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Relation	

Ontogeny of gene expression of group IB phospholipase A_2 isoforms in the red sea bream, *Pagrus* (*Chrysophrys) major*

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Abstract

The red sea bream was previously found to express mRNAs for two group IB phospholipase A_2 (PLA_2) isoforms, DE-1 and DE-2, in the digestive organs, including the hepatopancreas, pyloric caeca, and intestine. To characterize the ontogeny of the digestive function of these PLA_2s, the present study investigated the localization and expression of DE-1 and DE-2 PLA_2 genes in red sea bream larvae/juveniles and immature adults, by *in situ* hybridization. In the adults, DE-1 PLA_2 mRNA was expressed in pancreatic acinar cells. By contrast, DE-2 PLA_2 mRNA was detected not only in digestive tissues, such as pancreatic acinar cells, gastric glands of the stomach, epithelial cells of the pyloric caeca, and intestinal epithelial cells, but also in non-digestive ones, including cardiac and lateral muscle fibers and the cytoplasm of the oocytes. In the larvae, both DE-1 and DE-2 PLA_2 mRNAs first appeared in pancreatic tissues at 3 days post-hatching (dph) and in intestinal tissue at 1 dph, and expression levels for both gradually increased after this point. In the juvenile stage at 32 dph, DE-1 PLA_2 mRNA was highly expressed in pancreatic tissue, and DE-2 PLA_2 mRNA was detected in almost all digestive tissues, including pancreatic tissue, gastric glands, pyloric caeca, and intestine, including the myomere of the lateral muscles. In conclusion, both DE-1 and DE-2 PLA_2 mRNAs are already expressed in the digestive organs of red sea bream larvae before first feeding, and larvae will synthesize both DE-1 and DE-2 PLA_2 proteins.

1. Introduction

There is now a large amount of information available on the importance of dietary phospholipids for the growth of fish larvae (Koven *et al.* 2001; Tocher *et al.* 2008; Cahu *et al.* 2009). Supplementation with dietary phospholipids is potentially important in the formulation of practical diet (Coutteau *et al.* 1997). Microdiet supplemented with phospholipids, particularly phosphatidylcholine (PC), stimulated feeding activity and was incorporated with up to 45% efficiently in young larval seabream (Koven *et al.* 2001). Moreover, dietary PC appears to have a postprandial enhancing effect on lipoprotein synthesis (Tocher *et al.* 2008), resulting in an improved uptake of dietary lipids from the mucosa of the digestive tract to the circulatory system. Sargent et al. (1997, 1999) analyzed the lipid compositions of marine fish egg yolk, whole larvae, and zooplankton (their natural prey), and proposed that ideal diets for marine fish larvae should contain at least 10% n-3 highly unsaturated fatty acid (HUFA)-rich marine phospholipids in dry weight, with less than 5% triacylglycerols. Clearly, dietary phospholipids are important for the growth of marine fish larvae; however, specific nutritional aspects, such as the enzymatic processes responsible for lipid digestion in marine fish, especially in early stages of marine fish larvae/juveniles, remain unclear. Protease- and amylase-related digestive processes underlying protein and carbohydrate metabolism have been studied intensively in the larvae of several fish species. By contrast, very little information exists on the digestive enzymes of dietary phospholipids in fish larvae (Ozkizilcik *et al.* 1996; Evans *et al.* 1998; Buchet *et al.* 2000; Zambonino infante *et al.* 2001; Srivastava *et al.* 2002; Tocher *et al.* 2008).

Phospholipase A_2 (phosphatide 2-acyl hydrolase, EC 3.1.1.4, PLA_2) of animals is a representative and essential lipolytic enzyme that catalyzes the hydrolysis of the ester bond at the *sn*-2 position of glycerophospholipids. Mammalian pancreatic PLA_2s, which belong to group IB, are stored in the form of an inactive pro-PLA_2 (zymogen) in the secretory granules

of pancreatic acinar cells. Pro-PLA_2 is activated through limited tryptic proteolysis in the intestinal tract into its active form, which is capable of digesting dietary phospholipids (Verheij *et al.* 1981). The primary structures of mammalian group IB PLA_2s have been determined, and their physicochemical properties and reaction mechanisms as a lipolytic enzyme have been extensively studied (Verheij *et al.* 1981; Heinrikson 1991).

By contrast, few studies have been performed on fish PLA_2s (Audley *et al.* 1978; Neas *et al.* 1985; Aaen *et al.* 1995). Zambonino Infante and Cahu (Zambonino Infante *et al.* 1999) determined the primary structure of seabass (*Dicentrarchus labrax*) PLA_2 and found that the seabass enzyme belongs to the group IB PLA_2s. They also found that PLA_2 mRNA expression and PLA_2 activity in seabass and red drum (*Sciaenops ocellatus*) larvae were stimulated by increased phospholipid contents in the diet (Zambonino Infante *et al.* 1999; Buchet *et al.* 2000). In addition, Sæle *et al.* (Sæle *et al.* 2011) very recently determined the sequence for the group IB PLA_2 gene in Atlantic cod *(Gadus morhua)* and described its ontogeny at the genetic and protein levels. We investigated the localization and ontogeny of digestive PLA_2 in the red sea bream, *Pagrus* (formerly *Chrysophrys*) *major,* using an antiserum against *Naja naja* venom PLA_2, and found that a PLA_2-like protein was distributed in the pancreatic acinar cells and certain epithelial cells of epithelial crypts in the pyloric caeca of the red sea bream (Uematsu *et al.* 1992). We further purified six low molecular weight Ca^{2+}-dependent PLA_2s from the pyloric caeca (Iijima *et al.* 1997), the hepatopancreas (Iijima *et al.* 1990; Ono *et al.* 1998) and the gills (Iijima *et al.* 2000) of the red sea bream and found three distinct isoforms of group IB PLA_2: DE-1and DE-2 PLA_2s in the hepatopancreas and G-3 PLA_2 in the gills. We also recently isolated a cDNA encoding a novel PLA_2 isoform from the intestine, denoted as IN PLA_2, that showed 49–75% homology to DE-1, DE-2, and G-3 PLA_2s, that exists only in a single fish species, the red sea bream (Iijima *et al.* 2000; Iijima *et al.* 2001; Iijima *et al.* 2009). In addition, RT-PCR experiments revealed that, in the red sea bream (200 g in body mass),

mRNAs of DE-1 and DE-2 PLA_2s were expressed in digestive systems, such as the hepatopancreas, pyloric caeca, intestine, and stomach, whereas G-3 PLA_2 mRNA was expressed only in the gills and gonads (Iijima *et al.* 2001; Uchiyama *et al.* 2002). These distributions suggest that DE-1 and DE-2 PLA_2s predominantly work as digestive enzymes of the dietary phospholipids. However, the significance of these broadly localized expression patterns of these isoforms among the digestive organs remains unclear. We therefore tried to detect the mRNAs of DE-1 and DE-2 PLA_2s in the various organs of adult and larval/juvenile red sea breams by *in situ* hybridization, in order to comprehend the ontogeny of the phospholipid digestive enzymes.

2. Materials and Methods

2.1. Materials

The DIG RNA Labeling Kit (SP6/T7), tRNA (from brewer's yeast), blocking reagent, and sheep anti-digoxigenin-Fab fragments, conjugated with alkaline phosphatase (anti-DIG-AP Fab fragments), were obtained from Roche Diagnostics K. K. (Tokyo, Japan). The DC Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was purchased from Dojindo Laboratories (Kumamoto, Japan), and *o*-phenylenediamine dihydrochloride and (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (*p*-APMSF) was purchased from Wako Pure Chemicals (Tokyo, Japan). TITANIUM^{TM} Taq DNA polymerase was obtained from Clontech (Palo Alto, CA), Isogen from Nippon Gene (Tokyo, Japan), and pBluescript SK (+) vector from Stratagene (La Jolla, CA).

2.2. Fish and tissue preparation

Immature adult red sea bream with an average body mass of 200 g were obtained from Hiroshima Fisheries Experimental Station, Ondo, Japan. The hepatopancreas, stomach, pyloric caeca, anterior intestine, posterior intestine, gills, heart, spleen, kidney, brain, lateral muscles, and gonads were dissected from three adult fish and were immediately frozen in liquid nitrogen for Northern hybridization and reverse-transcription polymerase chain reaction (RT-PCR) analysis. In parallel, parts of these organs were dissected from five other adults and were fixed in Bouin's solution overnight at 4°C for *in situ* hybridization.

Larvae and juveniles of the red sea bream were obtained from a stock that were naturally fertilized, hatched, and reared at 20°C with rotifers, brine shrimp, and an artificial diet for larvae at Hiroshima Prefectural Fish Farming Center, Takehara, Japan. The fishes were fed four times a day with these foods, combinations of which were changed according to their growth. Larvae and juveniles from 0 until 32 days-post-hatching (dph) were preserved for later analyses by *in situ* hybridization, Northern hybridization, and RT-PCR using the same preservation procedures as were used for adults.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from frozen powders of hepatopancreas, pyloric caeca, intestine, spleen, gill, gonad, heart, brain, stomach, kidney, and lateral muscle tissue from the immatures, using Isogen, according to the manufacturer’s instructions. Total RNA from whole eggs and larvae/juveniles from 0 to 32 dph was also prepared as described above.

Total RNA (3 μg) was used as the template to synthesize the first strand of cDNA. Then, the cDNA fragments containing coding and noncoding regions of DE-1 (DDBJ, accession

number **AB050632**) and DE-2 (DDBJ, accession number **AB009286**) PLA_2 cDNAs were amplified by PCR from the first-strand cDNA using TITANIUM^{TM} Taq DNA polymerase as described previously (Iijima *et al.* 2001). The primers used were: DE-1 PLA_2, 5'-GCCTTATGGCAGTTTGGGAACA-3'(identical to nt 76–97) and 5'-CTCTAACTTCAACAAATCAGG-3' (complementary to nt 540–560); DE-2 PLA_2, 5'-GCACTCAACCAGTTCAGACAG-3' (identical to nt 70–92) and 5'-TAGTAGGGAATGATGGATGGC-3' (complementary to nt 572–592) (Iijima *et al.* 2001). A pair of primers (5'-CGGGATCCACTACCTCATGAAGATCCTG-3' and 5'-CCGCTCGAGTTGCTGATCCACATCTGCTG-3') specific for red sea bream heart ß-actin gene (DDBJ, accession number **AB050670**) was used to amplify a 478 bp fragment of red sea bream ß-actin as an internal control. PCR conditions were: an initial denaturation for 2 min at 94°C, followed by 35 cycles of amplification, with 30 s denaturation at 95°C, 10 s annealing at 58°C, and 30 s extension at 74°C. The reaction products were electrophoresed on a 1% agarose gel.

2.4. Northern hybridization

Northern hybridization was carried out as described previously (Iijima *et al.* 2009). In brief, a digoxigenin (DIG)-labeled cRNA probe was prepared with the DIG RNA Labeling Kit (Roche Diagnostics). The sequences of DE-1 PLA_2 cDNA corresponding to nt 78–562 and DE-2 PLA_2 cDNA corresponding to nt 103–625 were used for making the DIG-labeled cRNA probes. Total RNA for the red sea bream was isolated from various organs of the adult fish, whole eggs, and whole bodies of larvae/juveniles from 0 to 37 dph, as described in the RT-PCR methods. After separating 15 µg of total RNA on a 1% agarose gel containing formaldehyde and transferring onto a nylon membrane (Zetaprobe, Bio-Rad, Hercules, CA),

the total RNA was hybridized with the DIG-labeled antisense cRNA probe at 60°C overnight. The membrane was washed twice in washing buffer at 65°C, and the hybridization signals were detected using anti-DIG-AP-fragments (1:1,000 v/v) and CDP-Star (Roche Diagnostics), according to the manufacturer's instructions.

2.5. In situ hybridization

After fixation in Bouin's solution, tissues of the adults and whole bodies of the larvae/juveniles were dehydrated through a graded alcohol series (2 h each) at 4°C, cleared in toluene, and embedded in paraffin (Tissue-Tech Paraffin wax II60, Sakura Fine Tech Japan, Tokyo). Then, 5-μm serial sections were cut and attached to 3-aminopropyltriethoxysilane (APS)-coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan). Tissue sections were de-waxed and rehydrated. After a brief wash with 0.1 M phosphate buffer (pH 7.4), sections were hybridized with the DIG-labeled cRNA antisense or sense probe for either DE-1 PLA_2 or DE-2 PLA_2 and stained as described previously (Uchiyama *et al.* 2002).

3. Results

3.1. mRNA expression of DE-1 and DE-2 PLA_2*s in adult red sea bream*

The mRNA expression levels of DE-1 and DE-2 PLA_2s were compared among various tissues of the red sea bream by Northern blot analysis. As shown in Figure 1A, DE-2 PLA_2 mRNA was expressed in the hepatopancreas, pyloric caeca, intestine, gonad, heart, stomach, kidney, and lateral muscles, but not in the spleen and gill, whereas DE-1 PLA_2 mRNA was expressed only in the hepatopancreas. The expected cDNA corresponding to DE-1 PLA_2 (483

bp) was amplified by RT-PCR in the hepatopancreas, pyloric caeca, intestine, spleen, gonad, brain, stomach, and kidney of the immatures, and the DE-2 PLA_2 cDNA (522 bp) was amplified in the hepatopancreas, pyloric caeca, intestine, spleen, gill, gonad, heart, stomach, kidney, and muscle (Fig. 1B).

In situ hybridization with an antisense probe for DE-1 PLA_2 yielded intense positive signals in exocrine acinar cells of the hepatopancreas only (Fig. 2A). No signal was observed in the cells treated with a sense probe for DE-1 PLA_2 as a negative control (Fig. 2B). By contrast, DE-2 PLA_2 mRNA was detected not only in exocrine pancreatic cells (Fig. 3A), but also in other digestive organs, including the cytoplasmic perinuclear region of epithelial cells, notably in the cells located in the crypts of the pyloric caeca (Fig. 3B), the gastric gland of the stomach (Fig. 3C), and intestinal epithelial cells (Fig. 3D). Signals corresponding to the DE-2 PLA_2 mRNA were detected in non-digestive tissues, such as cardiac muscle cells (Fig. 3E), lateral muscles (Fig. 3F), and the cytoplasm of oocytes (Fig. 3G). An antisense probe for DE-2 PLA_2 revealed dense colored granules in the muscle fiber of the lateral muscles and the connective tissue cells between muscle cells (Fig. 3F), as well as the cytoplasm of oocytes in the perinucleolus stage (Fig. 3G). No positive signal was detected in these tissues with a sense primer for DE-2 PLA_2 (data not shown).

3.2. mRNA expression of DE-1 and DE-2 PLA_2*s in larvae/juveniles*

By Northern blot analysis, mRNA expression of DE-2 PLA_2 was first detected at 3 dph, while that of DE-1 PLA_2 was not detected until 32 dph (Fig. 4A).

By RT-PCR analysis, mRNA expression of DE-1 PLA_2 was already detected at 0 dph and that of DE-2 PLA_2 was detected from 1 dph. From 3 to 32 dph, the mRNA of both enzymes was continuously expressed (Fig. 4B). The mRNA expression of ß-actin was also detected

almost constantly in the larvae/juveniles from 0 to 32 dph.

In situ hybridization with an antisense probe yielded faint signals indicating expression of DE-1 PLA_2 mRNA in the exocrine pancreatic tissue from 3 dph (closed arrowheads in Fig. 5C), and the intensity of the signals gradually increased along with growth until 32 dph, the last sampling day (arrowheads in Fig. 5 D-G). At 32 dph, the pancreas was still independent from the liver, and the signals were detected only in the pancreatic tissue (Fig. 5G). No positive signal was detected in any organs of the whole body of larvae and juveniles from 0 to 32 dph with a sense probe (data not shown). Using the antisense probe for DE-2 PLA_2 cRNA, faint signals were first detected from several areas in intestinal tissue in the larvae on 1 dph (Fig. 6B). At 3 dph, signals were observed in almost all of the intestinal epithelial cells (closed arrowheads in Fig. 6C). Thereafter, the intensity of the staining in the intestine gradually increased (closed arrowheads in Fig. 6F), and positive signals were also detected in the lateral muscles of the larvae at 9 dph (open arrowheads in Fig. 6F). Signals were detected in the hepatic tissues of the larvae between 3 and 5 dph only (open arrowheads in Fig. 6C and D). At 32 dph, dense signals were also recognized in whole intestinal tissues (Fig. 6H) and around the myomere of the lateral muscles, especially near the vertebrae (Fig. 6I). No positive signal was detected in the whole bodies of larvae and juveniles from 0 to 32 dph with the sense probe for DE-2 PLA_2 (data not shown).

4. Discussion

We previously reported that both DE-1 and DE-2 PLA_2s purified from the hepatopancreas of the red sea bream could be classified into the mammalian pancreatic-type group IB PLA_2, but that the homology of DE-2 PLA_2 to DE-1 PLA_2 at the primary structure level was low (46.7%) (Iijima *et al.* 2001). The existence of multiple isoforms of group IB PLA_2 in a single

species has never been reported in mammals. In addition, the physiological differences between the two PLA_2 isotypes were unclear. In this study, we investigated the mRNA expression of DE-1 and DE-2 PLA_2 in adults and larvae/juveniles of the red sea bream. In common with many other fishes, red sea bream has four major sites containing digestive enzymes, the stomach, the pancreatic portion of the hepatopancreas, the pyloric caeca and the intestine (Fänge *et al.* 1979; Bakke *et al.* 2011). As DE-1 PLA_2 mRNA was principally expressed in the exocrine acinar cells in the hepatopancreas (Figs. 1A and 2), it would be expected to be secreted from the pancreas and act as a digestive enzyme for dietary phospholipids in the adults. On the other hand, DE-2 PLA_2 expression was detected not only in the acinar cells of the hepatopancreas, but also in epithelial cells of the pyloric caeca and intestine, and gastric glands of the stomach (Figs. 1A and 3), indicating that DE-2 PLA_2 is secreted into the digestive tract, where it would be expected to hydrolyze dietary and biliary phospholipids, in collaboration with DE-1 PLA_2.

Pancreatic-type group IB PLA_2 is secreted from the pancreatic tissue into the intestinal tract and digests dietary phospholipids (Verheij *et al.* 1981; Murakami *et al.* 2002). Recently, group IB PLA_2 mRNA and protein were also found in non-digestive organs, including spleen, lung, kidney, and ovary (Six *et al.* 2000). Furthermore, mammalian group IB PLA_2 is recognized to cause various biological responses through binding to the PLA_2 receptor (Valentin *et al.* 2000). These additional aspects of group IB PLA_2 could be applicable to the fish DE-2 PLA_2 found in non-digestive tissues of the adult red sea bream. As with the mammalian group IB PLA_2 isoform, DE-2 PLA_2 may also elicit any number of unknown physiological responses through binding to the PLA_2 receptor in fishes.

We further tried to investigate the sequence of mRNA expression for both enzymes in red sea bream larvae/juveniles through the developmental stages, and summarized this study into the Figure 7. DE-2 PLA_2 mRNA first appeared in the intestine on 1 dph, and DE-1 PLA_2 was

observed in the pancreatic acinar cells from 3 dph. The expression of mRNA for both enzymes gradually increased in these organs along with growth of the fish until 32 dph. The mouths of larvae open on 4 dph, and at this point they started to feed on rotifers (Fukuhara 1985). In addition, the same study found that yolk and oil droplets were exhausted by 4 and 6 dph, respectively. The present results demonstrate that both DE-1 and DE-2 PLA_2 mRNAs are already being expressed in the digestive organs of red sea bream larvae before the onset of feeding, and indicate that larvae synthesize both enzymes in the digestive organs. This idea is supported by the positive correlation between expression of group IB PLA_2 mRNA and protein in the digestive organs of Atlantic cod (Sæle *et al*. 2011). However, it remains unclear why DE-2 PLA_2 mRNA was expressed in the hepatic tissues of the larvae between 3 and 5 dph only. Phospholipids are the major lipid constituent of marine fish eggs and account for roughly 10% of total egg dry weight; triacylglycerols usually are a minor component (Sargent *et al.* 1999). This indicates that phospholipids are one of the important endogenous nutrients for the newly hatched larvae. Pre-larvae of the red sea bream must completely rely on endogenous yolk phospholipids as an energy source until 5 dph, when they start feeding. It is very interesting to note that the liver synthesizes and secretes DE-2 PLA_2 only during this short, non-feeding period in the larval development. The DE-2 PLA_2 that originates in the liver might act in combination with other PLA_2s of different origins to digest endogenous phospholipids in the yolk sac. In other words, the liver could be a supplementary organ secreting PLA_2 only during an important larval stage; the observation that PLA_2 mRNAs were not expressed in the liver of larvae from 0 to 2 dph supports this idea.

In mammals, pancreatic-type group IB PLA_2, synthesized as prepro PLA_2, is processed with signal peptidase in the rough endoplasmic reticulum and then stored as inactive proPLA_2 in the zymogen granules (Verheij *et al.* 1981). After secretion in the intestinal lumen, pro PLA_2 is activated by limited tryptic proteolysis into a form capable of digesting dietary

phospholipids. Both DE-1 and DE-2 PLA_2s of the red sea bream have peculiar prepro sequences (Iijima *et al.* 2001), and both PLA_2s, secreted in the digestive tract as proenzymes, must be activated by limited proteolysis with trypsin, in the same way as mammalian pancreatic group IB PLA_2s. Therefore, it is essential to investigate the ontogeny of the proteolytic enzyme trypsin to understand when red sea bream larvae become capable of digesting dietary phospholipids. In addition, we are intrigued by the question of why two isoforms of PLA_2, DE-1 and DE-2, are expressed as functional enzymes in the various tissues or organs of red sea bream larvae/juveniles and adults. Although PLA_2 activity has been detected in the spleen, gonad, heart, kidney, and lateral muscles (Iijima *et al.* 2001), we have not yet analyzed histologically the distribution of the two isoforms of this protein in these organs. To clarify the above questions, it is necessary to investigate the distribution of DE-1 and DE-2 PLA_2 by immunoblotting and immunohistochemistry. As we have already succeeded in producing recombinant DE-1 and DE-2 PLA_2 (Fujikawa *et al.* 2003) and preparing specific antibodies against DE-1 and DE-2 PLA_2s, we will next investigate the localization and ontogeny of both PLA_2 proteins in adults and larvae/juveniles of the red sea bream.

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FIG. 1. Northern blot (A) and RT-PCR (B) analyses of mRNA expression of DE-1 and DE-2 PLA_2 in the adult red sea bream. (A) Total RNA from the various organs of the adult red sea bream was loaded in each lane. Each blot was hybridized with DIG-labeled probes as described in the materials and methods section. Lane 1, hepatopancreas; lane 2, pyloric caeca; lane 3, intestine; lane 4, spleen; lane 5, gill; lane 6, gonad; lane 7, heart; lane 8, stomach; lane 9, kidney; lane 10, lateral muscle. Total RNA is shown in the third panel, as visualized by ethidium bromide staining of the gel from which the blots shown above were made. (B) RT-PCR analysis of mRNA expression of DE-1 and DE-2 PLA_2s in the various tissues. Lane 1, hepatopancreas: lane 2, pyloric caeca: lane 3, intestine: lane 4, spleen; lane 5, gill; lane 6, gonad; lane 7, heart; lane 8, brain; lane 9, stomach; lane 10, kidney; lane 11, lateral muscle. β-actin was used as an internal standard.

FIG. 2. Detection of DE-1 PLA_2 mRNA in the hepatopancreas of the adult red sea bream by *in situ* hybridization. Paraffin sections of the organs were incubated with DIG-labeled cRNA probes for DE-1 PLA_2 and developed with alkaline phosphatase-conjugated, anti-DIG antibodies. Signals were detected in the exocrine pancreatic tissue (EP) of the hepatopancreas with an antisense probe (A). No positive signal was detected in either tissue of the hepatopancreas with a sense probe (B). Abbreviations: He, hepatic tissue; EP, exocrine pancreatic tissue. Calibration bars in all panels = 100 μm.

FIG. 3. Detection of DE-2 PLA_2 mRNA in the various organs of the adult red sea bream by *in situ* hybridization. Paraffin sections of the hepatopancreas (A), pyloric caeca (B), stomach (C), intestine (D), heart (E), lateral muscle (F), and ovary (G) were incubated with DIG-labeled cRNA probes for DE-2 PLA_2 and developed with alkaline phosphatase-conjugated, anti-DIG antibodies. Intense signals were detected in the pancreatic

acinar cells of the hepatopancreas (A) and the cells located in the crypts of the pyloric caeca (B). Intense signals were also detected in the gastric glands of the stomach (C), the intestinal epithelial cells (D), almost all of the cardiac muscle cells (E), the lateral muscle cells (F), and the cytoplasm of oocytes (G). No positive signal was detected in any tissue tested with sense probe for DE-2 PLA_2 (data not shown). Abbreviation: Ga, gastric gland. Calibration bars in all panels = 100 μm.

FIG. 4. Northern blot (A) and RT-PCR (B) analyses for mRNA expression of DE-1 and DE-2 PLA_2s in eggs and larvae/juveniles of the red sea bream. (A) Total RNA extracted from several developmental stages of whole eggs and larvae/juveniles was loaded in each lane. Each blot was hybridized with the DIG-labeled probes as described in the materials and methods section. Total RNA is shown in the third panel, as visualized by ethidium bromide staining of the gel from which the blots shown above were made. (B) Total RNA from several developmental stages of the red sea bream were used as templates for RT-PCR. The cDNAs encoding DE-1 and DE-2 PLA_2s were amplified with a single round of PCR as described in the materials and methods section. ß-actin was used as an internal standard. Lane 1, egg; lane 2, 0 dph; lane 3, 1 dph; lane 4, 3 dph; lane 5, 5 dph; lane 6, 7 dph; lane 7, 9 dph; lane 8, 11 dph; lane 9, 13 dph; lane 10, 15 dph; lane 11, 17 dph; lane 12, 22 dph; lane 13, 27 dph; lane 14, 32 dph.

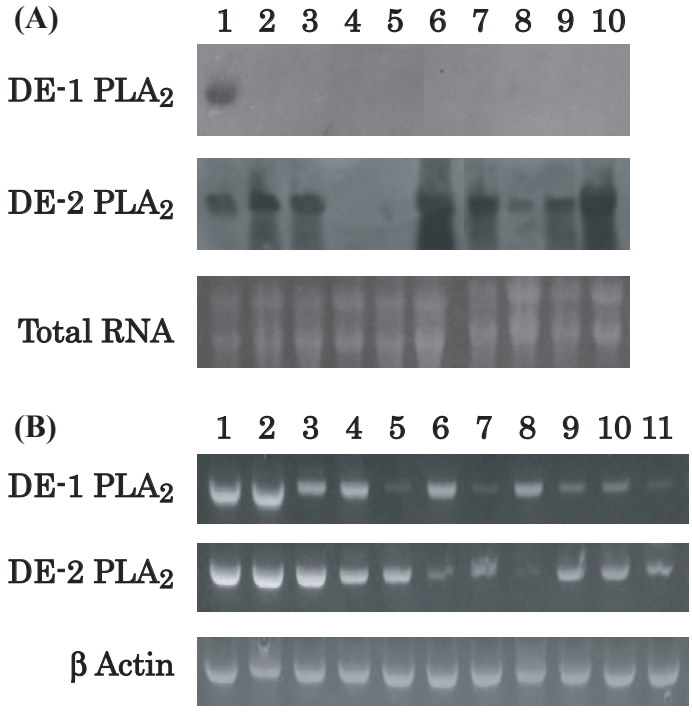
FIG. 5. Detection of DE-1 PLA_2 mRNA by *in situ* hybridization in red sea bream larvae and juveniles at 0 dph (A), 1 dph (B), 3 dph (C), 5 dph (D), 7 dph (E), 27 dph (F), and 32 dph (G). Serial sagittal sections of the whole body were incubated with a digoxigenin-labeled cRNA antisense probe for DE-1 PLA_2 and developed with alkaline phosphatase-conjugated, anti-DIG antibodies. No positive signal was detected in the whole bodies of larvae at 0 and 1

dph with an antisense probe for DE-1 PLA_2 (A and B). At 3 dph, faint signals were detected in the exocrine pancreatic tissue (arrowheads) (C). Thereafter, intensity of the staining gradually increased (D), especially after 7 dph (E and F). Even at 32 dph, the pancreas was still independent from the liver (G). No positive signal was detected in the whole body of larvae and juveniles in any of the stages (data not shown). Abbreviations: He, hepatic tissue; In, Intestine. Calibration bars in all panels = 100 μm.

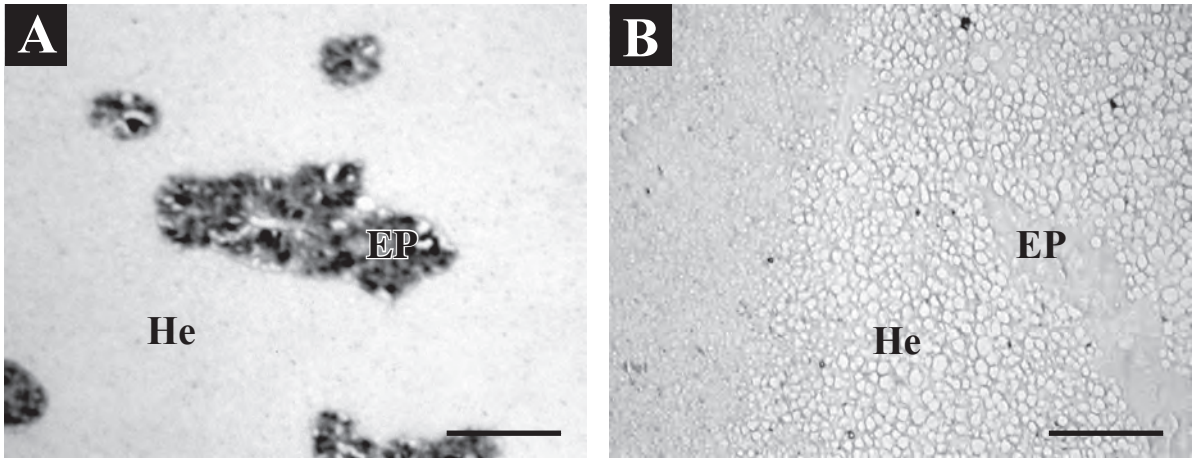
FIG. 6. Detection of DE-2 PLA_2 mRNA in the whole bodies of the red sea bream larvae and juveniles at 0 dph (A), 1 dph (B), 3 dph (C), 5 dph (D), 7 dph (E), 9 dph (F), 27 dph (G), and 32 dph (H and I). Serial sagittal sections of the whole body were incubated with a DIG-labeled cRNA antisense probe for DE-2 PLA_2 and developed with alkaline phosphatase-conjugated anti-DIG antibodies. No positive signal was detected in the whole bodies of larvae at 0 dph with an antisense probe for DE-2 PLA_2 (A). From 1 dph, faint signals were detected in the intestinal tissue (arrowheads) with the antisense probe (B). At 3 dph, signals were detected in almost all of the intestinal epithelial cells (C). Thereafter, the intensity of the staining gradually increased (D and E), and positive signals were also detected in the lateral muscles of the larvae at 9 dph, in addition to the intestinal epithelial cells (F). Signals were detected in the hepatic tissues of larvae at 3 and 5 dph only (C and D). At 32 dph, dense signals were recognized in whole intestinal tissues (H) and around the myomere, especially near the vertebra (I). No positive signal was detected in the whole bodies of larvae and juveniles from 0 to 32 dph with the sense probe for DE-2 PLA_2 (data not shown). Abbreviations: He, hepatic tissue; EP, exocrine pancreatic tissue. Calibration bars in all panels = 100 μm.

Fig. 7. Development of the digestive system and gene expression of DE-1 and DE-2 PLA_2s in

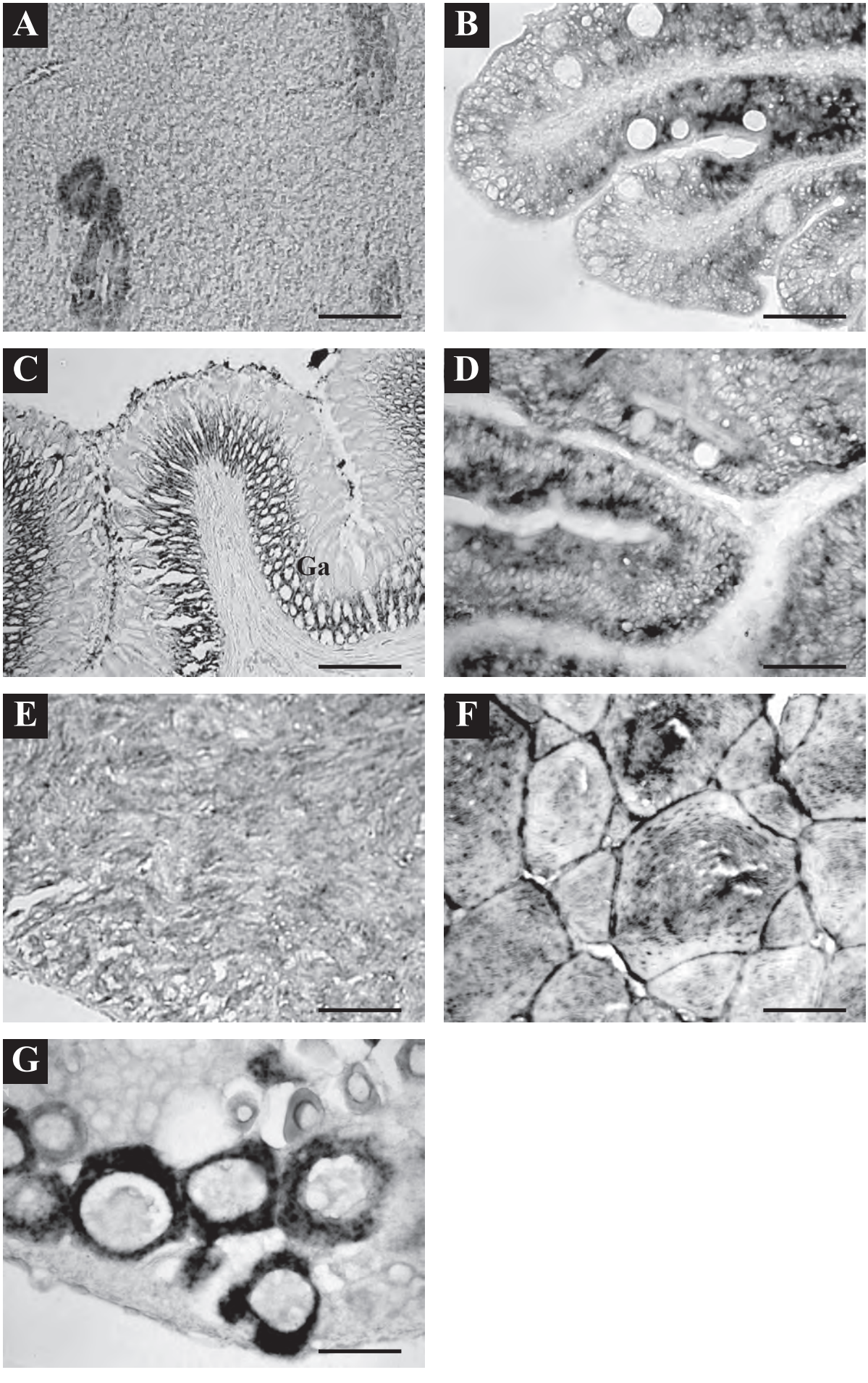
larval/juvenile and adult red sea bream. The development of the digestive system in the larval/juvenile red sea bream is accompanied by secretion of bile acid into the gallbladder, zymogen granule formulation in the pancreatic cells, and differentiation of the epidermal cells of the intestine by 5 dph. DE-1 PLA_2 mRNA was already expressed in the exocrine pancreatic tissue at 3 dph. The mRNA expression of DE-1 PLA_2 was increased from 7 to 32 dph and detected in the pancreatic cells of the adult red sea bream. DE-2 PLA_2 mRNA was detected at 1 dph and then expression was gradually increased. In the adult, DE-2 PLA_2 mRNA was detected in the pancreatic cells, pyloric caeca, intestine, and stomach. The DE-1 and DE-2 PLA_2 proteins were stored as inactive proPLA_2 in these tissues, and the proPLA_2 was then activated by limited tryptic proteolysis into a form capable of digesting dietary phospholipids. Abbreviation: BM, body mass.



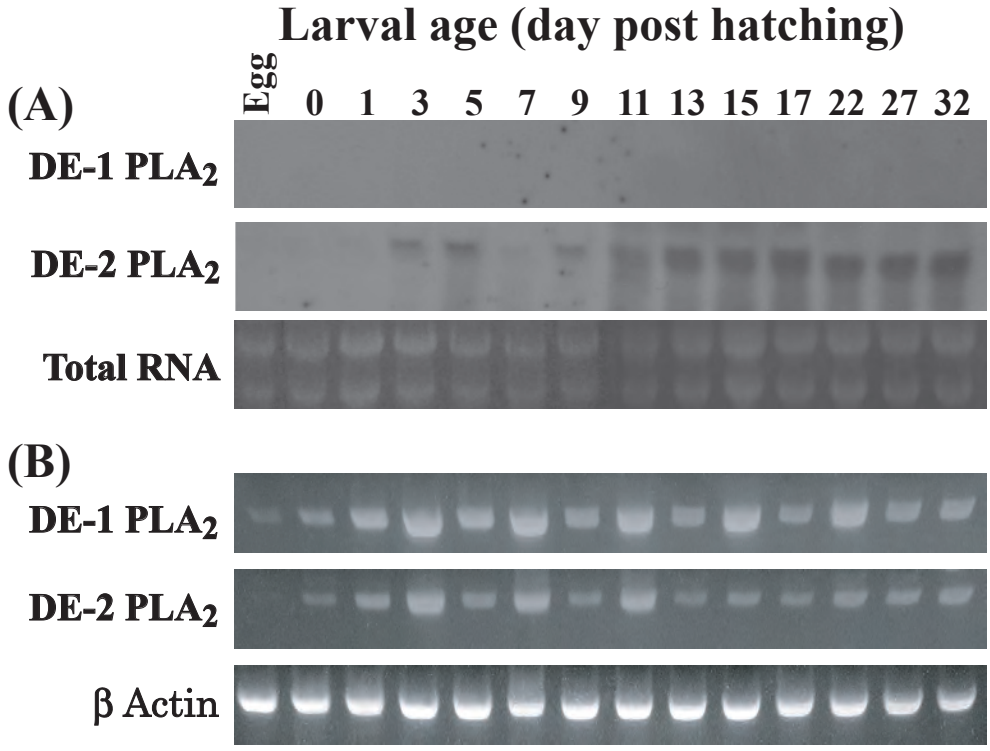
Fujikawa et al. Figure 1



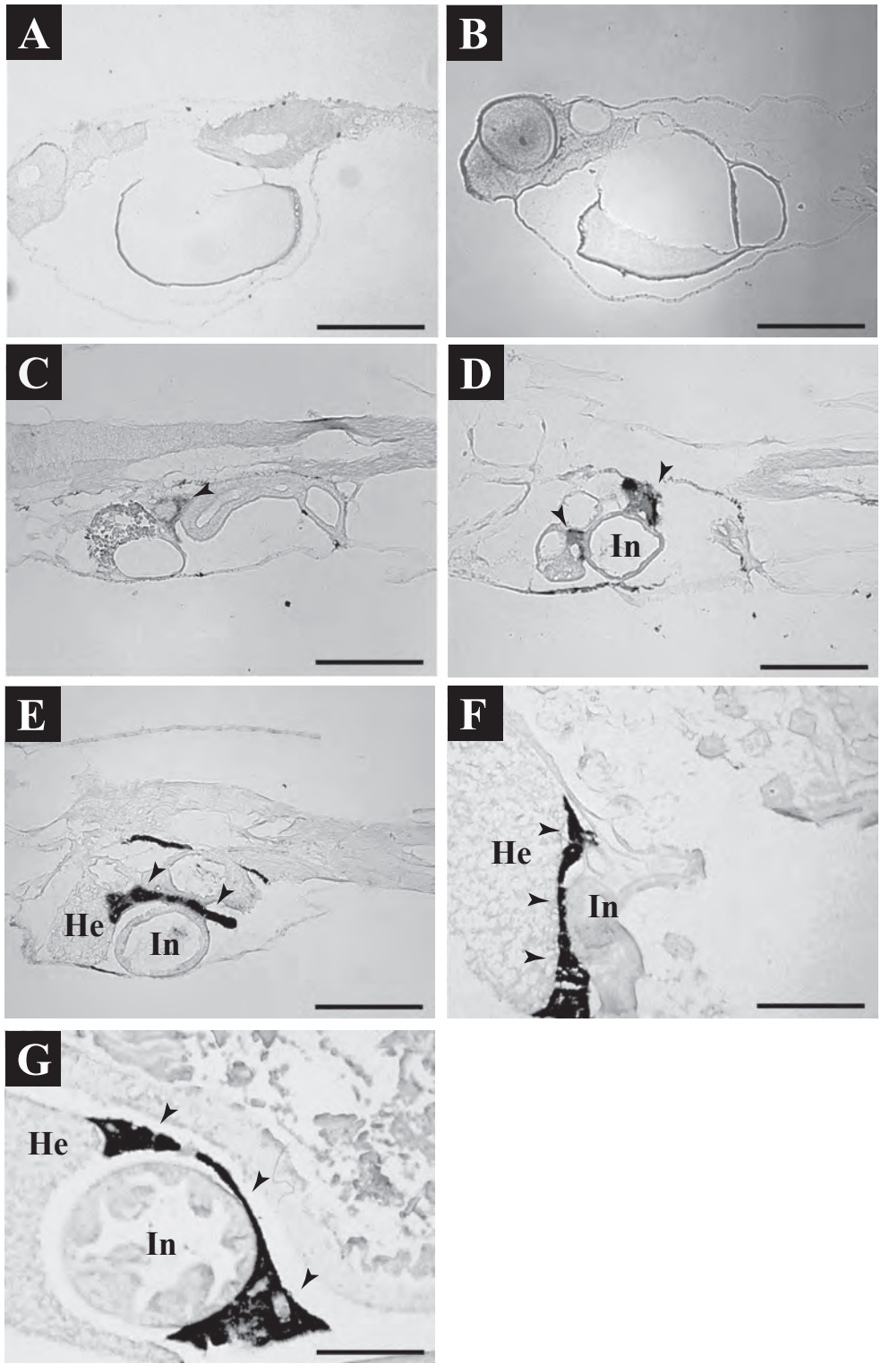
Fujikawa et al. Figure 2



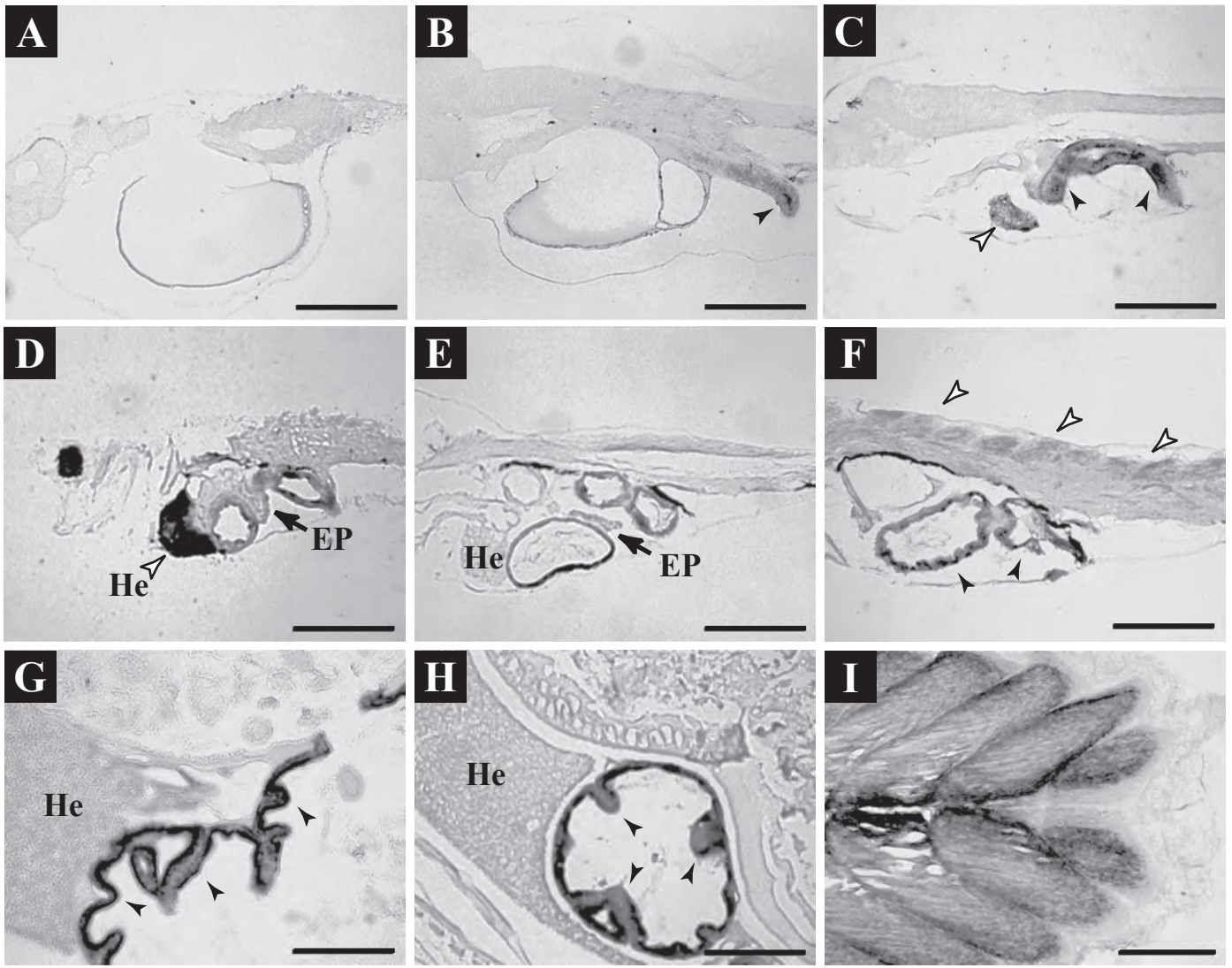
Fujikawa et al. Figure 3



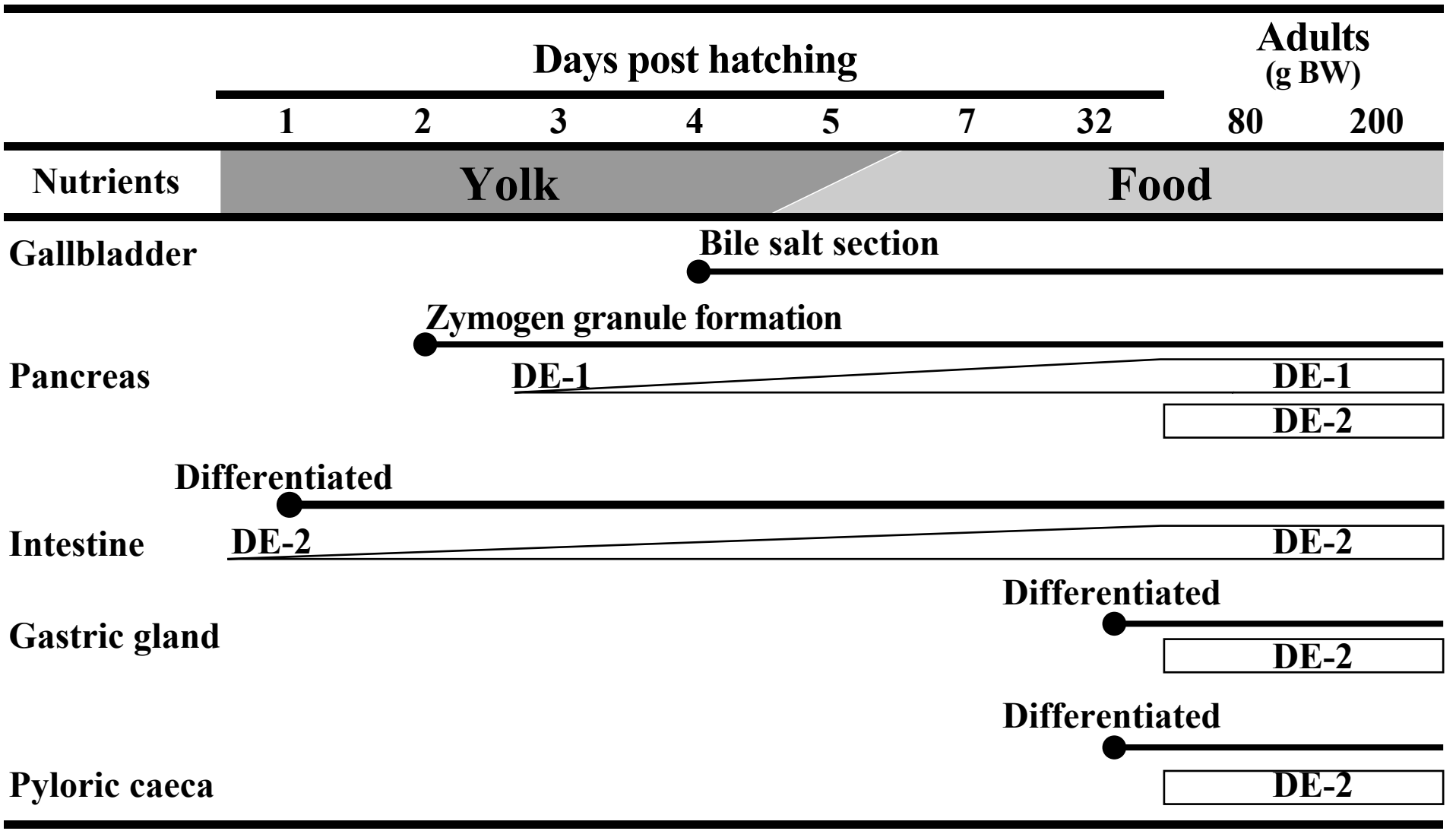
Fujikawa et al. Figure 4



Fujikawa et al. Figure 5



Fujikawa et al. Figure 6



Fujikawa et al, Figure 7