Histological investigations of organs and tissues development of ballan wrasse larvae during ontogenesis

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Preface

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Abstract

The main objective of the present work was to study the larval development of the most promising cleaner fish *Labrus bergylta* which have not been described previously. The present study provides valuable information on its structural status during ontogeny, and it can be useful for establishing the functional systemic capabilities and physiological requirements of larvae for optimal welfare and growth.

Gross morphology of the larvae was examined using a stereomicroscope. Organs and tissues of ballan wrasse larvae of different ages were studied with light microscopy. For light microscopic studies, Haematoxilin and eosin staining, Alcian blue-PAS (pH 2.5) techniques were used. The ontogeny of the Ballan wrasse larvae was studied by means of morphological and histological approaches from 0 until 49 days after hatching (DAH). Larvae were hatched form eggs obtained by natural spawning from a wild caught broodstock held in captivity. With reference to the main external morphological characteristics and source of food, larva development was divided into four stages: (1) Yolk sac larva (0-9 DAH), (2) Preflexion larva, (10-25 DAH); (3) Flexion larva (26-33 DAH); (4) Postflexion larva (34-49). The organ development in larvae was very prominent during the first three stages. Development during stage 4 was characterized by the proliferation and growth of existing structures. At hatching, the mouth and anus were closed, eyes were not pigmented, and digestive tract was an undifferentiated and straight tube. The majority of the organs were observed as undifferentiated groups of cells or primordial structures. Pericardic cavity, urinary bladder and exocrine pancreas anlages were seen. During stage 1 both the mouth and the anus opened in conjunction with the differentiation of the digestive tract. Buccopharyngeal cavity, oesophagus, stomach midgut and hindgut were distinguished. Primordial structures of liver, swim bladder, gall bladder, gills, pituitary and kidney appeared. Lecitoexotrophic period started when eyes were pigmented and larvae were ready for capture of the prey and feeding. As ingestion of prey began, the digestive processes continued developing with the appearance of mucous cells in the oesophagus, gut folds and brush border in the intestine. The circulatory system became functional, with the compartmentalization of the heart. During stage 2 (and 3) first haematopoietic tissue appeared. Endocrine part of pancreas – Langerhans islet was evident. Taste buds were seen in the oesophagus and skin. Throughout stage 3 thyroid follicles appeared, gill structures continued developing. Number of mucous cells in the oesophagus increased and first mucous cells appeared in the gill opening. During stage 4, gill filaments and lamellae proliferated, the number of mucous cells in gill opening region increased. Most organs essentially exhibited an increase in tissue structure and size.

1. Introduction

1.1. Ballan wrasse: identification, distribution and habitat

Ballan wrasse belongs to the wrasse family (devision Teleostei, order Perciformes, family Labridae) the second largest of marine fishes and the third largest perciform family, with at least 60 genera. The established name is *Labrus bergylta* Ascanius, 1767. Ballan are the largest of the north European wrasses, and may attain a total length of 600 mm, though lengths of 300-500 mm are more common (Sayer, et al., 1996). It reaches 3.5 kg and 25 years of age (Munk, et al., 2005).

The ballan wrasse has a robust body and a small, thick-lipped mouth with large conical teeth. It has a very variable coloration dominated by green and brown, without a black spot on the caudal peduncle (Munk, et al., 2005). The body coloration is not dependent on season or sex, and may possibly change to match the background color to its immediate surroundings. Ballan are recorded on eastern Atlantic coasts from Morocco to Norway. They are found in the North Sea and western parts of the Baltic, but may be rare or even absent from the Mediterranean (Darwall, et al., 1992). Ballan occur on stony and rocky shores (Munk, et al., 2005). Juveniles are sometimes found in the interidial; adults presence may extend below 30 m (Darwall, et al., 1992). It feeds mainly on crustaceans and mollusks (Munk, et al., 2005).

Wrasses have territorial behavior. Some aggressive behaviour in the sea may be associated with territorial defence, especially during the breeding season. A large male of wrasse will defend a teritory of 200 m^2 to 400 m^2. Females and smaller males may also use the territory and be aggressive to similarly sized fish (Darwall, et al., 1992).

A “harem” of 6 to 8 smaller female fish is associated with each ballan territory. It is hypotisized that successful protection of a harem by the dominant male would select for monandric protogyny. If larger males have greater spawning success, then it may be an advantage to be a breeding female when younger (Darwall, et al., 1992).

Fish attend a sexual maturity at about 6-9 years of age and 16-18 cm for females and 28 cm length for males. It spawn on gravel or rock place, female build the nest and male take parental care (Darwall, et al., 1992). When the egg hatch, after about one week, the males moves to another territory and repeats the behavior (Anne Berit Skiftesvik, 2003).

Ballan is monandric, changing sex when over six years of age, protogynous species (Darwall, et al., 1992).

On the Atlantic coasts spawning of ballan occurs from April to August. However, on the west coast of Sweden records indicate spawning to occur from the end of May to the end of June. Breeding generally begins earlier in the Mediterranean. Ballan are synchronous spawner - spawn

once within a breeding season (Darwall, et al., 1992). The individuals spawn in pairs, courtship takes place above the nest.

Within a territory defended by a single male clearing of the rock surfaces with subsequent spawning on the rock by female were described for ballan (Darwall, et al., 1992).

It is possible to strip ballan wrasse, but farming is dependent on natural spawning. The fish develop gonads, then they must be offered an environment that triggers spawning behavior, where a suitable substrate for the eggs must be present (Anne Berit Skiftesvik, 2003).

Ballan wrasse is the most effective cleaner fish used for big salmon (more than 2 kg), which can be active down to +3°C, and therefore may be used through winter in many localities (Per Gunnar, 2003).

1.2. General information about ballan wrasse larvae

The demersal and sticky eggs of *L. bergylta* 0.7-1.15 mm in diameter, spherical, creamy-white with no oil globule (Fives, 1976) exists in nest of algae in the crevices with spherical shape.

Hatching take place when the larva is approximately 2.75 to 3 mm long. Body of newly-hatched larva heavily pigmented with chromatophores from posterior to the head to approximately the eight post-anal segment. Head pigment restricted to two crescent-shaped areas running longitudinally on either side of the mid-dorsal line. Anal fin membrane with a scattering of stellate chromatophores. There may be some small pigment spots along the posterior ventral margin of the body and on the primordial fin membrane in that region (Fives, 1976).

Development of larva occurs planktonic. Larvae have a big and ovoid formed and unsegmented yolk sac. Preanal swimming pattern is clearly seen, the body is surrounded by dorsal, caudal and postanal swimming patterns (Artuz, 2005).

Pre-flexion larva has accentuated pigmentation on the body, while there are no pigment in the caudal region, on the snout or on the lower jaw. The pigmentation of the anal fin is restricted to the anterior region. The head pigment is distributed in two or semi-circular areas running longitudinally on either side of the mid-dorsal line (Munk, et al., 2005).

Flexion stage is characterized by apparent inter-spineous area of the caudal fin at a larval size of 5 mm, and at 7mm the urochord is fully bent upwards. The anal and dorsal fins develop from a larval length of 8 mm melanophores appear anteriorly on the anal fin (Munk, et al., 2005).

At the post-flexion stage both spines and soft rays develop in the dorsal and anal fins (Munk, et al., 2005).

1.3. Organs and tissues development in teleost fish

All teleosts have the same mechanisms of ontogenesis with differences in timing of developmental events. But large difference exist between species with regard to egg size, yolk composition, cleavage and developmental rates, egg incubation time, developmental status and size at hatch and the timing of development and functionality of various organs and organ system. Direct comparisons of organogenesis are complicated due to large variations in egg sizes and incubation temperatures between species. Developmental status at hatch differs between species and the duration of the yolk sac period varies. Main organs and organ systems become functional by first feeding and differentiate during the larval stage and metamorphosis (Falk-Petersen, 2005).

The larval ontogenesis could be divided on different stages according to the main developmental events (Blaxter, 1988).

Egg stage - from spawning to hatching. Yolk sac stage – from hatching to complete absorption of yolk sac. Preflexion stage – from complete yolk-sac absorption to start of notochord flexion. Flexion stage - start of notochord flexion to completion of notochord flexion. Postflexion stage – from completion of notochord flexion to start of metamorphosis. Metamorphosis – transformation stage until completion of fin-ray development and start of squamation.

1.3.1. The yolk sac stage

The duration of the yolk sac period depends not only upon species and temperature but also on egg size. Atlantic cod *Gadus morhua* larvae for example hatch after 70-80 d° from 1.2 to 1.4 mm large eggs (Falk-Petersen, 2005), at lengths of 3-5 mm (Morrison, 1987). Atlantic salmon *Salmo salar* larvae hatch after 510 day-degree (d°) from 5-7 mm eggs, at lengths of 17-20 mm. Larvae of Atlantic halibut *Hippoglossus hippoglossus* at size 6 mm hatch after 82-85° from 3.0-3.8 mm eggs (Moksness, et al., 2004).

The epidermis of the newly hatched marine larvae consists of two layers of metabolically active squamous cells, including chloride cells and secretory cells. The number of secretory cells and chloride cells increase during the yolk sac stage, after 2 to 4 days after hatch in cod, but the thickness of the epidermis remains the same. Both mucous cells with membrane-bound, secretory vesicles with dense contents and sacciform secretory cells with less dense secretory granules have been described in cod (Morrison 1987) and halibut yolk sac larvae, these cells have been associated with the non-specific immune system during early life stages (Ottesen, et al., 1997).

The myotomes are simple, segmental and V-shaped at hatch in most species. Further myotomes may be added posteriorly during early growth and they act against the notochord to provide a hydrostatic skeleton. The notochord is formed of vacuolated cells separated by thin cell membranes (Morrison, 1987).

The heart of newly hatched cod larvae has not distinct division into atrium and ventricle and opens directly into the yolk sac sinus and also receives blood from the pronephros region. The heart of cod larvae is differentiated into four compartments after 4 days, the first trabeculae are seen in the ventricle after 8 days (Morrison, 1987). In newly hatched halibut the heart is a primitive tube as well and the bulbus arteriosus thickens first 20 days after hatch and slowly forms the four chambers (Pittman, et al., 1990).

Gas exchange takes place cutaneously in small yolk sac larvae (and larvae) and functional gills do not exist at hatch (Falk-Petersen, 2005). Gill anlage is visible in pharyngeal region at hatching in common dentex *Dentex dentex*. At the beginning of the exogenous feeding primordial gill arches are visible and first chondroblasts appear in first arches (Santamaría, et al., 2004).

The pharynx of newly hatched cod larvae contains four gill arches without gill filaments, each with a cartilaginous core (Morrison, 1987) and there is one small branchial artery in each arch. Branchial cartilage is not seen until 16 days after hatch in halibut larvae, when also precursors of the lamellae on the gill arch are noted and branchial blood vessels are first seen on day 26 (Falk-Petersen, 2005).

The sensory epithelia of embryos and larvae often have chemosensory cilia directly exposed to the exterior (Blaxter, 1988). The olfactory placodes (the sensory epithelium of the olfactory organ), are located posterior to the mouth and dorsal to the eye of the yolk sac larvae, while the olfactory organs are located inside the nasal cavities at later stages. In cod, as well as in other species, the area increases during the yolk sac stage and the sensory epithelium gradually becomes more depressed (Morrison, 1987). Taste buds seem to appear later in small larvae (Falk-Petersen, 2005).

The eyes of indirectly developing species are often slightly pigmented or non-pigmented at hatch and probably non-functional. The larval retina generally contains only cones, and rods are reported to appear during metamorphosis. In all species the eyes are pigmented at the late yolk sac stage, i.e. by first feeding. Pure-cone retina appears to be sufficient for that purpose. Rods seem to be involved in movement perception and may be particularly important in predator avoidance (Falk-Petersen, 2005).

In most species the digestive tract first appeared as a straight undifferentiated tube attached dorsally to the yolk sac (Santamaría, et al., 2004), often with ciliated cells (Morrison, 1987). No

mouth is present just after hatch in codfishes, flatfishes (Falk-Petersen, 2005); only rudiments of the jaw bones are present and the anus is closed (Morrison, 1987).

The mouth of cod larvae opens after about 2 days, that of halibut after 15-20 days. After 5 days the cod jaw becomes functional. The buccopharyngeal apparatus, which is required for feeding and later respiratory functions are now fully formed (Falk-Petersen, 2005). In halibut, this occurs first after 15 days, with full jaw functionality after 25 days (Pittman, et al., 1990).

The oesophagus of the cod yolk sac larvae is narrow and surrounded by a circular layer of striated muscle. The first part of the midgut is wider than the rest and the hindgut (rectum) is separated from the posterior part of the midgut by a circular fold that develops into a constriction (sphincter) after 3 days. The gut is lined with columnar epithelial cells with microvilli at the surface (Morrison, 1987). There are no mucous cells in the midgut of the cod larva. The foregut of the halibut larvae is less differentiated at hatch than the rest of the gut and contains sparsely developed microvilli during the first three weeks (Falk-Petersen, 2005).

Peristaltic movements are noted after about 5 days in cod larvae, when feeding may be initiated. In halibut yolk sac larvae persistalsis is first noted after 23 days (first feeding) (Falk-Petersen, 2005).

Salmonids appear to have a functional stomach prior to the change from endogenous to exogenous nutrition (Falk-Petersen, 2005).

A liver, pancreas and gallbladder (between liver and pancreas) are present at hatch in cod (Falk-Petersen, 2005). The pancreas has one islet of Langerhans near the surface. The first granules of exocrine part appear three days after hatch (Morrison, 1987).

In newly hatched common dentex a gallbladder anlage is visible and the liver is just a mass of undifferentiated cells, exocrine pancreas differentiates after 2 days (Santamaría, et al., 2004).

In the halibut only a liver anlage is present during the first days after hatch. The beginning of liver segmentation appears after 16 days and is completed one week later when a significant size increase is noted. Gallbladder and bile duct first appear 20 days after hatch, the bladder seems fully formed after 23 days. Pancreatic tissue with small amounts of zymogen granules is present 20 days after hatch (Falk-Petersen, 2005).

In cod larvae at hatch the pronephric tubule of each pronephric organ forms several loops anteriorly. An excretory duct with a small lumen leads to the body surface (Morrison, 1987).

The swimbladder anlage is attached to the dorsal part of the front midgut at hatch in cod larvae (Morrison, 1987). The swimbladder, which acts as a hydrostatic organ is filled in many species soon after hatch (cod 8-10 days) whereas others may be several weeks months old (salmonids) before the swimbladder is inflated (Falk-Petersen, 2005).

The kidney, excretory organ and the primary lymphoid organ in fish, is present at hatch with undifferentiated stem cells in many fish species (Falk-Petersen, 2005). The kidney also consists of two pronephric organs in cod yolk sac larvae, the tubule of each forms several loops anteriorly (above the oesophagus) and the tubules are surrounded by haemopoietic and lymphomyeloid tissue (Morrison, 1987). The pronephric tubules join pronephric ducts which end in a thin-walled urinary bladder (Falk-Petersen, 2005).

The pituitary gland may occur a few days after hatch in some species, but the three parts of the pituitary generally develop by the end of yolk absorption, concomitant with full eye pigmentation (Kjørsvik E., 2004). Thyroid follicles appear functional in some species at hatching, or even before, as in some salmonids. In small pelagic larvae the thyroid follicle is generally apparent, but not necessarily functional, during the first few days after hatch (Falk-Petersen, 2005).

1.3.2.The larval stage

The epidermis is still thin (as at hatch) but numerous chloride cells (not on head) are noted. More cartilage has formed in the head region. The notochord is larger and the actinotrichia of the pectoral fins larger. The larvae have one continuous finfold supported by dermotrichia (unjointed slender horny finrays or actinotrichia), most noticeable in the tail. The pectoral fin is simple and rounded with a central region containing a thin sheet of cartilage (Falk-Petersen, 2005).

There are four blocks of larval muscle; two dorsal and two ventral. The myotomes become progressively more complex in shape as they interdigitate with growth. In the first developmental phases of most teleosts, the myotomes are composed of presumptive white immature fibers surrounded by a monolayer of small embryonic red fibers, these can be more differentiated in some species. The red muscle is initially present as a superficial cylindrical sheath around the body, but it later becomes concentrated in a midlateral strip (Falk-Petersen, 2005).

The otocysts have thicker cartilaginous walls and are divided into three chambers and with three otoliths. The olfactory epithelium is already more depressed and occupies a larger area. Additional paired neuromasts are noted along the lateral line (Morrison, 1987). Lateral line canals develop at the late larval stage. The eyes are larger and the inner plexiform layer, pigment layer and iris more developed (Falk-Petersen, 2005).

A transparent swimbladder is developed in cod larvae at the age of 10 days (Morrison, 1987). After 17-20 days the swimbladder is more elongate and oval and the dorsal surface covered with melanocytes (Morrison, 1987).

In cod larvae an operculum gradually covers the gill slits in the pharynx region and chloride cells proliferate in the posterior part and the floor of the pharynx. Gill arches with cartilaginous rods and branchial artery appear, while no gill filaments are formed yet. In halibut, only gill arch lamellae precursors appear as late as 16 days after hatching (Pittman, et al., 1990).

The oesophagus of cod larvae is initially narrow with longitudinal folds and a circular layer of striated muscle cells. Mucous cells appear later, first in the oesophagus and then in the intestine of larvae. In the midgut columnar epithelial cells with microvilli are noted and in the hindgut the mucosa is narrower but more convoluted and entero-endocrine cells are present (Morrison, 1987). After 11 days the oesophagus epithelium has differentiated and folds have increased in the intestine. After 17 days the gut starts to form a loop and changes in the digestive and absorptive abilities as well as nutritional needs take place. The gut wall is more folded and the epithelial cells of the oesophagus and midgut contain increased numbers of granules (Falk-Petersen, 2005).

The liver is first a rounded mass, the hepatocytes larger than those of the yolk sac larvae. The gallbladder (between liver and pancreas) has a larger lumen and the wall has become thicker than at hatch. The pancreas still has one islet of Langerhans, the exocrine part has extended (Morrison, 1987).

The interrenals, thyroid follicles and pituitary are present and apparently functional near first feeding in marine larvae (Falk-Petersen, 2005).

The lymphoid organs in cod are present, but not functional at the late larval stage, in 9 mm cod larvae (Falk-Petersen, 2005).

1.3.3.Metamorphosis

Larval characters disappear during the transitional period and stomach, pyloric caecae (in many species), calcified skeleton, lateral line, vision, functional gills, final fins and fin rays, thick skin and scales appear and behavioral changes occur (Falk-Petersen, 2005).

At metamorphosis the skin of cod is several layers thick (stratified squamous epithelium), the swimbladder is more elongated and most larvae are found in mid-water (Morrison, 1987). A few teeth are noted on the premaxilla and dentary, a pharyngeal tooth plate is established and teeth are also found along the gill arches. Later the gill arch has two rows of filaments with well-developed lamellae (Falk-Petersen, 2005).

The olfactory epithelium is convoluted in a partly covered pit. The lense is no longer connected to the posterior retina and cones are noted in the visual cell layer (Blaxter, 1988). The swimbladder is elongate with wide lumen, convoluted gas gland and rete mirabile (Morrison, 1987).

Mucous cells are numerous in cod in the oesophagus, the liver is large, small pyloric caecae are observed, the anterior midgut has folded mucosa and the beginning of a stomach are seen as well. The stomach and pyloric caecae develop during metamorphosis (Morrison, 1987). The stomach develops first as a dorsal pouch-like extension in the caudal part of the oesophagus in cod larvae and then increases in size with increasing fish size. Gastric glands are prominent. The pyloric caecae grow proportionally faster than the fish length (Falk-Petersen, 2005).

The pancreas is further extended during metamorphosis (Falk-Petersen, 2005).

Haematopoietic tissue increases substantially in size and concentrates in the cranial and caudal regions (Falk-Petersen, 2005).

The lymphoid organs are fully developed. The number of cells increased during the first four weeks after hatch (Falk-Petersen, 2005).

Progressive development of the rete mirabile in swim bladder takes place (Santamaría, et al., 2004).

2. Materials and methods

2.1. Fish husbandr y

The study of grass morphology and histological development of ballan wrasse, *Labrus bergylta* was conducted at Mørkvedbukta Research Station of Bodø University College, Bodø, Norway. Ballan wrasse eggs were collected from a broodstock kept in Hall 3 at Mørkvedbukta Research Station in July 2009.

The broodstock was wild caught fish from Agder (Sørlandet, Norway) adapted to captivity. Females were kept in captivity for one year, and males were caught in Agder and transferred to the tanks in June 2009. Each of the tanks was set with a numbers of females range from 10 to 20 and 1 – 2 males. The broodstock were kept under natural photoperiod conditions in big (5000 l) round and small (2 × 2 m) square tanks. Temperature was around 8°C during the year and increased from June to 11°C in some tanks during the spawning season. Salinity was 34 ‰, oxygen level 8.3 mg l^{-1}. The tanks were equipped with pieces of plastic pipes (30 – 50 cm length, Ø = 90 – 110 mm), serving as shelters or houses for the fish. In addition, artificial seaweeds made from black plastic bags were put in the tank, serving as shelters. The fish were fed to apparent satiation 3 times per week with a feed composed of shrimps, fish meal and fish oil.

2.2. Collection and incubation of eggs

The ballan wrasse has benthic eggs. The eggs were transparent and sticky and were difficult to observe and collect from the bottom. Curved plates made of transparent plastic were used as a spawning substrate, and most of the eggs were spawned on these plates.

The tanks were inspekted daily during spawning season. Spawning occurred usually in the morning, between 9 and 11 a.m. Eggs were checked for fertilization ratio at the collection from the tank.

Plates with spawned fertilized eggs were transferred to 350 l incubators with 12°C temperature and the water flow rate 3 l min^{-1} and incubated in darkness. The hatching was examined in 72°D (6 days in 12°C).

2.3. Larvae rearing

First larvae started to hatch under the microscope observation, when eggs were checked on stage of development. Main hatching started during eggs transportation to the rearing hall. Probably, mechanical, oxygen or light stress induced hatching process. Hatching was not synchronic and took about 2 days.

Newly hatched larvae were transferred in 100-l polyethylene tanks with flow rate of water 200 ml min^{-1} and gradually increased to 600 ml min^{-1} at day 7. The experiment was carried out

under an artificial photoperiod. Photoperiodicity was 18 h light (1100 lux), 6 h total darkness. The water was gently aerated from the bottom of the tank. The water temperature was variable (11-14.5°C), with overage temperature 13.9ºC ± 0.9ºC.

Larvae were first fed from 4-5 days after hatching (DAH) with cultured rotifers *Brachionus plicatilis* (5-7 ind ml^{-1}) enriched with red pepper. Algae (*Chlorella* sp., 20 ml/8 l of rotifers) were added to the rotifers before they were distributed to the first feeding tanks. Rotifers were added to the tanks three times a day. *Artemia* (about 1 ind ml^{-1}, also enriched with red pepper) was introduced to the larvae at 20 day. Rotifers were given until day 27, so that co-feeding was performed for 7 days.

2.4. Sampling

All ballan wrasse larvae were prepared for morphological investigations and light microscopy (LM) at Mørkvedbukta Research Station of Bodø University College, Bodø, Norway.

Larvae were taken from tank in first-feeding Hall 6 with conditions described before. Larvae were sampled daily between 0 and 9 DAH and every 4 days between 9 and 49 DAH, and anesthetized in 70 mg/l tricaine methanesulfonate (MS-222, Sigma). Each sample contained 4-6 larvae.

Anesthetized larvae were fixed in a mixture of 10% paraformaldehyde and 25% glutaraldehyde in cacodylate buffer (pH 7.2) (Appendix 1) and stored at 12°C until subsequent inquiry.

Sampling data, day after hatching, day-degree was noted for each sample. Because the chronological age of the larva does not necessarily indicates its physiological age, which depends mainly on water temperature (Blaxter, 1988), the larvae were staged on the basis of external morphological features and histological observations were related to these stages and day-degree calculation.

2.5. Gross morphology

Fixed larvae were staged on the basis of main external morphological features.

Larvae were viewed and photographed using stereomicroscope with digital camera Olympus (Olympus, Tokyo, Japan). Each fish was measured for morphometric characteristics using Cell^A software.

Standard length was measured from the tip of the snout to the end of notochord for preflexion larva (Santamaría, et al., 2004) or to the end of the vertebral column after flexion (Chen, et al., 2006) to the nearest 0.01 mm on each sampling day.

Myotome height was measured along a line set across the notochord in 90° angle. The ventral position of the line was placed where the posterior wall of the urinary bladder meets notochord (Skålsvik, 2008).

Description of pigmentation of newly-hatched, yolk-sac and on-growing larvae were made according to the definitions of body regions (Fig. 1).

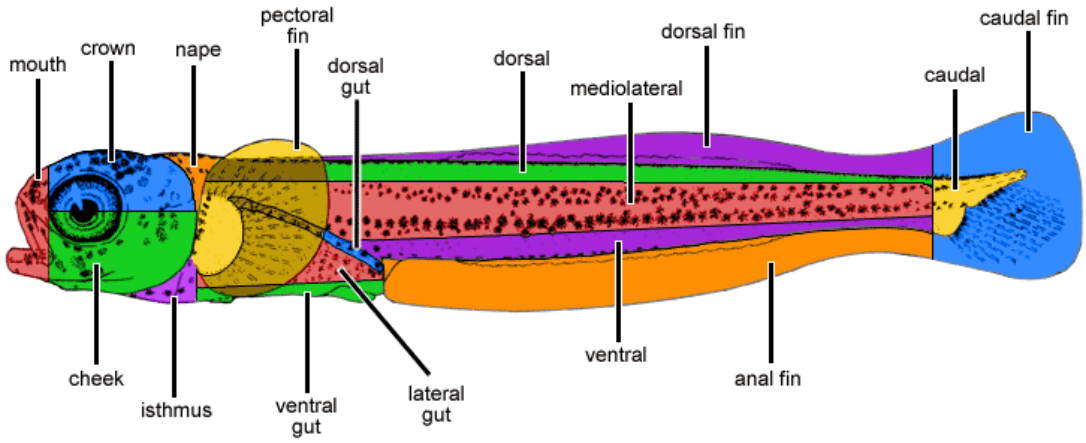

Fig. 1. Pigmentation region definitions of fish larva (http://access.afsc.noaa.gov/ichthyo/PigmentationDef.cfm).

2.6. Larval growth

For the mathematical and statistical analysis of larval growth mean of standard length was calculated ± standard deviation (SD) for each day.

Growth in length was assessed by measuring the absolute growth rate (AGR) as mm day^{-1} (Hopkins, 1992). Equation used was:

$$AGR_L = \frac{L_f - L_i}{t},$$

where L_f - the mean SL (mm) of the sample at the end of the each developmental stage, L_i - the mean SL at the end of the previous stage,

t – the length of the stage (days).

The same principle of AGR was used for myotome height (MH) as mm day^{-1} calculation. Equation used was:

$$AGR_H = \frac{H_f - H_i}{t},$$

where H_f - the mean MH (mm) of the sample at the end of the each developmental stage, H_i - the mean MH (mm) at the end of the previous stage,

t – the length of the stage (days).

2.7. Histology sample sectioning

Longitudinal sections of fixed larvae with a thickness of 3 µm were processed using a tissue processor (Shandon Citadel 2000, Thermo Electron Corp., Pittsburg, PA, USA) and embedded in paraffin wax by a paraffin dispenser (Tissue-Tec Wax-Dispenser WD-4, Kunz instruments A/S, Copenhagen, Denmark). The longitudinal sections were cut on a Shandon Finesse ME microtome (Shandon Lipshaw, Pittsburgh, PA, USA).

The longitudinal sections with thickness of 3 µm were stained with haematoxylin and eosin (H&E) (Appendix 2) for general staining and with AB-PAS (pH 2.5) (Appendix 3) to visualize neutral and acid glycoproteins.

Photographs from the sections were captured using a digital camera Olympus DP71 (Olympus, Tokyo, Japan) connected with Olympus BX51 microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Ballan wrasse growth

Changes in SL and MH of ballan wrasse larvae during their first 49 days of life are shown in Figure 2. During this period, fish growth was not uniform. Newly hatched larvae measured 3.64±0.05 mm, reaching 10.52±0.82 mm by the end of the study. The highest AGR_L values were obtained during stage 4, and lowest during stages 2 and 3 (Appendix 4). But the AGR_H increased from stage to stage (Appendix 4).

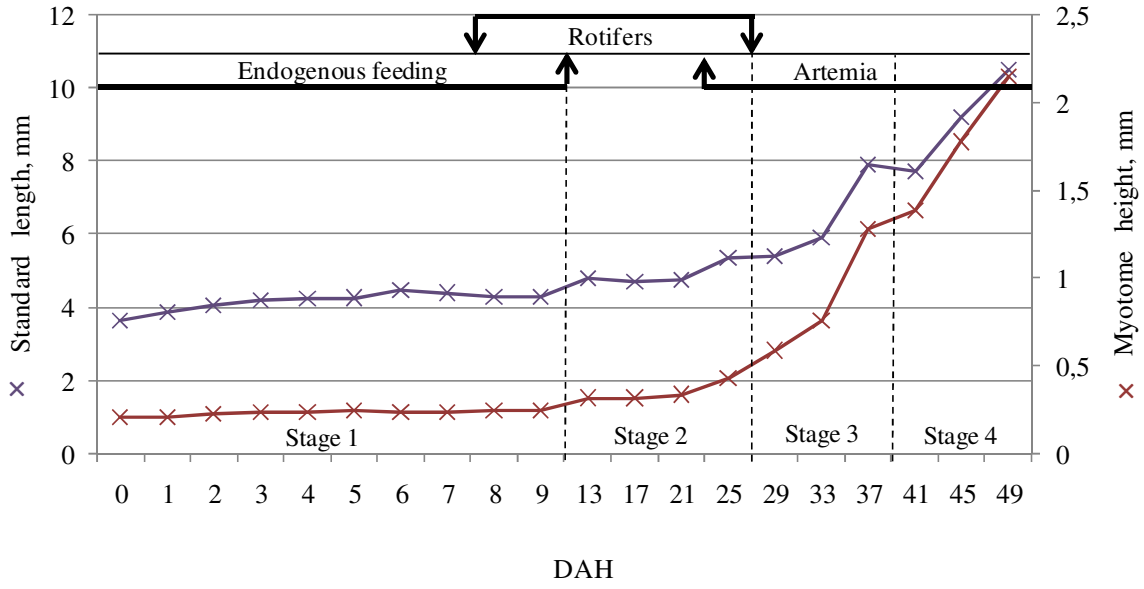

Fig. 2. Growth of *L. bergylta* larvae during first 49 after hatching. The main developmental stages and the larval feeding schedule are indicated.

During first 49 of life fish growth followed an exponential curve (r^2=0.8).

3.2. Morphological aspects of ballan wrasse development

Larvae were viewed under a light microscope, staged on the basis of main external morphological features and source of food. According to the source of food, the external morphological features, and the structural changes, four main stages were established during *L. bergylta* larval development (Table 1).

Table 1. Main developmental stages of ballan wrasse larvae defined on the basis of external morphological observations

Stage	External morphological observations	DAH	d°	T, °C (mean ± SD)	Food source
1	*Yolk sac larva* Mouth closed, eyes not pigmented.	0-6	10.5-71	11.83±1.13	exclusively endogenous
	Opening of the mouth, pigmentation of the eyes.	7-9	84.8-108	13.83±0.15	endo- and exogenous
2	*Preflexion larva* YS disappear. Inflated swim bladder became visible. Initial formation of caudal fin rays.	10-25	122.1-340.7	14.26±0.14	exclusively exogenous
3	*Flexion larva* Initial resorption of primordial finfold.	26-33	355-456.4	14.46±0.07	exclusively exogenous
4	*Postflexion larva* Dorsal, anal, caudal and pelvic fins develop.	34-49	470.8-685.7	14.33±0.08	exclusively exogenous

Stage 1. Yolk-sac larva.

Lecitotrophic period.

This period started from larvae hatching. The embryo freed itself from the egg membrane with rapid movements of the body and tail. The newly hatched larva (Fig. 3) was 3.64±0.05 mm long, has 18 myomeres. Larvae were transparent and floating at the surface with ovoid and unsegmented yolk-sac uppermost and sometimes in a lateral position. Head was pointed forwards and partly free from the yolk. The fin-fold invested much of the body and was subdivided in preanal fold, dorsal, caudal and postanal parts. The mouth was undeveloped.

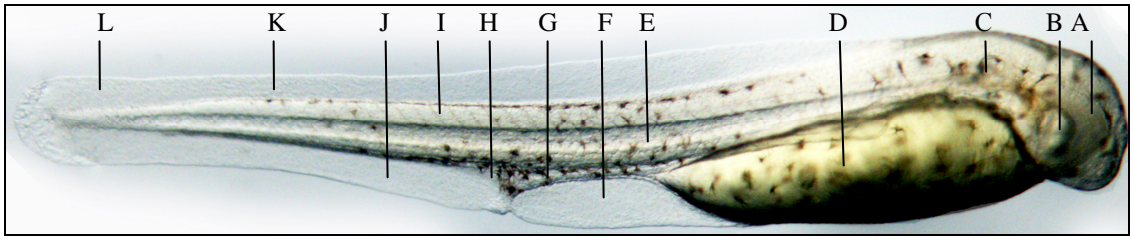

Fig. 3. Light microscopy of *L.bergylta* larva 0 DAH, 3.64 ± 0.05 mm SL. A, eye; B, lens; C, otic capsule; D, yolk sac; E, notochord; F, preanal fin fold; G, gut; H, urinary bladder; I, myotome; J, postanal part of the fin fold; K, dorsal part of the fin fold; L, caudal part of the fin fold.

Head pigment was restricted to crown region. Body was heavily pigmented with chromatophores from posterior of the head to approximately the eight post-anal segment and with melanophores on the yolk sac.

The large eyes were free of pigments and supposed to be non-functional. Otic capsules were seen.

At 3 DAH yolk sac became smaller and dorsal swimming pattern became much higher. The pigmentation was more concentrated in yolk sac and body. Black pigment was well-distinguished in the eyes (Fig. 4).

Fig. 4. Light microscopy of *L.bergylta* larva 3 DAH, 4.18 ± 0.06 mm SL. A, eye with pigment; B, resorption of yolk sac; C, pigment cells.

By the end of the stage the black pigments in eyes has gradually been increased and the eyes were black. Mouth began to open. The body and notochord were straight.

Lecitoexotrophic period.

At 7 DAH resorption of yolk sac was proceeding. Eyes were completely pigmented and became functional, so the larva could find and orientate on food particles. The mouth was fully opened and functional. Larvae were ready for exogenous feeding. But a small yolk sac was still present, so larvae at first was not totally dependent on food from outside. Initial pigmentation of ventral gut and postanal fin regions of finfold began (Fig. 5).

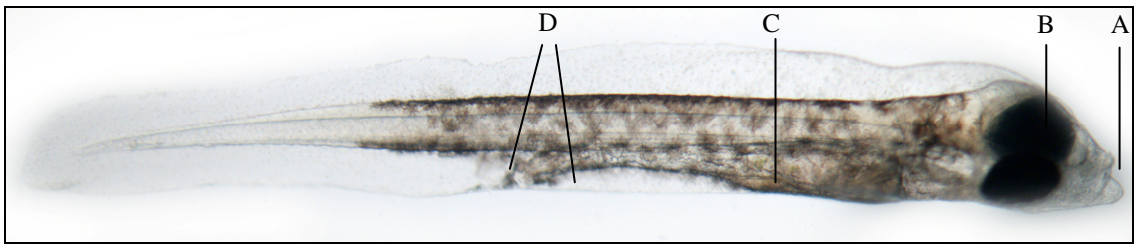

Fig. 5. Light microscopy of *L.bergylta* larva 7 DAH, 4.40 ± 0.15 mm SL. A, open mouth; B, pigmented eyes; C, rest of the yolk sac; D, pigment cells.

Moment of first exogenous feeding was noticed. Passage of food along the digestive tract and rotifers in the stomach of the larvae were clearly visible.

To 9 DAH larva still had some of the yolk left. Melanophores concentrated on nape, crown, isthmus regions and the trunk. Ventral gut region and 1/3 of anal fin region were also pigmented (Fig. 6).

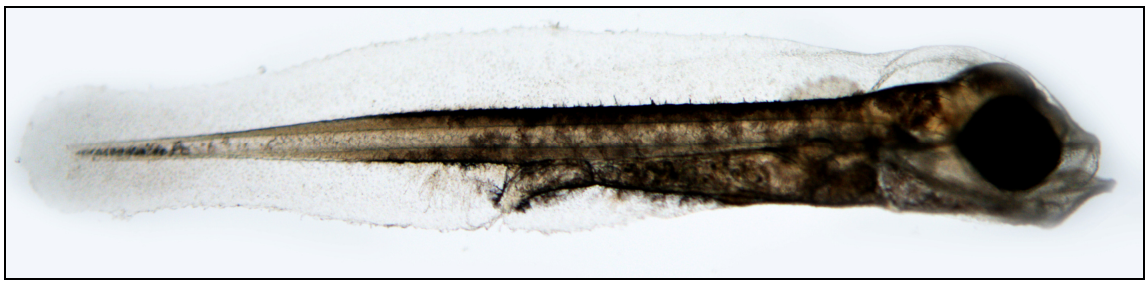

Fig. 6. Light microscopy of *L.bergylta* larva 9 DAH, 4.28 ± 0.11 mm SL.

Stage 2. Preflexion larva.

Exotrophic period.

During this developmental stage, the swim bladder was developing and it was filled with air by 13 DAH (Fig. 7).

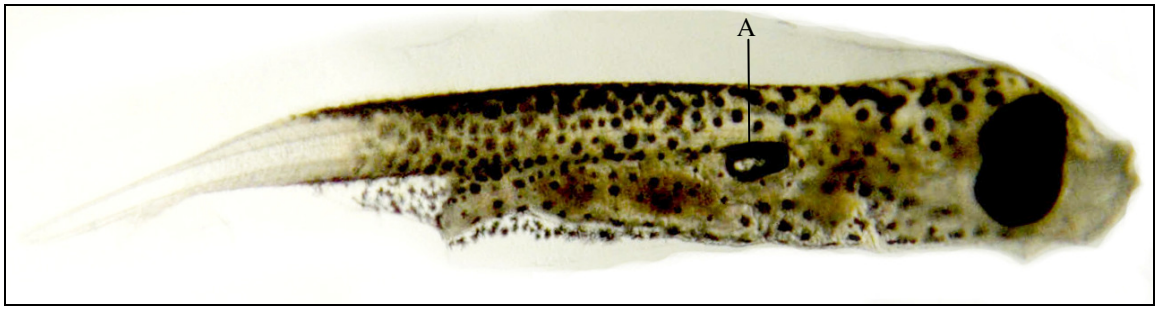

Fig. 7. Light microscopy of *L.bergylta* larva 13 DAH, 4.78 ± 0.19 mm SL. A, inflated swim bladder.

At 25 DAH caudal fin rays anlage appeared. Head, body, fins were heavily pigmented. Melanophores concentrated on 2/3 of the anal fin region (Fig. 8).

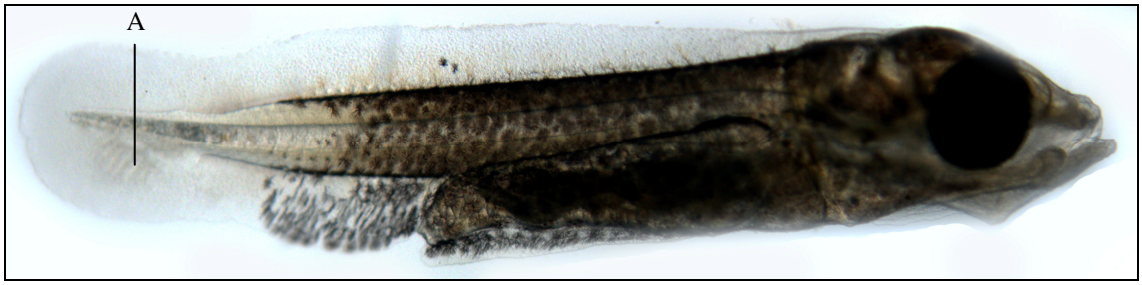

Fig. 8. Light microscopy of 7. *L.bergylta* larva 25 DAH, 5.35 ± 0.30 mm SL. A, caudal fin rays anlage.

Stage 3. Flexion larva. The upward inclination of the posterior part of urostyle presented (Fig. 9). At 29 DAH the small pelvic fins were visible.

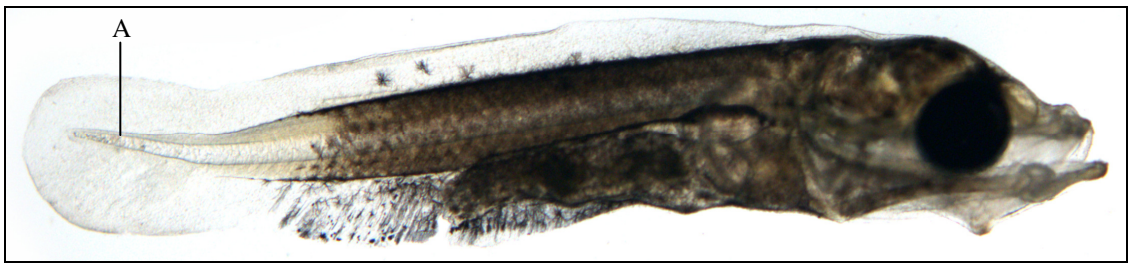

Fig. 9. Light microscopy of *L.bergylta* larva 29 DAH, 5.40 ± 0.66 mm SL. A, inclination of urostyle.

To the end of the stage the caudal fin starts to be separated from the dorsal fin as a discontinuity in the margin of the finfold. Anlage of the dorsal fin and the incipient rays of anal fin were visible (Fig. 10).

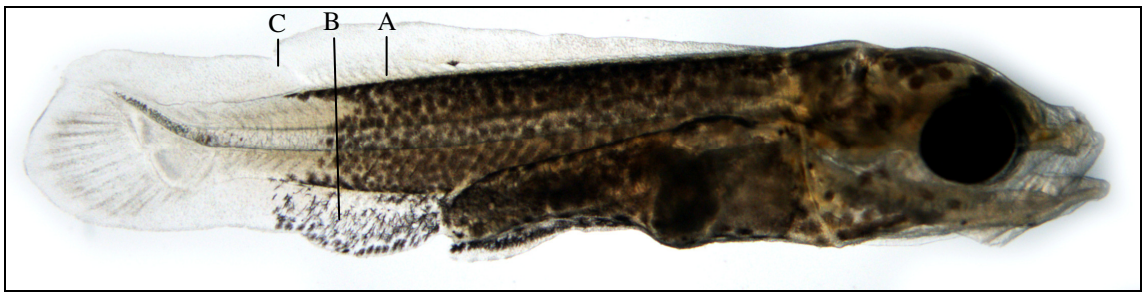

Fig. 10. Light microscopy of *L.bergylta* larva 33 DAH, 5.90 ± 0.78 mm SL. A, anlage of the dorsal fin; B, incipient rays of anal fin; C, discontinuity in the finfold.

Stage 4. Postflexion (transition) larva. By 37 DAH caudal, dorsal and anal fins were separated. Anal fin was pigmented and body coloration was also spread to the end of anal fin. All fin rays were formed (Fig. 11).

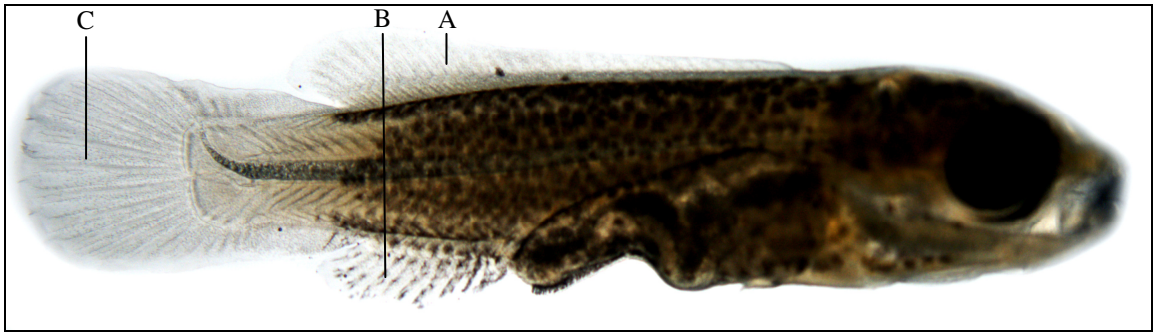

Fig. 11. Light microscopy of *L.bergylta* larva 37 DAH, 7.90 ± 0.41 mm SL. A, dorsal fin; B, anal fin; C, caudal fin.

To the end of stage larval body was fully pigmented, except urostyle region (Fig. 12).

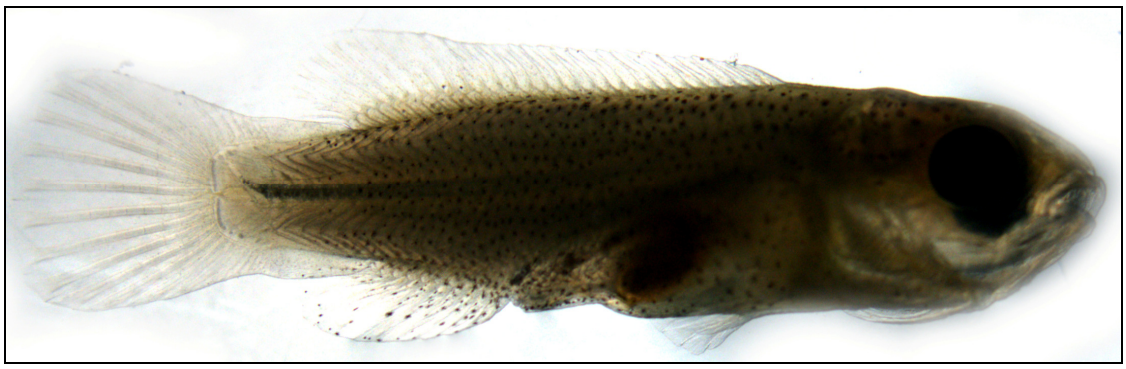

Fig. 12. *L.bergylta* larva 49 DAH, 10.52 ± 0.82 mm SL.

3.3. Histological aspects of ballan wrasse larvae development

3.3.1. Endogenous reserves

At hatching larvae showed a homogenous yolk sac matrix started with initial fragmentation due resorbtion (Fig. 13). The yolk was surrounded by a simple epithelium or syncytical layer of squamous cells, called periblast or vitelline envelope (Fig. 13, 40 a). This periblast layer formed the walls of the yolk sac.

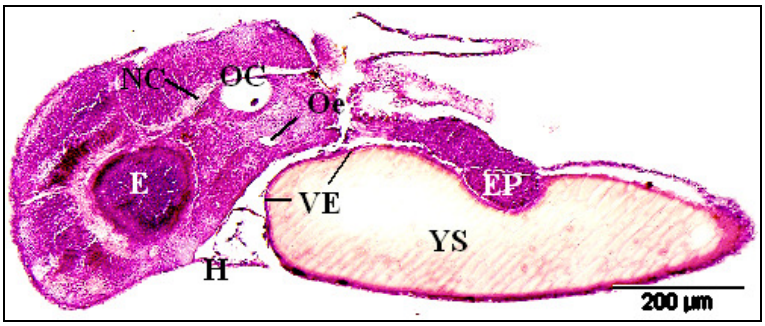

Fig. 13. Longitudinal section of 0 DAH ballan wrasse larvae (HE, × 10). E, eye; EP, exocrine pancreas; H, heart; NC, nervous cord; OC, otic capsule (with otholit); Oe, oesophagus; V, vitelline envelope; YS, yolk sac.

During the yolk resorption, yolk sac matrix showed appearance of the resorption granules at the periphery (Fig. 38).

Lecitoexotrophic period of the yolk sac stage started at 7 DAH with the moment of first feeding, when mouth and anus were opened, and this period was characterized by the coexistence of endogenous (yolk sac) and exogenous (rotifers) food sources. Resorption of endogenous resources proceeded. At 9 DAH the yolk sac presented as two separated parts (Fig. 19) fragmented into small round particles.

The yolk sac was completely resorbed by the beginning of preflexion stage at 10 DAH when exotrophic period began.

3.3.2.Digestive system

At hatching the digestive tract appeared as a straight undifferentiated tube attached dorsally to the yolk sac and lined by a single layer of epithelial cells (Fig. 14). Posterior part of the digestive tract was bended ventrally, mouth and anus were closed. The digestive tract lumen was narrow with a tendency to widen at posterior end.

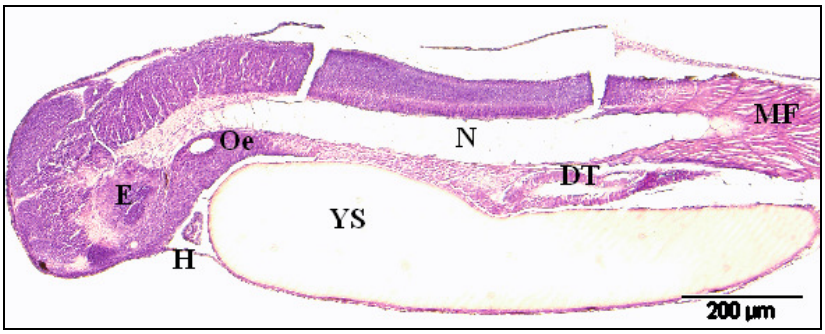

Fig. 14 . Longitudinal section of 0 DAH ballan wrasse larvae (HE, × 10). DT, digestive tract; E, eye; H, heart; MF, muscular fibres; N, notochord; Oe, oesophagus; YS, yolk sac.

At the 3 DAH lumen in the hindgut region became wider, the anal pore was opened (Fig. 15 b), the mouth and the pharynx started to be opened (Fig. 49 b) and were fully opened by 4 DAH (Fig. 16).

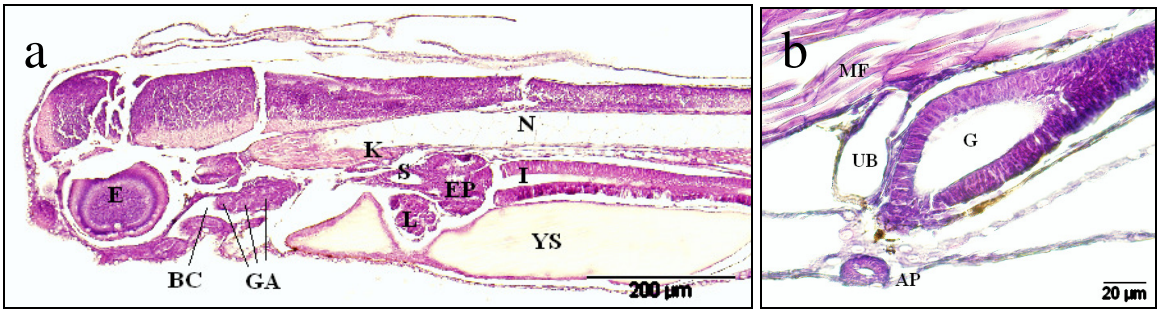

Fig. 15. Longitudinal section of ballan wrasse larvae 3 DAH. (a) Digestive system(HE, × 10). (b) Excretory system (HE, × 60). AP, anal pore; BC, buccopharyngeal cavity; E, eye; EP, exocrine pancreas; G, gut; GA, gill arches (anlage); I, intestine; K, kidney; L, liver; MF, muscular fibres; N, notochord; S, stomach; UB, urinary bladder; YS, yolk sac.

The formation of the intestinal valve on 4 DAH divided the incipient intestine into midgut and hindgut. Oesophagus and stomach were also distinguished (Fig. 16).

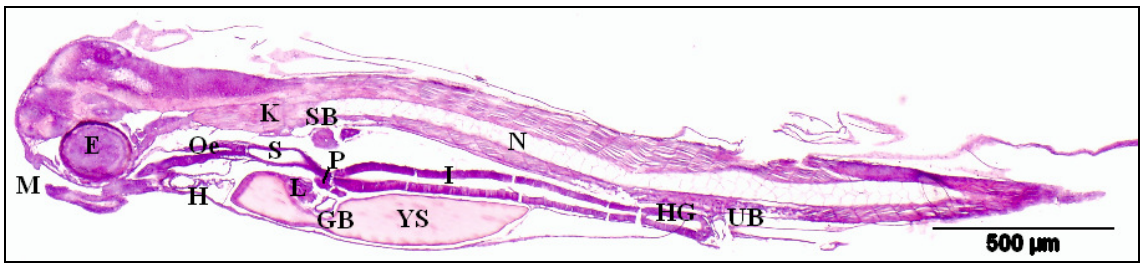

Fig. 16. Longitudinal section of ballan wrasse larvae 4 DAH (HE, × 10). E, eye; GB, gall bladder; H, heart; HG, hindgut; I, intestine; K, kidney; L, liver; M, mouth; N, notochord; Oe, oesophagus; P, exocrine pancreas; S, stomach; SB, swim bladder; UB, urinary bladder; YS, yolk sac.

After beginning of mouth opening on 5 DAH the lumen in the intestine became wider (Fig. 17).

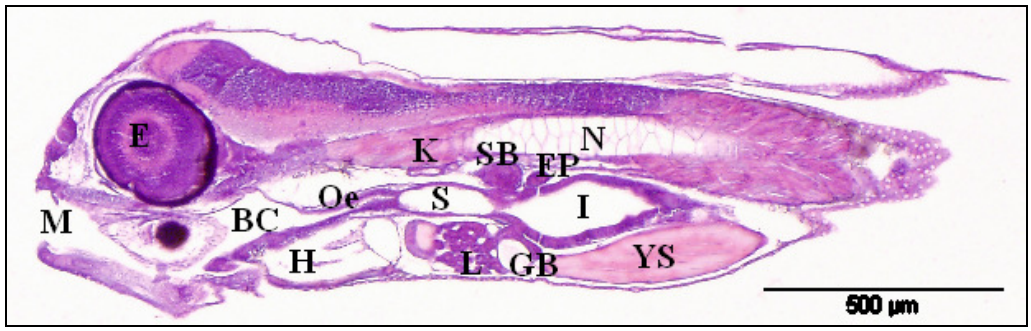

Fig. 17. Longitudinal section of ballan wrasse larvae 5 DAH (HE, × 4). BC, buccopharyngeal cavity; E, eye; EP, exocrine pancreas; GB, gall bladder; H, heart; I, intestine; K, kidney; L, liver; M, mouth; N, notochord; Oe, oesophagus; S, stomach; SB, swim bladder; YS, yolk sac.

Folds in the posterior part of midgut and in the hindgut started to form at 7 DAH, when the first digested rotifers in the midgut were observed (Fig. 18).

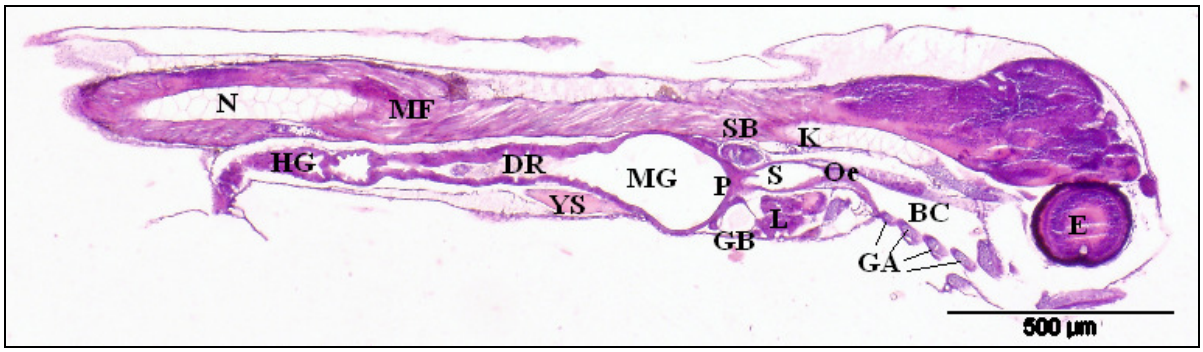

Fig. 18. Longitudinal section of ballan wrasse larvae 7 DAH (HE, × 4). BC, buccopharyngeal cavity; DR, digested rotifers; E, eye; P, exocrine pancreas; GA, gill arches; GB, gall bladder; HG, hindgut; K, kidney; L, liver; MF, muscular fibres; MG, midgut; N, notochord; Oe, oesophagus; S, stomach; SB, swim bladder; YS, yolk sac.

By 9 DAH brush border first appeared in the intestine and digested rotifers were seen along the whole digestive tract (Fig. 19). By 13 DAH first AB-positive cells appeared in the stomach.

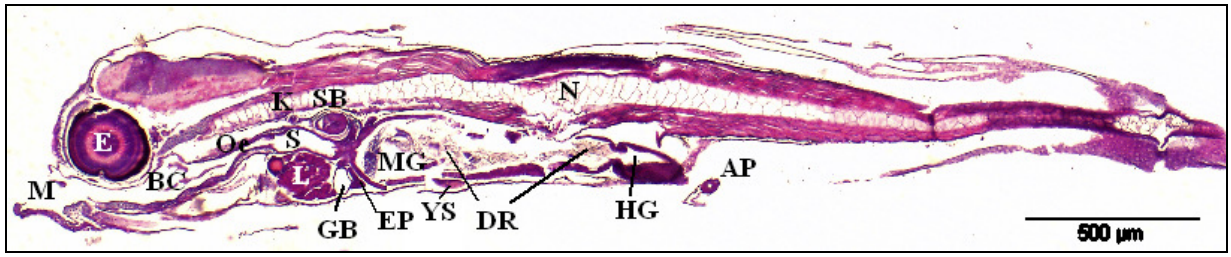

Fig. 19. Longitudinal section of ballan wrasse larvae 9 DAH (HE, × 4). AP, anal pore; BC, buccopharyngeal cavity; DR, digested rotifers; E, eye; EP, exocrine pancreas; GB, gall bladder; HG, hindgut; K, kidney; L, liver; M, mouth; MG, midgut; N, notochord; Oe, oesophagus; S, stomach; SB, swim bladder; YS, yolk sac.

Formation of evident intestinal folds, granules and brush border in the midgut epithelium were distinguished at 17 DAH (Fig. 20, 21).

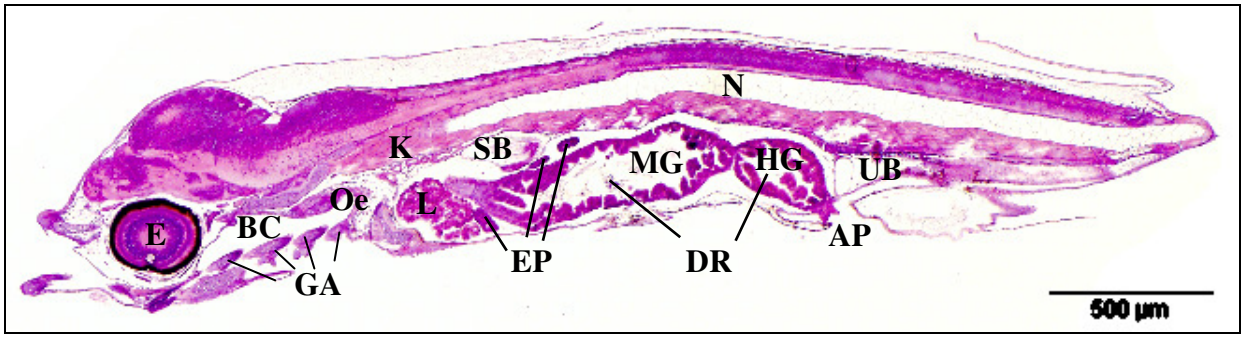

Fig. 20. Longitudinal section of ballan wrasse larvae 17 DAH (HE, × 4). AP, anal pore; BC, buccopharyngeal cavity; DR, digested rotifers; E, eye; EP, exocrine pancreas; HG, hindgut; K, kidney; L, liver; MG, midgut; N, notochord; Oe, oesophagus; SB, swim bladder; UB, urinary bladder.

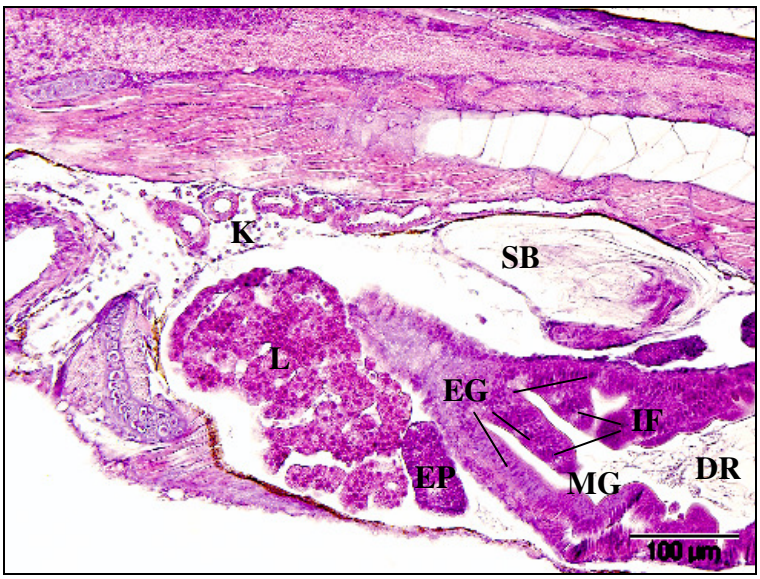

Fig. 21. Longitudinal section of the ballan wrasse larvae digestive tract at 17 DAH (HE, × 20). DR, digested rotifers; EG, epithelial granules; EP, exocrine pancreas; IF, intestinal folds; K, kidney; L, liver; MG, midgut; SB, swim bladder.

From the beginning of the co-feeding period at 25 DAH, only rotifers were digested in the intestine, but undigested artemia were observed in the digestive tract (Fig. 22). Brush border in the midgut shown PAS-positive staining (Fig. 23).

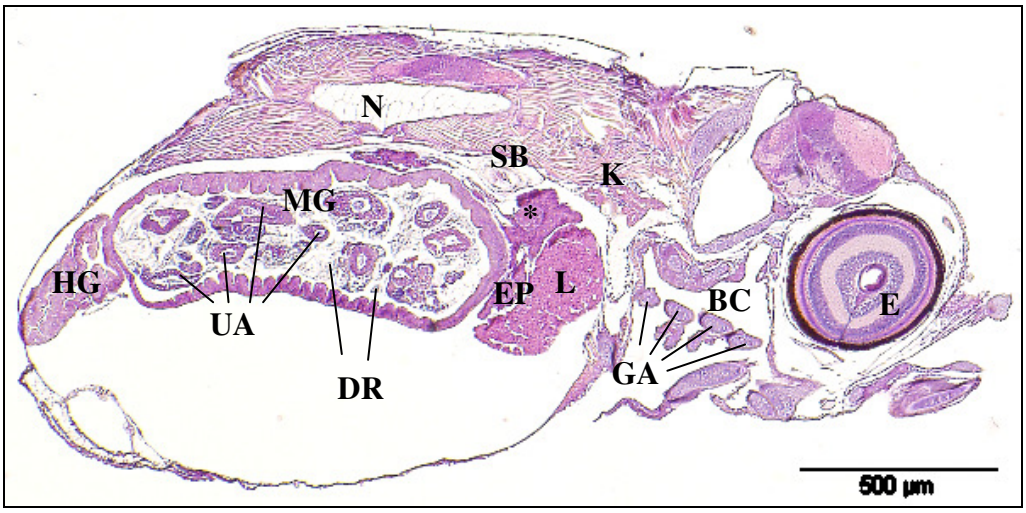

Fig. 22. Longitudinal section of ballan wrasse larvae 25 DAH (HE, × 4). BC, buccopharyngeal cavity; DR, digested rotifers; E, eye; EP, exocrine pancreas; HG, hindgut; K, kidney; L, liver; MG, midgut; N, notochord; Oe, oesophagus; SB, swim bladder; UA, undigested artemia; *, islet of Langerhans among the exocrine pancreas tissue.

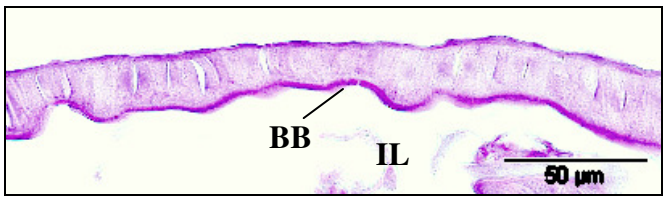

Fig. 23. Longitudinal section of the ballan wrasse larvae intestinal wall with brush border at 25 DAH. (AB-PAS, × 60). BB, brush border; IL, intestinal lumen.

At 29 DAH partly digested artemia were seen in the midgut. Numerous folds in the midgut and hindgut were observed (Fig. 24), where several AB-positive cells presented in the epithelial tissue (Fig. 25).

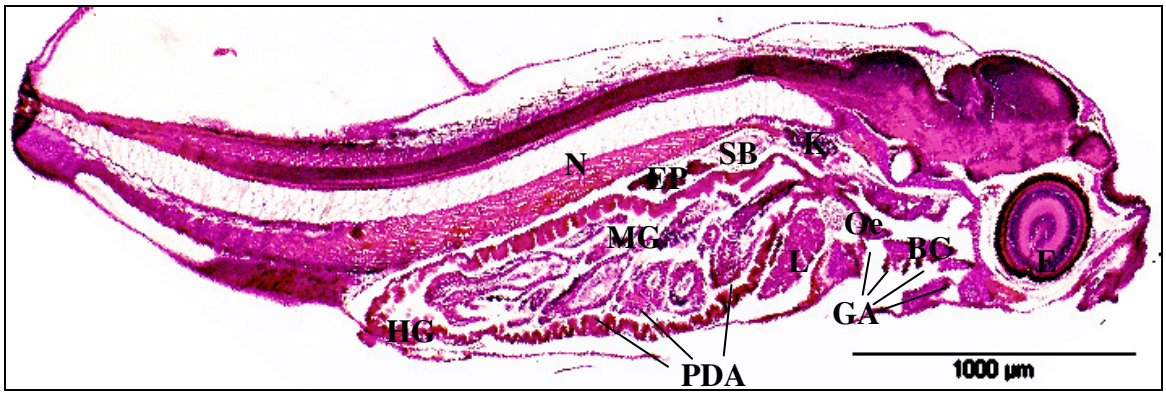

Fig. 24. Longitudinal section of ballan wrasse larvae 29 DAH (HE, × 4). BC, buccopharyngeal cavity; EP, exocrine pancreas; GA, gill arches; K, kidney; L, liver; MG, midgut; N, notochord; Oe, oesophagus; PDA, partly digested artemia; SB, swim bladder.

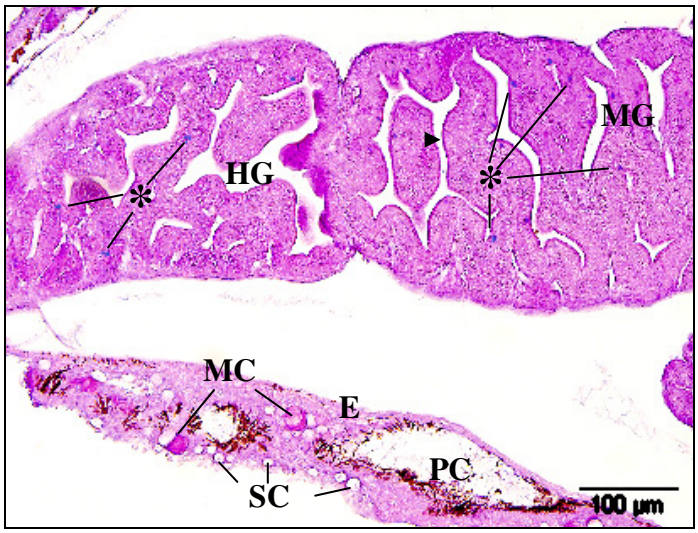

Fig. 25. Longitudinal section of ballan wrasse larvae digestive tract at 29 DAH (AB-PAS, × 20). E, epidermis; HG, hindgut; MC, mucous cell; PAS-positive mucous cells; MG, midgut; PC, pigment cell; SC, saccular cells; *, AB-positive cells.

First digested Artemia were observed in the posterior part of the midgut at 33 DAH (Fig. 26) and in the stomach at 37 DAH.

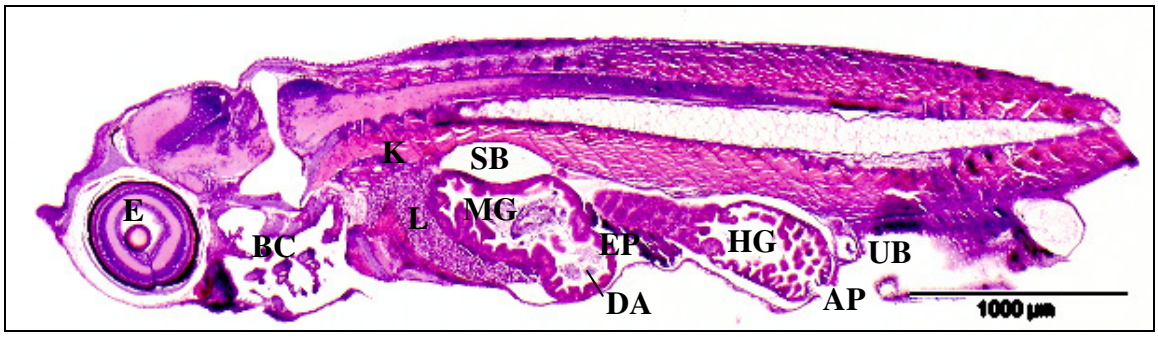

Fig. 26. Longitudinal section of ballan wrasse larvae 33 DAH (HE, × 2). AP, anal pore; BC, buccopharyngeal cavity; DA, digested artemia; E, eye; EP, exocrine pancreas; G, gut; HG, hindgut; K, kidney; L, liver; MG, midgut; N, notochord; SB, swim bladder, UB, urinary bladder.

Oesophagus

At hatching oesophagus was a small simple tube similar to incipient gut, closed digestive tract before the formation of the buccopharyngeal cavity (Fig. 13, 14). It was lined by a single layer of simple epithelium. No mucous cells were present.

On 3 DAH, the posterior oesophagus expanded to form the stomach (Fig. 15 a). At 4 DAH oesophagus lumen connected the buccopharyngeal cavity and intestine (Fig. 16).

Some mucous cells showed PAS-positive staining at first feeding on 7 DAH while first AB-positive mucous cells appeared at 13 DAH (Fig. 27). Mucous cells increased in number during following development (Fig. 29).

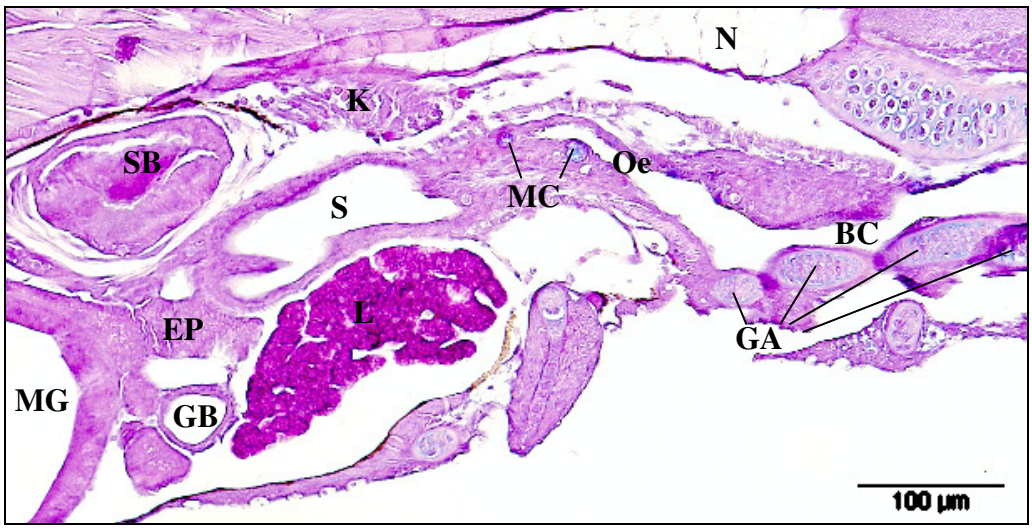

Fig. 27. Longitudinal section of ballan wrasse larvae digestive system at 13 DAH (AB-PAS, × 20). BC, buccopharyngeal cavity; EP, exocrine pancreas; GA, gill arches; GB, gall bladder; K, kidney; L, liver; MC, mucous cells; MG, midgut; N, notochord; Oe, oesophagus; S, stomach; SB, swim bladder.

At 17 DAH formation of taste bud in the oesophagus was noticed (Fig. 28).

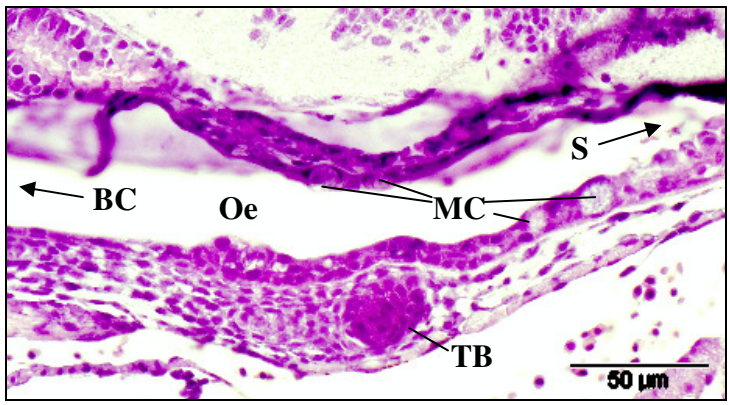

Fig. 28. Longitudinal section of the 17 DAH ballan wrasse larva oesophagus (HE, × 40). BC, buccopharyngeal cavity; MC, mucous cells; Oe, oesophagus; S, stomach; TB, taste bud.

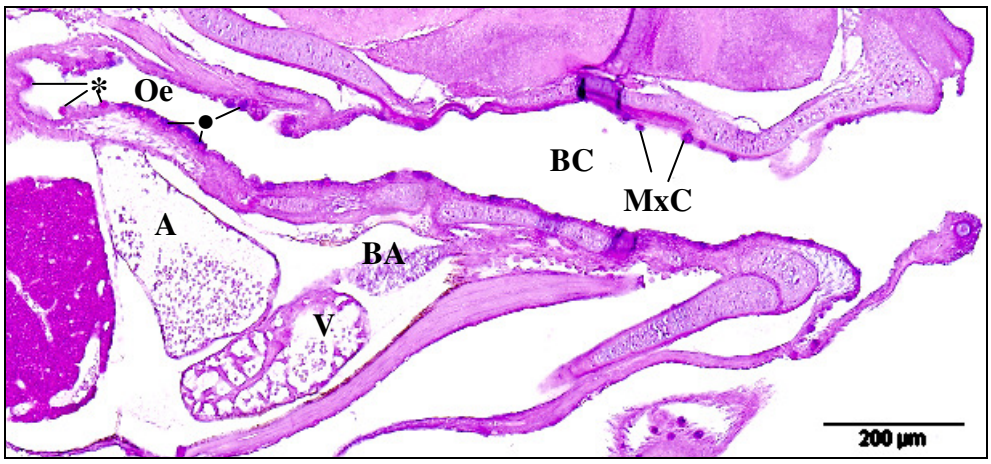

Fig. 29. Longitudinal section of the ballan wrasse larva head region at 29 DAH (AB-PAS, × 10). A, atrium; BA, bulbus arteriosus; BC, buccopharyngeal cavity; MxC, mucous cells, showing mixed staining; Oe, oesophagus; V, ventricle; *, PAS-positive mucous cells; ●, AB-positive mucous cells.

3.3.3.Skin

The skin of newly hatched larvae was well-developed. The number of cell layers in epidermis varied from 1 to 2. Epithelial, saccular and few mucous cells were found in the epidermis by LM.

The newly hatched larva presented melanophores in the skin, under the skin, at the head region and around the yolk sac. A thin layer of pigment cells was seen outside the eyes.

At 1 DAH numerous mucous cells were observed in the skin of the larvae. The cells could be distinguished from others by PAS staining reactions. Dye stained the content of mucous cells in purple colour. The cells were of oval or round shape with round or irregular compactly distributed globules (Fig. 30).

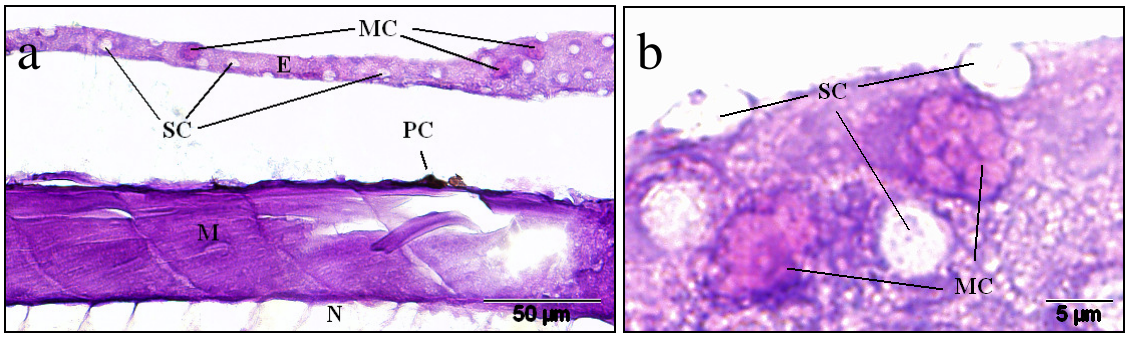

Fig. 30. Longitudinal section of the ballan wrasse larvae 1 DAH of the body dorsal part. (a) General structure of epidermis (AB-PAS, × 40). (b) Mucous and saccular cells in the epidermis (AB-PAS, × 60). E, epidermis; MC, mucous cells; M, myotome; N, notochord; PC, pigment cell – melanophore; SC, saccular cells.

During larval development pigmentation became more intense.

First mucous cells with PAS-positive staining in the gill opening and pharynx appeared at 29 DAH (Fig. 31 a). First AB-positive mucous cells in the gill opening were observed by 33 DAH (Fig. 31 b). From this time amount of AB-positive mucous cells in the gill opening increased (Fig. 31 c, d).

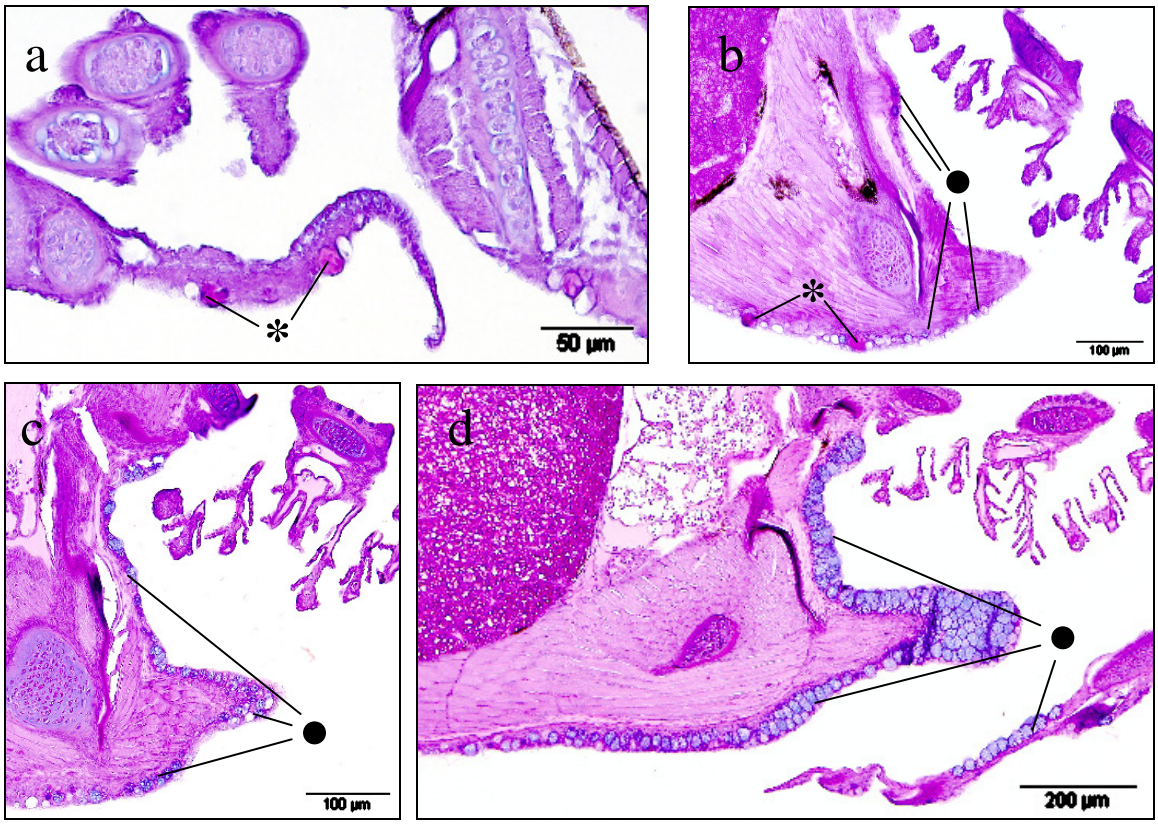

Fig. 31. Mucous cells in the gill opening of ballan wrasse larva (AB-PAS). (a) Larva at 29 DAH (× 40).(b) Larva at 33 DAH (× 20). (c) Larva at 37 DAH (× 20). (d) Larva at 41 DAH (× 10). *, PAS-positive mucous cells; ●, AB-positive mucous cells.

3.3.4.Muscular structure and notochord

The skeletal muscular fibres were arranged in V-shaped myomers on either side of the multicolumnar notochord. The notochord was formed of vacuolated cells separated by thin cell membranes.

3.3.5.Sensory and nervous system

Eyes

The newly hatched larva had immature, unpigmented eyes (Fig. 32). The following layers were distinguished in the eye: lens, ganglion layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, photoreceptor layer. The lens was spherical. Optic nerves were recognized.

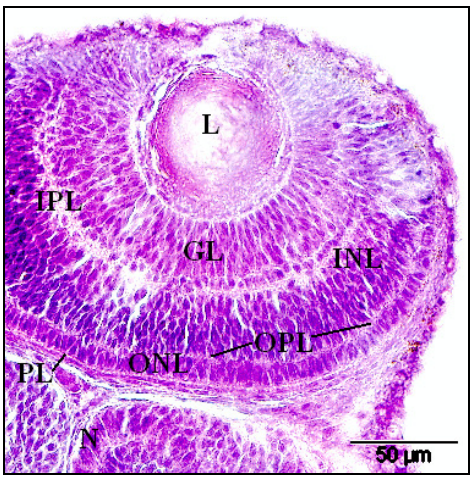

Fig. 32. Eye in newly hatched larva (HE, × 40). G, ganglion layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; N, nerves; ONL, outer nuclear layer; OPL, outer plexiform layer; PL, photoreceptor layer.

Pigmentation of the eyes started at 1 DAH and was completed by 7 DAH.

Inner ear

Newly hatched larvae had only otic capsules in which otolith was recognized (Fig. 33 a). Ear semicircular canals were seen at 3 DAH. At the 5 DAH sensory cells and tissue dividing otocysts into chambers were seen in the otic capsule (Fig. 33 b). During larval development canals ossified and increased in size.

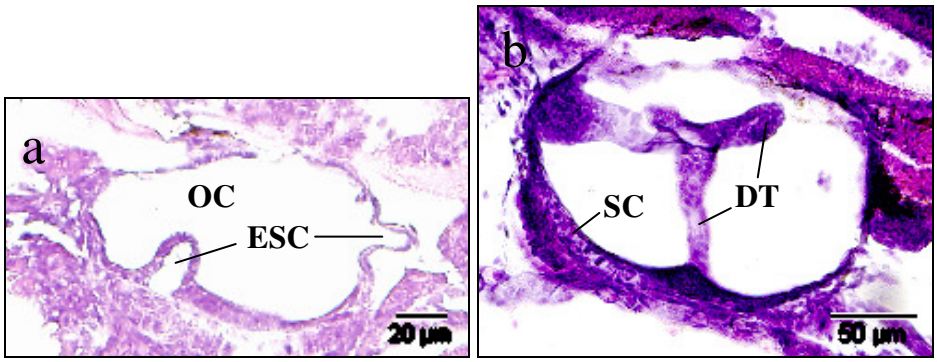

Fig. 33. Development of ballan wrasse larva inner ear (HE). (a) Larva at 3 DAH (× 20). (b) Larva at 5 DAH (× 40). DT, dividing tissue; ESC, ear semicircular canals; OC, otic capsule; SC, sensory cells.

Olfactory organ

Olfactory pits were closed at hatching. No evidence of olfactory epithelium or bulb was recognized. At 1 DAH bilateral regions of the olfactory epithelium fused with olfactory bulbs were distinguished anterior to the eyes (Fig. 34 a). Started from 3 DAH the olfactory bulbs, separated from the olfactory lobes, were seen (Fig. 34 b).

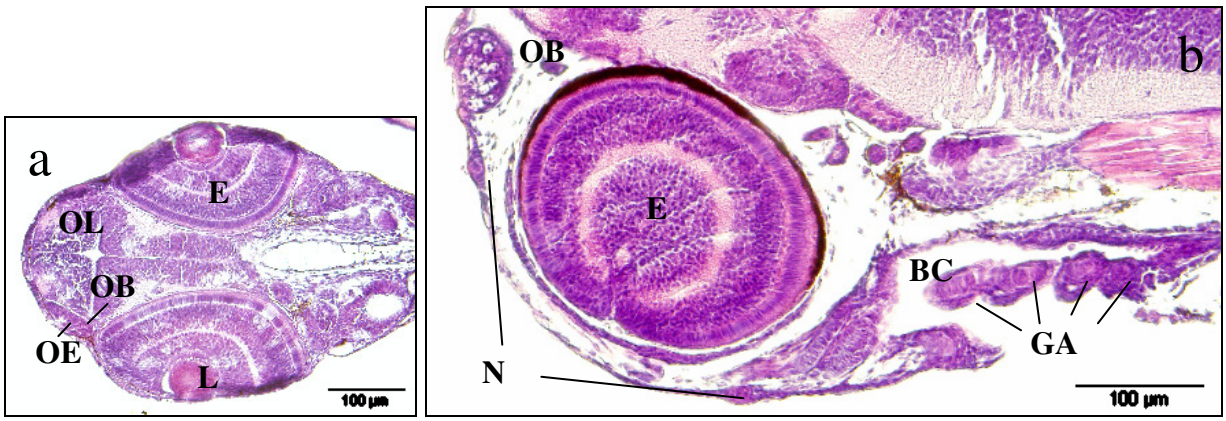

Fig. 34. Sections of the ballan wrasse larva head. (a) Larvae at 1 DAH (HE, × 20). (b) Larvae at 4 DAH (HE, × 20). BC, buccopharyngeal cavity; E, eye; GA, gill arches anlage; L, lens; N, neuromast; OB, olfactory bulb; OE, olfactory epithelium; OL, olfactory lobe.

With larval growth the olfactory area became larger, area of sensory epithelium increased and sensory epithelium sunk under the skin (Fig. 35).

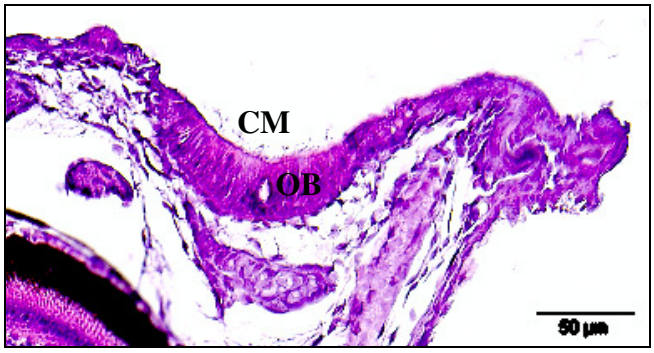

Fig. 35. Longitudinal section of the ballan wrasse larva olfactory organ on 25 DAH (HE, × 20). CM, cilia and microvilli; OB, olfactory bulb.

3.3.6.Accessory glands

Pancreas

At hatching the incipient pancreas was detected between the yolk sac and developing digestive tract (Fig. 36). It was formed by irregularly shaped cells.

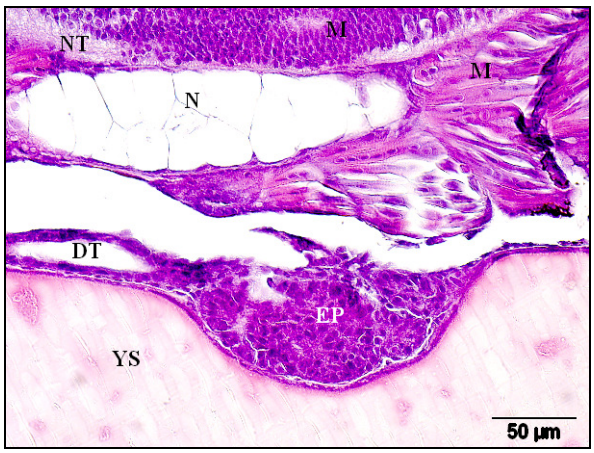

Fig. 36. Longitudinal section of the digestive tract of ballan wrasse larva. (HE, × 40) DT, digestive tract; EP, exocrine pancreas; M, myomeres; N, notochord; NT, nervous tissue, YS, Yolk sac.

On 4 DAH, the exocrine pancreatic pyramidal cells concentrated between the swim bladder, the yolk sac and the intestine. First zymogen granules were apparent in the central part of the organ (Fig. 46).

After beginning of first feeding, the size of pancreas, and the number of zymogen granules increased (Fig. 37). At 17 DAH the endocrine cells could be distinguished as islet (islet of Langerhans) inside the exocrine pancreas (Fig. 44 a, 37 b). From then on, the exocrine pancreas increased notably in size, surrounding the different structures within the visceral cavity.

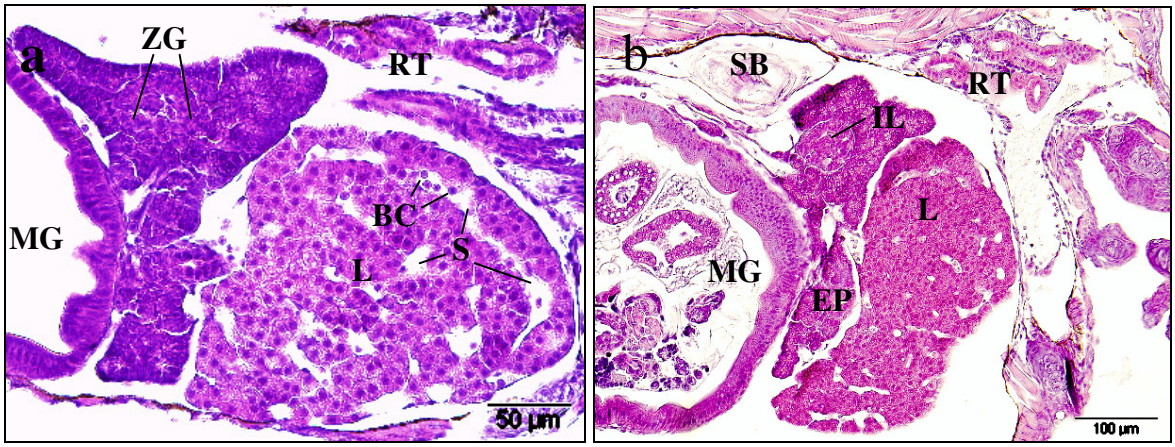

Fig. 37. Longitudinal section of accessory glands of ballan wrasse larvae. (a) Larvae 9 DAH (HE, × 40). (b) Larvae 25 DAH (HE, × 20). BC, blood cells; EP, exocrine pancreas; IL, islets of Langerhans; L, liver; MG, midgut; RT, renal tubules; S, sinusoid; SB, swim bladder; ZG, zymogen granules.

Liver

At hatching the liver was not evident. At 3 DAH primordial liver was first observed as a small patch of undifferentiated rounded cells behind 1/4 part of the yolk sac (Fig. 38 a). By

5 DAH hepatic tissue started to differentiate and polyhedral PAS-positive hepatocytes with granular cytoplasm were clearly distinguishable (Fig. 38 b).

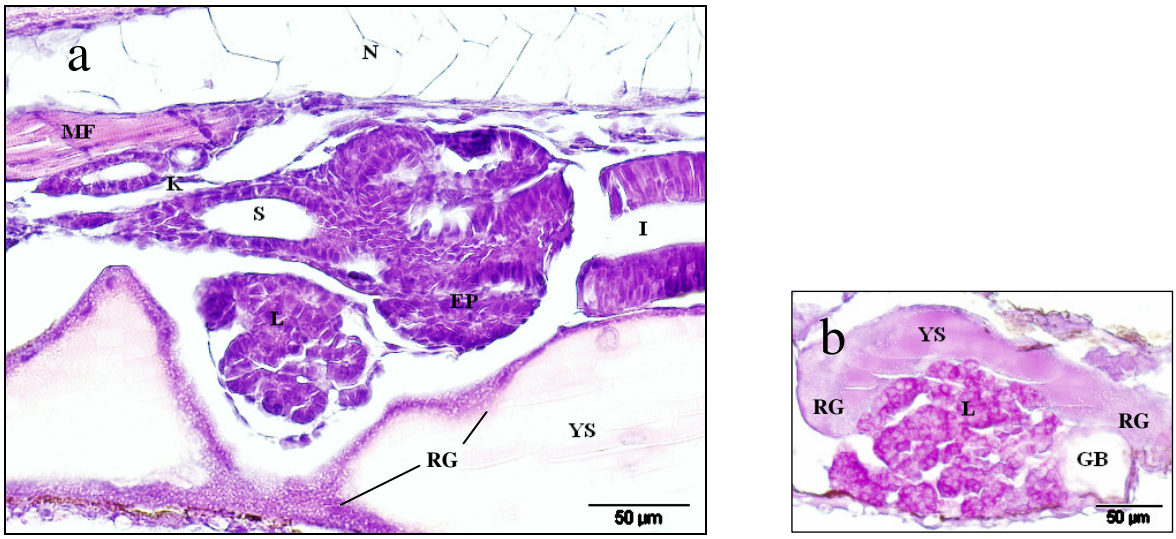

Fig. 38. Longitudinal section of ballan wrasse larvae. (a) Larvae 3 DAH (HE, × 40). (b) Larvae 5 DAH (AB-PAS, × 40). EP, exocrine pancreas; GB, gall bladder; I, intestine; K, kidney; L, liver; MF, muscular fibres; N, notochord; RG, resorption granules; S, stomach; YS, yolk sac.

From the 7 DAH and during further development liver showed strong PAS-positive staining evident feeding behavior of the larvae and storage glycogen in the liver. At 9 DAH the hepatocytes were tightly packed between sinusoids and showing a prominent central nucleus and abundant glycogen containing granules within their cytoplasm. Blood cells were seen inside the sinusoid (Fig. 37 a). The liver increased in size during following stages. The vacuolization of the hepatocytes and the proliferation of sinusoid begin its most notable features.

Gall bladder

The gall bladder was first seen at 3 DAH, lying between the liver, pancreas and yolk sac (Fig. 39 a). It was lined by a single layer of cells, which appeared flattened and thin when the bladder was distended (Fig. 39 b). At 13 DAH the epithelium of the gall bladder appeared thicken, with cuboidal and cylindrical cells (Fig. 39 c).

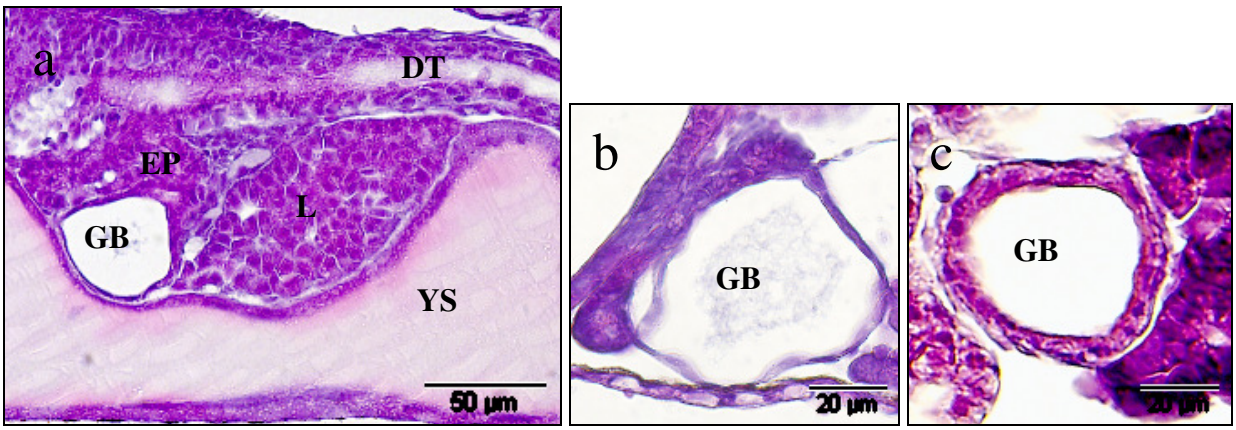

Fig. 39. Longitudinal section of ballan wrasse larvae. (a) Larvae 3 DAH (HE, × 60). (b) Distended gall bladder of larvae 7 DAH (HE, × 40). (c) Thickened gall bladder of larvae 13 DAH (HE, × 40). DT, digestive tract; EP, exocrine pancreas; GB, gall bladder; L, liver, YS, yolk sac.

3.3.7.Heart

At hatching the heart was detected as undifferentiated tubular structure, known as pericardic cavity, located in the anterior zone near to the yolk sac. In the lumen of the pericardic cavity, some undifferentiated cells, the primordium of the three cardiac compartments, were observed (Fig. 13, 40 a).

At 7 DAH first blood cells were seen within the chambers (Fig. 40 b). At the flexion stage three defined compartments (bulbus arteriosus, ventricle and atrium) and sinus venosus were completely differentiated and clearly distinguished (Fig. 41).

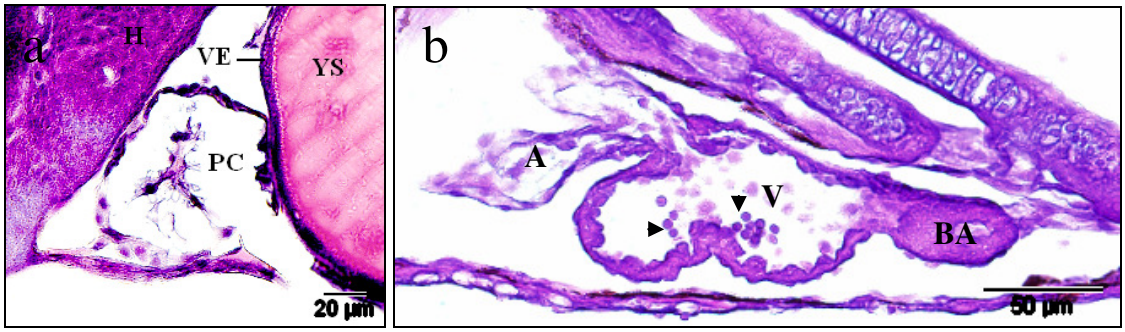

Fig. 40. Longitudinal section of the heart of ballan wrasse larvae. (a) Larval heart presented by tubular, undifferentiated pericardic cavity at 0 DAH (HE, × 40). (b) Heart at 7 DAH (AB-PAS, × 40). Arrow head, blood cells; A, atrium; BA, bulbus arteriosus; H, head; PC, pericardic cavity; V, ventricle; VE, viteline envelope; YS, yolk sac.

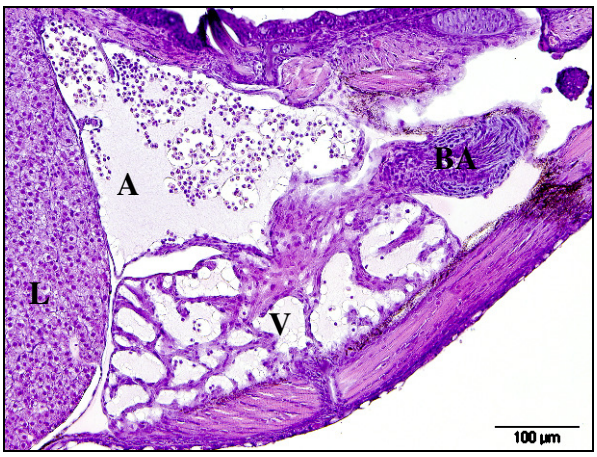

Fig. 41. Longitudinal section of the heart of ballan wrasse larvae 29 DAH (HE, × 20). A, atrium; BA, bulbus arteriosus; L, liver; V, ventricle.

3.3.8.Swim bladder

At 4 DAH the primordial swim bladder was differentiated from the dorsal wall of the digestive tube. This structure possessed a thick epithelial wall consisting of columnar cells, small irregular lumen and connective tissues aroung (Fig. 42).

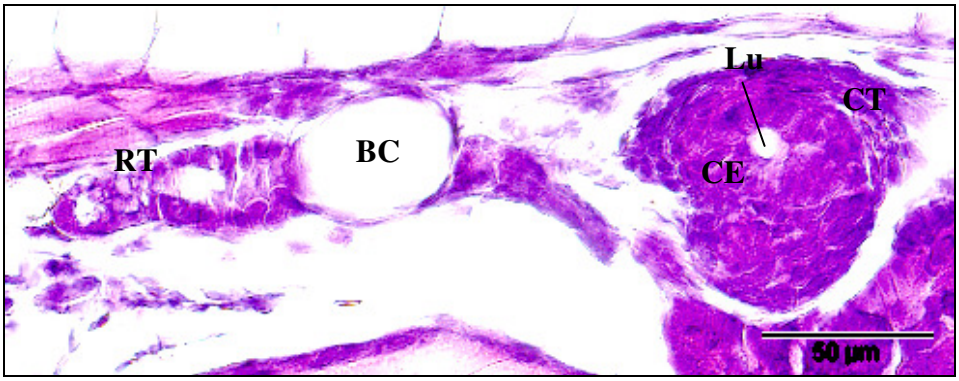

Fig. 42. Longitudinal section of ballan wrasse larvae 4 DAH (HE, × 40). BC, Bowman's capsule; CE, columnar epithelium; CT, connective tissue; Lu, lumen; RT, renal tubules.

At the beginning of lecitoexotrophic period (7 DAH) swim bladder was more structured and the gas gland in its anterior zone was visible. Pneumatic duct connecting the swim bladder with the digestive tube wall also was seen in the posterior region (Fig. 43).

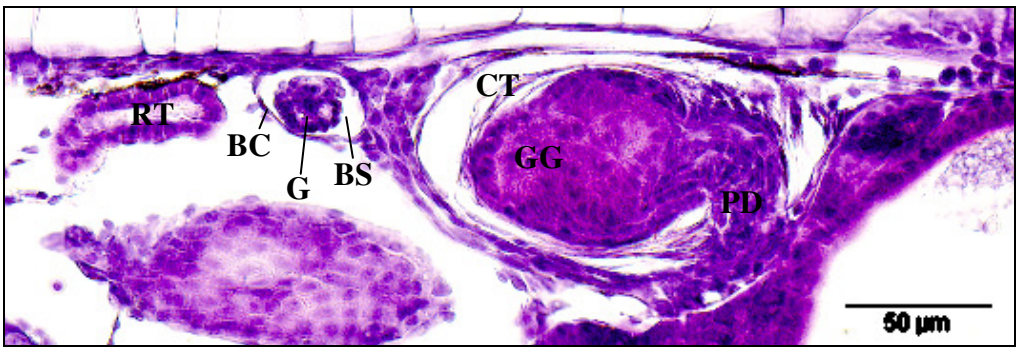

Fig. 43. Longitudinal section of ballan wrasse larvae 7 DAH (HE, × 40). BC, Bowman's capsule; BS, Bowman space; CE, columnar epithelium; CT, connective tissue; G, glomerulus; GG, gas gland; L, lumen; PD, pneumatic duct; RT, renal tubules.

The swim bladder was first filled with air at 9 DAH (Fig. 47). From this moment corresponding with the bladder inflation (Fig. 44 a), the cells of the gas gland arranged in a single layer at a ventral zone of the chamber (Fig. 44 b).

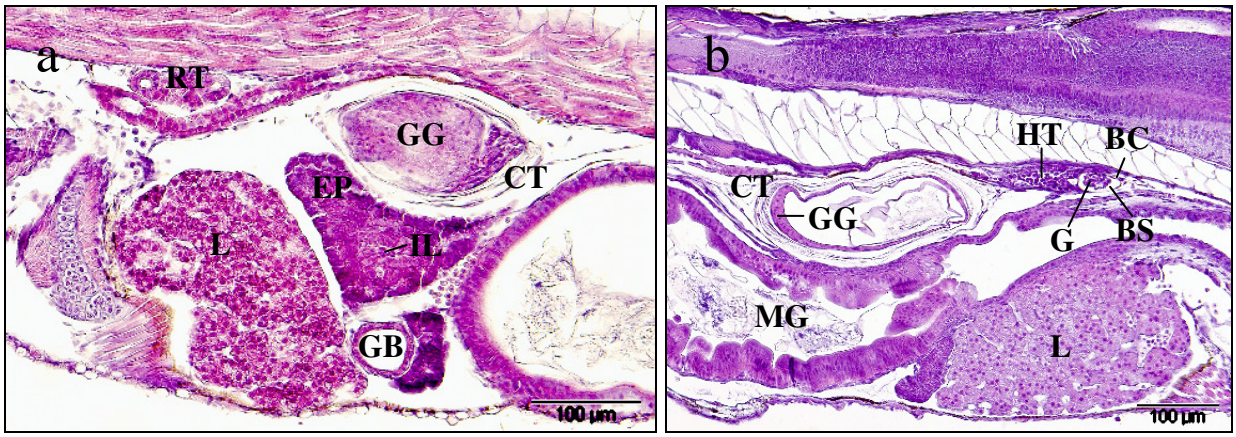

Fig. 44. Longitudinal section of ballan wrasse larvae. (a) Larvae at 17 DAH (HE, × 40). (b) Larvae at 19 DAH (HE, × 40). BC, Bowman's capsule; BS, Bowman space; CT, connective tissue; EP, exocrine pancreas; G, glomerulus; GB, gall bladder; GG, gas gland; HT, haematopoietic tissue; IL, islet of Langerhans; L. liver; MG, midgut; RT, renal tubules.

Rete mirabile was observed at 29 DAH.

3.3.9.Excretory system

The excretory kidney was already seen at hatching in the posterior region. It consisted of the primordial collecting straight tube running just below the notochord axis and above the gut. It was connected to the urinary bladder. The urinary bladder anlage was visible at the vicinity of the anus as a closed wall sac lined by a simple layer of epithelial cells (Fig. 45).

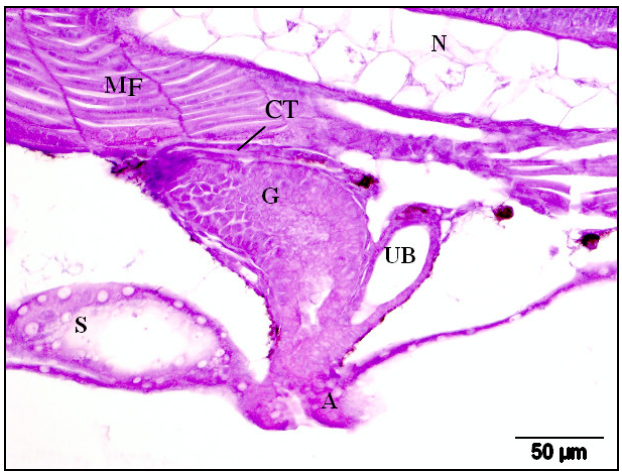

Fig. 45. Longitudinal section of ballan wrasse larvae 0 DAH (HE, × 40). A, anus; CT, collecting tube; G, gut; MF, muscular fibres; N, notochord; S, skin; UB, urinary bladder.

At 3 DAH the epithelial cells that lined the lumen of the urinary bladder appeared flattened (Fig. 15 b).

Started by 3 DAH and during yolk sac stage primary renal tubules appeared and began convolution (Fig. 38 a), which increased during exotrophic period being more pronounced in the cephalic region. The first renal corpuscle connected to its proximal convoluted renal tubule was visible at 4 DAH. Corpuscle was formed by a glomerulus and Bowman's capsule (Fig. 46).

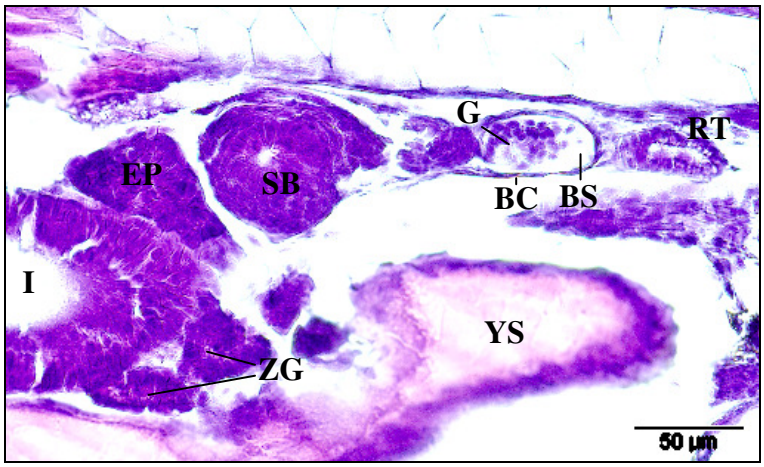

Fig. 46. Longitudinal section of ballan wrasse larvae 4 DAH (HE, × 40). BC, Bowman's capsule; BS, Bowman space; EP, exocrine pancreas; G, glomerulus; GG, gas gland; I, intestine; RT, renal tubules; SB, swim bladder; YS, yolk sac; ZG, zymogen granules.

At 9 DAH the proximate renal tubules were seen to be connected to the collecting duct (Fig. 47).

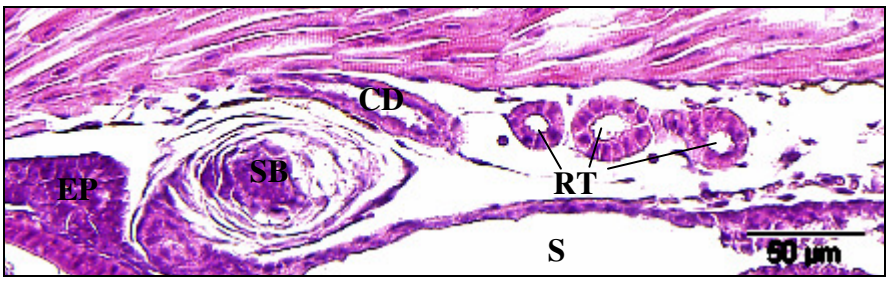

Fig. 47. Longitudinal section of ballan wrasse larvae 9 DAH (HE, × 20). CD, collecting duct; EP, exocrine pancreas; RT, renal tubules; S, stomach; SB, swim bladder.

At 17 DAH haematopoietic cells (interstitial tissue) appeared in the kidney around the renal corpuscle (Fig. 44 b) and between the renal tubules (Fig. 48).

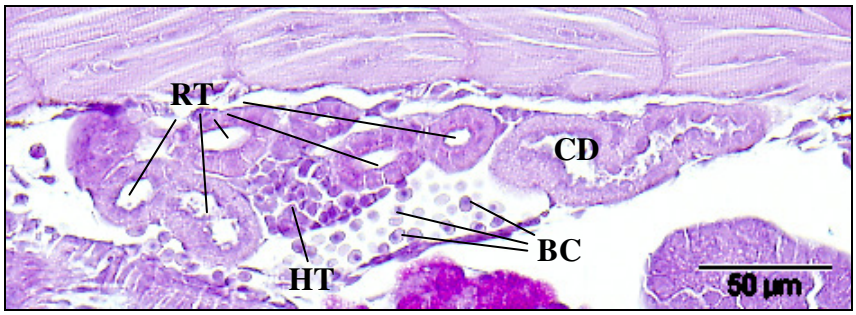

Fig. 48. Longitudinal section of ballan wrasse larvae 17 DAH (AB-PAS, × 20). BC, blood cells; CD, collecting duct; HT, haematopoietic tissue; RT, renal tubules.

The following days the proximal renal tubules and the haematopoietic tissue proliferated and originated the notable increase in kidney size.

3.3.10.Respiratory system

Buccopharyngeal cavity was not formed at hatching. It was not possible to distinguish gill anlages (Fig. 13, 14).

Gill anlage (Fig. 49 a) was visible in the buccopharyngeal cavity (Fig. 49 a) at 3 DAH as a four primordial gill arches formed by cores of chondroblast covered with an undifferentiated epithelium. Oral valve was seen as dorsal epithelial fold (Fig. 49 b).

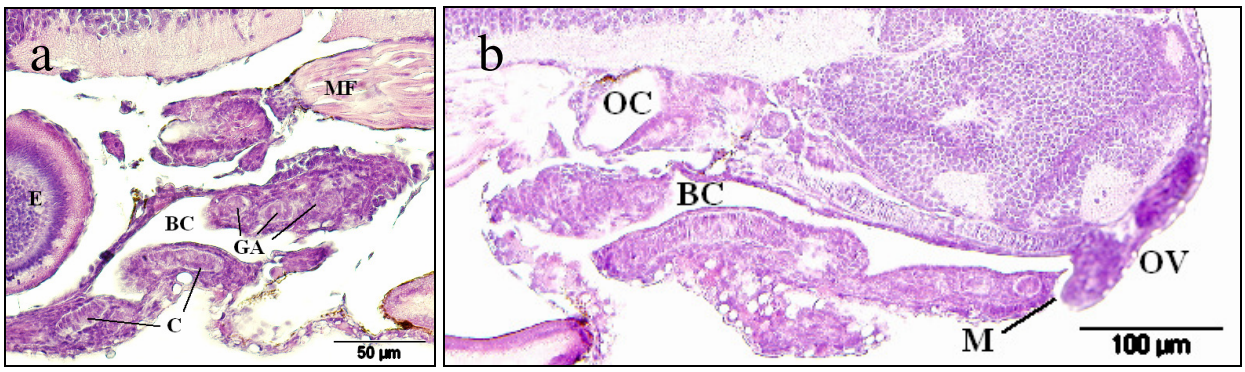

Fig. 49. Longitudinal section of ballan wrasse larvae head at 3 DAH. (a) Buccopharyngeal cavity with gill arches anlage (HE, × 40). (b) Opening of the mouth and buccopharyngeal cavity (HE, × 20). BC, buccopharyngeal cavity; C, cartilages; E, eye; GA, gill arches; M, mouth; MF, muscular fibres; OC, otic capsule, OV, oral valve.

By the 4 DAH pharynx was opened (Fig. 34 b). At 7 DAH 4 separate gill arches were clearly distinguished (Fig. 17). AB-positive staining of cartilage in the arches evident about their continuous development.

Primary lamellae in the gill filaments started to form at 17 DAH and were clearly seen by 25 DAH (Fig. 50 a). AB-positivity was seen in immature cartilages in this period. By 29 DAH anlages of secondary lamellae in gill filaments were seen (Fig. 50 b, c).

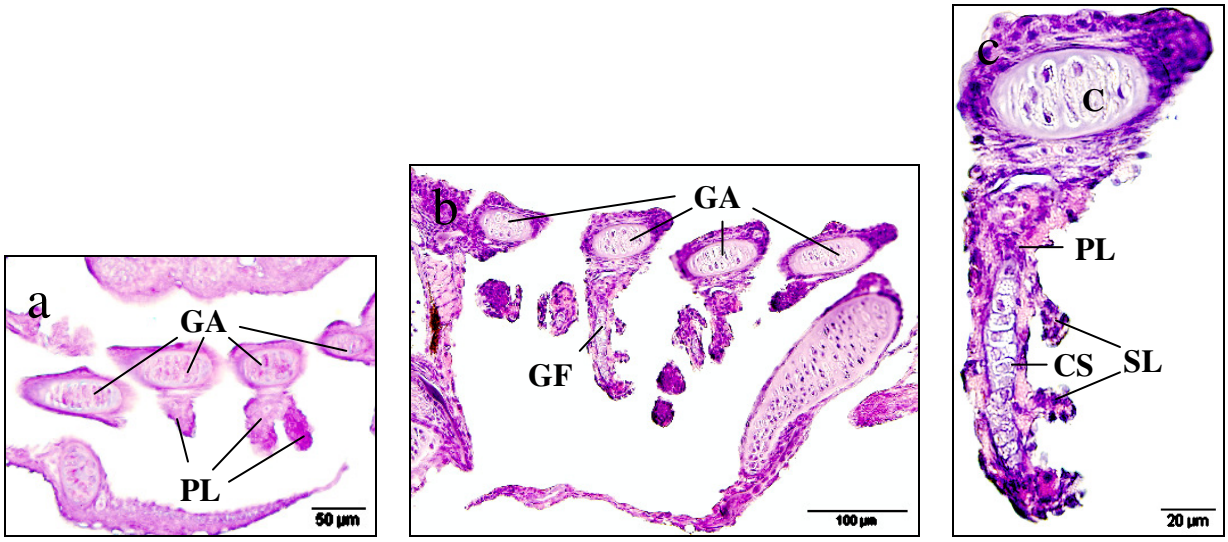

Fig. 50. Gills development of ballan wrasse larvae. (a) Longitudinal section of 25 DAH larva gills (AB-PAS, × 40). (a) Longitudinal section of 29 DAH larva gills (AB-PAS, × 40). (b) Longitudinal section of 29 DAH larva gill arch (AB-PAS, × 60). C, cartilage; CS, cartilaginous support; PL, primary lamellae; SL, secondary lamellae.

3.3.11.Thyroid

Thyroid gland consisted of a some follicles located under the lower jaw was seen at 29 DAH (Fig. 51 a). Each follicle was covered by cubic epithelial cells and contained colloid – storage form of the thyroid hormone (Fig. 51 b).

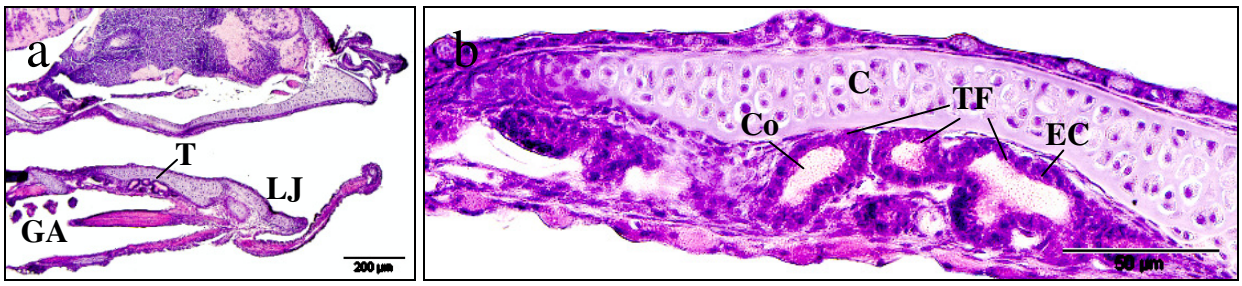

Fig. 51. Longitudinal section of ballan wrasse larva at 29 DAH. (a) Larvae head (AB-PAS, × 10). (b) Thyroid structure (AB-PAS, × 40). C, cartilage; Co, colloid; EC, epithelial cells; GA, gill arches; LJ, lower jaw; T, Thyroid; TF, thyroid follicles.

The number of thyroid follicles increased progressively during the following days (Fig. 52).

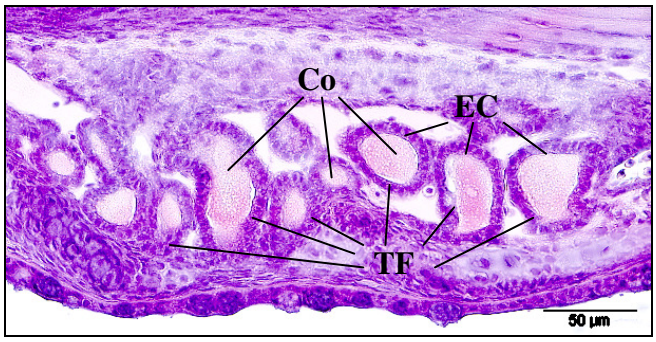

Fig. 52. Thyroid structure on 33 DAH (AB-PAS, × 40). Co, colloid; EC, epithelial cells; TF, thyroid follicles.

3.3.12.Pituitary

The pituitary gland first was observed at 5 DAH. It was located in the ventral surface of the brain, in the mid-line (Fig. 53 a). The pituitary was relatively unspecialized and closely attached to the brain (Fig. 53 b).

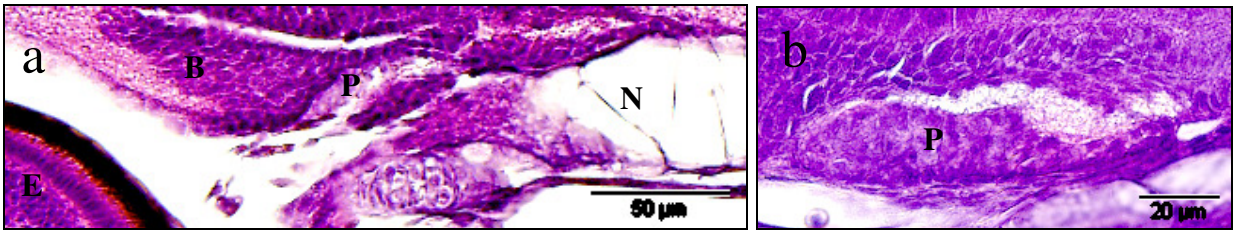

Fig. 53. Longitudinal section of ballan wrasse larvae head. (a) Larva at 5 DAH (HE, × 10). (b) Pituitary on 17 DAH (HE, × 60). B, brain; E, eye; N, notochord; P, pituitary.

4. Discussion

4.1. Ballan wrasse growth

Ballan wrasse larvae were 3.64 ± 0.05 mm length at hatching. The length of newly hatched larvae of wrasse was significantly larger than those of the other labrid species (Dulcic, et al., 1999; Kimura, et al., 1993), but smaller than of brown wrasse *Labrus merula* (Dulcic, et al., 1999).

Compared with data of the length at hatching (2.7 ± 0.2 mm) from Artuz (2005) ballan wrasse larvae from this work also shown higher length parameters. This difference in larval size could be explained by origination of the eggs, environmental conditions of broodstock living and larvae rearing parameters such as temperature, salinity, water flow, oxygen level and light regime.

The lowest AGR_L values were recording during stages 2 and 3 (Appendix 4), when the all energy and building molecules obtained from external feeding were likely used to construct tissues, organs and systems. It is recognized that this period, particularly as Artemia is introduced as feed in the rearing system, is critical for marine larviculture (Blaxter, 1988; Santamaría, et al., 2004). In accordance with obtained data on wrasse larval growth, other marine species such as Australasian snapper *Pagrus auratus* (Pankhurst, et al., 1991), pink dentex *Dentex gibbosus* (Fernandez-Palacios, et al., 1994), brill *Scophthalmus rhombus* (Hachero-Cruzado, et al., 2009) have also been reported to exhibit the lowest AGR_L during the same larval period. In the contrary, stage 4 was not characterized by the appearance of new structural elements, but by quantitative changes in pre-existing structures, and by a significant increase in AGR_L value.

One interesting thing in larval growth was that the AGR_H increased from stage to stage (Appendix 4). So, during stage 2 and 3 fish grew more in height than in length and the introducing of Artemia in the rearing system affected more on the capability of the larvae to digest this feed and survive. But feeding and survived organisms continued to growth.

During studied periods wrasse larvae growth followed an exponential curve. The same relationship were shown for other marine teleosts such as Senegalese sole *Solea senegalensis* (Ribeiro, et al., 1999), spotted sand bass *Paralabrax maculatofasciatus* (Peña, et al., 2003), common dentex *Dentex dentex* (Santamaría, et al., 2004), common pandora *Pagellus erythrinus* (Micale, et al., 2006), yellowtail kingfish *Seriola lalandi* (Chen, et al., 2006), brill *S. rhombus* (Hachero-Cruzado, et al., 2009).

4.2. Morphological aspects of ballan wrasse development

The present study provides integrated information about the morphological and histological characteristics of the organogenesis during *L. bergylta* larval development (from hatching to 49 DAH). The development of ballan wrasse larvae was divided into four ontogenic stages: yolk-sac, preflexion, flexion and postflexion larva. These stages also were divided into lecitotrophic, lecitoexotrophic and exotrophic periods according to the food source. Similar periods are often reported as stages of development for other species, i.e. gilthead seabream *Sparus aurata* (Elbal, et al., 2004), Australasian snapper *P. auriga* (Sánchez-Amaya, et al., 2007), brill *S. rhombus* (Hachero-Cruzado, et al., 2009), wedge sole *Dicologoglossa cuneata* (Herrera, et al.).

There are only few sources of the information about wrasse larval development and especially about ballan wrasse. Artuz (2005) has studied ballan gross morphology and shown the same ontogeny of organs and appearance of the morphological features as in the present work with the main variability in the timing of organ development and features appearance. It was difficult to compare developmental level because of absence common scheme of presenting age of larvae. Artuz (Artuz, 2005) used only length development of larvae without calculation of day-degree.

4.3. Histological aspects of ballan wrasse development

Although the basic mechanisms of larval development do not differ greatly among teleosts, the timing of developmental events and the duration of stages are variable (Blaxter, 1988; Falk-Petersen, 2005).

4.3.1. Endogenous reserves

The newly hatched larva of *L. bergylta* presented, as the most teleosts, the yolk surrounded by the periblast (syncytical layer of squamous cells) (Falk-Petersen, 2005; Kjorsvik, et al., 1992; Morrison, 1987; Sánchez-Amaya, et al., 2007). By endocytosis of the yolk sac through a syncytical layer endogenous nutrition in fish larvae occurred (Balon, 1986).

The yolk sac of ballan wrasse larva was ovoid in shape and medium in size. There were no oil globules or oil droplets in the sac as seen in other species such as Atlantic cod *G. morhua* (Morrison, 1987), Atlantic halibut *H. hippoglossus* (Haug, et al., 1989), common wolffish *Anarchicas lupus* (Falk-Petersen, et al., 2001).

The yolk was completely resorbed at 10 DAH (4.3 mm length) similarly with the data from Artuz (2005) work (4.1 mm length). This process takes nearly 50 DAH in Atlantic halibut

H. hippoglossus (Lein, et al., 1992), 8 DAH in gilthead seabream *S. aurata* (Elbal, et al., 2004), 9 DAH in Atlantic cod *G. morhua* (Morrison, 1993).

4.3.2.Digestive system

At hatching the digestive tract of ballan wrasse larvae was an undifferentiated staright tube, as reported in other teleost species like spotted sand bass *P. maculatofasciatus* (Peña, et al., 2003), redbanded seabream *P. auriga* (Sánchez-Amaya, et al., 2007), brill *S. rhombus* (Hachero-Cruzado, et al., 2009).

The differentiation of the digestive tract in *L. bergylta* larvae started before the onset of exogenous feeding since the buccopharyngeal cavity, oesophagus, primordial stomach and the intestine, divided into midgut and hindgut, were distinguished 4 DAH with the moment of mouth opening. These main developmental events are in agreement with previous reports in other species (Elbal, et al., 2004; Micale, et al., 2006; Sarasquete, et al., 1995). All these changes were important for adaptation to exogenous feeding (Segner, et al., 1993). During this period ballan wrasse larvae advanced the digestive system in structure and function for successful ingestion, digestion and assimilation on exogenous food before the depletion of endogenous reserves.

The first functional, PAS-positive mucous cells in the digestive tract were detected in the oesophageal epithelium at 7 DAH, at the moment of first feeding. The presence of digestive mucous cells in ballan larvae at this developmental stage was similar to that of other species, as dover sole *Solea solea* (Boulhic, et al., 1992), brill *S. rhombus* (Hachero-Cruzado, et al., 2009).

The folds, cytoplasmic granules (17 DAH) and PAS-positive brush border (25 DAH) in the epithelial layer of the intestine suggested that ballan larvae were able to digest and absorb carbohydrates at the time of exogenous feeding and co-feeding (Micale, et al., 2006).

At 17 DAH first taste buds were detected in the oesophagus in *L. bergylta* larvae. It is a chemosensory organ that consisted of modified epithelial cells, which are implied in the selection of the food and play a crucial role in gustation: foraging and food-recognition (Sánchez-Amaya, et al., 2007).

At the beginning of the cofeeding ballan larvae could not digest artemaia. But at 37 DAH digested artemia were observed along the whole digestive tract. It was linked with development of the intestinal folds and appearance of AB-positive cells in the intestinal epithelium.

4.3.3.Skin

As in other marine yolk-sac larvae (Morrison, 1987; Ottesen, et al., 2000) epidermis of *L. bergylta* yolk sac larva consisted of 1-2 layers of cells that developed multilayer tissue consisting of many epithelial, mucous and saccular cells during studied stages.

4.3.4.Muscular structure and notochord

The myotome and notochord structure in ballan larvae was the same as in most species (Kjørsvik, et al., 2007). Myotomes were V-shaped, simple and segmental. Notochord

4.3.5.Sensory organs

L. bergylta hatched with undeveloped sensory organs. The sensory organs, however, developed rapidly with fish growth. At the moment of first feeding (7 DAH) the larvae already had developed eyes, olfactory organs, free neuromasts and inner ears. All these organs, especially eyes play an important role in orientating in the environment, predating and catching the prey.

In ballan wrasse larvae eyes were unpigmented and non-functional at hatching like in many species, e. g. Atlantic halibut *H. hippoglossus* (Pittman, et al., 1990), common dentex *D. dentex* (Santamaría, et al., 2004). The main layers in the eye could be distinguished.

Inner ear development of *L. bergylta* was closely related to their swimming behaviour. At hatching otic capsule was round-shaped, co-occurring with an inability to swim horizontally but just up and down in the tank. Following days larvae swam more active and horizontally for a short time. It was linked with the beginning development of semicircular canals. The similar signs of organ development and behavior were common for other species, e. g. Atlantic cod *G. morhua* (Fridgeirsson, 1978; Morrison, 1987), brown trout *Salmo trutta* (Becerra, et al., 1993), African catfish *Clarias gariepinus* (Mukai, et al., 2008), bluefin tuna *Thunnus orientalis (Kawamura).*

4.3.6.Accessory glands

As in many teleosts (Chen, et al., 2006; Hachero-Cruzado, et al., 2009) the liver in *L. bergylta* larvae was not evident at hatching. Primordial liver could be observed at 3 DAH, while is later as to other species (Morrison, 1993; Sánchez-Amaya, et al., 2007; Santamaría, et al., 2004). However, the following development of the liver followed in common pattern.

Similar to Atlantic cod *G. morhua* (Morrison, 1993), but in contrast with other species (Hachero-Cruzado, et al., 2009; Sánchez-Amaya, et al., 2007), the exocrine pancreas was detected at hatching in ballan larvae. Endocrine part of the pancreas – the islet of Langerhans, was observed comparatively later, in the preflexion stage (13 DAH), than in other teleosts (Micale, et al., 2006; Sánchez-Amaya, et al., 2007) during yolk sac stage.

Development of gall bladder was the same as in many teleosts.

4.3.7.Heart

As in many species (Hachero-Cruzado, et al., 2009; Santamaría, et al., 2004) in ballan wrasse larva heart at hatching was presented by undifferentiated pericardic cavity. But at the beginning of first feeding the heart was divided into the three defined compartments and first blood cells appeared within the chambers. So, circulatory system was ready to supply the organs involved in digestive process.

4.3.8.Swim bladder

The primordial swim bladder appeared in *L. bergylta* larvae few days after hatching (4 DAH) as was reported also for other species (Hachero-Cruzado, et al., 2009; Sánchez-Amaya, et al., 2007; Santamaría, et al., 2004).

At 7 DAH pneumatic duct was visible, gas gland appeared and inflation of the ballan wrasse swim bladder occurred at 9 DAH. It is also filled in many species soon after hatching: European seabass *Dicentrarchus labrax*, Atlantic cod *G. morhua*. But it needs several weeks for gilthead sea bream *S. aurata* (Elbal, et al., 2004), Atlantic herring *Clupea harengus,* Californian anchovy *Engraulis mordax* or months for salmonids (Blaxter, 1986).

4.3.9.Excretory system

During first stage of development (0-9 DAH), the excretory system became functional in *L. bergylta* larvae, mainly mostly because of development of renal corpuscles and tubules joined to the collecting duct towards the urinary bladder. Similar functionalities were reported for Atlantic cod *G. morhua* (Morrison, 1993), common dentex *D. dentex (Santamaría, et al., 2004)*, redbanded seabream *P. auriga* (Sánchez-Amaya, et al., 2007), flatfish brill *S. rhombus* (Hachero-Cruzado, et al., 2009).

4.3.10.Respiratory system

Contrarily to some species (Sánchez-Amaya, et al., 2007; Santamaría, et al., 2004), gill arches anlage in *L. bergylta* were not distinguished at hatching, but at 3 DAH gill arches anlage were seen in the buccopharyngeal cavity.

It is known that gills become functional when lamellae develop (Hoar, et al., 1969). In ballan larvae, as in most marine teleost (Hachero-Cruzado, et al., 2009; Santamaría, et al., 2004), primary lamellae appeared during preflexion stage. Wolf fish larvae have functional gills at hatch (Falk-Petersen, et al., 2001; Sánchez-Amaya, et al., 2007).

4.3.11.Endocrine organs

It was suggested that thyroid follicles and pancreatic islets first appeared at about the same time, and pituitary gland differentiation coincided with the time of eye pigmentation (Tanaka, et al., 1995). But in ballan wrasse larvae thyroid follicles could be first observed only during flexion stage, while endocrine pancreas was seen at the preflexion stage. Meanwhile, pituitary gland was distinguished at the same time with eye pigmentation during the yolk sac stage.

In other species (Sánchez-Amaya, et al., 2007) earlier development of thyroid hormone was shown and seems important for the general metabolism from the first feeding onwards, and thus for the further larval development and metamorphosis.

5. Conclusion

Ballan wrasse larvae hatched at 72°d and at 3.64±0.05 mm length and reached 10.52±0.82 mm at the end of the study. Larval growth followed an exponential curve. During stages 2 and 3 larvae undergo intense organogenesis and the all energy and building molecules obtained from external feeding were likely used to construct tissues, organs and systems. In the contrary, stage 4 was not characterized by the appearance of new structural elements, but by quantitative changes in pre-existing structures.

Although the basic mechanisms of *L. bergylta* larvae development do not differ greatly from other teleosts, there is some interspecific variability in the timing at which different ontogenic events occur.

During stage 1 the majority of the organs were observed as undifferentiated groups of cells or primordial structures. At hatching, the mouth and anus were closed, eyes were not pigmented, and digestive tract was an undifferentiated and straight tube. Pericardic cavity, urinary bladder and exocrine pancreas anlages were seen. By the first feeding eyes were pigmented, both the mouth and the anus opened in conjunction with the differentiation of the digestive tract. Buccopharyngeal cavity, oesophagus, stomach midgut and hindgut were distinguished. Primordial structures of liver, swim bladder, gall bladder, gills, pituitary and kidney appeared. As ingestion of prey began, the digestive processes continued developing with the appearance of mucous cells in the oesophagus, gut folds and brush border in the intestine. The circulatory system became functional, with the compartmentalization of the heart.

During stage 2 (and 3) development of organs and structures continued. Haematopoietic tissue and endocrine part of pancreas – Langerhans islet were evident. In the oesophagus and skin were seen first taste buds.

Throughout stage 3 thyroid follicles appeared, gill structures continued developing. Number of mucous cells in the oesophagus increased and first mucous cells appeared in the gill opening. Liver and pancreas proliferated in size.

Stage 4 was characterized by developing of existence organs: gill filaments and lamellae proliferated, the number of mucous cells in gill opening region increased. Most organs essentially exhibited an increase in tissue structure and size.

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7.Appendices

Appendix 1

EM-fixative

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SOLUTIONS:

A. Buffer

Caccodylic acid sodium salt trihydrate (Sigma-Aldrich CO250)	20.8 g
Sucrose, $C_{12}H_{22}O_{11}$	140 g
1% Calcium Chloride, $CaCl_2$	20 ml
Distilled water	2 l

Mix and adjust the pH to 7.2 with 1 N HCl

B. Stem solution

Paraformaldehyde	25 g
Distilled water	250 ml
Buffer	1 l

Dissolve paraformaldehyde in distilled water by heating (not more than 80°C), using an exhaust hood. Add concentrated NaOH (few droplets) until pH becomes 7.1-7.3. Cool and mix with buffer. Store the solution at 4°C.

FIXATION:

Mix stem solution and 25% glutaraldehyde in proportion 12.5:1 (stem solution (12.5 ml), glutaraldehyde (1 ml).

Appendix 2

Haematoxylin and eosin (H&E) (Amin, et al., 1991) staining technique

SOLUTIONS:

A. Haematoxylin

Aluminium potassium sulphate-12-hydrate, $AlK(SO_4)_2 \times 12H_2O$	125 g
Haemotoxylin, $C_{16}H_{14}O_6$	7.5 g
Sodium iodate, $NaIO_3$	0.625 g
Distilled water	2.5 l

Dissolve aluminium potassium sulphate-12-hydrate in water under mixing. Add haematoxylin, then – sodium iodate. Parboil the solution a bit and leave for "maturation". The solution should have a metallic gleam on the surface. Filter the solution before use.

B. 1% Erythrosine

Erythrosine B	25 g
Distilled water	2.5 l

STAINING PROCEDURE:

1. Deparaffinization and hydration:
   heating at 60°C for 30 min;
   2 × xylen for 5 min;
   xylen for 1.5 min;
   2 × 100% ethanol for 1.5 min;
   96% ethanol for 1.5 min;
   80% ethanol for 1.5 min;
   50% ethanol for 1.5 min;
   rinsing for 1.5 min.
2. Staining with haematoxylin solution for 1.5 min.
3. Rinsing in tap water for 3 min.
4. Staining with eosin solution for 2 min.
5. Rinsing in tap water for 1.5 min.

6. Dehydration:

   50% ethanol for 1.5 min;

   70% ethanol for 1.5 min;

   96% ethanol for 1.5 min;

   2× 100% ethanol for 1.5 min;

   xylen for 1.5 min.

7. Mounting of sections with mounting medium (Pertex).

RESULTS:

Nuclei – blue-black, cytoplasm – pink.

Appendix 3

Periodic acid Schiff (PAS)-Alcian Blue (AB) (pH 2.5) staining technique (Amin, et al., 1991)

SOLUTIONS:

A. Alcian Blue solution (pH 2.5)

B. 0.5% Periodic Acid

C. Schiff's reagent

D. Haematoxylin

STAINING PROCEDURE:

1. Deparaffinization and hydration (see H&E staining).
2. Dripping of Alcian Blue (pH 2.5) for 5 min.
3. Rinsing in tap water for 10 min.
4. 1 % Periodic Acid for 5 min.
5. Rinsing in distilled water for 10 min.
6. Schiff's reagent in the oven for 5-10 min (until proper coloration of specimens).
7. Rinsing in tap water for 10 min.
8. Haematoxylin for 15 sec.
9. Rinsing in tap water for 10 min.
10. Dehydration (see H&E staining).
11. Mounting of sections with mounting medium (Pertex).

RESULTS:

Acidic mucins – blue, neutral mucins – magenta, nuclei – deep blue.

Appendix 4

Table 2. Main morphological measurements during *L. bergylta* larval development.

Day	d°	Mean SL	SD	AGR_L	Mean MH	SD	AGR_H	Type of feed
0		3,64	0,05		0,207	0,007		YS
1	10,5	3,85	0,03		0,213	0,009		YS
2	22,2	4,06	0,12		0,226	0,011		YS
3	33,1	4,18	0,06		0,241	0,006		YS
4	45	4,24	0,08		0,241	0,011		YS
5	57,3	4,26	0,05		0,246	0,011		YS
6	71	4,46	0,04		0,241	0,023		YS
7	84,8	4,40	0,15		0,242	0,005		Rotifers
8	98,5	4,29	0,37		0,245	0,037		Rotifers
9	108	4,28	0,11	0,072	0,253	0,017	0,005	Rotifers
13	164,3	4,78	0,19		0,321	0,029		Rotifers
17	225,9	4,69	0,28		0,319	0,039		Rotifers
21	269	4,75	0,44		0,336	0,068		Rotifers, Artemia
25	340,7	5,35	0,30	0,067	0,431	0,057	0,007	Rotifers, Artemia
29	398,5	5,40	0,66		0,587	0,114		Artemia
33	456,4	5,90	0,78	0,068	0,763	0,184	0,042	Artemia
37	514	7,90	0,41		1,275	0,139		Artemia
41	571,2	7,72	0,88		1,391	0,195		Artemia
45	628,2	9,21	0,33		1,782	0,100		Artemia
49	685,7	10,52	0,82	0,289	2,151	0,252	0,087	Artemia