Cathepsin B differential expression and enzyme processing and activity during *Fundulus heteroclitus* embryogenesis

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ABSTRACT

The role of lysosomal proteases such as cathepsin B (Ctsb) and one of the paralogs of cathepsin L (Ctsla) during yolk metabolism in fish oocytes is well established. However, the function of Ctsb during embryogenesis, particularly in marine teleosts, has been poorly documented. In this study, the spatio-temporal expression of Ctsb and Ctsla, their enzymatic activities, and the processing of the Ctsb and its cellular localization, was investigated in developing embryos of *F. heteroclitus*. Both *fhctsb* and *fhctsla* transcript levels, as well as cathepsin B- and L-like activities, gradually increased in embryos from the 2-4 cell stage up to 7 dpf. During the morula to gastrula transition an increase of the active FhCtsb single chain form was followed by a rise in cathepsin B activity, which were apparently regulated by post-transcriptional mechanisms. During neurulation, a 8-fold increase in cathepsin B activity was accompanied by a more moderate increase in cathepsin L activity, which was 6-fold enhanced by 7 days post-fertilization. These increased catalytic activities were well-correlated to changes in the electrophoretic pattern of yolk proteins and a strong expression of *fhctsb* and its protein product in the yolk syncytial layer. The increase of cathepsin B activity was further correlated with an increment of the relative amount of the FhCtsb single and double chain forms, both active forms of FhCtsb. These results suggest that FhCtsb may be involved in the mechanisms underlying the onset of gastrulation in *F. heteroclitus* embryos, and may play complementary roles with FhCtsla during yolk metabolism.

Key words: Embryo, yolk metabolism, gastrulation, cathepsin, enzyme processing

1. Introduction

Acidic proteases, generally known as cathepsins, are members of the endosomal/lysosomal protease family and now include more than 21 proteases (Roberts, 2005). They can be divided into four families according to the identity of the critical residue in the active site, cysteine, aspartate, serine, and metal ions in metalloprotease. Cathepsins are widely distributed in animal and plant tissues and in microorganisms, and are thought to play a wide range of biological functions, including protein turnover (Callegari et al., 2005), antigen processing (reviewed by Hsing and Rudensky, 2005), proenzyme activation (Azaryan and Hook, 1994), hormone maturation (Yasothornsrikul et al., 2003) or epidermal homeostasis (Roth et al., 2000; Tobin et al., 2002; Lecaille et al., 2002). In teleosts, the majority of cathepsins identified in mammals are also found (Carnevali et al., 1999a; Kwon et al., 2001; Fabra and Cerdà, 2004; Tan et al., 2006; Tingaud-Sequeira and Cerdà, 2007; Kao and Huang, 2008; Ahn et al., 2008, 2009; Yeh and Klesius, 2008, 2009; Jia and Zhang, 2009; Park et al., 2009; Je et al., 2009), and some of these are represented in the genome by duplicated or triplicated isoforms possibly as a result of the whole genome duplication event that occurred in the teleost lineage (Meyer and van de Peer, 2005). Thus, cathepsin B may remain in duplicate (Ctsba and Ctsbb) in some species (Tan et al., 2006; Tingaud-Sequeira and Cerdà, 2007), whereas cathepsin L may be present in triplicate (Ctsla, Ctslb, and Ctslc), as in the zebrafish (*Danio rerio*) (Tingaud-Sequeira and Cerdà, 2007). However, little information on the physiological functions of cathepsins in teleosts is yet available.

The role of cathepsins during yolk metabolism in fish has been however investigated more extensively (reviewed by Carnevali et al., 2006; Cerdà et al., 2007; Finn and Fyhn, 2010). Several cathepsins have been implicated in yolk processing, although experimental evidence is available only for a few of them. Thus, the aspartic protease cathepsin D (Ctsd) seems to be involved in the processing of the vitellogenins (Vtgs) into yolk proteins (YPs; lipovitellins, phosvitins, the β' -component, and the C-terminal peptide [Campbell and Idler, 1980; Matsubara and Sawano, 1995; Carnevali et al., 1999a]) in the oocyte during vitellogenesis (oocyte growth), whereas cysteine proteinases such as Ctsla, Ctsb and/or cathepsin Z (Ctsz) possibly participate in the subsequent hydrolysis of YPs during oocyte maturation (Sire et al., 1994; Carnevali et al., 1999b, 2008; Kwon et al., 2001; Hiramatsu et al., 2002; Matsubara et al., 2003; LaFleur et al., 2005; Raldúa et al., 2006; Kao and Huang, 2008). This additional cleavage of YPs produces a free amino acid (FAA) pool essential for the hydration of the oocytes, the subsequent buoyancy of spawned eggs in seawater, and as

energy source for developing embryos (Ohkubo and Matsubara, 2002; Cerdà et al., 2007; Finn, 2007a; Amano et al., 2008). However, the metabolism of Vtgs and YPs in the oocyte is most likely a complex and well coordinated process in which multiple cathepsins including Ctss, Ctsh and Ctsf may also be involved (Fabra and Cerdà, 2004; Raldúa et al., 2006; Kao and Huang, 2008).

Cathepsins also play a role during the mobilization and hydrolysis of the stored YPs in fish embryos, which is critical to embryonic development. Some studies have suggested that *ctsla*, which is maternally inherited and further highly expressed in the yolk syncytial layer (YSL) or periblast (Sire et al., 1994; Tingaud-Sequeira and Cerdà, 2007), may be the major protease in fish embryos and larvae (Murakami et al., 1990; Sire et al., 1994; Kestemont et al., 1999; Carnevali et al., 2001; Tingaud-Sequeira and Cerdà, 2007). The YSL consists of a vitellolysis zone and a cytoplasmic zone surrounding the yolk, and is therefore considered to play an integral role in the absorption of yolk in fish (Walzer and Schonénberger, 1979ab; Hemming and Buddington, 1988). The essential function of Ctsla in yolk hydrolysis during embryogenesis is further supported by the observation that genetic mutants of the *Caenorhabditis elegans* cathepsin L gene *cpl-1* show aberrant processing and/or conformational changes in YPs, resulting in abnormal yolk platelet fusion (Britton and Murray, 2004). In the zebrafish *ctsb* is also highly expressed in the YSL (Alt et al., 2006), and in some species of insects, the sea urchin and in salmonids, cathepsin B-like cysteine proteinases and/or serine proteases have been suggested as the enzymes involved in the processing of yolk materials during early embryogenesis (Mallya et al., 1992; Cho et al., 1999; Known et al., 2001; Hiramatsu et al., 2002). Therefore, it is possible that mechanisms for yolk processing during fish development are mediated by both Ctsla and Ctsb. However, the potential function of Ctsb, particularly in marine teleosts, has been poorly documented.

In the marine killifish *Fundulus heteroclitus*, which produces benthic eggs, a limited hydrolysis of YPs occurs during oocyte maturation, in which Ctsb rather than Ctsla is likely the major protease involved (LaFleur et al., 2005; Raldúa et al., 2006). In this species, there is no evidence for the expression of a second isoform of Ctsb in oocytes or embryos (Fabra and Cerdà, 2004). *F. heteroclitus* thus offers a good model to investigate the role of Ctsb during early development in fish. In the present study, we have determined the spatio-temporal changes in *F. heteroclitus ctsb* gene expression and enzyme activity during embryonic development in relation to those of Ctsla. These processes were correlated with developmental changes in the synthesis and processing of Ctsb and the occurrence of yolk proteolysis.

2. Materials and methods

2.1. Animals and embryo collection

Adult *F. heteroclitus* were obtained from saltmarshes in the bay of Cádiz (southern Spain), transported to the laboratory, and maintained as described (Cerdà et al., 1996; Fabra and Cerdà, 2004). Embryos were collected daily from naturally spawning fish using plastic trays placed at the bottom of the tanks (Tingaud-Sequeira et al., 2009), and were staged after Armstrong and Child (1965). Embryos were incubated in seawater (38‰ salinity; 1,110 mOsmol/kg H₂O) at 25°C in a temperature-controlled incubator for up to 14-15 days post-fertilization (dpf), when most embryos hatched. Procedures related to the care and use of fish were approved by the Ethics Committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) in accordance with the “Guiding Principles for the Care and Use of Laboratory Animals”.

2.2. Real-time quantitative PCR

The temporal expression pattern of *F. heteroclitus* *ctsb* (*fhctsb*) and *ctsla* (*fhctsla*) genes during embryonic development was assessed by real-time quantitative PCR (qPCR). Total RNA was extracted by using the RNAeasy minikit (Qiagen) and treated with Turbo-DNase (Ambion) as indicated by the manufacturer. An aliquot corresponding to 1 µg was reverse transcribed using 0.5 µg oligo-(dT)₁₇, 1 mM dNTPs, 40 IU RNase inhibitor, and 10 IU MMLuV-RT enzyme (Roche), for 1.5 h at 42°C. Real-time PCR was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Power SYBR[®] Green PCR Master Mix (Applied Biosystems). For *fhctsb* (GenBank accession No. AY217741), the forward and reverse primers were 5'-GCTTAACACGACCTGGAAGG-3' and 5'-ACAGGAGCCTTGGTCTCTGA-3' (in exon 1 and 3, respectively, based on the genomic sequence of zebrafish *ctsb* gene). For *fhctsla* (GenBank accession No. AY212286), the forward and reverse primers were 5'-TGGAAGAGCTGGCACAGTAA-3' and 5'-TTTGTGCTTGCTAGCCGTTCA-3' (in exon 1 and 3, respectively, based on the genomic sequence of zebrafish *ctsla* gene). *F. heteroclitus* ornithine decarboxylase (*odc*) mRNA (GenBank accession No. CN977303) was used as a reference gene, employing a forward primer 5'-GCTGCGTCCTCCACCTTCAT-3', and a reverse primer 5'-GGGCATGTCCAAGCTGCTCT-3' (Tingaud-Sequeira et al., 2009). The amplification reactions were carried out in a final volume of 20 µl, containing 10 µl of the master mix, 2 µl

of cDNA diluted 1:10 and 0.2 μ M of each specific primer. The cycle was set as follows: activation for 120 s at 50°C and initial denaturation for 10 min at 95°C, followed by 40 cycles at 95°C for 10 s (denaturation) and 62°C for 60 s (annealing and extension). A final step with a decrease and increase of the temperature from 95°C to 60°C and from 60°C to 95°C, respectively, was applied to determine the melting curve. The threshold cycle number (Ct) was determined for all PCR reactions. Formation of primer dimers was checked by adding water instead of the cDNA in the reaction mixture. The relative transcript level was calculated as fold change using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.3. Whole-mount *in situ* hybridisation

Whole-mount *in situ* hybridization on *F. heteroclitus* embryos was carried out as previously described (Tingaud-Sequeira and Cerdà, 2007). Digoxigenin (DIG)-labeled sense and antisense riboprobes were synthesized from the FhCtsb cDNA previously cloned (Fabra and Cerdà, 2004). Pre-hybridization and hybridization were carried out at 62°C. After colorimetric reaction, embryos were transferred to glycerol solutions (25%, 50%, 75%, and then 100%), and pictures were taken on 100% glycerol-embedded embryos by using a Nikon SMZ1000 stereomicroscope. Some labeled embryos were transferred to 100% ethanol, then 100% xylol, and finally embedded into paraplast (Sigma) for sectioning. Sections (14 μ m) were examined and photographed with a Zeiss Axioskop 2 plus microscope.

2.4. Cathepsin L- and B-like enzyme assays

Groups of embryos ($n = 30$) at different developmental stage were mechanically homogenized in 400 μ l distilled water and the homogenate centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was recovered for each sample and the protein content measured using the Bio-Rad Protein Assay kit (Bio-Rad) using bovine serum albumin as standard. Cathepsin B- (EC 3.4.22.1) and L-like (EC 3.4.22.15) enzymatic activities were determined using the same colorimetric methods described by Zhang et al. (2008). For cathepsin B-like activity, Z-Arg-Arg-NNap (alpha-N-benzyloxycarbonyl-L-Arg-L-Arg-2-naphthylamide) was used as the specific synthetic substrate, whereas Z-Phe-Arg-NNapOMe in the presence of 4 M urea in the reaction buffer, which inhibits cathepsin B activity, was used to determine cathepsin L-like activity. The specific activity of cathepsin B-like was expressed as μ mol of 2-naphthylamine released/min/mg of protein at 40°C, whereas that of cathepsin L-like was expressed in μ mol of 4-methoxy-2-naphthylamine released/min/mg of protein at 45°C. Each assay was repeated 3 times with different batches of embryos.

2.5. SDS-PAGE of YPs

Embryos at different developmental stages, 2-4 cells, morula, blastula, gastrula, neurula and 7 dpf, were mechanically homogenized in 1 x Laemmli sample buffer (Laemmli, 1970) and denatured at 95°C for 5 min. After treatment with 10 U of the endonuclease Benzonase (Sigma) for DNA digestion (20 min at room temperature), the samples were centrifuged 10 min at full speed and processed for electrophoresis. A volume corresponding to 0.025 embryo equivalents was loaded in each lane for YP profile determination. SDS-PAGE was performed in 15% acrylamide minigels (7 x 3 x 10 cm) and electrophoresed at constant voltage (130 V) for 1.5 h. After fixation in 12.5% trichloroacetic acid, the gel was stained overnight in 0.2% Coomassie blue in 30% methanol and 10% acetic acid solution. The gel was progressively destained in 25% methanol and 7% acetic acid solution until the bands were clearly revealed. Previously identified YPs in *F. heteroclitus* oocytes and eggs (LaFleur et al., 2005) are related to the precursor Vtg nomenclature proposed by Finn and Kristoffersen (2007) and Finn et al. (2009).

2.6. Immunoprecipitation and immunoblotting

Ten embryos for each developmental stage (2-4 cell, morula, blastula, 50% epiboly, late neurula, and 7 dpf) were homogenized on ice in 500 µL of NP-40-containing lysis buffer (1% NP-40, 1 mM CaCl₂, 150 mM NaCl, 10 mM Tris-HCl pH 7.4) in PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) supplemented with a cocktail of protease inhibitors (mini-EDTA-free; Roche). Immunoprecipitation using 10 µg of anti-*F. heteroclitus* Ctsb rabbit antiserum was performed as described (Raldúa et al., 2006). Aliquots of 10 µg of immunoprecipitated protein for each embryo stage were separated by SDS-PAGE (15%) and electroblotted onto PVDF membranes (Bio-Rad) using glycine transfer buffer (190 mM glycine, 25 mM Tris pH 8.6, 20% methanol). Membranes were blocked with TBST (20 mM Tris, 140 mM NaCl, 0.1% Tween, pH 7.6) containing 5% non-fat dry powder for 1 h, and then incubated overnight with 1:500 diluted anti-*F. heteroclitus* Ctsb antisera in TBST with 5% milk powder at 4°C. As secondary antibody, a 1:8000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma) was used. Reactive protein bands were detected using enhanced chemiluminescence (Amersham). Three independent experiments on different batches of embryos were performed for quantification of band intensity using the Gel Doc software (Bio-Rad).

2.7. Whole-mount immunocytochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C, and subsequently washed in PBST (PBS, 0.8% Triton X-100) and manually dechorionated. After five washes in PBST, non-specific sites were blocked by incubation in PBST containing 10% goat serum for 2 h at room temperature. Embryos were transferred to 1% goat serum PBST with anti-*F. heteroclitus* Ctsb antiserum diluted 1:500 and incubated overnight at 4°C. Negative controls were probed with the antisera preincubated with 100 µg of the synthetic peptide used for immunization (Raldúa et al., 2006) for 1 h at 37°C. After several washes of 15 min each with PBST, embryos were incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:200 diluted) in 1% goat serum PBST for 4 h at room temperature. Embryos were washed with PBST and transferred to 0.1 M Tris pH 7.4 for 15 min. 3,3'-Diaminobenzidine (DAB) revelation was performed as recommended by the manufacturer (SIGMAFAST™, Sigma) until reactive signals were visible, and embryos were subsequently stored in glycerol at 4°C. For sectioning, embryos were washed with PBS containing decreasing concentration of glycerol, dehydrated, and embedded into Paraplast. Sections (15 µm) were deparaffinized in xylene, mounted with Eukitt mounting medium (Fluka), and photographed as indicated above.

2.8. Immunofluorescence microscopy

Chorion-bearing embryos at the gastrula stage (50-70% epiboly) were permeabilized with 3% NaOCl for 1 min, and fixed with 4% PFA in PBS for 4 h at room temperature. After fixation, embryos were manually dechorionated and refixed with 4 % PFA for 1 h. Embryos were then washed, dehydrated and embedded into Paraplast. Sections (15 µm) were rehydrated and blocked with 5 % goat serum in PBSTB (0.1% BSA, 0.05% Tween 20 in PBS) for 1 h, and incubated with anti-*F. heteroclitus* Ctsb antisera (1:100) in 1% goat serum PBSTB overnight at 4°C. After washing, sections were incubated with a secondary anti-rabbit IgG FITC-coupled antibody (Sigma) for 1 h, and subsequently with 4',6-diamidino-2-phenylindole (DAPI; Sigma) diluted 1:3000 in PBS for 3 min. Sections were mounted with fluoromount aqueous anti-fading medium (Sigma), and immunofluorescence was observed and documented with a Zeiss imager.z1 microscope.

2.9. Statistics

The values presented are mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA followed by the Tukey's multiple-range test. When data were not

normally distributed, a non-parametric Kruskal-Wallis test, followed by a Mann-Whitney test, was used to detect statistical differences. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Pattern of *fhctsb* and *fhctsla* gene expression during embryogenesis

The changes in *fhctsb* and *fhctsla* mRNA abundance from the 2-4-cell stage up to 7 dpf was determined by qPCR (Fig. 1). Expression of *fhctsb* and *fhctsla* was detected at the 2-4-cell stage indicating that both transcripts were maternally inherited. However, subsequent changes in the relative amount of *fhctsb* and *fhctsla* during development were different. The expression of *fhctsb* significantly ($p < 0.05$) dropped at the morula stage, presumably prior to the activation of zygotic expression, and progressively increased thereafter from the blastula stage until 7 dpf (Fig. 1A). In contrast, the levels of *fhctsla* remained unchanged until the blastula stage, increasing exponentially from the gastrula stage up to 7 dpf (Fig. 1B). However, the relative increase of *fhctsla* from the 2-4-cell stage up to 7 dpf was about the double of that of *fhctsb*.

To investigate the cellular localization of *fhctsb* expression during early embryogenesis, whole-mount *in situ* hybridization was carried out (Fig. 2). Contrary to the results obtained by qPCR, *fhctsb* transcripts could not be detected by *in situ* hybridization until the neurula stage, approximately at 24 h postfertilization (hpf) (Fig. 2A-C). By this time, a strong signal for *fhctsb* was obtained in the YSL surrounding the yolk sac (Fig. 2D-E), which remained through the 19-somite stage, approximately 48 hpf (Fig. 2F), up to 5 dpf (data not shown). The high level of *fhctsb* expression in the YSL was confirmed by immunolocalization of the protein product by whole-mount immunocytochemistry as well as by immunofluorescence microscopy using a specific anti-FhCtsb antiserum (Fig. 3). In both types of experiments, specific immunoreactions were seen in the cytoplasm of the YSL, whereas the more external blastomeres and the enveloping layer were negative (Fig. 3A).

3.2. Developmental heterochrony of cathepsin B- and L-like activities

In order to investigate if the pattern of *fhctsb* and *fhctsla* expression during embryo development correlated with differences in enzyme activation, cathepsin B- and L-like activities were assayed in embryos at different stages of development (Fig. 4). The developmental pattern observed for the cathepsin B-like activity approximately followed that of *fhctsb* expression. At the 2-4-cell stage, cathepsin B-like activity was very low and

progressively increased as the embryo developed from the morula stage, showing a 8-fold increase as embryos reached the neurula stage (Fig. 4A). Cathepsin B-like activity increased more moderately until 7 dpf, and then remained stable until the time of hatching, when a low but significant increase in activity was noted (Fig. 4). The changes in cathepsin L-like activity during development were delayed with respect those of cathepsin B-like (Fig. 4B). Thus, cathepsin L-like activity remained low until the gastrula stage and increased by about 2-fold at the neurula stage. By 7 dpf, a 6-fold elevation in activity was observed, which continued to increase until 14 dpf (Fig. 4B). Therefore, both cathepsin B- and L-like activities showed a marked increase (8- and 6-fold, respectively) during development, but that of cathepsin B-like occurred earlier.

3.3. Processing of FhCtsb during embryogenesis

To estimate the rate of FhCtsb synthesis and activation during embryonic development, Western blot analysis on immunoprecipitated FhCtsb was performed. In mammals, it is well established that cathepsin B is translated as a preproenzyme, and then transferred to the Golgi Complex as a proenzyme with very weak activity (reviewed by Ishidoh and Kominami, 2002). The mature cathepsin B enzyme is localized in lysosomes where it is found as both single and double chain forms. The double chain form is made up of two components originated from digestion of the single chain linked by disulfide bonds. In rats, peptide monomers of the procathepsin B form, the single chain active form, and the double chain form, have molecular masses of 45, 32 and 28 kDa, respectively (Authier et al., 1995). In the present study, three independent Western blots were performed to quantify the ratio between FhCtsb single chain and proFhCtsb (i.e., active and inactive forms, respectively). FhCtsb polypeptides were immunoprecipitated using the FhCtsb antiserum raised against the C terminus of the protein (Raldúa et al., 2006), which reduced further contamination with YPs and improved the immunodetection of FhCtsb.

The results of these experiments showed that just after fertilization (2-4 cell stage), three FhCtsb immunoreactive bands could be detected, which were identified as preproFhCtsb, proFhCtsb, and FhCtsb active single chain form, with apparent molecular masses of 47, 45 and 30 kDa, respectively (Fig. 5A). At the morula stage, a significant ($p < 0.05$) increase in the ratio of active FhCtsb single chain with respect the proenzyme was noted, but at subsequent blastula and gastrula stages the ratio decreased again to the levels observed in 2-4-cell embryos (Fig. 5B). By the neurula stage, a significant ($p < 0.05$) increase of the active FhCtsb single chain form with respect to the proenzyme was further observed

(Fig. 5B), and a fourth 28 kDa immunoreactive band, most likely corresponding to the FhCtsb heavy chain of the double chain form, became apparent (Fig. 5A). By 7 dpf, the FhCtsb double chain form appeared to increase, whereas the single chain FhCtsb form was almost double that of the proenzyme (Fig. 5B). These data thus positively correlated with the increased cathepsin B-like activity observed from the gastrula stage up to 7 dpf.

3.4. Pattern of YP hydrolysis during *F. heteroclitus* embryo development

To investigate if the changes in FhCtsb and FhCtsla gene expression and enzyme processing and activity correlated with yolk proteolysis during embryogenesis, the changes in the major YPs were characterized by SDS-PAGE followed by Coomassie blue staining (Fig. 6). In 2-4-cell embryos, a total of 11 different bands were revealed by SDS-PAGE, which were named after their apparent molecular mass. Nine of these YPs, YP122, YP103, YP77, YP75, YP69, YP45, YP42, YP26 and YP19, have been reported in *F. heteroclitus* unfertilized eggs (Lafleur et al., 2005), whereas the YP50 and YP32, which seems to be absent before fertilization, appeared as a very tiny bands. No major band shifts were detected until the neurula stage, when the YP122 completely disappeared, and a new YP with an apparent molecular mass of approximately 25 kDa was detected (Fig. 6). At the same time, the amount of YP103 decreased slightly, which corresponded to an increased intensity of YP77. These altered electrophoretic profiles during neurulation correlated well with the 10-fold increase in cathepsin B-like activity. By 7 dpf, when the activities of both cathepsin B- and L-like were almost asymptotic, the hydrolysis of YPs was more pronounced. At this stage, a marked decrease of the YP103 and YP69 bands, and increase of the YP77 band was, observed, while the YP25 band, which originated by the neurula stage, and the YP42 band disappeared. Concomitantly, newly formed YPs, YP40, YP22, YP21, YP18 and YP15, were detected, as well as an increase of the intensity of the YP32 band and a decrease of that of YP19. The YP50 and YP45 remained essentially unaltered at 7 dpf.

4. Discussion

By using N-terminal microsequencing of isolated YPs, Lafleur et al., (2005) described the model of Vtg/YP precursor/product relationship and further processing during oocyte maturation in *F. heteroclitus*, which may be similar for other teleosts producing benthic eggs (LaFleur et al., 2005; Cerdà et al., 2007; Finn, 2007b). Thus, in *F. heteroclitus* most of the YPs identified in post-vitellogenic oocytes are derived from VtgAa (Vtg1), whereas YP69 is

originated from the hydrolysis of VtgAb (Vtg2), which is also proteolytically cleaved into small peptides during vitellogenesis (LaFleur et al., 2005). During the transformation of oocytes into mature, ovulated eggs, one of the most pronounced changes in the oocyte YPs is the drastic reduction of the YP122, which corresponds to the heavy chain of VtgAa lipovitellin (Wallace and Begovac, 1985; Wallace and Selman, 1985; Greeley et al., 1986; McPherson et al., 1989; LaFleur et al., 2005), while YP45 is partially degraded (LaFleur et al., 2005). In the present study we show that no major YP proteolysis occurs until after gastrulation. Thereafter, significant proteolysis commences during neurulation and becomes more prominent by 7 dpf. Interestingly, the VtgAb-derived YP69 was maintained more or less intact until 7 dpf, suggesting that VtgAa-derived YPs are the initial nutritive sources during *F. heteroclitus* early embryogenesis. Nevertheless, the delay in yolk resorption during embryogenesis, which in *F. heteroclitus* was not detected until 24 hpf, has also been described in salmonids such as masu salmon (*Oncorhynchus masou*) and Sakhalin taimen (*Hucho perryi*) in which proteolysis of YPs does not occur until mid-embryogenesis, by 14 and 22 dpf, respectively (Hiramatsu et al., 2002). In barfin flounder (*Verasper moseri*), utilization of VtgAb-derived lipovitellin occurs also late from 16 dpf, and correlates with the depletion of the FAA pool originated from the hydrolysis of YPs during oocyte maturation (Ohkubo and Matsubara, 2002), suggesting that amino acids from the YP pool are recruited when the FAA pool cannot fulfill anymore the nutritional requirements of developing embryos (Finn et al., 1996; Ohkubo and Matsubara, 2002; Finn and Fyhn, 2010). A similar scenario may be proposed for *F. heteroclitus* embryos, although some hydrolysis of YPs occurring at earlier stages producing peptides that do not appear on the gels as bands either because they do not stain well with Coomassie blue or because they are too small to resolve on the gel, cannot be ruled out.

Since FhCtsb has been suggested as the major enzyme involved in the oocyte maturation-associated proteolytic processing of YP122 in *F. heteroclitus* (LaFleur et al., 2005), in the present study we investigated in detail developmental changes in *fhctsb* gene expression and enzyme activity. In comparison, we also determined the changes in *fhctsla* expression and cathepsin L-like activity since this protease is likely to be involved in yolk proteolysis during fish embryogenesis (Murakami et al., 1990; Sire et al., 1994; Kestemont et al., 1999; Carnevali et al., 2001; Tingaud-Sequeira and Cerdà, 2007). Our data indicate that both *fhctsb* and *fhctsla* transcripts in embryos progressively increased during development from the 2-4 cell stage up to 7 dpf. Accordingly, cathepsin B- and L-like activities increased with development, but a 8-fold increase in cathepsin B activity, which was accompanied by a

more moderate increase in cathepsin L activity, was observed at the neurula stage, when hydrolysis of YPs start to be visible. Such an increase of cathepsin B activity correlated with an increment in the embryo of the relative amount of the active FhCtsb single chain form with respect to the proenzyme. These observations, together with the fact that *fhctsb* and the protein product are strongly expressed in the YSL, suggest that FhCtsb, possibly together with Ctsla, may play a role in the initial mechanisms of yolk resorption in *F. heteroclitus*.

Western blot analysis of immunoprecipitated FhCtsb also revealed that this protease possibly acts in two-chain form as described for mammals and in teleosts such as the common carp (*Cyprinus carpio*) (Ishidoh and Kominami, 2002; Tan et al., 2006). This notion is strengthened by comparing the amino acid sequences flanking the cleavage site between the light chain and heavy chain of mammalian, common carp and *F. heteroclitus* cathepsin B, in which a basic residue and a Val are conserved (Arg⁴⁹-Val⁵⁰ in mouse and bovine cathepsin B [Ferrara et al., 1990; Bechet et al., 1991], and Lys⁴⁹-Val⁵⁰ in carp and *F. heteroclitus* cathepsin B [Fabra and Cerdà, 2004; Tan et al., 2006]). In common carp muscle, the N-terminal amino acid sequence of the putative Ctsb heavy chain shows high homology with those of mammalian cathepsin B, suggesting that the common carp Ctsb most likely works in two-chain form, consisting of a light chain and a heavy chain bound by disulfide bond (Tan et al., 2006). In contrast, common carp and *F. heteroclitus* Ctsla are likely to act only as single chain forms since the P1 position at the cleavage site, which contains a hydrophylic amino acid (Ser) in mammalian cathepsin L, is a hydrophobic residue (Val) in both common carp and *F. heteroclitus* Ctsla (Fabra and Cerdà, 2004; Tan et al., 2006). The appearance of the FhCtsb double chain form in embryos occurred at neurula stage and appeared to increase later in development, being however absent at earlier stages. The physiological significance of this differential processing of FhCtsb during embryogenesis remains intriguing and requires further investigation.

A heterochronic increase in cathepsin B-like activity, but not of cathepsin L-like activity was observed from the blastula stage, and was preceded by an elevation of the active form of FhCtsb at the morula stage. The elevation of both FhCtsb active form and further enzyme activity may have been derived from maternal mRNA, but later it was likely regulated by post-transcriptional mechanisms, since the *fhctsb* transcript levels in the embryo markedly decreased at the morula and blastula stages. The levels of maternal *fhctsb*, which are reported to be accumulated in ovarian follicles throughout vitellogenesis (Fabra and Cerdà, 2004), were apparently quite low during early embryogenesis since they could only be detected by qPCR. Maternal levels of *fhctsb* therefore may be much lower than those of *fhctsla* which

have been shown to increase approximately by 3-fold during oocyte maturation (Fabra and Cerdà, 2004) and are highly expressed at the morula stage of *F. heteroclitus* and zebrafish (Tingaud-Sequeira and Cerdà, 2007). Nevertheless, the specific activation of FhCtsb during the blastula and gastrula stages suggest that maternal *fhctsb* may play a critical function in *F. heteroclitus* early embryos unrelated to yolk mobilization. In European sea bass (*Dicentrarchus labrax*), maximum cathepsin B activity occurs at the morula stage (Carnevali et al., 2001), which may be a consequence of the different development rates of these embryos when compared with those of *F. heteroclitus* (e.g. European sea bass embryos hatch at about 2 dpf at 16-17°C [Devauchelle and Coves, 1988], whereas water-immersed *F. heteroclitus* embryos hatch at 14-15 dpf at 25°C [e.g., Tingaud-Sequeira et al., 2009]).

It is well established that proteases play distinct roles in the processing of signalling molecules involved in mesodermal patterning in various embryos (Yajima and Kawashima, 2002; Blomberg et al., 2008). Thus, for instance, precursor molecules of latent transforming growth factor beta (TGF- β) have been reported to be activated by lysosomal proteases (Oursler et al., 1993), and the receptor for the platelet-derived growth factor (PDGF), which is involved in pathways controlling the migration, differentiation and function of a variety of specialized mesenchymal and migratory cell types during gastrulation (Hoch and Soriano, 2003), is cleaved by a thiol protease to enable it to bind PDGF (Ek and Heldin, 1986). In *Xenopus laevis* embryos, disruption of cathepsin L with an antibody against cathepsin L-like protease induces a defective gastrulation leading to an abnormal mesodermal structure (Miyata and Kubo, 1997). Therefore, it is possible that the activation of FhCtsb and gradual increment of its activity during the blastula and gastrula stages in *F. heteroclitus* embryos are related with a role of this protease at the onset of gastrulation. However, it has been reported that Ctsb may regulate the activation of cathepsin L- and caspase-3-like activity in zebrafish ovarian follicles (Carnevali et al., 2006; Eykelbosh and Van der Kraak, 2010), and consequently whether the action of Ctsb on embryonic signalling pathways is direct or through the activation of other proteases such as Ctsla is unknown.

In summary, this study provides evidence for the potential role of FhCtsb during gastrulation and yolk metabolism in *F. heteroclitus* embryos. The present observations that stage-specific yolk proteolysis coincides with increased activity of FhCtsla, which is also expressed in the YSL of developing embryos (Tingaud-Sequeira and Cerdà, 2007), suggest that FhCtsla plays a complementary catalytic role to that of FhCtsb during yolk mobilization. Whether the specific role of Ctsb in this mechanism is the activation of Ctsla (Carnevali et al., 2006) remains to be investigated. However, in other teleosts, such as the zebrafish, *ctss*

and *ctsc* mRNAs are also localized in the embryonic YSL (Thisse et al., 2001; Rauch et al., 2003). These findings thus suggest that yolk degradation during embryogenesis in teleosts may possibly require the combined action of different proteolytic enzymes in addition to Ctsb and Ctsla (Sire et al., 1994; Hiramatsu et al., 2002). Further studies will be necessary to elucidate the specific role of the different embryonic cathepsins including Ctsb, as well as their interactions, during early embryogenesis and yolk metabolism in teleosts.

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Figure Legends

Fig. 1. Expression of *fhctsb* and *fhctsla* in *F. heteroclitus* embryos at different developmental stages determined by quantitative real-time PCR. Transcript levels of the *F. heteroclitus* ornithine decarboxylase (*odc*) gene were used as reference, and relative transcript levels were calculated as fold change using the $2^{-\Delta\Delta Ct}$ method. Data are presented as means $\pm$ SEM ($n = 3$ different batches of embryos). Values with a different superscript are significantly different (Kruskal-Wallis test, $p < 0.05$).

Fig. 2. Expression of *fhctsb* during *F. heteroclitus* early embryogenesis up to the late neurula stage (18-19 somites) at 48 h postfertilization (48 hpf) by whole-mount *in situ* hybridization using antisense DIG-labelled riboprobes (blue color). Lateral views of embryos with rostral to the left (A-C and F-G), and cross-section (D). Transcripts were not detected at morula stage (A) or at 50% epiboly (B). The arrowheads in B indicate the marginal region of the gastrula (Gs). In morula embryos, oil globules (Og) react unspecifically with the alkaline phosphatase substrate precipitating BM purple. At the late neurula stage (C), *fhctsb* expression (arrows) was found in the yolk syncytial layer (YSL) surrounding the yolk sac (Ys). The cross-section (D) and enlarged view of the Ys (E) shows the specific labelling around the nuclei in the yolk syncytium (arrows). The expression in the yolk syncytium is prolonged up to the neurula stage (F). The staining using sense riboprobes was negative in all stages (G shows the negative controls at the neurula stage). Scale bars, 500 μm (A-C, F, G), 100 μm (D, E).

Fig. 3. Immunolocalization of FhCtsb in *F. heteroclitus* embryos at the gastrula stage (50-70% epiboly) by immunofluorescence (A; green color), or at the neurula stage by whole-mount immunocytochemistry (C; brown color). The nuclei of blastomeres (Bl) are stained in blue with DAPI (A and B). Negative controls (B and D) were incubated with the anti-FhCtsb antiserum preadsorbed with the synthetic peptide. At the gastrula and neurula stages a strong immunoreactive signal is noted in the yolk syncytial layer (YSL) nearby the yolk sac (Ys), whereas the enveloping layer (Evl) is negative. Note in A the presence of an oil globule (Og) not stained. Scale bars, 20 μm (A, B), 10 μm (C, D).

Fig. 4. Cathepsin B- (A) and L-like (B) enzyme activity in *F. heteroclitus* embryos at different developmental stage. Data are presented as means $\pm$ SEM of 3 separate experiments performed on different batches of embryos. Values with different superscript are significantly different (ANOVA, $p < 0.05$).

Fig. 5. Immunodetection of FhCtsb in *F. heteroclitus* embryos. (A) Representative FhCtsb immunoblot of FhCtsb immunoprecipitated proteins from embryos at different developmental stages as indicated on the top. The same amount of protein (10 µg) was loaded in each lane. The arrowheads point to the position of the preproenzyme (Pre-proFhCtsb), proenzyme (ProFhCtsb), single chain active form (FhCtsbSC), and heavy chain of the double-chain active form (FhCtsbHC). Apparent molecular mass (M_r) values are given in kDa on the left. (B) Ratio of the intensities of the FhCtsbSC/FhProCtsb immunoreactive bands for each embryo stage. Data are mean $\pm$ SEM ($n = 3$ independent immunoblots). Values with different superscript are significantly different (ANOVA, $p < 0.05$).

Fig. 6. Major yolk proteins (YPs) of *F. heteroclitus* embryos at different developmental stages resolved by 15% SDS-PAGE and stained with Coomassie blue R-350. A volume corresponding to 0.025 embryo equivalents was loaded in each lane. The YPs (arrowheads) are named based on their apparent molecular mass after LaFleur et al. (2005). YPs formed during embryo development are in cursive. Apparent molecular mass (M_r) values are given in kDa on the left.