes (Dratz and Deese, 1986). A functional link has not yet been established between this specialisation and the molecular species of the phospholipids forming the membrane's bilayer. Nevertheless, experimental evidence shows that the main HUFA extracted from fish brains and retina is docosahexaenoic acid (DHA) (Bell and Dick, 1991; Sargent *et* al., 1993; Bell *et al.*, 1994; Shields *et al.*, 1997).

7.1.4. Objectives

The aim of this experiment was to improve the nutritional value of *Artemia* by mean of HUFA-rich enrichments. The performances of the larvae fed the resulting dietary treatments were monitored in order to isolate potential effects of the HUFA on their development and metamorphosis. Analysis of the lipid class and fatty acid composition of the enriched *Artemia* and of the larvae were performed on samples generated during

this experiment by Dr. J.G. Bell from the NERC Unit of Aquatic Biochemistry at the University of Stirling.

7.2. Materials and Methods

7.2.1. Experiment 3

7.2.1.1. Experimental design

7.2.1.1.1. Materials

* tanks: 16 black circular 100 l tanks with an external stand pipe and a water jacket.
* water: constant temperature (11.4 ± 0.6° C), salinity (33.4 ± 0.7 ppt), and pH (8.2 ± 0.9).
* temperature control: the fresh water filling the water jackets was recirculated and chilled and the room was equipped with two air chillers.
* lighting: dimmable PAR 38 tungsten floodlights (Osram Concentra, 80 watt), placed above each tank providing a maximum of 1200 lux on the water surface.
* aeration: single air stone placed in the centre of the tank.
* turbidity: 1 to 2 l of algae (*Nannochloris atomus*) added daily (maximum turbidity = 3 NTU) until a majority of the larvae became demersal.
* water flow: at the onset of the experiment, a 20% water exchange was performed daily with a flow of 200 ml min^{-1} switched on for 1h45. Once the majority of the larvae became demersal, the flow was maintained for longer to allow 50% of the water volume to be replaced daily. The flow was then progressively increased replacing 75% of the water volume daily by the end of the experiment.

7.2.1.1.2. Methods

Four different *Artemia* enrichments were selected. They were SSF, Herring Oil, Super Selco and Tuna Orbital Oil (see section 2.1.1.5 for details on the enrichment procedure).

Each of these dietary treatments was conducted in four replicate tanks. Using 2 l plastic beakers, 1600 larvae aged 8 days PFF were scooped out of the first feeding tank and randomly distributed between the 16 experimental tanks. All the tanks were treated identically in terms of water quality, lighting, and sampling.

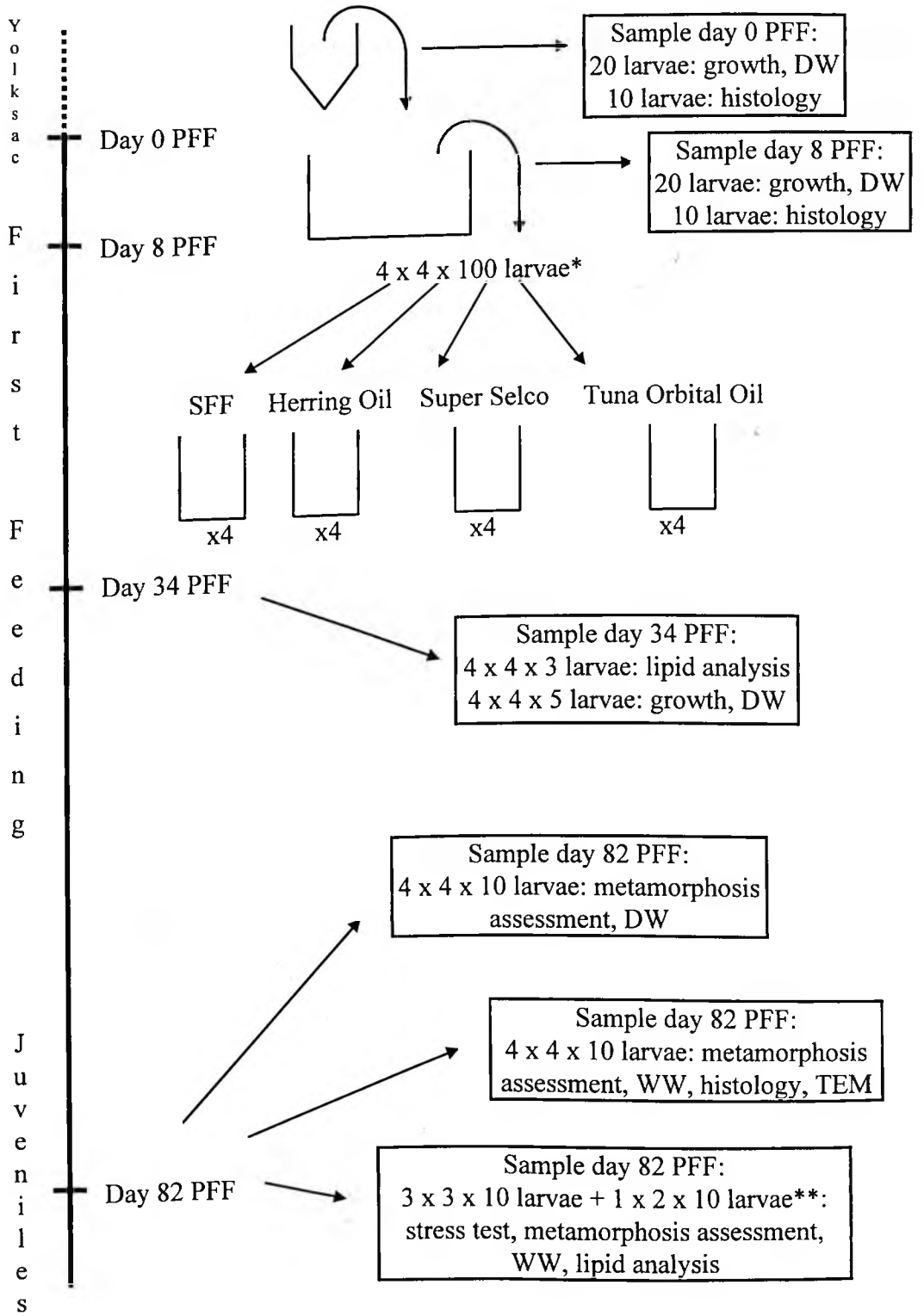
The larvae were fed twice daily to satiation with *Artemia* enriched for 18 h with appropriate enrichments. A daily count was performed on each *Artemia* culture tank before the morning feed. In addition, the larval tanks were cleared weekly (starting on day 42 PFF) of all the algae in order to allow a precise count of the surviving larvae. This information was used to determine the daily food ration of *Artemia* larva^{-1} day^{-1}.

7.2.1.2. Sampling schedule

From the batch of yolksac larvae to be transferred to the first-feeding tank, 30 larvae were randomly sampled using 2 l plastic beakers. They were killed with an over-dose of MS222. Twenty of them were separated for growth measurements and dry weight measurements. The remaining 10 were used for histological observations. An identical sampling procedure was applied 8 days later (day 8 PFF) before the larvae were transferred to the experimental tanks (Figure 7.5).

During the experiment, one intermediate sample was taken 34 days PFF. It consisted of 7 larvae randomly selected from each tank (4 x 7 larvae per dietary treatment). For each treatment, three larvae per tank were pooled, measured for growth and sent to the NERC Unit of Aquatic Biochemistry (University of Stirling) for lipid analysis. The remaining 4 x 5 larvae per treatment were used for growth and dry weight measurements.

*Figure 7.5: flow diagram of the sampling procedure applied during experiment 3. (*the numbers separated by x represent the number of dietary treatments, the number of replicates and the number of larvae) (** in this case only 3 replicates were sampled from 3 dietary treatments and only 2 from the Herring Oil-fed group).*



At the end of the experiment, 82 days PFF, a first group of 10 larvae were randomly selected from each tank (4 x 10 larvae per treatment). They were assessed for pigmentation and eye migration characteristics and for dry weight measurements. A further 10 larvae were then sampled from 3 out of the 4 tanks corresponding to each dietary treatment except those fed Herring Oil-enriched *Artemia*. The high mortality observed in this group only allowed the sampling of 10 larvae from 2 out of the 4 tanks. These larvae were used for the assessment of pigmentation and eye migration, for wet weight measurements and for the high salinity stress test. Finally a pooled sample of 10 larvae per treatment was collected and used for pigmentation and eye migration assessment, wet weight measurements and histological investigation. The remaining larvae from all the treatments were pooled and transferred to a weaning tank.

7.2.1.3. Lipid class and fatty acid composition

Lipid analyses were carried out on the larvae sampled for experiment 2 (see Chapter 5), as described in Chapter 2 (see section 2.6).

7.3. Results

7.3.1. *Artemia* enrichment

The fatty acid content of the total lipid fraction of the different groups of *Artemia* fed to the larvae are presented in Table 7.1. They revealed that the nature of the enrichment was the main factor affecting the fatty acid compositions of the *Artemia*, while the duration of the enrichment (18 or 42 h) had little effect. The main differences appeared in the percentage of EPA, DHA and their ratio. *Artemia* enriched with Super Selco contained the highest percentage of EPA while those enriched with Tuna Orbital Oil contained the highest percentage of DHA. As a result only the *Artemia* enriched with Tuna Orbital Oil had a DHA/EPA ratio above 1.0.

7.3.2. Lipid class and fatty acid composition

Analysis of the lipid class and fatty acid composition of the larval eyes and livers fed the different diets as performed by Dr. J.G. Bell. The main variation between treatments in term of lipid class of the livers is presented in Figure 7.6. It shows an increased level of triglycerides (TAG) in livers from larvae fed the diets richer in PUFA. Indeed, livers of larvae fed SSF-enriched *Artemia* contained the smallest amount of TAG (11.0 %) while the livers of larvae fed Tuna orbital oil-enriched *Artemia* contained the highest amount of TAG (36.9 %).

The fatty acid composition of the eyes revealed a higher DHA content in the larvae than in the *Artemia* used to feed them. Nevertheless, a relation between the content of HUFA in the eyes and the HUFA content of the *Artemia* appeared. Indeed, the eyes of

Table 7.1: fatty acid compositions of total lipid from Artemia *enriched with either SSF, Super Selco, Herring Oil, or Tuna Orbital Oil for 18 or 42 h. Values are in weight % of total fatty acids. The analyses were performed at the NERC Unit of Aquatic Biochemistry of the University of Stirling by Drs. J.G. Bell and L.A. McEvoy.*

Fatty acid/ Sample	SSF		Herring Oil		Super Selco		Tuna Orbital Oil	
	18h.	42h.	18h.	42h.	18h.	42h.	18h.	42h.
14:0	1.1	1.0	2.8	2.2	0.6	0.5	1.1	1.1
16:0	12.0	10.9	11.7	11.1	9.4	8.4	11.6	10.9
18:0	5.7	5.6	3.8	4.2	5.0	4.7	5.2	5.4
Total saturates[1]	19.1	18.2	19.0	18.2	16.5	15.4	19.7	18.9
16:1n-7	5.2	4.7	4.4	4.4	3.7	3.6	4.6	4.6
18:1n-9	20.8	20.3	17.0	18.1	17.4	17.0	19.7	20.0
18:1n-7	9.3	8.9	6.0	6.4	7.7	7.8	7.3	7.9
20:1n-9	1.7	3.2	6.4	6.5	1.3	1.8	1.0	1.7
22:1n-11	1.2	4.3	10.4	10.2	0.5	1.4	0.6	1.4
24:1	0.1	0.3	0.4	0.3	0.1	0.1	0.2	0.3
Total monoenes[2]	38.6	42.7	45.8	47.5	31.2	32.4	33.9	36.8
18:2n-6	4.7	3.8	4.3	4.0	4.6	4.3	7.4	6.8
20:2n-6	0.1	0.1	0.2	0.2	0.2	0.3	0.3	0.3
20:4n-6	0.8	1.1	0.8	0.9	1.2	1.3	1.4	1.9
22:5n-6	0.0	0.1	0.1	0.2	0.2	0.3	0.4	0.6
Total n-6[3]	5.6	5.1	5.4	5.3	6.2	6.2	9.5	9.6
18:3n-3	21.7	15.6	14.7	13.6	17.2	16.2	16.1	12.8
18:4n-3	2.5	1.7	2.6	2.2	1.7	1.5	1.7	1.3
20:3n-3	0.6	0.3	0.3	0.3	0.4	0.4	0.4	0.4
20:4n-3	0.5	0.4	0.3	0.5	0.6	0.6	0.4	0.4
20:5n-3	7.1	8.3	4.8	5.7	15.0	15.0	6.0	6.9
22:5n-3	0.1	0.3	0.3	0.3	1.5	1.7	0.5	0.6
22:6n-3	2.7	4.0	2.5	3.1	5.9	7.6	7.9	10.0
Total n-3	35.2	30.6	25.7	25.7	42.8	43.0	33.0	33.4
Total PUFA	40.8	35.7	31.1	31.0	49.0	49.2	42.5	42.0
DHA/EPA	0.4	0.5	0.5	0.5	0.4	0.5	1.3	1.5
EPA/AA	8.9	7.5	6.0	6.3	12.5	11.5	4.3	3.6

[1]includes 15:0 and 20:0. [2]includes 20:1n-11, 20:1n-7, 22:1n-9 and 22:1 n-11. [3]includes 18:3n-6 and 20:3n-6

Figure 7.6: triglyceride (TAG) composition (weight %) of livers of larvae aged 82 days PPF as a function of the enrichment used for the Artemia *with which they were fed.*

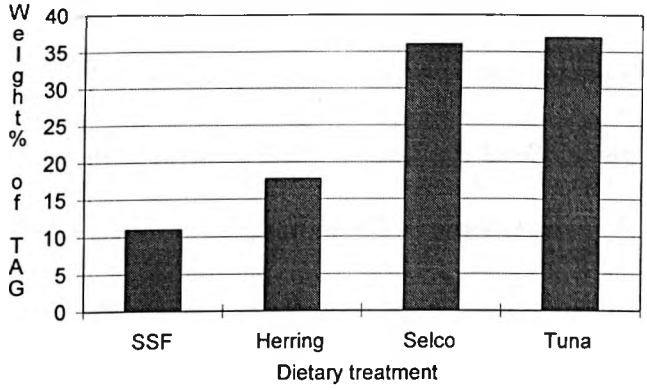


Table 7.2: fatty acid composition (± standard deviation) (weight %) of the eyes of larvae (82 days PFF) fed Artemia *enriched with either SSF, Super Selco, Herring Oil, or Tuna Orbital Oil for 18 or 42 h. The analyses were performed at the NERC Unit of Aquatic Biochemistry of the University of Stirling by Drs. J.G. Bell and L.A. McEvoy.*

Fatty acid/Sample	SSF	Herring Oil	Super Selco	Tuna Orbital Oil
14:0	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
16:0	14.7 ± 0.4	14.7 ± 0.5	13.1 ± 0.4	14.9 ± 1.0
18:0	10.4 ± 0.2	9.7 ± 0.5	9.1 ± 0.5	9.7 ± 0.7
Total saturates[1]	26.2 ± 0.6	25.5 ± 0.9	23.0 ± 0.8	25.5 ± 1.8
16:1n-7	2.7 ± 0.2	3.1 ± 0.2	2.7 ± 0.2	3.0 ± 0.2
18:1n-9	14.0 ± 0.4	14.1 ± 0.8	11.6 ± 1.0	12.6 ± 0.5
18:1n-7	5.9 ± 0.3	5.3 ± 0.5	5.1 ± 0.4	5.4 ± 0.2
20:1n-9	1.2 ± 0.2	1.4 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
22:1n-11	0.1 ± 0.1	0.6 ± 0.2	0.3 ± 0.2	0.2 ± 0.1
24:1	0.9 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.2 ± 0.1
Total monoenes[2]	24.8 ± 1.0	25.4 ± 1.1	20.8 ± 1.5	22.2 ± 1.1
18:2n-6	2.8 ± 0.7	3.1 ± 0.2	1.9 ± 0.1	3.6 ± 0.2
20:2n-6	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
20:4n-6	3.1 ± 0.2	2.9 ± 0.1	1.9 ± 0.0	2.6 ± 0.1
22:5n-6	trace	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.1
Total n-6[3]	6.3 ± 0.5	6.6 ± 0.2	4.6 ± 0.1	7.3 ± 0.3
18:3n-3	4.5 ± 0.4	4.8 ± 0.7	4.1 ± 0.6	4.1 ± 0.6
18:4n-3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
20:3n-3	1.9 ± 0.1	1.6 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
20:4n-3	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0
20:5n-3	16.8 ± 0.3	13.5 ± 0.4	12.7 ± 0.9	8.0 ± 0.8
22:5n-3	4.7 ± 0.2	4.2 ± 0.2	5.6 ± 0.3	2.7 ± 0.4
22:6n-3	9.0 ± 0.3	11.5 ± 0.3	21.3 ± 1.1	22.9 ± 1.3
Total n-3	37.4 ± 0.5	36.2 ± 1.0	45.4 ± 1.3	39.4 ± 1.4
Total PUFA	43.7 ± 0.9	42.8 ± 0.9	50.0 ± 1.4	46.7 ± 1.4
DHA/EPA	0.6 ± 0.1	0.9 ± 0.1	1.7 ± 0.2	2.9 ± 0.4
EPA/AA	5.5 ± 0.3	4.8 ± 0.2	6.7 ± 0.4	3.1 ± 0.3

[1,2,3]see table 7.1 for details

'Tuna-fed' larvae showed the highest DHA/EPA ratio (2.9), followed by the 'Selco-fed' (1.7), the 'herring-fed' (0.9) and the SSF-fed' larvae (0.6). Similarly, the EPA/AA ratio in the eyes corresponded, although being lower, to those of the *Artemia*. Overall, the eyes had a higher content of saturated fatty acid, a lower content of monounsaturated fatty acid and similar contents of n-6 and n-3 polyunsaturated fatty acid in comparison to the composition of the *Artemia*.

7.3.3. Morphological parameters

At the end of the experiment, 83 days PFF, a total count of the larvae in each tank gave the final survival figures (Figure 7.7). Although, the groups fed Super Selco and Tuna orbital oil enriched *Artemia* indicated a better survival with respectively 48 and 45% compared to 32% for the other two groups, the analysis of the variance (ANOVA I) did not reveal any significant differences. Indeed, the variability between replicates was very high.

The average body length of these larvae revealed significant differences between some of the treatments (Table 7.3). The larvae fed Super Selco enriched *Artemia* had the longest average body lengths at 25.52 ± 2.92 mm; there were significantly longer than the larvae from all the other groups. The groups fed Tuna Orbital Oil and Herring Oil enriched *Artemia* had similar average lengths at 21.73 ± 2.27 and 22.23 ± 3.08 mm respectively. However, those fed Tuna Orbital Oil were not significantly longer than those fed SSF enriched *Artemia* which averaged 19.50 ± 1.98 mm (Figure 7.8).

Figure 7.7: average survival, expressed in %, of the larvae at the end of the experiment (83 days PFF). The bars on top of each column represent the standard deviation calculated from the survival count from the four replicates. To improve the clarity of the chart, the names of the Artemia enrichments have been shorten: Herring = Herring oil, Selco = Super Selco, Tuna = Tuna orbital oil.

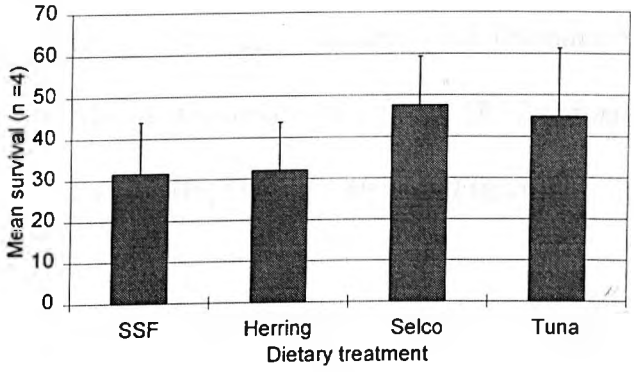
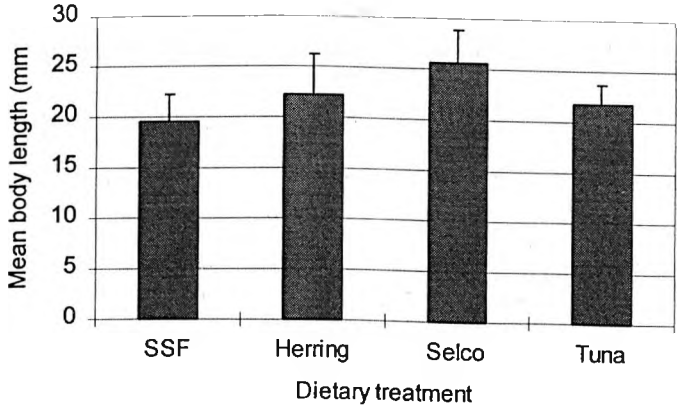


Table 7.3: comparison of the means using the Tukey's comparison, showing the significance of differences between the body length of larvae from the different dietary treatment groups. The test was run after an ANOVA 1 showed a highly significant difference (p = 4.07E-05) between the groups variances. NS stands for non significant and S for significant with $\alpha = 0.05$.

	SSF	Herring	Selco	Tuna
SSF				
Herring	S			
Selco	S	S		
Tuna	NS	NS	S	

Figure 7.7: average body length, expressed in mm, of the larvae at the end of the experiment (83 days PFF). The mean and the standard deviation represented were calculated from four replicated groups of 10 to 20 larvae.



Unexpectedly, the results of body length were not confirmed by the dry weight measurements. Indeed, the larvae fed *Artemia* enriched with Tuna Orbital Oil displayed the higher average weight (52.15 ± 17.57 mg), followed by those fed *Artemia* enriched with Herring Oil (45.50 ± 16.80 mg). The larvae fed *Artemia* enriched with Super Selco came third with an average dry weight of 37.87 ± 19.12 mg still well above the larvae fed *Artemia* enriched with SSF (23.82 ± 7.69 mg) (Figure 7.9).

Despite a relatively high variability within each group as represented by the standard deviation, a one-way ANOVA revealed a highly significant effect of the dietary treatments on the weight ($p = 3.76E-07$). When the mean of the replicates of each group were tested again each other using a Tukey's comparison of means, significant weight differences ($P = 0.05$) were highlighted (Table 7.4).

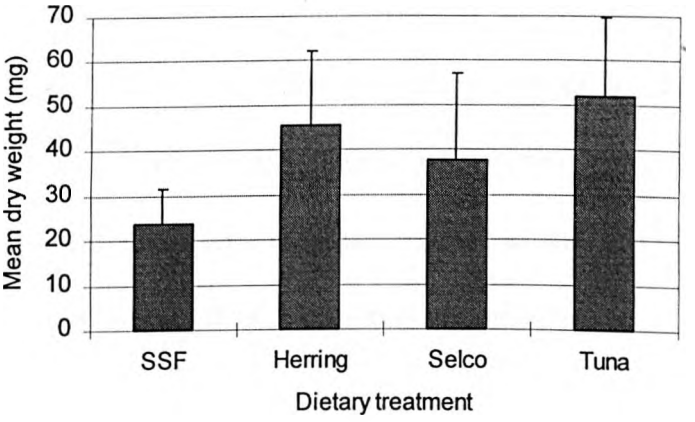
7.3.4. Assessment of metamorphosis

As described in details in Chapter 2, the metamorphosis assessment of larvae was performed categorising individual larva according to the extent of eye migration and pigmentation. As a reminder, 5 categories were set to describe the pigmentation, ranging from 1 (albinism) to 5 (full pigmentation). Furthermore, four categories described the migration of the eye, ranging from 0 (no migration) to 3 (complete migration of the eye).

Table 7.4: comparison of the means using the Tukey's comparison, showing the significance of differences between dry weight of larvae from the different dietary treatment groups. The test was run after an ANOVA 1 showed a highly significant difference (p = 3.76E-07) between the groups variances. NS stands for non significant and S for significant with $\alpha = 0.05$.

	SSF	Herring	Selco	Tuna
SSF				
Herring	S			
Selco	S	S		
Tuna	S	NS	S	

Figure 7.9: average dry weight, expressed in mg, of the larvae at the end of the experiment (83 days PFF). The mean and the standard deviation represented were calculated from four replicated groups of 10 larvae.



7.3.4.1. Pigmentation

Each larva sampled was categorised for pigmentation. The results were expressed in several ways. Firstly, global categories assessing the overall metamorphosis (see Chapter 2 for details) were determined. The frequency of occurrence of each category was calculated for each treatment and statistically tested. Table 7.5 presents the results of the ANOVA 1 performed on frequencies (using arcsin transformation) for each category between the 4 dietary treatments. Only the categories characterising larvae whose pigmentation patterns were either those of an albino, ambicolor or normal larva are shown. Other pigmentation patterns such as pigmentation of the head only (pigmentation category 2) were rarely encountered during this experiment. This approach only revealed that a significantly higher number of larvae fed *Artemia* enriched with Tuna Orbital Oil belonged to the category 251. This category represented the larvae that performed best in term of metamorphosis as only one larva was described as having its eye fully migrated and a normal pigmentation pattern out of all the treatments (category 351).

A second approach consisted of calculating the frequency of occurrence of the two most common types of pigmentation, either fully pigmented or albinos. This approach takes into account the pigmentation on both sides of the larvae separately. It showed that generally, the level of pigmentation was very similar on the dorsal and on the ventral sides suggesting that very little differentiation of the skin occurred in any of the groups. However, the larvae fed *Artemia* enriched with either SSF or Herring Oil were more pigmented than their counterparts fed *Artemia* enriched with Super Selco or Tuna

Table 7.5: statistical results (ANOVA 1) testing the differences of frequencies of metamorphosis categories between groups of larvae. Only the larvae fed tuna orbital oil were significantly more often of the category 251 (close to complete eye migration and proper pigmentation). NS stands for non significant with $\alpha = 0.05$.

Categories	Significance
11	NS
51	NS
55	NS
111	NS
151	NS
155	NS
211	NS
251	0.009731
255	NS
311	NS

Orbital Oil (Figure 7.10). This observation must be analysed with care as no significant difference was found between the groups when tested using ANOVA 1. This lack of differences can be attributed to the high variability within the replicates of each group.

7.3.4.2. Eye migration

As stated earlier, only one larva was described as having completed its eye migration. Therefore only the first three categories of eye migration were taken into account. Furthermore, as the comparison between groups considering all the aspects of metamorphosis was commented on the previous section, only the results showing the frequency of occurrence of each eye migration category are presented hereafter (Figure 7.11).

The average proportion of larvae whose eyes had not migrated at all was highest when the larvae were fed *Artemia* enriched with SSF (31.25 ± 13.15%) and lowest when fed *Artemia* enriched with Tuna Orbital Oil (7.5 ± 5.00%) (Figure 7.11). However, the analysis of the variance (ANOVA 1) after arcsin transformation of the data did not reveal any significant differences. Independently of the dietary treatment, the largest proportion of larvae had their eye only slightly migrated (category 1). When considering the category 2, Tuna Orbital Oil enrichment induced a significantly greater number of larvae to come close to a complete migration of the eye (Table 7.6). Indeed, no differences were revealed by the Tukey's comparison of means between the three other treatments.

Figure 7.10: average frequency of occurrence of different pigmentation categories on the dorsal side of the larvae at the end of the experiment (83 days PFF). The mean and standard deviation represented were calculated from four replicated groups of 10 to 20 larvae. Only the 2 categories are represented (white = albinism; grey = full pigmentation).

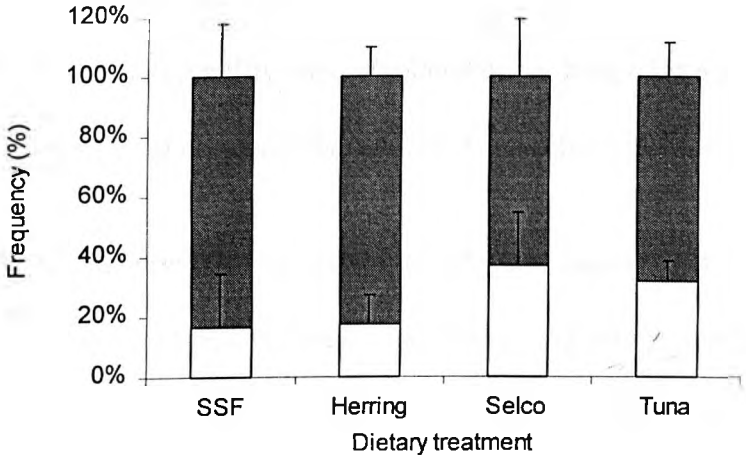


Figure 7.11: average frequency of occurrence of different eye migration categories of the larvae at the end of the experiment (83 days PFF). The mean and the standard deviation represented were calculated from four replicated groups of 10 to 20 larvae. Only the 3 categories are represented (0 = no migration; 1 = one eye slightly shifted upwards; 2 = the two eyes partially visible on the dorsal side of the head).

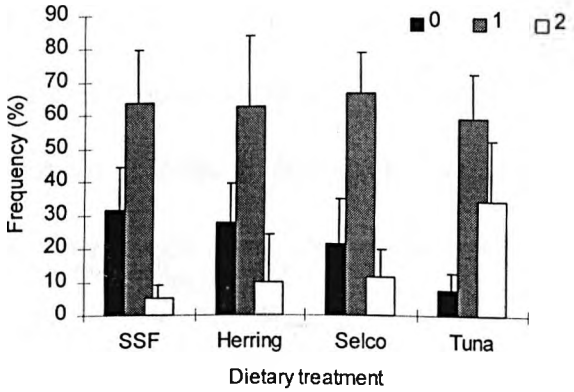


Table 7.6: comparison of the means using the Tukey's comparison, showing the significance of differences between larvae whose eye migrated almost completely (eye migration category = 2)from the different dietary treatment groups. The test was run after an ANOVA 1 showed a significant difference (p = 0.028652) between the groups variances. NS stands for non significant and S for significant with $\alpha = 0.05$.

	SSF	Herring	Selco	Tuna
SSF				
Herring	NS			
Selco	NS	NS		
Tuna	S	S	S	

7.3.5. Stress resistance

Three of the four replicated tanks for each dietary treatments (only two for the Herring Oil group) were sampled and each time, 10 larvae were transferred into the hypersaline solutions. The average mortality was calculated every hour after exposure to 65ppt salt water (see section 2.5 for details of the stress test procedure) (Figure 7.12).

The curves showing mortality as a function of time appeared as sigmoid for all the treatments, with those corresponding to the larvae fed *Artemia* enriched with Super Selco and Tuna Orbital Oil shifted to the right. Indeed, 50% of the mortality level was reached after approximately 9 hours for these groups while it was reached after less than 5 hours for the groups fed *Artemia* enriched with either SSF or Herring Oil.

The conversion of the mortality rates into a stress index confirmed the strength of two of the groups over the others. However, when including the standard deviation between the replicates, the indices revealed a difference between the two strong and weak groups. Indeed, in both cases, one group showed increased variability in comparison to the others (Figure 7.11).

Overall only the larvae fed *Artemia* enriched with Super Selco consistently showed a longer resistance to the hyper-salinity test and were in fact significantly stronger than those fed *Artemia* enriched with either Herring Oil or SSF (Table 7.7).

Meanwhile, the larvae fed *Artemia* enriched with Tuna Orbital Oil averaged a similarly low stress index (33.3 ± 24.9) but showed high variability between replicates. This

Figure 7.12: average mortality of larvae fed different diets and exposed to a hyper salinity (65ppt) test at the end of the experiment (day 83 PFF). Each value represents the mean mortality of three groups of 10 larvae per dietary treatment.

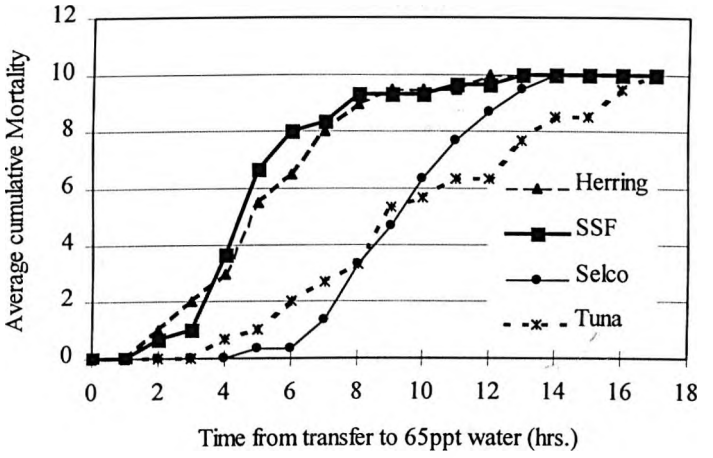


Figure 7.13: average stress index of larval groups fed different diets. The indices represent the cumulative count of mortalities over a period of 12 hours following the exposure of the larvae to 65ppt sea water.

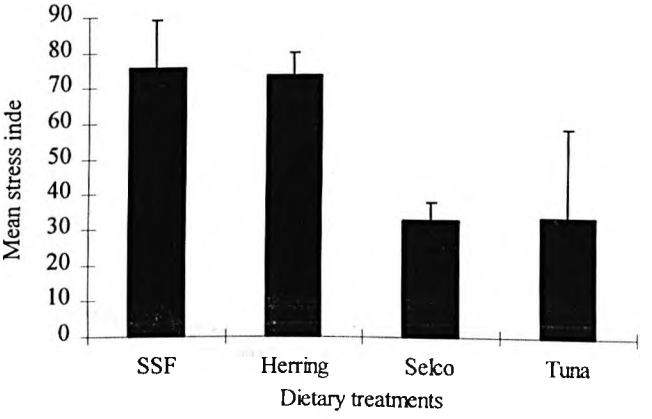


Table 7.7: comparison of the means using the Tukey's comparison, showing the significance of differences between stress indexes based on the larval resistance to a hyper salinity test and according to different dietary treatment groups. The test was run after an ANOVA 1 showed a significant difference ($p = 0.019471$) between the groups variances. NS stands for non significant and S for significant with $\alpha = 0.05$.

	SSF	Herring	Selco	Tuna
SSF				
Herring	NS			
Selco	S	S		
Tuna	NS	NS	NS	

variability is believed to have reduced the power of the statistical test. Consequently, these larvae, in comparison to those fed *Artemia* enriched with Herring Oil (stress index = 73.5 ± 13.7), could not be considered as statistically more resistant to the stress test.

7.4. Discussion

Broodstock fish preferentially transfer (n-3)PUFA from their body adipose tissues to the eggs (Henderson *et al.*, 1984). As a result eggs of marine species have high levels of DHA and EPA at hatch, accounting for example in Atlantic halibut for up to 42% and 13% respectively, of the polar lipid fraction (Parrish *et al.*, 1995). This process has been used to improve the (n-3) lipid content of eggs by supplementing the diets of broodstock fish (Cahu *et al.*, 1991; Abi-Ayad *et al.*, 1995). Furthermore, roe from halibut have even been used as *Artemia* enrichment for turbot larval culture (Reitan *et al.*, 1994) due to a DHA/EPA ratio superior to 3 (Falk-Pertersen *et al.*, 1986). Despite the beneficial effects of the high PUFA content in the eggs, the consequently improved survival and development is limited to the embryonic stages. After the onset of exogenous feeding and until weaning, the larvae largely depend on the nutritional value of their zooplankton diet. Thus the need to improve the lipid composition of *Artemia* when using those instead of wild plankton such as copepods as first-feeding diets (Skjolddal *et* al., 1990; Næss *et al.*, 1995).

The use of commercially available enrichments, justified by the industry's urgent need for improving the developmental successes of larval culture, enabled the production of a selection of *Artemia* presenting various lipid profiles. Their effect on the lipid composition of larval tissues, especially the eyes, was obvious despite the generally poor metamorphic performances of the larvae. Tuna Orbital Oil emulsified with 12% soya phosphatidylcholine (PC) provided the *Artemia* with the higher DHA/EPA ratio in agreement with the findings of McEvoy *et al.* (1995a). The high EPA level induced by

Super Selco was responsible for the low DHA/EPA ratio of this group despite the high DHA level. In comparison, the use of SSF and Herring Oil as enrichments resulted in low DHA/EPA ratio (0.5 or less).

The resulting dietary treatments induced a wide variety of growth related differences on the metamorphosing larvae. The high levels of DHA in the eyes confirmed that the retina is capable of selectively accumulating this fatty acid. Levels of DHA in the eye increased according to the level in the diet. Similarly, the level of AA was higher in the eyes than in the respective diets. It resulted in a low EPA/AA ratio for the larvae fed Tuna Orbital Oil. As AA is a more effective precursor of prostaglandins (Sargent *et al.*, 1997b), the eicosanoid synthesis may have been more effective in this group. Further evidence would have to be acquired from other specific experiments to substantiate this hypothesis. In the group of larvae fed Super Selco, the high EPA level of this commercial product contributed to the reduction of the DHA/EPA ratio. The best DHA/EPA ratio was that induced by Tuna orbital oil but it was still lower than those observed in eyes of rainbow trout (Bell and Tocher, 1989), Japanese flounder (Estevez and Kanazawa, 1996) or copepod-fed halibut larvae (Shields *et al.*, 1997). It can be suggested that despite the improved HUFA content of *Artemia* when using Tuna orbital oil as enrichment, the larval requirements were still not met. Based on Bell *et al.* (1995b) it could be suggested that the visual acuity of the larvae in all groups was impaired. This would account for the relatively low growth and feeding rates. On the other hand, the TAG content of livers from larvae fed *Artemia* enriched with tuna orbital oil was high. Triglycerides are preferentially used in marine fish as energy resources

while phospholipids are used mainly for structural purposes (Sargent *et al.*, 1993). The accumulation of TAG in the livers could reflect the high energetic value of the diet.

In terms of survival, despite the great differences between the average mortality in each group, the high variability accounted for the lack of significant differences. Nevertheless, apart from Koven *et al.* (1992), who found a significant effect of n-3 fatty acids supplementation on the larval survival of the gilthead seabream, most authors found no relation between the n-3 fatty acid content of the diet and survival (Dhert *et al.*, 1990; Koven *et al.*, 1990; Izquierdo *et al.*, 1992; Ashraf *et al.*, 1993; Reitan *et al.*, 1994).

The size of the larvae aged 83 days PFF expressed either as body length (in mm) or as dry weight (in mg) revealed some surprising results. While Tuna Orbital Oil induced the significantly best weight gain, Super Selco was responsible for significantly best growth in body length. In both cases, larvae from the Herring Oil group came second better while those from the SSF group were significantly smaller and lighter. In the case of larvae fed *Artemia* enriched with Herring Oil, the average mortality was higher, suggesting that only the larvae in the best conditions survived until the end of the experiment. This may have accounted for the good growth observed amongst the larvae. Nevertheless, the effect of the dietary treatments on either weight or body length could be related to the specificity of the enrichments. Tuna Orbital Oil emulsified with soya PC is a pure lipidic product shown to be highly energetic. The composition of Super Selco could not be known in great detail due to commercial secrecy. However, the product is advertised as boosted with vitamin C (ascorbic acid). On the other hand

SSF is derived from herring meal and therefore is expected to contain a variety of other components beside lipids such as proteins, carbohydrates and vitamins. When considering strictly the n-3 HUFA content of the diets fed to the larvae, the lipid profile of the Tuna Orbital Oil enriched *Artemia* was the closest to that recommended. It may explain the improved performances of the larvae from that group. In fact, DHA and EPA have a crucial role as components of biological membranes (Watanabe and Kiron, 1994) and n-3 HUFA have beneficial effects on larval development (Tuncer *et al.*, 1993). The larval ability to absorb the enrichments should also intervene with their beneficiary role, and it has been reported that lecithin improves the lipid digestibility (review by Krogdahl, 1985; McEvoy *et al.*, 1995b). The lecithin content of the Tuna Orbital Oil, used as an emulsifier, could have been of significant importance.

In addition to its relatively good HUFA profile, the vitamin C reported to be added to Super Selco may have accounted for the enhanced growth which this enrichment induced. Deficiency in this vitamin has been well documented and is known to provoke, amongst other effects, spinal deformities such as lordosis and scoliosis. A supplementation of ascorbate in the diet was also responsible for an improved growth rate and lower condition factor in *Oreochromis mossambicus* in a study by Soliman *et al.* (1986). In addition, fish have a limited ability to synthesise vitamin C (Watanabe and Kiron, 1994) but find it easily in their natural diet at first feeding as phytoplankton is a rich source of ascorbate for herbivorous zooplankton (Dabrowski, 1992). *Artemia* enrichment without an additional dose of vitamin C could be detrimental to the larval growth, especially to their skeletal growth as vitamin C has a functional role in the synthesis of collagen (Ross *et al.*, 1995). Furthermore, an hereditary defect in ascorbic

acid synthesis in rats induced bone deformities without any weight alterations (Togari *et al.*, 1995).

Irrespective of the dietary treatments, two types of pigment disorder appeared with either albinism on the ocular side or hypermelanosis on the blind side. The larvae fed with *Artemia* enriched with SSF or Herring Oil showed the lowest occurrence of albinism on the ocular side, but the highest occurrence of hypermelanosis on the blind side. These features are characteristic of hatchery-reared flatfish. Seikai and Matsumoto (1994) suggested that the ocular side albinism was the result of a disruption of the mechanism of skin differentiation. Kanazawa (1993) attributed the albinism of the ocular side to a disruption of the melanophore-stimulating hormone regulation (for further details, see Chapter 3). Furthermore, a series of authors recorded improvements of the pigmentation in relation to nutritional factors. Fukusho *et al.* (1987) suggested an unidentified trace substance present in eggs as a factor reducing the appearance of albinism in flounder. The same group of researchers also envisaged without success the role of tank aeration (Fukusho *et al.*, 1986). Studies on the use of different prey attributed beneficial effects to some strains of *Artemia* rather than others (Seikai, 1985a) and to wild zooplankton rather than *Artemia* (Seikai, 1985b; Næss *et al.*, 1995). Complementary research revealed that the fatty acid content of the prey organisms justified the differences between larvae. When feeding turbot or flounder, DHA/EPA ratios were reported as being more important than the overall n-3 HUFA content (Devresse *et al.*, 1994; Reitan *et al.*, 1994). When feeding plaice, the duration of n-3 PUFA feeding was reported as the main factor over the timing of the supplementation

(Dickey-Collas, 1993). Besides the fatty acids, vitamin A was also found as an important player in pigmentation induction in flounder (Miki *et al.*, 1990).

The limited knowledge of composition of commercial enrichments like those used in this experiment restricted the conclusions than were extracted from the data, especially when dealing with a process as complex as pigmentation. However, it can be suggested that the nutritional requirements of halibut larvae are greater than those of other flatfish species. Indeed, in the studies described above, supplementation of n-3 HUFA such as those provided by Super Selco and Tuna Orbital Oil fed *Artemia* appeared sufficient to improve the pigmentation. However, it is still unclear whether other nutritional factors are of importance in successfully rearing larval halibut. Growth and developmental rate have already been suggested earlier.

One of the most striking features of metamorphosis in flatfish larvae is the migration of one eye until both are on the same side of the head (for further details, see Chapter 3). Another important process occurring during larval development is the ossification. The cranial ossification in particular has a great impact on the eye migration as the two processes occur simultaneously during metamorphosis in flatfish (Brewster, 1987; Wagemans *et al.*, 1998). An incomplete migration of the eye by the completion of metamorphosis will therefore be irreversible once the cranium is fully ossified. In most studies investigating the larval development of flatfish, both the pigmentation and the eye migration have been considered simultaneously as part of the metamorphosis. However, the present results showed little correlation between the two processes as highlighted by the appearance of almost all combinations of categories according to the

visual assessment of the metamorphosis. Indeed, only larvae of the category 251 (eye almost completely migrated and proper pigmentation) were significantly more abundant when feeding *Artemia* enriched with Tuna Orbital Oil. All the other categories were equally represented in all the groups. As suggested in Chapter 3, these observations may confirm that growth rate and eye migration are closely linked, while pigmentation may be more related to other physiological events taking place during larval development. As such, it can be suggested that any dietary improvement may influence the two processes simultaneously. Optimised HUFA ratios and increased energy supply may provide the ideal nutritional conditions.

When the eye migration was considered separately, greater differences appeared between treatments. The Tuna Orbital Oil group had the lowest appearance of eye either not migrated (category 0) or slightly migrated (category 1) while it showed significantly higher appearance of eye almost fully migrated (category 2). However, with only 32% of those larvae having both eyes on the ocular side, it seems reasonable to say that the supplementation of n-3 HUFA and the increased DHA/EPA ratio resulting from the Tuna Orbital Oil enrichment was not sufficient. However, recent studies have clearly shown that the growth obtained during this experiment was low (Shields *et al.*, 1997). As the occurrence of eye migration has been suggested to be influenced mainly by growth (Policansky, 1982; B. Gara, Personal communication), it might explain the poor eye migration observed for all the treatments.

Stress tests, and in particular hypersalinity tests have been shown as good practical tools to measure larval vitality. The results obtained from the different groups showed clearly

an improved stress tolerance of the larvae fed *Artemia* enriched with either Super Selco or Tuna Orbital Oil. However, the statistical tests revealed that although the Super Selco group was significantly more resistant than the Herring Oil or SSF groups, the Tuna Orbital Oil group was only significantly more resistant than the SSF group. Although, larger fish would be expected to be more resistant, the trends in stress tolerance did not relate to the size of the larvae at the end of the experiment except for the SSF group that performed badly in both cases. The good tolerance of the Tuna Orbital Oil group could be attributed to the improved DHA/EPA ratio and n-3 HUFA diet that they received. Indeed, higher food content of n-3 HUFA has been held responsible for the increased stress resistance in perch larvae (Abi-Ayad *et al.*, 1994), in sea bass (Dhert *et al.*, 1990) and in sturgeon (McKenzie and Taylor, 1995). To a lesser extent, the same explanation could be applied to the Super Selco group. But, the vitamin C content of Super Selco could also be responsible for a significant improvement of the hypersalinity tolerance. In stressful situation, the elevated production of corticosteroids creates an increased demand for vitamin C. Therefore, a greater supply of vitamin C can minimise the damage caused by the stress (Steffens, 1989).

The different dietary treatments envisaged in this study have revealed that, on one hand, the fatty acid composition of the diets, and more importantly their n-3 HUFA content, is of great importance in improving the performance of larval halibut during their development. On the other hand, the lipid composition of larval tissues when compared to those of copepod-fed larvae highlighted the gap remaining between these two invertebrates in fulfilling the larval requirements.

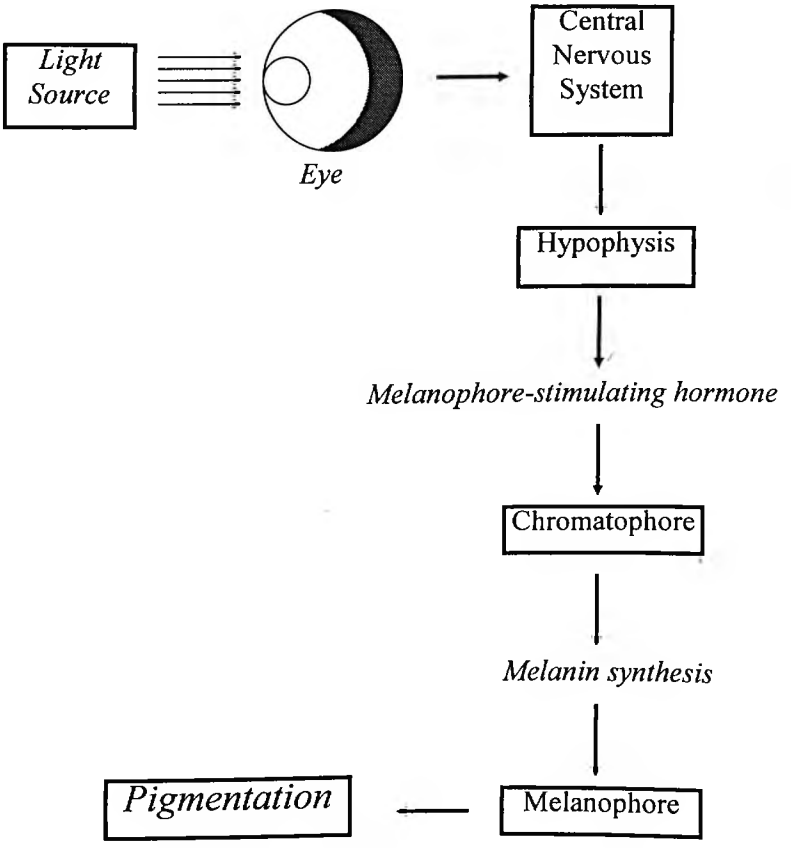
Chapter 8. Effects of light on the metamorphosing larvae

8.1. Introduction

Results presented earlier have shown amongst other things that feeding halibut larvae with *Artemia* resulted in poor pigmentation and reduced recruitment of rod photoreceptors along with an apparent lack of DHA accumulation in the eyes. Furthermore, the digestibility of *Artemia* and the ability of the larvae to benefit fully from the lipid enrichment were questioned notably by the present study of the digestive tract. Nonetheless, weaning of *Artemia*-fed larvae has proven successful (N. Brown, personal communication). At present, the rearing of copepods remains limited for commercial purposes due to the high cost and the seasonal nature of their production. An improvement of the larval metamorphosis must be achieved while feeding the larvae with *Artemia*.

It is presumed that pigmentation depends greatly on the secretion of melanocyte-stimulating hormone that is assumed to be stimulated by visual transmission from the photoreceptors (see section 8.1.2 for details). In that line of thinking, Kanazawa (1993) suggested that an insufficiency of the photoreceptor pigment, rhodopsin, was responsible for albinism in flounder (Figure 8.1). The authors related the lack of rhodopsin with nutritional deficiencies in vitamin A, and DHA. Indeed, vitamin A is a precursor for the formation of rhodopsin and DHA has been shown to be a major component of photoreceptors outer segment membranes. A deficiency in the former would be responsible for a lack of rhodopsin while a deficiency of the latter would alter the properties of the outer segment membranes thereby affecting the transmission of light.

Figure 8.1: schematic representation of the cascade of events from the stimulation by light of the retinal photoreceptive cells to the synthesis of melanin. (adapted and redrawn from Kanazawa, 1993).



It was shown in Chapter 5 that the retina of *Artemia*-fed larvae is structurally altered and the number of rod cells is reduced. It can be postulated that the occurrence of malpigmentation in halibut larvae may be partially due to an improper visual transmission of light by the retinal photoreceptors and more particularly the rod cells. Furthermore, it has been shown in other species that rhodopsin is most sensitive to short wave-length light at the blue end of the spectrum (Bowmaker, 1990). An improvement of the larval metamorphosis and more particularly of the pigmentation can therefore be expected rearing the larvae under different light environments.

8.1.1. Halibut's life history in relation to light

At hatching, the eyes of halibut are poorly developed with no pigmentation and an undifferentiated retina. The appearance of all the types of retina cells occurs half way through the yolk-sac stage, but only one type of photoreceptor can be seen, the cones (Kvenseth *et al.*, 1996). Their outer photosensitive segments are present, which suggests that the eye may be capable of photo detection (Kvenseth *et al.*, 1993). Almost simultaneously, around 23 days after hatching, the larvae start to display negative phototaxis to dim lights (< 1 lux) and positive phototaxis to slightly brighter lights (> 10 lux) (Naas and Mangor-Jensen, 1990; Mangor-Jensen and Naas, 1993). This trend, which continues during the rest of the yolk-sac stage, can be interpreted as a pre-adaptation to first feeding and relates to the larval vertical migration. Nevertheless, yolk-sac larvae cultured from day one post hatch under light are subject to high percentages of abnormalities and their survival rates are decreased (Bolla and Holmefjord, 1988).

Generally, it appears that feeding decreases with drops of light intensity. Although this is contradictory to the findings of Gulbrandsen (1991) for halibut first feeding larvae. The higher feeding success was found to be at 0.5 lux and declined rapidly as the intensity increased to 50 lux. For higher intensities from 50 to 1000 lux, there was a slight, but not significant increase. Beside, first feeding is enhanced when the larvae are reared in green water. This can be partially attributed to the variation of the light regime (Naas *et al.,* 1992; Gulbrandsen *et al.*, 1996).

Similarly, the largest growth and lowest mortality of juveniles were obtained at low light intensities (1-10 lux) (Hole and Pittman, 1993). Nevertheless, unpublished results obtained at Ardtoe showed good larval performances (growth, metamorphosis, stress resistance) at higher light intensities such as those used in the present study.

8.1.2. Light and pigmentation

There are three types of pigment cells in fish, the melanocytes, the xanthocytes and the iridocytes; melanocytes being the most common and containing black pigments called melanin. The biological functions of melanin include a protection against photic damage by absorbing the shorter wavelengths of light. Melanin reacts to neutralise the reactive chemical moieties that are generated by photic exposure. This pigment is not only confined to the skin but appears throughout the internal organs where it prevents oxidative damage. The immune system of fish also relies on melanin (Grønås *et al.,* 1993; for more details see Chapter 3).

The onset of pigmentation is therefore an essential step in larval development. However, under culture conditions, malpigmentation, especially in flatfish, has been a

major problem (Fernando and Grove, 1974; Seikai, 1980; Lagardere *et al.*, 1993). Several hypotheses have been suggested to explain this phenomenon. Kanazawa (1993) considered that the chomatophores, which are responsible for the normal pigmentation on the ocular side of flatfish, are controlled by the melanocyte-stimulating hormone secreted from the pituitary. The regulation of which is assumed to be stimulated by visual transmission from retinal rod cells. Previously, Narayan and co-authors (1985) localised the cells containing the melanocyte-stimulating hormone in the *pars intermedia* of the adenohypophysis. This region of the hypophysis happens to be the most innervated region of the neurohypophysis supporting the hypothetical nervous role in the control of pigmentation (Fernando and Grove, 1974; Joss, 1985).

Seikai *et al.* (1987b) suggested that the occurrence of albinism could be caused by the disorder of normal organogenesis. This suggestion is based on their hypothesis stating that albinism corresponds to the distribution of internal organ systems which induce the differentiation of pigment cells. The same authors along with others also highlighted the importance of lighting conditions for flounder larvae to achieve normal pigmentation (Seikai, 1985a; Nakano *et al.*, 1992).

The effect of light can also influence the pigmentation process via the skin rather than through visual stimulation. Indeed, the formation of the melanin itself occurs in the skin's melanocytes. Hydroxylated tyrosine compounds are cycled and polymerised to form melanin (Grønås *et al.*, 1993). One of the most important enzymes involved in this process is a tyrosinase, which is promoted by superoxide radicals. Besides, riboflavin, which is known to bind to melanosomes, produces superoxide radicals easily

under UV radiation. These radicals are thought to be scavenged and regulated by superoxide dismutase (SOD) in the skin. And in fact, a higher SOD activity in the pigmented parts of the skin of larval flounder. Knowing that SOD is activated by zinc and that tyrosinase is a copper containing enzyme (Jacobson, 1996) and having mentioned earlier the role of melanin in preventing oxidation in tissues, it becomes clear that an appropriate balance of all these components is essential for the regulation of pigmentation. The interaction between light and the quality of the food then becomes crucial.

8.1.3. Light and nutritional deficiencies

Preliminary experiments revealed the importance of the live feed quality in relation to pigmentation (Seikai and Sinoda, 1981; Seikai, 1985a). In fact the feeding duration and origin of *Artemia* influence the pigmentation of larval flounder. Later, it was stated that the proximate, mineral and amino acid composition of cultured food organisms are not so different from those of the wild, while fatty acid composition of the former is very different from the latter (Seikai, 1985b). The attention was thereafter focused on the fatty acid compositions of the live food.

Quickly, the importance of polyunsaturated fatty acid (PUFA) rich food was highlighted (Dickey-Collas, 1993). More precisely, the DHA/EPA ratio was shown to be of greater impact than the overall PUFA content (Devresse *et al.*, 1994; Reitan *et al.*, 1994).

The neural tissues of fish have DHA/EPA ratios of approximately 4:1 to 8:1 whereas a ratio of 2:1 is normally found in other tissues (Bell and Tocher, 1989). Besides, the amounts of di 22:6 n-3 molecular species of phospholipids in the retina of herring larvae

increase during development as rods appear. These molecular species appear as primordial in the adaptation to dim light environment (Bell and Dick, 1993). In fact, nutritional deficiencies for these fatty acids, result in their replacement in the retina by other PUFA molecular species but the vision remains impaired (Bell *et al.*, 1995b). So, as mentioned in previous sections, the nutritional requirements for DHA, phospholipids and vitamin A are directly related to the proper development of the retina and the acuity of the vision (Kanazawa, 1993; Estevez and Kanazawa, 1996).

8.1.4. Other parameters affected by the light

Other physiological functions are regulated by light, and the effect of photoperiod is well known for the fauna and flora. But as the pigmentation is part of the metamorphosis process, the relation between light and thyroid hormone secretion could also be of great interest. In fact, Flett and Leatherland (1989) showed that conversion between the two thyroid hormones, T4 and T3, occurring in the liver, is affected by light. In a reverse effect, thyroid stimulating hormone (TSH) increase the cyclic AMP levels of the pigment epithelium cells of the retina (Koh and Chader, 1984). Cyclic AMP is thought to play a role in the regulation of the pigment epithelium and photoreceptor cell functions. Besides, a lack of thyroid hormone affects the neural tissues development in human (Ford and Cramer, 1977). However, to our knowledge, no experiments have been conducted on fish in order to establish the links between photic stimulation and thyroid hormone secretion.

8.1.5. Objectives

The aim of this experiment was to test whether the quality of light provided to halibut larvae at the time of pigmentation and rod cell recruitment could influence the

occurrence of albinism. The focus was on the effect of light providing photons of short wavelengths such as blue light and daylight.

8.2. Materials and Methods

8.2.1. Experiment 4

8.2.1.1. Experimental design

8.2.1.1.1. Materials

* tanks: 15 black circular 100 l tanks with external stand pipes and water jackets.
* water: constant salinity (33.8 ± 0.3 ppt), dissolved oxygen (8.8 ± 0.4 g l^{-1}), and pH (8.2 ± 0.9). The temperature was slowly raised from 11.5 to 13.9° C during the course of the experiment.
* temperature control: the fresh water filling the water jackets was recirculated and chilled and the room was equipped with two air chillers.
* lighting: two banks of 3 tanks were equipped with blue fluorescent lights (Philips Super Actinic TL40W/03, Philips, UK) two with daylight fluorescent lights (Hagen Sunglo 40W, Rolf C; Hagen USA Corp., UK) and one with the standard tungsten floodlight. For each of the two fluorescent light treatments, one group was set to receive a 4 h period of darkness while all the other, as well as the tungsten control group, received 24 h of light. As the intensity of light produced by fluorescent or tungsten tubes vary during the course of their life, each tube was stabilised before its use for the experiment.
* aeration: single air stone placed in the centre of the tank.
* turbidity: the daily addition of algae (*Nannochloris atomus*) varied and the turbidity was closely monitored. It was set at 3.20 ± 0.34 NTU for the first week (days 30 to

36 PFF) then decreased to 1.50 ± 0.38 NTU for the following 20 days (days 37 to 57 PFF). By that time as the majority of the larvae were demersal, no more algae were added.

* water flow: at the onset of the experiment, a 20% water exchange for performed daily with a flow of 200 ml min^{-1} switched on for 1 h 45. By day 58 PFF, as no more algae were added, the flow was switched on longer to assure a 50% exchange of the tanks water volume.

8.2.1.1.2. Methods

The effects of the different light sources and photoperiod regimes on the metamorphosing larvae were tested. Five treatments were selected. Only the spectral emission of the different lights and the photoperiod varied between groups as the photic energy was set to identical values for all the treatment. At the onset of the experiment (day 30 PFF) the photic energy was 35 μW cm^{-2}; corresponding to the light regime applied to the larvae prior to the start of the experiment. Using dimable lights, the photic energy was increased to a constant value of 375 ± 36 μW cm^{-2} by day 35 PFF. All the measurements were taken at the water surface. The photoperiod and light spectrum characteristics of each treatment are presented in Table 8.1.

Using 2 l plastic beakers, 300 larvae aged 30 days PFF were scooped out of the first feeding tank and randomly distributed between the 5 sets of 3 experimental tanks. All the tanks were treated identically in terms of water quality, feeding, and sampling. Each bank of 3 tanks corresponded to one light regime and was isolated from the others using black plastic sheets.

Table 8.1: light spectrum and photoperiod characteristics of the different light regimes

Light regime	Photoperiod	Light spectrum
Blue 24:0	24h. light : 0h. dark	blue (max. at 475 nm.)
Daylight 24:0	24h. light : 0h. dark	complete sun light spectrum
Tungsten 24:0	24h. light : 0h. dark	shifted towards the red
Daylight 20:4	20h. light : 4h. dark	complete sun light spectrum
Blue 20:4	20h. light : 4h. dark	blue (max. at 475 nm.)

The larvae were fed twice daily to satiation with two population of *Artemia*, one enriched for 18 h with Algamac 2000 and one enriched for 18 h with Super Selco. This combination of enrichments was the most efficient diet established at Ardtoe at the time of the experiment. As in experiment 3, *Artemia* and mortality counts were used to calculate the daily rations.

8.2.1.2. Sampling schedule

Due to the limited numbers of larvae available, samples were only collected at the end of the experimental period (75 days PFF). Ten larvae were randomly collected for the stress test from two out of the three tanks corresponding to each light regime. These larvae were later assessed for eye migration and pigmentation. A pooled selection of 10 larvae from each treatment was also collected for the behavioural observations (see section 8.2.1.3). These larvae were later used for growth and wet weight measurements, eye migration and pigmentation assessment and finally preserved for histological observations. The remaining larvae from each tank were measured for growth and wet weight and assessed for eye migration and pigmentation.

8.2.1.3. Behavioural observations

8.2.1.3.1. Aim

An attempt was made to establish whether the larvae were actively feeding in the light only and whether the light regime under which they were reared affected such behaviour. For this purpose, small observation tanks containing a light and a dark room were made. The movement of the larvae from one chamber to the other was recorded over a 24 h period.

8.2.1.3.2. Materials

* observation tank: 10 l transparent rectangular container covered with black plastic sheets on all sides. The container was separated into 2 chambers by 2 black plastic panels of 80% of its width. Each panel was positioned on either side of the container at its centre and separated by a gap of 30 mm. As a result, the two chambers communicated by a small 'passway' with an opening on one side towards the dark chamber and on the other side towards the light chamber.
* lighting: the light regime in the light chamber was identical to that of the tanks from which the larvae were sampled.
* aeration: one air stone was placed in each chamber and set in order to assure an equal distribution of *Artemia* in the whole container.
* temperature: identical to that of the tank from which the larvae were sampled.

8.2.1.3.3. Methods

Groups of 10 larvae collected from each treatment of the experiment were transferred using a small hand net into different observation tanks. The cover of the dark chamber had been removed so that the larvae could be randomly placed with the container. Once 10 larvae had been transferred, they were all described precisely using schematic drawings on which all the larval characteristics were recorded. This enabled each larva to be rapidly identified. The cover of the dark chamber was then sealed and the larvae were left to adapt for 24 h without food.

After the 24 h adaptation period, the observations started. A detailed note of the number and the identity of larvae present in the light chamber was performed every 1 h for 24 h

and every 1/2 h for 2 h after each meal. The meals were delivered at the same time and with the same ration as during the experimental period that preceded. The *Artemia* were added to the tank in the space between the separation panels via a temporary opening in the cover. This procedure had been previously tested in absence of larvae to ensure that the aeration was appropriate to disperse the *Artemia* in the whole tank. At the end of the observation period, the larvae were killed using an overdose of MS222.

8.2.2. Experiment 5

An experiment similar to experiment 4 except for the lack of replication was initiated with larvae aged 14 days PFF. However, massive mortalities occurred within the first 10 days amongst the larvae reared under blue light and daylight. As a result, this experiment was aborted before its completion as only a very small number of larvae survived. However, the mortality rates observed at the end of the experiment are presented in section 8.3.

8.3. Results

The data presented hereafter was generated by the experiment 4 described in section 8.2. As the experiment 5 was aborted before completion, only survival data are presented. Some suggestions were made relative to the high mortalities experienced during that experiment and they are discussed in section 8.4 along with the discussion of the other results.

8.3.1. Growth and survival

On a daily basis, the number of *Artemia* fed to the larvae was established in order to feed to satiation while avoiding over-feeding. These feeding rations were determined and are presented in Figure 8.3. In all treatments, the feed intake increased quickly, from 500 to >1500 *Artemia* larva^{-1} day^{-1}, during the first 20-25 days of the experiment before reaching a plateau. During this period, larvae reared under blue light had the highest feeding rate. The plateau of feeding corresponded to the time during which the larvae started to become demersal, between days 50 to 60 PFF. The larvae reared under tungsten light reached this plateau the latest and at the highest level of feed intake. By day 60-65 PFF, when most of larvae had become demersal, the increase in feed intake started again with the larvae reared under daylight showing the highest increase.

In terms of weight (Figure 8.4), these similarities in feed intake patterns between the treatments resulted in mean wet weight between 190 and 255 mg without any statistically significant differences between groups. However, the two groups reared under blue light, with or without photoperiod showed the best mean growth.

Figure 8.3: feeding rates of the larvae reared under different light regimes, expressed as a mean number of Artemia *eaten per larvae per day.*

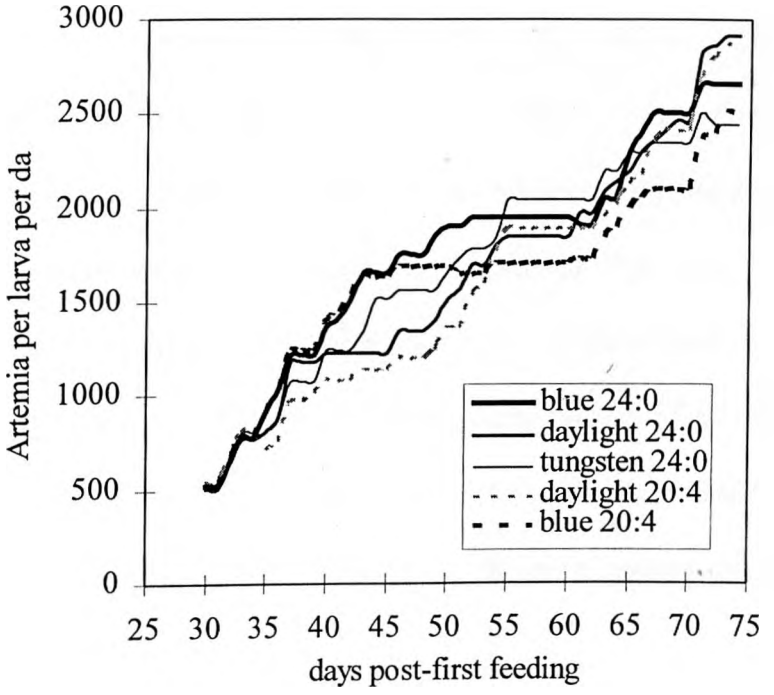
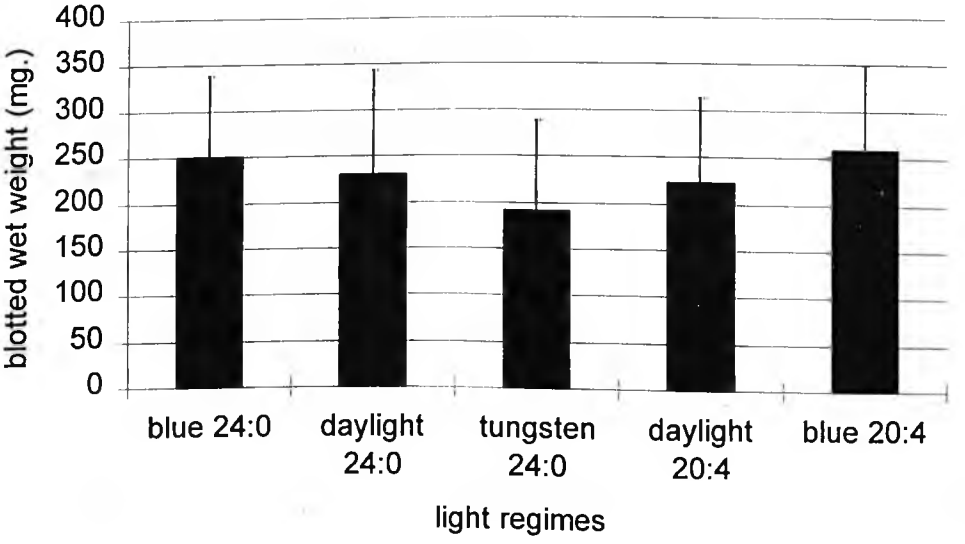


Figure 8.4: mean blotted wet weight of larvae (75 days PFF) reared under different light conditions (n = 3).



These same two groups showed also the best survival at the end of the experiment, with 83 ± 7% for the larvae reared under blue light with a 20L:4D photoperiod and 85 ± 8% for those reared under blue light with a 24L:0D photoperiod (Figure 8.5). This time, the data tested using a two way ANOVA (light environment = factor 1; time = factor 2) revealed a highly significant difference between treatments ($p = 0.001744$). This statistical method was preferred to a multiple linear regression although both tests gave significant differences between the survival in the groups over time. Indeed, using ANOVA, one does not make any assumption concerning the linearity of the relation between the mortality rate and the time. When testing only the final survival figures with an ANOVA 1 after an arcsine transformation of the value expressed as percentage of survival, no significant differences emerged between groups due to the high variability.

Concerning experiment 5, only the larvae reared under tungsten floodlight showed an 'expected' survival rate (62.5%), while all the other light regimes and most particularly the constant light regimes induced high mortalities. Indeed, only 15% of the larvae survived in the groups reared under constant blue light or daylight while the survival reached 35 and 40% in the same groups submitted to 4 h of darkness daily.

8.3.2. Metamorphosis

Concerning the assessment of the metamorphosis, the scoring system described previously (see Chapter 2) was used. However, there were no significant differences due to a very high variability. However, looking at the mean percentage of appearance for each category, some trends appeared.

Figure 8.5: mean survival of larvae reared under different light regimes.

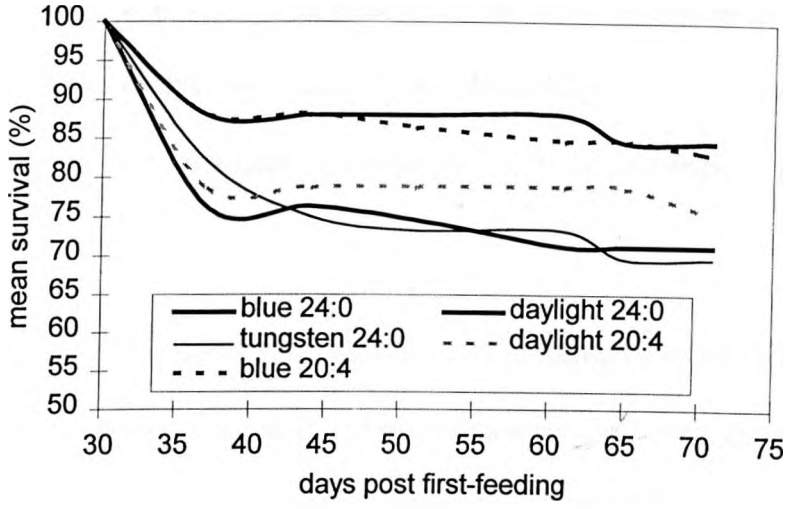
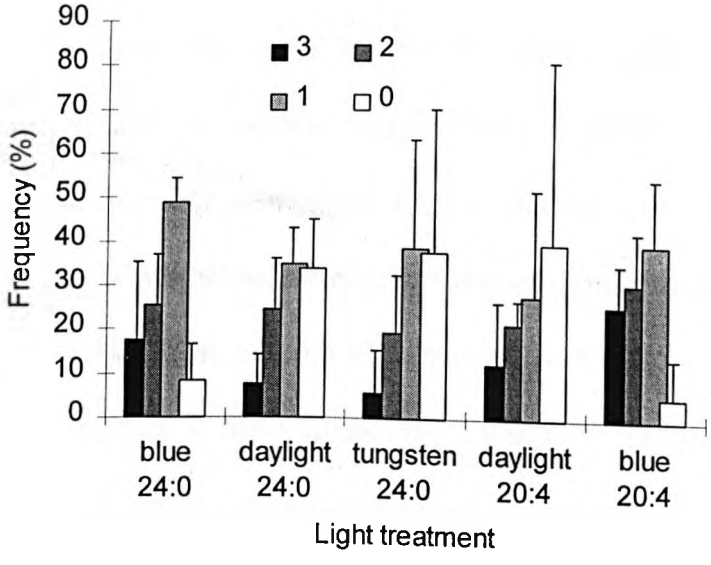


Figure 8.6: average frequency of occurrence of different eye migration categories of larvae at the end of the experiment (75 days PFF). The mean and standard deviation represented were from three replicated groups of 10 larvae. (0 = no migration; 1 = one slightly shifted upwards; 2 = the eyes partially visible on the ocular side of the head; 3 = complete eye migration).



In terms of eye migration, the blue light seemed to have enhanced the migration as the two groups it affected showed the highest levels of fully migrated eyes and the lowest of non-migrated eyes. Within the light treatments, the larvae reared under constant light showed a lower percentage, by 6 and 7% for the daylight and the blue light groups respectively, of full eye migration in comparison to their counterparts which had a 4 hour night (Figure 8.6).

For the assessment of the dorsal pigmentation, little differences could be observed with an overall high proportion of the larvae being malpigmented in every group. Indeed, the percentage of full pigmentation reached a maximum of only 37% for the larvae reared under blue light and subjected to a photoperiod (Figure 8.7).

8.3.3. Stress resistance

The stress resistance was quantified using an index calculated as the cumulative mortality of 10 larvae kept in 65ppt water over a 12 hour period. A low index indicated that the larvae survived longer and therefore were more stress resistant or more fit. In this case, the small number of replicates, only 2, affected greatly the power of the statistical tests and is probably responsible for the lack of significant differences between the treatments. Nevertheless, the earlier trends showing better performances of the larvae reared under blue light, in terms of growth and survival, were confirmed by the increased stress tolerance of these larvae (see Table 8.1). For the other results, except the frequency of larvae showing fully migrated eye, the 4 hours of darkness did not show any consistent effect on the larvae reared under daylight or blue fluorescent light. While such a photoperiod regime has improved the stress resistance of the group

Figure 8.7: average frequency of occurrence of different pigmentation categories on the dorsal side of larvae at the end of the experiment (75 days PFF). The mean and standard deviation represented were calculated from 3 replicated groups of 10 larvae. Only 2 categories are represented (white = albinism; grey = full pigmentation).

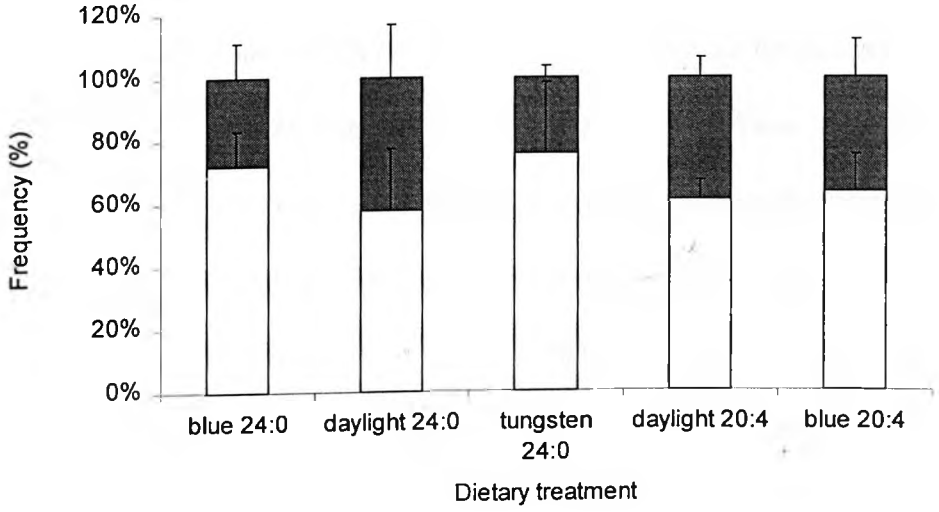


Table 8.1: average (n = 2) stress index of group of larvae reared under different light conditions. The indices represent the cumulative count of mortality over a period of 12 hours following the exposure of larvae (75 days PFF) to 65ppt sea water.

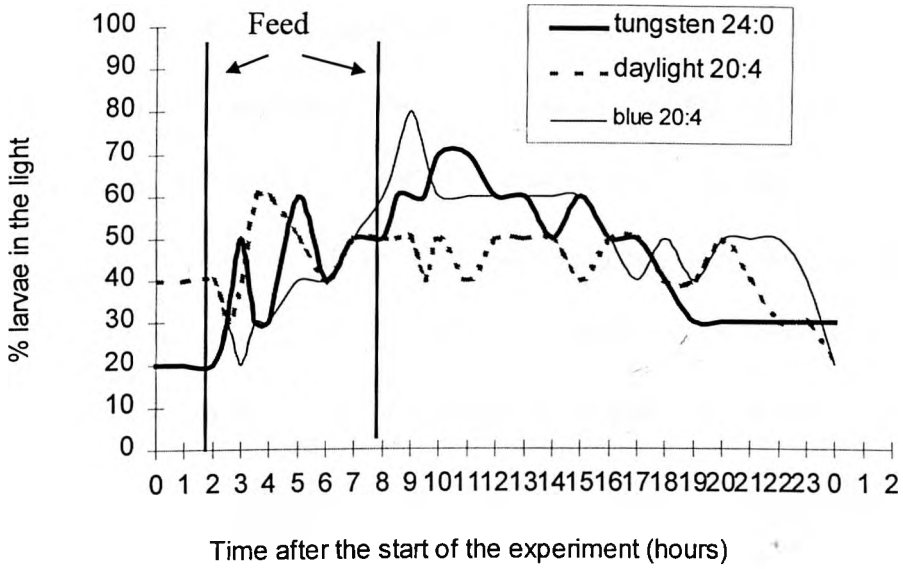
Light regime	Blue 24:0	Daylight 24:0	Tungsten 24:0	Daylight 20:4	Blue 20:4
Stress index	22.0	23.0	34.5	30.0	17.5

reared under blue light it has decreased the resistance of those reared under daylight. However, the control group (tungsten light regime) showed the lowest stress resistance.

8.3.4. Behaviour

In all groups except those reared under constant blue light, a general trend was observed, despite variations from hour to hour. The number of larvae, present in the light chamber, increased after both meals (Figure 8.8). Two hours after the second meal, the number of larvae feeding had slightly decreased. During the following 10 to 15 h, between 40 and 60% of the larvae were observed in the light chamber. By the end of this long feeding period most of the *Artemia* had been eaten and a majority of the larvae returned to the dark chamber (Figure 8.8). The total number of hours that each larva spent in the light chamber was tested statistically against their individual weight. No significant results were obtained, suggesting that the larval size did not affect their presence or absence in one or the other chamber. The larvae reared under constant blue light displayed a different trend as their number had not decreased in the light chamber by the end of the 24 h period although they had also eaten all the *Artemia*.

Figure 8.8: frequency of appearance of the larvae (75 days PFF) in the light chamber of the 'behaviour box'. The results from only 3 of the treatments are shown in order to improve the clarity of the figure.



8.4. Discussion

The light irradiance in terms of energy flux density chosen for the experiment (375 ± 36 $\mu W\ cm^{-2}$) corresponded to the photosynthetically active radiation (PAR) observed at depth around 12 m on a clear day of the end of May by the island of Easdale (56°17 N), just south of Ardtoe (56°46 N) (Tett, 1990). Knowing that the maximum photosynthetic activity is occurring at this depth, and that halibut larvae would be expected to have reached the first-feeding stage by May in their natural environment, these conditions were considered as optimal for a study of the effect of light. Furthermore, the light was provided either by fluorescent tubes or flood light avoiding concentrated beams of light which may have affects the spatial distribution of prey organisms and interfered with the feed consumption of larvae (Gulbrandsen, 1996).

No differences in pigmentation were observed irrespective of the light regimes applied in the present study. It is known that light can enhance the skin pigmentation in two ways: by direct stimulation of the melanophores and the activation of photolabile-aggregating (or -dispersing) compound (Daniolos *et al.*, 1990); and by stimulation of the MSH via the stimulus perceived by the eye (Kanazawa, 1993). In the former, as the influence is direct, the impact of nutritional factors could be neglected. In the latter, results presented in previous sections have shown that the adequacy of the diet in providing sufficient quantities of PUFA is of importance in the perception and transmission of the light stimulus by the retina. Consequently, our results tend to show that the blue light did not increase the visual stimulus as suggested.

The diet was selected according to the best *Artemia* enrichments available at Ardtoe at the time of the experiment in order to provide a good HUFA supplementation to the larvae. It proved successful to some extent as the stress indices for this experiment were comparable or lower than the best indices recorded for the experiment dealing with lipid enrichments (see Chapter 7). However, the performances of the control group demonstrated that the lipid quality of the diet was either not appropriate or that some other dietary components of importance were missing or provided in inappropriate quantities.

As the photic energy, at the surface of the water, was identical for all the treatments, it may suggest that at this early stage of development, the intensity of light may be of greater impact on the pigmentation than the wavelengths. Indeed, at the onset of first feeding, halibut larvae greatly modulate their feed intake according to light intensity (Gulbrandsen, 1991). In seabream, decreased light intensities were shown to improve the daily food-intake and consequently the growth and survival (Buchet *et al.*, 1993).

Naas *et al.* (1992) demonstrated in a very conclusive experiment that the suspension of algae in the water was crucial in allowing a good survival, feed consumption and growth of halibut larvae at the time of first-feeding. However, no nutritional role was associated with the algae although it has been shown in turbot that algal addition improved or maintained the fatty acid profile of rotifers (Reitan *et al.*, 1993). In the case of halibut, it was suggested that the algal suspension benefited the larvae by increasing the visual contrast and consequently the visibility of the prey organisms (Naas *et al.*, 1993; Gulbrandsen *et al.*, 1996). In addition, the algae increase the

absorption and scattering of light, which reduces the light intensity with increasing depth by absorption (Naas *et al.*, 1996). As a result of these findings the larvae reared in this experiment were kept in water of constant turbidity until they became demersal. The subsequent removal of algae was performed for husbandry reasons. Beside, it has been shown (R. Shields, personal communication) that at that stage the larvae are less sensitive to modification of their light environment.

In the presence of their natural prey, larval herring have been shown to increase their activity. In the presence of *Artemia*, the increase in activity only occurred once the larvae had ingested some *Artemia*, even at light intensities below the feeding threshold (Batty, 1987). Indeed, gustation and olfaction are the two main ways of detection of chemical stimuli by fish, even at the larval stage (Hara and Zielinski, 1989). Based on these observations, it could be speculated that halibut larvae transferred to the behaviour chamber could have been feeding in the dark as a result of chemoreceptive stimulation. However, the present results suggest that feeding took place preferably in the light. This pattern was repeated amongst larvae of the different groups suggesting that the light environment did not affect the feeding behaviour. This may be linked to the results shown in previous studies on the nutritional effects on retinal morphology of a dietary regime based on enriched-*Artemia* only. Indeed, the suggested impairment of the photoreceptor layer of such larvae, and particularly their poor recruitment of rod, would affect their vision under low light intensities. However, further experiments would be required to eventually link the quality of the diet, the rod recruitment and the preference of light regime when feeding.

On the other hand, the calculation of the feeding rates showed that the larvae reared under blue light had the higher feed consumption rate during the pre-metamorphic stage and up to the peak of metamorphic climax (day 45 PFF). Even if the feeding behaviour was not affected, the rate of success with which the larvae captured the *Artemia* seemed increased. Similarly, the mortalities in these groups were the lowest during that same period. The blue light could have had a beneficiary effect by increasing the contrast between the prey organisms and the environment. The stabilisation of the feed intake during the metamorphic climax is in agreement with earlier studies on flatfish (Laurence, 1977). The author suggested that the ingestion rate declines at metamorphosis due to the high efficiency of converting food to growth at that time of development and to the visual adaptation required during the process of eye migration.

When facing external stressors, such as the hypersalinity of the stress test, fish have to maintain homeostasis through mechanisms enabled by the general adaptation syndrome (GAS). In other words, anabolism is changed in favour of catabolism to allow the mobilisation of extra energy resources (for details see: Mazeaud *et al.*, 1977; Dhert *et al.*, 1990; Schreck, 1990; Briggs, 1992; Sampath-Kumar *et al.*, 1993; Iwama, 1995; Perry, 1997).

Although, the larvae reared under blue light resisted to the hyper-salinity test better than the others, no significant differences were noted between treatments. It shows at least that these larvae did not suffer from additional stress during the experiment, especially as they showed the best survival. This observation contrasts with the second experiment (experiment 5) that was aborted due to high mortalities amongst these larvae. The only

difference between the two experiments was the age of the larvae at the onset of the experiment, 14 days PFF in the latter and 30 days PFF in the former. Indeed, the high mortalities observed in the second experiment were attributed to excessive stress due to the light environment and more particularly to the constant light as the larvae reared under a photoperiod survived slightly better and those reared under tungsten floodlight did not show similar mortality rates. As the treatments in experiment 5 were not replicated, no strong conclusions can be drawn from these observations. However, it tends to suggest that the ability of the larvae to live in an environment characterised by photon of short wavelength may be detrimental at a time when the rod recruitment had not started. In humans for example, whose eyes are not adapted to blue light, intense light of short wavelengths has damaging effects on the retina (Rózanowska *et al.*, 1995). Whether these observations reflect a difference in the natural environment, suggesting vertical migrations during the first-feeding stage remains to be investigated. Such migrations have been documented for the yolksac stage (Naas and Mangor-Jensen, 1990; Mangor-Jensen and Naas, 1993).

The light treatments applied for this experiment did not include ultraviolet (UV) radiation. Although, in humans UV light induces pigmentation (see review by Gilchrest *et al.*, 1996) and in halibut it affects the spatial distribution of larvae (Naas *et al.*, 1995), the penetration of UV light in water is very limited (Denton, 1990) and the use of UV lights in hatchery conditions would lead to husbandry problems. Furthermore, UV light has a strong oxidative potential and has been associated with fat necrosis in fish reared outdoors (Bricknell *et al.*, 1996).

Globally, none of the treatments applied during this experiment significantly improved the performances of halibut larvae except for the survival rate. Furthermore, no effect of the photoperiod could be identified. However, trends appeared suggesting that the blue light was beneficial to the larval feeding success during some part of the first-feeding stage. It suggested that environment parameters such as light may not be optimised at present in halibut hatcheries. However, it also showed that the main problems relating to the completion of the metamorphosis remained unsolved. The quality of the diet still appeared as the main bottleneck in successfully producing metamorphosed halibut larvae.

Chapter 9. Conclusions

The present investigations tried to fulfil two major objectives. The first, covered in Chapters 3 to 6, aimed at improving the current knowledge of the developmental biology of halibut first-feeding larvae in order to provide a description of its critical stages. The second, covered in Chapters 7 and 8, aimed at applying the research findings in order to improve the rearing success of larvae through the first-feeding stage.

The search for the identification of critical stages of halibut larval development was tackled from a multi-disciplinary approach. All the observations were based on the comparison of larvae reared in conditions approaching those used in commercial hatcheries, *i.e.* relying on enriched-*Artemia* only; and larvae reared in conditions chosen to approach those experienced by larvae in the wild. Therefore, a dietary regime dominated by a marine copepod, *Eurytemora velox*, supplemented by enriched-*Artemia* was used. It was meant to provide a positive control on which to base all the comparisons. This appeared to be the most restrictive aspects of this thesis. Indeed, the intensive culture of *Eurytemora velox* is not sufficiently reliable to provide a constant source of live food. The fluctuation in copepod provision and the need for the use of enriched-*Artemia* as a dietary supplement has partially jeopardised the outcome of the comparisons. Indeed, most observations could hardly provide the basis for any extrapolation as the control did not always stand the comparison with similar experiments performed elsewhere. Consequently, our main recommendation in relation to the study of the developmental biology of halibut larvae is that either a negative control be found or that a reliable source of marine copepod, possibly from the wild, be secured prior to any experimentation. In addition, some potential restriction appeared in the timing of the experiments. Indeed, it could be suggested that the experiment started

too late to reverse some detrimental effects associated with the use of enriched-*Artemia* as the sole source of live food between the initiation of first-feeding and the beginning of our experiments.

Nevertheless, the experiments designed to study the morphology, the digestive ontogeny, the development of the retina, and the role of thyroid hormones provided some interesting data. Indeed, the description of the morphological parameters affected, during the first-feeding stage only, confirmed the specificity of flatfish in terms of pigmentation patterns and body growth. Such standardisation of the descriptive approach proved valuable in many cases. It generated the basis for more detailed studies. Indeed, only three stages of development could be identified in relation to the timing of the metamorphosis. These observations would need to be refined, taking into account not only the age but also the size of the larvae in order to provide a good reference for future work.

The description of the digestive ontogeny located, in time, developmental thresholds such as the appearance of the stomach and related them to external features. It added precision to the timing of the metamorphic climax. Differences in the sites of absorption and lipid digestion were observed. They were associated with the use of *Artemia* and suggested that the fatty acid profile and more particularly the PUFA profile of *Artemia* were deficient. It indirectly implied that *Artemia*, irrespective of the quality of enrichment, may not be a proper source of food to the larvae until the functionality of the stomach has been acquired.

Measurements of thyroid hormone concentrations and observations of the follicular structure at different time confirmed the involvement of thyroid activity during the larval development. Indeed, there was a clear variation in the cellular characteristics of the follicular cells supporting the analytical data and suggesting a peak of T3 and T4 at the time of metamorphosis. However, differences between *Artemia* and copepod-fed larvae could not be clearly established.

The description of the structure and ultrastructure of the retinal photoreceptors provided clear evidence of the impairment of the visual process resulting from the use of *Artemia* as prey organisms. It also provided further evidence of the importance of PUFA in the larval diet. Indeed, the recruitment of rods was significantly reduced and the structure of the photoreceptors outer segment membranes as well as the lipid recycling process within the retinal pigment epithelium was altered. The eyes of larvae fed enriched-*Artemia* only were characterised by lower levels of DHA partially compensated by higher levels of 22:5 n-6 and 22:5 n-3. These findings suggest some disruption of the transmission of the visual stimulus that could have wide ranging implications. These could go from reduced rates of feeding success to alterations of the control of melanophore-stimulating hormone secretion.

Resulting from the above mentioned observations and hypothesis, two applied experimental designs were established in order to achieve the second major objective of improving the rearing success of halibut larvae through first-feeding. On one hand, a series of four *Artemia* enrichments were chosen for their lipid and more particularly their PUFA content. The dietary experiment that followed confirmed the importance of

PUFA for the development of halibut larvae, and more particularly for growth and stress resistance. However, the high percentage of larvae that did not fully pigment and whose eyes did not complete their migration highlighted the limitations of an approach dealing with one parameter at a time. Indeed, it was felt that other parameters, such as the culture conditions may have affected the development of the larvae, hence restricting the potential effects of dietary improvement. In this case, as mentioned earlier, the need for a reliable positive control would have eased the comparison and added to the applicability of the observations.

Similarly, in another experiment designed to improve the light environment used in hatcheries, the effects triggered by different light regimes, ranging from blue to daylight spectrum, could not be attributed to a single factor. Only the exposure to blue light seemed to have some beneficial effects on survival mostly by increasing the food consumption during the metamorphic climax.

In conclusion, the present investigations provided a strong basis for future experiments. Indeed, they highlighted the importance of nutritional, environmental and physiological factors on the larval development and metamorphosis in Atlantic halibut. It also supported the need for adequate provision of copepods in order to provide a reliable positive control and the potential importance of the timing of the experimentations.

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