

Ontogeny of Lymphoid Organs in the Asian Sea Bass (*Lates calcarifer*, Bloch)

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Abstract

Ontogeny of the lymphoid organs of hatchery-reared larvae/juveniles *Lates calcarifer* (Bloch) was studied from zero day post hatch (dph) to 60 dph. Histological sections of the samples, collected at daily intervals from zero dph till 25 dph, and at weekly intervals till 60 dph were examined. The thymus was first noticed, at 2 dph, as a bi-lobed organ, situated dorso-posteriorly in the oro-pharyngeal cavity, in the angle between the opercular bone and the head bone. The lymphoid kidney (pronephros) was seen with undifferentiated stem cells at 2 dph, though the excretory kidney tubules were noticed at zero dph. The developed kidney runs ventral to the vertebral column all along the length of the peritoneal cavity, distinctly bi-lobed at its proximal end forming the head-kidney and fused in the mid and tail kidney portions. The spleen, seen mostly as an erythropoietic organ, is capsulated and appeared, at 2 dph, attached to the mesogastrium. The sequence of development of these lymphoid organs was: the thymus (2 dph), the kidney (excretory kidney-0 dph and lymphoid kidney -2dph), the spleen (2 dph) and the gut associated lymphoid tissue (GALT, 5 dph). Various cellular components of these lymphoid organs and their probable role in immune response of the fish have been discussed.

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Introduction

Setback due to the diseases of predominant aquaculture commodity of Asia, the shrimp, is one of the factors responsible for the diversification in the coastal aquaculture. As an alternative, fish is considered as an important group of species for culture in coastal and brackishwater areas. The Asian sea bass, *Lates calcarifer* (Bloch) (Centropomidae), is one of the most promising species for coastal aquaculture, especially in India after the significant achievement in captive breeding of the species at the Central Institute of Brackishwater Aquaculture (CIBA), Chennai. Aquaculture involving the sea bass, in various parts of the world, has also been facing problems of diseases. Early stages of larval development are susceptible to diseases of varying etiology (Chong and Chao 1986; Munday et al. 1992). Preventive and prophylactic measures, to minimize losses due to diseases, can be effectively carried out with the basic information on the onset of innate and acquired immunity of the species (Ellis 1988). Not many species of fish have been investigated related to the ontogeny of lymphoid organs. Information on the ontogeny of lymphoid organs in tilapia (Sailendri and Muthukkaruppan 1975), rainbow trout (Grace and Manning 1980), common carp (Botham and Manning 1981), rutilus and goby (Zapata 1981), yellowtail, red sea bream and Japanese flounder (Chantanachookhin et al. 1991), sea bream (Jósefsson and Tatner 1993) and turbot (Padros and Crespo 1996) and Indian major carps (Kalita 1998) is available. However, information on the ontogeny of lymphoid organs of the Asian sea bass is lacking. Hence, the present work was taken up to study the sequence of development of the lymphoid organs in this important fish species.

Materials and Methods

Fish sampling periodicity

Hatchlings of the sea bass produced at the Fish Hatchery of CIBA, Chennai, were used in the present study. Fertilized eggs from a single spawning mother were hatched in fibre glass tanks (0.5 to 1.0 tonne capacity) filled with filtered sea water (salinity: 30 to 32 ppt; temperature: 28 to 30°C). Thorough aeration was provided during the experiment and desirable water quality parameters were maintained. The larvae were reared on live rotifers (*Brachionus plicatilis*) up to 9 days post hatch (dph) and on

gradual weaning from rotifers to *Artemia* larvae from 10 to 21 dph. Minced clam/fish meat was given during the next stages of development.

The hatchlings were sampled daily from 0 to 25 dph, and weekly from 30 to 60 dph. Random samples of hatchlings were drawn, a minimum of 30 hatchlings during the daily sampling and 10 each from then onwards. The fish were initially fixed in 10% (v/v) buffered formalin and the fixative changed twice before final fixation. Larger fish were cut open at the abdomen facilitating proper penetration of the fixative.

Tissue processing and light microscopy

Processing and paraffin embedding of the samples were carried out following standard procedures. Embedding of the whole body of small hatchlings and individual organs of fish, when dissection of the organs was possible, was carried out. Tissue sections of 5-6 μ m thickness were used for staining using haematoxylin and eosin (H & E). Light microscopy was carried out to record the observations.

Results

Thymus

The thymus of the sea bass is a paired organ located dorso-posteriorly in the oropharyngeal cavity at the angle between the opercular bone and the head bone (Fig. 1a). The primordial thymus, appearing at 2 dph, is composed of several darkly staining lymphoblast-like cells. The thymus at 40 dph (Fig. 1b) was seen with clear dark and light zones of cells. A thin layer of epithelium composed of many mucus cells (Fig. 1c) separated the thymus from the gill chamber. Organelle, with reticulated margins (Fig. 1d), similar to those of the Hassle's corpuscle were seen at this stage of development. Trabeculae (Fig. 1e) that seem to divide the thymus into different capsules are also noticed at this stage. Myoid cells (Fig. 1f) were noticed in the sections thymus at 30 dph.

Kidney

Though the kidney appeared as a simple excretory tube at 1 dph (Fig. 2a), undifferentiated stem cells, sparsely distributed between the excretory tubules of the head kidney, were noticed at 2 dph (Fig. 2b). Large number of actively dividing and darkly staining lymphoblasts were

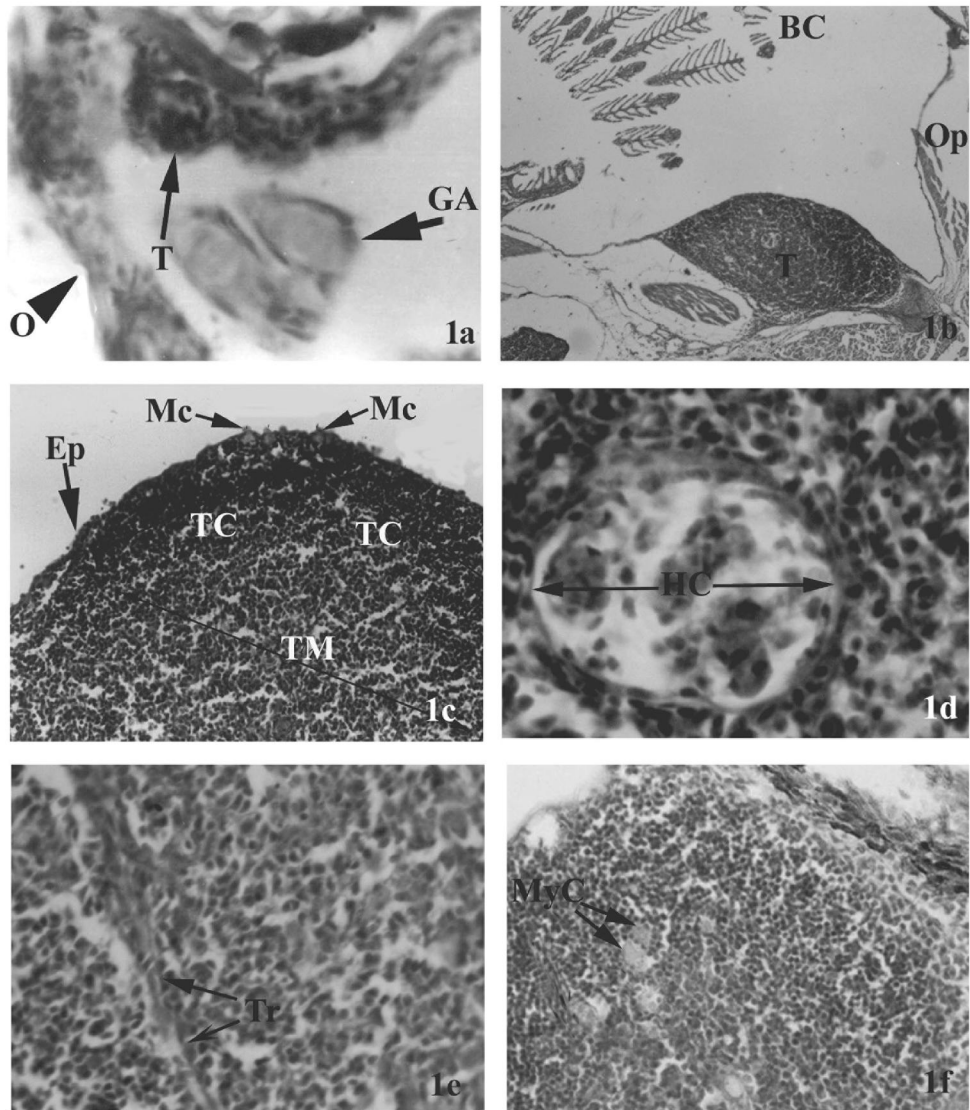


Figure 1. Haematoxylin and eosin stained sections of Asian seabass larvae showing the histogenesis of different lymphoid organs. 1a. Cross-section in the head region of 2 day-old fry primordial thymus with darkly staining (arrow) thymocytes, 400x; 1b. Fully developed thymus at 40 dph showing the position of the thymus in the bronchial chamber, 200x; 1c. Differentiation of cortical and modularly zones in the fully developed thymus of 40 day-old seabass fry. Note a thin layer of epithelium (thick arrows) with mucus cells (thin arrows), 400 x; 1d. Hassle's corpuscles in the thymus of a 40 day-old fry, 1000 x; 1e. Trabeculae (arrow) in the fully developed thymus, 1000 x; 1f. Myoid cell (arrows) in thymus of a 30 day-old fry distributed in the modularly zone, 400 x.

noticed at 6 dph (Fig. 2c) and at this stage, the number of tubules were fewer than those observed in the early stages. Macrophages, melanomacrophage centres (MMC), well differentiated haematopoietic and lymphopoietic cells were observed at 8 dph (Fig. 2d). Lymphocytes, macrophages, blood vessels, MMC and degenerating tubules were clearly shown at 40 dph (Fig. 2e). Thymus and head kidney were noticed in close proximity to each other and a rich supply of blood through the renal arteries was clearly visible at this stage (Fig. 2f).

Spleen

The first evidence of the appearance of spleen was recorded at 2 dph (Fig. 3a). At this stage the spleen appeared like a small bunch of darkly staining haematopoietic cells attached to the mesogastrium. At 6 dph the spleen appeared with a distinct capsule and variably stained zones of cells (Fig. 3b). Fully developed spleen, as seen at 40 dph (Fig. 3c), was characterized by the presence of MMC, the red and the white pulp, macrophages and the ellipsoids. Spleen from the adult fish was an elongated, capsulated distinct organ.

Gut associated lymphoid tissue

Gut associated lymphoid tissue (GALT) in the form of intraepithelial and intralaminal lymphocytes were noticed at 5 dph. Mature and darkly staining lymphocytes were noticed in the lamina of the hind gut at 10 dph (Fig. 3d).

The sequence of appearance of the lymphoid organs of *L. calcarifer* during the ontogeny was: lymphoid kidney (2 dph), thymus (2 dph), spleen (2 dph) and the GALT (5 dph). A comparative analysis of the appearance of lymphoid organs in some teleosts is given in table 1.

Discussion

The sequence of development of lymphoid organs of *L. calcarifer* is similar to that of the migratory salmon, *Salmo gairdneri* (Ellis 1977), rainbow trout, *Oncorhynchus mykiss* (Grace and Manning 1980), carp, *Cyprinus carpio* (Botham and Manning 1981) and Indian major carps (Kalita 1998). However, in yellow tail (*Seriola quinqueradiata*), red sea bream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*) and the turbot (*Scophthalmus maximus*), the thymus

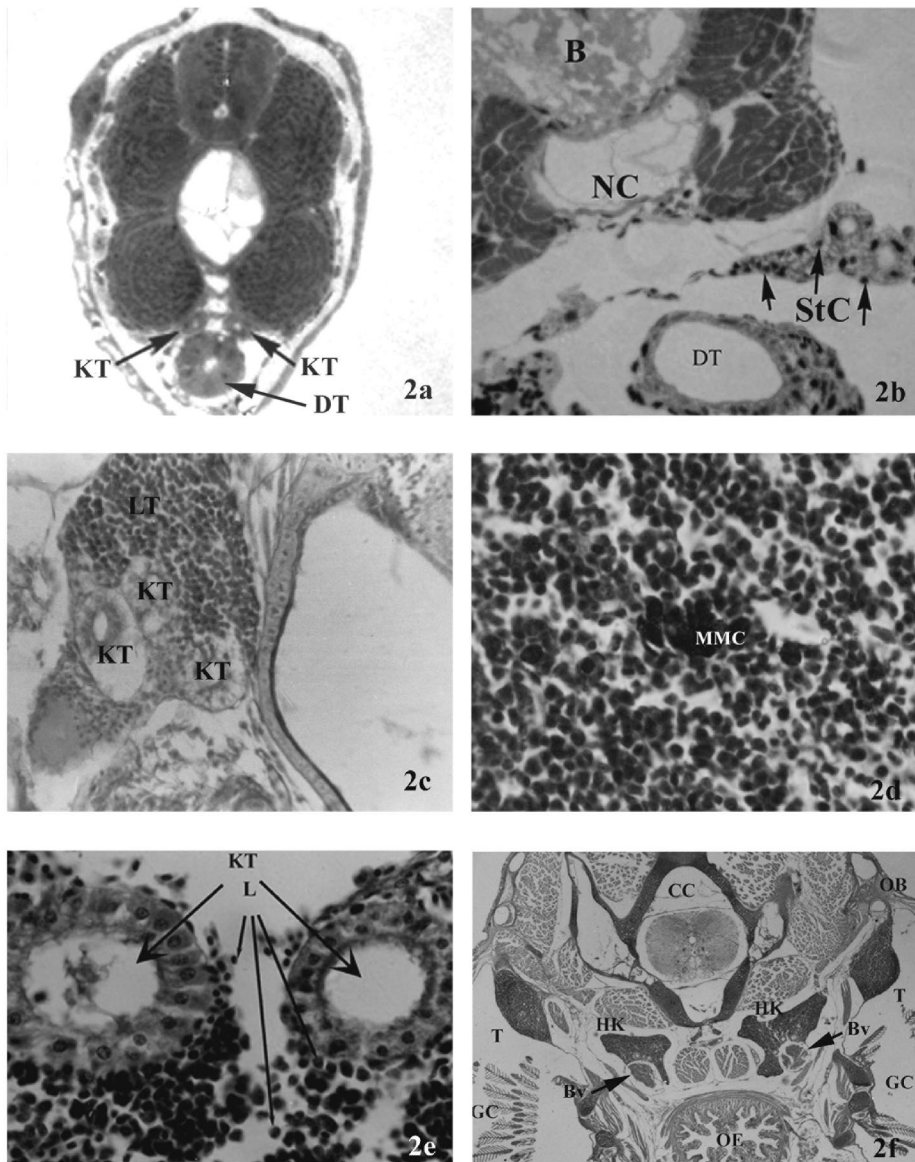


Figure 2. Haematoxylin and eosin stained sections of Asian seabass larvae showing the histogenesis of different lymphoid organs. 2a. Larvae (0 dph) showing excretory kidney tubule (KT) and digestive tube (DT), 400x; 2b. Undifferentiated stem cells (arrows) in the pronephros of 2 day-old larvae, 400x; 2c. Head kidney of 6 day-old larvae with fully developed lymphoid tissue and fewer kidney tubules, 400x; 2d. Melanomacrophage centres in the pronephros of 40 day-old fry, 1000x; 2e. Mature lymphocytes in the pronephros, 1000x; 2f. Relative position of thymus and pronephros in the 40 day-old fry, 200x.

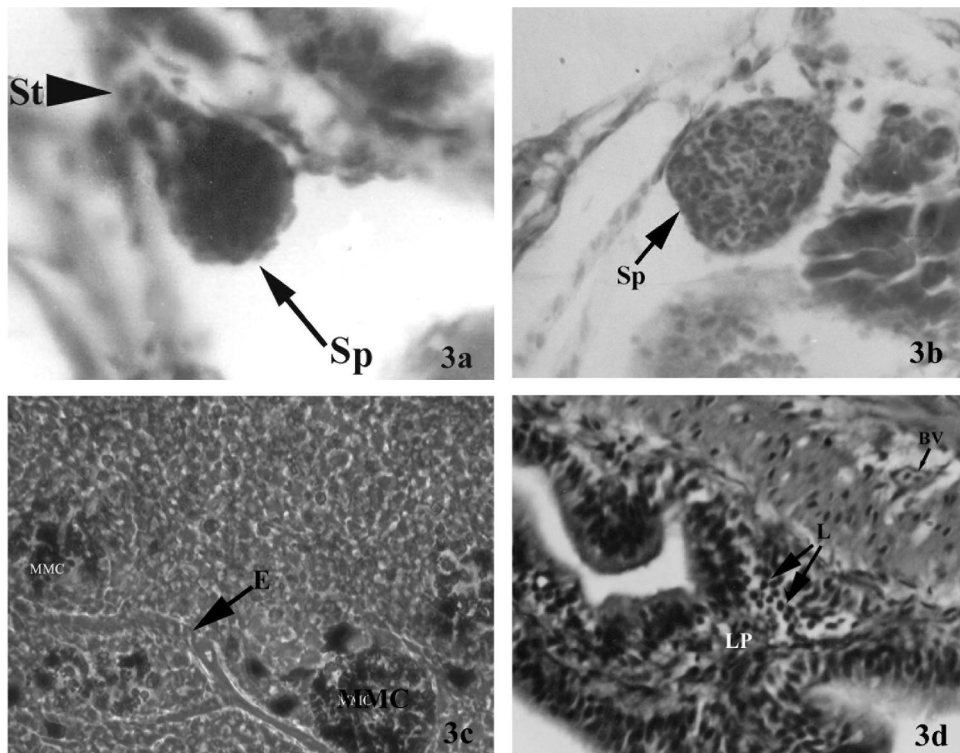


Figure 3. Haematoxylin and eosin stained sections of Asian seabass larvae showing the histogenesis of different lymphoid organs. 3a. First appearance of spleen in a 2 day-old sea bass larvae. Note the erythropoietic spleen with a stalk attached to the mesogastrium, 1000x; 3b. Spleen cell types getting differentiated in 6 day-old larvae, 400x; 3c. Well developed spleen showing ellipsoids and the MMC, 1000x; 3d. Fully developed GALT in seabass larvae (6 dph), note the blood vessel (BV) in the mucosa and the lymphocytes (L) in the lamina propria of the hindgut, 400x. O-opercular bone; T-thymus; GA-gill arch; BC-bronchial chamber; Op-operculum; Ep-epithelium; Mc-mucus cells; TC-thymic cortex; TM-thymic medulla; HC-Hassle's corpuscle; Tr-trabeculae; MyC-myoid cells; KT-kidney tubule; B-brain; NC-notocord; StC-stem cells; LT- lymphoid tissue; MMC-melanomacrophage centres; L-lymphocytes; CC-cranial chamber; OB-opercular bone; GC-gill chamber; BV-blood vessel; OE-oesophagus; St-stalked spleen; Sp-spleen; E-ellipsoid; LP-lamina propria

was the last to appear (Chantanachookhin et al. 1991; Padros and Crespo 1996).

Appearance of the undifferentiated stem cells in the primordial thymus (2 dph) of the Asian sea bass is similar to the observations on the rudiments of the thymus at 5 days pre-hatch in the rainbow trout (Grace and Manning 1980). Darkly staining undifferentiated stem cells in the

Table 1. Appearance of lymphoid organs in different fish species during the early developmental stages

| Species | Thymus | Spleen | Kidney | References |
|-------------------------------|---------|---------|---------|---|
| <i>Cyprinus carpio</i> | 5 dph | 8-9 dph | 7-8 dph | Botham and Manning (1981) |
| <i>Salmo salar</i> | 22 dprh | 42 dph | 14 dprh | Ellis (1977) |
| <i>Salmo gairdneri</i> | 3-5 dph | 21 dph | 5-6 dph | Grace and Manning (1980) Tatner and Manning (1985) |
| <i>Catla catla</i> | 2-3 dph | 4-5 dph | 2-4 dph | Kalita (1998)* |
| <i>Labeo rohita</i> | | | | |
| <i>Cirrhinus mrigala</i> | | | | |
| <i>Seriola quinqueradiata</i> | 11 dph | 3 dph | 1 dph | Chantanachookin et al. (1991) |
| <i>Pargus major</i> | 11 dph | 3 dph | 0 dph | Chantanachookin et al. (1991) |
| <i>Paralychthys olivaceus</i> | 10 dph | 8 dph | 7 dph | Chantanachookin et al. (1991) |
| <i>Scophthalmus maximus</i> | 3-4 dph | 5-6 dph | 0 dph | Padros and Crespo (1996) |

dph: days post hatch; dprh: days pre-hatch; * First appearance of the organs

thymic primordium, at 2 dph, of the Asian sea bass is an important observation as the previous workers have opined on the probable recruitment of virgin pool of lymphocytes from the thymus followed by the development of other lymphoid organs (Cooper 1973; Zapata et al. 1996) and the *Ikaros* gene is known to be an essential factor in the differentiation of the B and T lymphocytes (Georgopoulos et al. 1997). At this stage, though very few undifferentiated stem cells appeared in the head kidney, the thymocytes were in an advanced stage of development with more and distinct actively dividing cells. Further studies with the thymocyte labelling using *Ikaros* gene probes might probably throw more light on the likely time of recruitment of thymocytes into the developing immunity of Asian sea bass. Investigations on the *Ikaros* expression as a marker for lymphoid progenitors during zebra fish development have shown that the *Ikaros*-expressing cells were found in the pharyngeal region, adjacent to the developing thymus (Willett et al. 2001). Later these cells were found in the pronephros. The thymus showed a positive expression for this gene in developing zebra fish

at 96 h (Willett et al. 1999). There are clear evidences indicating that the thymus plays a key role in the ontogeny of immunological competence (Cooper and Hildemann 1965; Cooper 1973). Thymus is thought to be the major organ for storage and maturation of T cells and the first lymphoid organ that acquires lymphocytes during the histogenesis of the lymphoid tissue (Manning 1994; Zapata et al. 1996). Cellular characteristics of the thymus of the Asian sea bass are more or less similar to those recorded for the other teleosts (Chantanachookhin et al. 1991; Padros and Crespo 1996; Kalita 1998). Inner light zone and an outer dark zone representing, respectively, the differentiating and differentiated thymocytes and the presence of trabeculae were the other morphological characteristics of the fully-grown thymus of the sea bass. Padros and Crespo (1996) recorded similar observations in the turbot. There are very few reports on the presence of Hassall's corpuscles in the teleostean thymus. The present study, however, is in agreement to the observations of Sailendri and Muthukkaruppan (1975) and Fishelson (1995) who reported that the thymus of the cichlid teleost, *Tilapia mossambica*, contained Hassall's corpuscles in the inner zone. Hassall's corpuscles are probably of epithelial origin and may serve to remove cellular debris from apoptotic thymocytes (Picker and Siegelmen 1993). Our observations indicating well developed Hassall's corpuscles suggest that the thymus of seabass might also undergo involution following ageing. The appearance of Hassall's corpuscles and the epithelial cells has been associated with the involution process of the thymus in aged or older fish (Luer et al. 1995). Though, we observed the Hassall's corpuscles in the thymus of a 40-day old fish, it is not known whether the involution process has already started at 40 dph or not.

Kidney, in the very early stages of development, was represented mainly by the excretory tubules and only at 2 dph; a few of the undifferentiated stem cells in the head kidney were noticed. As the stages of development advanced the darkly staining stem cells, later the lymphocytes, increased in number replacing the degenerating tubules. Similar observations were made by Chantanachookhin et al. (1991) working on three marine fish species comprising of yellow tail, red sea bream and the Japanese flounder. In the embryonic condition, the head kidney (pronephros) is involved in excretory function, but in the later stages it becomes modified into a specialized lymphoid tissue (Sailendri and Muthukkaruppan 1975). Though, kidney appeared first, the stem cells representing the primordial thymus were the first to be seen in the thymus of the sea bass. This could suggest that the thymus probably contributed to the virgin pool of lymphocytes. Working on the elasmobranches, Beard (1894) suggested that the

thymus is the source of lymphoid cells that ultimately get distributed in other peripheral lymphoid organs. This observation is different from those of Ellis (1977) and Chantanachookhin et al. (1991) who said that the kidney could be regarded as the source of stem cells.

The spleen and the kidney were found to be rich sources of MMC, indicating that the non-specific defences in the Asian sea bass could have taken care of the invading foreign particles before the specific immunity became competent. The spleen of Asian sea bass has a thin fibrous connective tissue forming a capsule. The MMC and haemosiderin deposits seen in the spleen of *L. calcarifer* are probably indicative of the antigen trapping and haemocyte-residue recycling mechanisms which have been suggested in several other fish species (Ferguson 1976; Agius 1979). Probable role of the spleen as an organ responsible for phagocytosis before immune competence is attained has also been suggested (Tatner and Manning 1985; Chantanachookhin et al. 1991).

The role of GALT in fish vaccination was not very well understood till recently. It was the effort of Duff (1942), reporting a high protective response in orally vaccinated cutthroat trout against *Aeromonas salmonicida*, that attracted the attention of fish immunologists. Immunological importance of gut reflecting the potentialities of oral vaccines in aquaculture has been demonstrated in carps (Rombout et al. 1986; Azad et al. 1999; 2000). The GALT, popularly referred to as Payer's Patches, play an important role in the mammalian defense against the antigens entering the gastrointestinal tract. Sub-epithelial lymphoid aggregates have been recorded in the perch, *Perca fluviatilis* (Zapata 1979). Davina et al. (1982) reported that the epithelium and lamina propria of the carp gut contained scattered lymphoid-like cells and based on the present observations it can only be said that in the Asian sea bass the GALT could play an important role in oral vaccination. With the increasing importance of minimizing stress during various aquaculture operations and success in enhanced immune response of protected oral antigens, the role of GALT in oral vaccination of fish will be much more focused in the years to come. Hence, the information on the ontogeny of GALT in fish is the pre-requisite for designing the nature of oral vaccines in fishes like the Asian sea bass.

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