Characterisation and expression of secretory phospholipase A₂ group IB during ontogeny of Atlantic cod (Gadus morhua)

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Abstract
The pancreatic enzyme secretory phospholipase A₂ group IB (sPLA₂ IB) hydrolysates phospholipids at the sn-2 position, resulting in a NEFA and a lyso-phospholipid, which are then absorbed by the enterocytes. The sPLA₂ IB is a member of a family of nineteen enzymes sharing the same catalytic ability, of which nine are cytosolic and ten are secretory. Presently, there are no pharmacological tools to separate between the different secretory enzymes when measuring the enzymatic activity. Thus, it is important to support activity data with more precise techniques when isolation of intestinal content is not possible for analysis, as in the case of small teleost larvae, where the whole animal is sometimes analysed. In the present study, we characterise the sPLA₂ IB gene in Atlantic cod (Gadus morhua) and describe its ontogeny at the genetic and protein level and compare this to the total sPLA₂ activity level. A positive correlation was found between the expression of sPLA₂ IB mRNA and protein. Both remained stable and low during the larval stage followed by an increase from day 62 posthatch, coinciding with the development of the pyloric ceaca. Meanwhile, total sPLA₂ enzyme activity in cod was stable and relatively high during the early stages when larvae were fed live prey, followed by a decrease in activity when the fish were weaned to a formulated diet. Thus, the expression of sPLA₂ IB mRNA and protein did not correlate with total sPLA₂ activity.

Key words: Phospholipid digestion: Pancreas: Western blot: Quantitative PCR: Enzyme activity

Phospholipids (PL) are an essential nutrient for fish larvae (see review by Coutteau et al.(1)), and it has been demonstrated in fish larvae that increasing dietary PL levels can improve growth rates, increase survival(2–4), reduce deformation(4) and improve stress tolerance(5). Generally, the dietary requirement of PL is high in fish larvae at the start of feeding and decreases as the fish approach the juvenile stage(5,5,6). Although it is well known that the digestive system in altricial fish is still developing during the larval stage, it is still uncertain why there is such a high PL requirement during this period. Furthermore, it has been demonstrated that the digestive tract of sea bass (Dicentrarchus labrax) larvae matured faster and the larvae had increased growth, survival and decreased malformations when fed compound diets containing high levels of dietary PL from first feeding(7). The same study also suggests that sea bass utilise dietary PL more efficiently than neutral lipids.

Digestion of PL is mainly catalysed by pancreatic phospholipase A₂ (phosphatidate 2-acyl hydrolase, EC 3.1.1.4; PLA₂). The PLA₂ hydrolysates the fatty acid ester bond at the sn-2 position of PL and produces a NEFA and a lysophospholipid(8). Altogether, nineteen enzymes with PLA₂ activity have been described in mammals. Ten of these are of the secretory type and nine are cytosolic. One of the secretory PLA₂ (sPLA₂) is produced in the pancreas for the purpose of PL digestion: the sPLA₂ IB(9). All the sPLA₂ are Ca²⁺ dependent and are recognised by a conserved Ca²⁺-binding loop as well as a conserved catabolic site. The distinctive feature separating the pancreatic form sPLA₂ IB involved in PL digestion from the other sPLA₂ is a domain called the pancreatic loop.

Unfortunately, there are no methods available today to distinguish between the enzymatic activities of the different PLA₂; at best, it is possible to discriminate between the cytosolic and secretory forms(10,11). This presents a challenge when analysing small marine larvae where dissection of the digestive tract may be challenging, and therefore whole fish must be analysed. The analysis of PLA₂ activity in whole fish larvae will provide information of PL hydrolysis in all tissues involving a multitude of sPLA₂ enzymes, which will obscure information of the digestive capability

Abbreviations: dph, post hatch; GI-tract, gastrointestinal tract; PL, phospholipids; PLA₂, pancreatic phospholipase A₂; SL, standard length; sPLA₂, secretory phospholipase A₂.

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of specific phospholipases. It is therefore vital to accompany enzymatic activity analysis with more specific protein and mRNA quantification methods describing the enzyme in question.

Two low-molecular-weight sPLA₂ group IB have been characterised in the hepatopancreas of red sea bream (Pagrus major)(12), and there is also a description of a PLA₂ in sea bass(13). Little information on the ontogeny of PLA₂ activity in marine fish larvae is available, but Uematsu et al.(13) did not find PLA₂ in acinar cells of the hepatopancreas in red sea bream before 13 d post hatch (dph). To shed light on the dynamics of sPLA₂ IB hepatopancreas in red sea bream before 13 d post hatch (dph). To shed light on the dynamics of sPLA₂ IB activity in fish larvae, we have analysed the enzyme at the mRNA and protein level and compared this to total sPLA₂ activity. Due to multiplicity of enzymes in the PLZ family, we have emphasised the description of the distinctive parts of the pancreatic form of PLA₂. Furthermore, we discuss what different analytical tools may reveal about the production and activity of this one specific enzyme out of the multitude of other similar enzymes.

Material and methods

Biological parameters

Atlantic cod (Gadus morhua) larvae were sampled from three production tanks at the commercial hatchery Cod Culture Norway, in Rong, Norway, according to their fish holding routines, which are in accordance with the Animal Welfare Act of 12 December 1974, nr 73, §§22 and 30, amended 17 November 1998. Eggs were collected from natural spawning brood stock and incubated for 15 d at 7°C. Hatched embryos were transferred to three start feeding tanks (diameter: 3 m, depth: 1 m) and fed rotifers (Brachionus plicatilis) from 3 dph. Formulated diet (Gemma Micro, Skretting, Norway) was introduced to larval tanks at 18 dph and co-fed with rotifers until 30 dph. From 31 dph and onwards, larvae were fed solely on formulated diet. Fertilised eggs used for the production of larvae used in this work came from a brood stock tank containing fifty-five females and thirty-nine males. The batch of eggs may therefore come from multiple parents. The temperature in the three sampling tanks was 7±1°C at 3 dph and was raised incrementally to 11°C by 17 dph. The temperature was kept constant at 11±2 ± 0.5°C for the rest of the sampling period. Oxygen was measured and delivered continuously to larval tanks via an automated system with oxygen saturation ranging from 84 to 97% within tanks during the sampling period.

Biological sampling

Pooled samples of whole larvae and/or larvae gastrointestinal tract (GI-tract) were taken from the three tanks at every sampling point (Fig. 1) from 3 to 62 dph. At 74 dph, the three fish tanks were graded and approximately 35% of the smallest fish were removed. This was routinely done at the commercial facility where we sampled the larvae. The retained fish were sorted into a medium- and large-sized group. At 83 and 97 dph, fish were sampled from both the medium- and large-size group. Until 56 dph, the fish were collected using a 60 cm long glass pipette. From 63 dph, the fish were collected using a fish net. Fish were collected from approximately 40 cm below the surface and from the areas in the tank with the highest density of fish. The larvae were filtered using a plankton net screen which was patted dry from underneath with a paper towel to remove excess water from the larvae. All the larvae were killed by inserting a needle through the brain before dissection. The whole larvae and larvae gastro intestinal tract (GI-tract) were taken from the three tanks at every sampling point. GI-tract samples were pooled and homogenised with a pestle mortar in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). The samples were then put on dry ice for approximately 3–5 h before storing at −80°C until RNA extraction. For enzyme activity and Western blot, larvae and GI-tract samples were flash-frozen in liquid N₂ without any treatment. At every

Fig. 1. Trial and sampling scheme. The arrow gives the type of diet during the sampling period, numbered circles indicate the number of pooled individuals in each sampling and the age of larvae at sampling is given in days post hatch (dph). GI, gastrointestinal tract.
sampling point, standard length (SL) (minimum ten fish/tank) was measured. Fish weight was measured from 56 dph (fifteen fish/tank). The fish were dried with paper and weighed individually. All the fish sampled for measuring length (SL) and weight were anaesthetised and killed with an overdose of metacaine (MS-222®; Norsk medisinaldepot AS, Bergen, Norway) dissolved in seawater. Dry weight was calculated according to Finn et al.\(^\text{[14]}\) based on SL. A size- and temperature-based growth model was used to evaluate the growth performance according to Folkkvord\(^\text{[15]}\).

**RNA extraction and quantitative PCR**

The homogenised and stored in Trizol samples were thawed and thoroughly homogenised a second time, using a Precell 24 (Bertin Technologies, Montigny le Bretonneux, France). Total RNA was extracted from whole fish and GI-tract with Trizol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. The RNA-isolated samples were diluted in RNase-free double-distilled H\(_2\)O to 200 mg/ml and treated with DNA-free TM kit (Ambion, Austin, TX, USA) according to the manufacturer’s descriptions. The quality of the RNA was assessed with the NanoDrop\(^\text{[16]}\) ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Optical density 260 nm/optical density 280 nm ratios for all total RNA samples ranged between 1.87 and 2.06.

Integrity of the RNA was controlled in twelve randomly chosen samples out of the thirty-six using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA 6000 Nano LabChip\(^\text{[17]}\) kit (Agilent Technologies, Palo Alto, CA, USA). RNA integrity numbers\(^\text{[16,17]}\) were between 8.9 and 10.

RT-PCR were run in duplicates on a ninety-six-well plate. For efficiency calculations, a twofold serial dilution of total RNA, mixed from all samples, ranging from 1000 to 31 ng/µl, was run in triplicates. All reactions were synthesised with a 500 ng total RNA input. Each plate included a no template control and RT-control (a reaction without RT enzyme). Plates were run on a GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA) using TaqMan Reverse Transcription Reagent containing Multi-scribe RT (50 U/ml; 833.5 nkat/µl) (Applied Biosystems).

Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2.5 µM) for all the genes in 30 µl total volume. The final concentrations of the other chemicals in each reaction were MgCl\(_2\) (5.5 mM), dNTP (500 mM of each), 10 X TaqMan RT buffer (1 X), RNase inhibitor (0.4 U/µl) and Multiscribe RT (1.67 U/µl).

From each RT reaction, 2 µl of complementaryDNA and then 18 µl consisting of 7.6 µl double-distilled H\(_2\)O, 10 µl TaqMan\(^\text{[18]}\) Universal PCR Master Mix (Applied Biosystems), 0·2 µl of each primer (50 µM forward: GTCAGGGTCTTGTTGCTCTTG and reverse: GCCAACTCATGACCATTGCT) were pipetted in duplicate to 384-well plates. Pipetting was performed automatically by a robot (Biomek 3000; Beckman Coulter, Fullerton, CA, USA). Real-time PCR was run on the LightCycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland), with a 5 min activation and denaturing step at 95°C, followed by forty cycles of a 15 s denaturing step at 95°C, a 60 s annealing step and a 30 s synthesis step at 72°C. The annealing temperature was 60°C for all primer pairs.

For the mean normalised expression calculations of the target genes, the geNorm VBA applet for Microsoft Excel 97 was used to calculate a normalisation factor based on three reference genes\(^\text{[18]}\). Ubiquitin, RPL 4 and RPL 37 were the selected reference genes based on Olsvik et al.\(^\text{[19]}\) and Sæle et al.\(^\text{[20]}\), and the normalising factor calculated from these genes was used to calculate the mean normalised expression for each of the target genes.

**Homogenisation**

Whole larvae and dissected GI-tracts were homogenised in 4 X sample wet weight PBS with an Ultra – Turax\(^\text{[20]}\) 20000 (Upn, Janke u. Kunkel KG, Germany) followed by a second homogenisation in a ball mill (Retsch\(^\text{[21]}\) MM301; Retsch GmbH, Haan, Germany), for 2 X 2 min (thirty shakes/s). Homogenates were centrifuged at 10000 g for 15 min at 4°C and the supernatants were measured for total protein with the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA), according to the manufacturer’s instructions. The supernatant was used for analysis of enzyme activity and for Western blot analysis.

**Enzyme activity**

A sPLA\(_2\) assay kit (product no. 765001; Cayman Chemical Company, Ann Arbor, MI, USA) was used for enzyme activity calculations. The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine which serves as a substrate for most PLA\(_2\) (for example, bee and cobra venoms, pancreatic fluid, etc.) with the exception of cytosolic PLA\(_2\)\(^\text{[10,11]}\). Upon hydrolysis of the thio-ester bond at the sn-2 position by PLA\(_2\), free thiols are detected using 5,5-dithio-bis-(2-nitrobenzoic acid). Plates were read every 15 s for 30 min at 414 nm with a microreader (Labsystems iEMS Reader MF, Helsinki, Finland). Enzymatic activity was calculated accordingly:

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s_{\text{PLA2}} = \frac{\Delta A_{414/min}}{10.66 \text{ per mM} \times \text{ sample dilution}} \times 0.225 \text{ ml} \times 0.01 \text{ ml}
\]

\(\Delta A_{414/min}\) is the change in absorbance/min, 10.66 per mM is the extinction coefficient for 5,5-dithio-bis-(2-nitrobenzoic acid) under the assay conditions.
**Western blot**

Polyclonal antibodies were produced in rabbits immunised with the PLA2 peptide sequence: NPYTHGYHQTCDKSTK (Inbiolabs, Tallin, Estonia). Samples were resolved by denaturing SDS-PAGE using 12% polyacrylamide gels (BioRad, Hercules, CA, USA) and transferred to a polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA). The membrane was blocked with 3% non-fat milk in TPBS (PBS, 0.1% Tween 20, pH 7.4) for 1 h, rinsed with TPBS and subsequently incubated with the primary antibody. Membranes were probed with primary anti-PLA2 rabbit (dilution: 1:2000; Dako, Glostrup, Denmark) in blocking buffer. Membranes were then treated with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) for 5 min and photographed on hyperfilm (GE Healthcare) in the dark. The negatives were scanned and the area of sPLA2 IB protein bands was estimated using ImageJ software (http://rsb.info.nih.gov).

**Open reading frame, alignments and statistics**

Open reading frame sPLA2 IB for Atlantic cod was generated with Baylor College of Medicine HGSC Search Launcher (http://searchlauncher.bcm.tmc.edu/cgi-bin/seq-util/sixframe.pl). Alignments were done in ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Potential differences were tested with one-way ANOVA. Newman–Keuls post hoc test was used to test for significant differences between group means in an ANOVA. Correlations were tested with multiple regression analysis. Differences and effects were considered significant at $P<0.05$ for all tests and differences were annotated with different letters in figures. ANOVA and post hoc analyses were performed on Statistica 9.0 (StatSoft, Inc., Tulsa, OK, USA).

**Results**

**Larvae growth**

Specific growth rate increased from 6.0% at 10 dph to peak at 11.9% at 34 dph when the SL was 7.75 mm. From 34 dph, there was a steady decrease in specific growth rate to 7.1% at 97 dph. To evaluate the growth performance of fish larvae used in the present study with potential growth in wild cod, a size- and temperature-based growth model by Folkvord$^{15}$ was used:

$$SGR = 1.08 + (1·79T) - 0·074 \times T(\ln DW) - 0·0965 \times T(\ln DW)^3 + 0·0112 \times T(\ln DW)^3$$

$T$ is temperature (°C) while DW is the dry weight (mg) of larvae at the first sampling point (3 dph). Dry weights were calculated based on SL according to the following equations from Finn et al.$^{14}$:

Size range : 0·031–0·175 mg DW : $\ln SL = \frac{(\ln (DW + 7·799))}{3·109}$

Size range : 0·175–1·829 mg DW : $\ln SL = \frac{(\ln (DW + 9·657))}{4·129}$

Size range : 1·829–409 mg DW : $\ln SL = \frac{(\ln (DW + 7·932))}{3·406}$

There were an increase in the difference between the observed length growth (SL) and modelled growth throughout the ontogeny series. The increase in percent difference between the modelled and observed growth had a slope of 0.7 ($y = 0·6859x - 6·026, R^2 = 0·95$); consequently, the fish were 61·02 mm at 97 dph, whereas the model predicted a SL of 95·86 mm, a difference of 57% (Fig. 2).

**Identification of the secretory pancreatic phospholipase A2 IB gene**

The expressed sequence tag (EST): EX726814 contained an open reading frame corresponding to a predicted amino acid sequence of matching sPLA2 group IB (Fig. 3). Atlantic cod open reading frame similar to sPLA2 group IB was aligned to sequences annotated to the PLA2 superfamily of *D. labrax* (CAA10765), *P. major* (BAA23737) and *Fundulus heteroclitus* (AAU50527). Sequences of *Danio rerio* (AA14294), *Sus scrofa* (NP_001004037), *Rattus norvegicus* (NP_113775) as well as *Homo sapiens* (NP_000919) annotated to sPLA2 group IB were also aligned.

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*Fig. 2.* Size at sampling. Standard length (SL) of Atlantic cod larvae at sampling points analysed (mean values with their standard errors). The curve gives the predicted growth based on the model: $SGR = 1·08 + (1·79T) - 0·074 \times T(\ln DW) - 0·0965 \times T(\ln DW) + 0·0112 \times T(\ln DW)^3$. The model is based on data from wild populations. There was a significant increase in SL from 20 days post hatch (dph) on (one-way ANOVA, $P<0.05$). SGR, Specific growth rate; DW, dry weight.
To demonstrate that the previously listed sequences had the pancreatic loop unique to IB sPLA2s, group V and X sPLA2s were included in the alignment: *H. sapiens* (AAG43522) and *Mus musculus* (NP_001116426) from group V and *H. sapiens* (AAH69539), *M. musculus* (AAG43522) and *D. rerio* (NP_001002350) from group X. The Ca\(^{2+}\)-binding loop (YGXXCGXGG) and the catalytic site (DXCCXXH) were conserved between all sequences aligned.

**Assay verification**

Atlantic cod sPLA2 IB mRNA expression was assessed by RT-quantitative PCR in GI-tract, including pancreas, and also in the intestine, muscle, liver, gill, kidney and brain. The RT-PCR was run twenty-six and thirty-five cycles. After thirty-five cycles, all tissues except muscle produced a product when run on an ethidium bromide gel (data not shown). When the PCR was run twenty-six cycles, only GI-tract containing pancreatic tissue showed a band; all other tissues were blank (Fig. 4(a)). Ubiquitin was used as reference\(^{19,20}\). An increase from twenty-six to thirty-five PCR cycles gives a difference in amplification by the power of seven (\(Y = x^7\)).

Western blot of GI-tract homogenates showed only one band with the approximate size of 14 kDa (Fig. 4(b)). Enzymatic activity was tested in tissues of adult Atlantic cod. Activity was detected in liver and brain (mean 0.27 (sd 0.05) \(\mu\)mol/min per ml) as well as in kidney and gill (mean 0.43 (sd 0.14) \(\mu\)mol/min per ml), but no activity was detected in skeletal muscle (Fig. 4(c)).

**Ontogeny of secretory pancreatic phospholipase A\(_2\) group IB**

The relative gene expression of PLA2 (quantitative RT-PCR) in homogenates of whole larvae was very low at the start of feeding (3 dph) until 48 dph, ranging from 0.0004 (sd 0.0002) to 0.04 (sd 0.01), respectively. There was a significant increase at 62 dph to 0.17 (sd 0.11). In GI-tract homogenates, there was significant increases from 0.0003 (sd 0.0001) at 34 dph to 0.586 (sd 0.192) at 62 dph when it plateaued (Fig. 5(A)).

Protein expression was not detectable in whole fish homogenates until 62 dph. In GI-tract homogenates, there was an increase in protein expression from 48 to 62 dph and then again from 83 to 97 dph (Fig. 5(B)).
There was a significant correlation between mRNA expression and protein expression both in larvae homogenates ($P<0.0001$, $r^2=0.9$) and in GI-tract ($P=0.004$, $r^2=0.5$) (Fig. 6).

Enzymatic activity was detectable already from first exogenous feeding. Homogenates of whole larvae had an $sPLA_2$ activity of $0.70 \mu$mol/min per g from 3 to 20 dph. At 34 dph, the activity decreased to $0.36 \pm 0.04 \mu$mol/min per g. There was no increase in activity from 34 to 62 dph in larvae homogenates. The activity in GI-tract homogenates was stable from 34 to 97 dph, with an average activity of $1.11 \pm 0.18 \mu$mol/min per g (Fig. 5(C)). Enzymatic activity did not correlate with mRNA nor with protein expression.

**Discussion**

The growth rate of cod larvae when fed natural zooplankton can be very high and is reported to range from 13 to 30% per day during the first 50 days (14–21). Atlantic cod larvae fed live feed-like enriched rotifers (*B. plicatilis*) and *Artemia* and formulated diets generally have poor growth compared with those fed wild collected zooplankton (22). For example, cod larvae fed *Artemia* and formulated feed had a growth rate ranging from 5.1 to 10.7% between 22 and 64 dph (23, 24). Fish in the present study grew well compared with those in previous experiments evaluated using the same growth model (15), by Busch et al. (22). The fish in the present study were 13.5 and 31.7% shorter than the model predictions at 9.9 mm SL (34 dph) and 23.8 mm SL (62 dph), respectively, while in another trial with a similar diet to that used in the present experiment, the larvae were 43.9 and 42.0% shorter than the model at a similar SL (24). We may therefore conclude that the ontogeny series of cod larvae analysed in the present study had good growth compared with other intensive rearing trials where larvae were start fed on rotifers and weaned at an early stage.

The family of $sPLA_2$ consists of the groups I, III, V and X, containing all together ten enzyme forms as described in mammals. The $sPLA_2$ IB belongs to a family of ten $sPLA_2$ that share a highly conserved region for a Ca$^{2+}$-binding loop (XCGXGG) and a catalytic site (DXCCXXH) (9). These sites are conserved between Atlantic cod and the evaluated fish, *D. labrax*, *P. major*, *D. rerio* and *F. heteroclitus* as well as in the mammals *S. scrofa*, *R. norvegicus*.
and *H. sapiens*. However, in front of the Ca²⁺ loop, there is also a conserved region of the amino acids tyrosine (Y), glycine (G) and cysteine (C) between all listed species in Fig. 8, with the exception of Atlantic cod that has a tryptophan (W) in place of the C. The codon for C is UGU or UGC and for W it is UGG. Since C domains are much conserved²⁵, it is likely that the third G in the codon is a sequencing error and should in fact be a U or a C. With this exception, all C domains are conserved between the fishes listed in Fig. 8.

Distinctive for the pancreatic form sPLA₂ group IB involved in PL digestion is the pancreatic loop containing the domain PYTX. This domain is conserved between all listed sPLA₂ with the exception of enzyme groups V and X and the *F. heteroclitus* sequence (Fig. 8). The sPLA₂ group V and X are not expressed in pancreatic tissue and are not involved in PL digestion²⁶. We may therefore conclude that the evaluated sequence in Atlantic cod most likely encodes for sPLA₂ IB. Further supporting this, the protein weight of sPLA₂ IB has previously been reported as 14 kDa⁹–²⁹, which was the size of the protein picked up by our antibody. In addition, we also provide proof that the PLA₂ sequences from *D. labrax* and *P. major* probably should be annotated sPLA₂ IB.

The primers used in the present study may amplify PLA₂ isoforms present in other tissues than in the pancreatic tissue. Iijima *et al.*²⁷ characterised three isoforms of group I sPLA₂ in the gills of red sea bream, out of which one belonged to group IB. It is plausible that there are similar isoforms in Atlantic cod. The sPLA₂ IB has also been found in trace amounts in other organs, such as lung, kidney and spleen, in rat⁹–²⁸. In the present study, mRNA expression of sPLA₂ IB in pancreas containing tissue was at least to the seventh power higher than in the liver, brain, kidney and gill. It is therefore likely that relative quantities of mRNA measured in GI-tract system as well as in whole larvae reflect the pancreatic production.

The enzyme activity assay used in the present study was specific for secretory enzyme forms, but cannot differentiate between different groups of sPLA₂. Analysing the homogenates of whole organisms and organ systems such as the GI-tract will therefore include the catalytic capability of PL sn-2 fatty acid hydrolysis of many tissues.
and enzyme isoforms \(^{(10,11)}\). Iijima et al. \(^{(27)}\) found that the PLA2 activity in the gills of red sea bream was seventy times higher than in the pancreatic tissue. In the present study, the activity in gill tissue of juvenile cod was the same as in kidney but less than half of the activity found in the GI-tract homogenate that included pancreatic tissue and gut content. Nevertheless, there is no doubt that enzyme activity data presented here and in other

![Fig. 7. The secretory phospholipase A\(_2\) activity in whole Atlantic cod larvae (●) and their gastrointestinal (GI)-tract (■). Inset shows correlation between percent size of GI-tract of whole larvae and percent activity in GI-tract to activity in whole larvae at ages 34, 48 and 62 d post hatch (dph).](image)

### Fig. 8. Alignment of the open reading frame amino acid sequences of phospholipase A\(_2\) with the known pancreatic PLA\(_2\) fish sequences: Dicentrarchus labrax (CA1A0765), P. major (BA423737), Fundulus heteroclitus (AAU50527), Danio rerio (XP_700448) and the secretory PLA\(_2\) (sPLA\(_2\)) mammalian sequences: Sus scrofa (NP_001004037), H. sapiens (NP_000919) and R. norvegicus (NP_113773). For visualisation of characteristics of the pancreatic form of sPLA\(_2\), sequences of the group V sPLA\(_2\): M. musculus (EDL13282) and H. sapiens (AAG3432) and the group X sPLA\(_2\): M. musculus (NP_001116426), Homo sapiens (AAH69539) and D. rerio (NP_0010023405) were included in the alignment. Accession numbers are given in parenthesis. Sequences for the Ca-binding loop (1), the catalytic domain of the enzyme (2), the pancreatic loop (3). *Indicates conserved amino acid residues identical to those of Atlantic cod, –indicates amino acid residues absent in the sPLA\(_2\) groups V and X.

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papers\(^{[30]}\) represent the activity of a cocktail of PLA\(_2\) types from various tissues. There have been attempts to minimise this problem. For example, Cahu \textit{et al.}\(^{[7]}\) divided the larvae in segments, but the pancreas segment probably still included the parts of kidney and liver, the important contributors to PLA\(_2\) activity.

The mRNA expression of sPLA\(_2\) IB in whole fish was stable but low at the start of feeding, but increased from 48 dph and 15·1 mm SL, and was higher again at 62 dph and 23·8 mm SL. Uematsu \textit{et al.}\(^{[113]}\) did not find a sPLA\(_2\) IB immunohistochemical signal until 13 dph in pancreatic acinar cells from red sea bream, which is 11–9·d post first exogenous feeding\(^{[31]}\). The intensity of staining then increased steadily during development. In the present study, sPLA\(_2\) IB Western blots of homogenates from larvae did not pick up any signal until 62 dph. This together with the findings of Uematsu \textit{et al.}\(^{[113]}\) demonstrates that the quantity of sPLA\(_2\) IB is under the detection limit of these assays and therefore quite low during the early larval stages of both red sea bream and Atlantic cod. However, Ozkizilcik \textit{et al.}\(^{[30]}\) measured PLA\(_2\) activity on whole eggs and larvae of striped bass (\textit{Morone saxatilis}) and found a steady increase in activity from fertilisation until the juvenile stage. As emphasised by the authors, activity assays register all types of PLA\(_2\) activity and are unsuitable for assessment of isoform-specific activity during development. This is further emphasised by the elevated PLA\(_2\) activities in starved larvae when compared with fed larvae\(^{[30]}\).

An important difference between the present data and those of Ozkizilcik \textit{et al.}\(^{[30]}\) is that while they analysed larvae with empty guts, we analysed larvae with filled guts. The latter would then include any sPLA\(_2\) activity of ingested rotifers in addition to increased enzymatic activity of the larvae. This is visualised by the significant drop in whole larvae sPLA\(_2\) activity after 20 dph when weaning of larvae onto the formulated diet began (Fig. 5(C)). This drop is probably due to the absence of rotifer enzyme and not the decreased production of sPLA\(_2\) since it is only seen in activity and not in mRNA status (Fig. 5). According to Infante & Cahu\(^{[32]}\), the levels of PL in the diet regulate sPLA\(_2\) mRNA in \textit{D. labrax} larvae. However, no such regulation was apparent in cod from the present trial. The formulated diet contained 14–15\% total lipid of DM of which 80–86\% was PL (Gemma micro), whereas the rotifers fed had a total lipid content of 8·8 (sd 1·88)\% of DM of which 37 (sd 3\%) is PL.\(^{[35]}\) Hence, when the larvae are weaned, they change from a low PL diet of 3–15\% to a high PL diet of 12\% of dry weight. Instead of up-regulating the sPLA\(_2\) IB production and sPLA\(_2\) activity to accommodate for the increase in dietary PL, the enzyme production is unaffected and the enzymatic activity is decreased.

There was a linear correlation between the mRNA expression and the protein expression in GI-tract homogenates, with a coefficient of determination of 0·51 (Fig. 6). The mRNA of sPLA\(_2\) IB is only found in pancreatic acinar cells, whereas the protein would be present in the intestinal lumen as well in the pancreas\(^{[9]}\). The remaining 49\% of the variation between the quantity of mRNA and protein can therefore be explained by post-transcriptional regulations of protein synthesis and by enzyme present in the gut lumen.

In Atlantic cod, we found a close relationship between the ratio of GI-tract to whole larval weight and the ratio of GI-tract to total larvae enzyme activity. In turbot larvae, 61\% of the neutral lipolytic activity came from the gut at 12 dph decreasing to 52\% at 24 dph\(^{[34]}\). This coincides with the trend shown in cod and is probably correlated with the decreasing proportion of the GI-tract to whole body mass during ontogeny.

A question that can be asked based on the present results is: what does the ontogeny of the digestive enzyme expression and activity tell us about the digestive capabilities of the fish larva? The answer is that it appears to tell only part of the story, i.e. when the adult form of digestion develops. Fish larvae require PL-rich diets\(^{[6,35]}\) and it has been demonstrated that intact PL is absorbed in intestinal epithelium in larval zebrafish\(^{[36,37]}\). We propose that in the early larval stages, the endogenous production of sPLA\(_2\) IB is low and exogenous enzyme contribution from live prey is important for PL digestion in addition to the ability of the larvae to absorb intact PL. During ontogeny, these mechanisms may disappear and/or become less important in parallel with an increase in the endogenous enzyme production as the fish acquire the adult digestion form.

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