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Post-translational protein deimination in cod (*Gadus morhua* L.) ontogeny novel roles in tissue remodelling and mucosal immune defences?

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ABSTRACT

Peptidylarginine deiminases (PADs) are calcium dependent enzymes with physiological and pathophysiological roles conserved throughout phylogeny. PADs promote post-translational deimination of protein arginine to citrulline, altering the structure and function of target proteins. Deiminated proteins were detected in the early developmental stages of cod from 11 days post fertilisation to 70 days post hatching. Deiminated proteins were present in mucosal surfaces and in liver, pancreas, spleen, gut, muscle, brain and eye during early cod larval development. Deiminated protein targets identified in skin mucosa included nuclear histones; cytoskeletal proteins such as tubulin and beta-actin; metabolic and immune related proteins such as galectin, mannan-binding lectin, toll-like receptor, kininogen, Beta2-microglobulin, aldehyde dehydrogenase, bloodthirsty and preproapolipoprotein A-I. Deiminated histone H3, a marker for anti-pathogenic neutrophil extracellular traps, was particularly elevated in mucosal tissues in immunostimulated cod larvae. PAD-mediated protein deimination may facilitate protein moonlighting, allowing the same protein to exhibit a range of biological functions, in tissue remodelling and mucosal immune defences in teleost ontogeny.

1. Introduction

Peptidylarginine deiminases (PADs) are a family of calcium dependent enzymes that are preserved throughout phylogeny from bacteria to mammals (Vossenaar et al., 2003). PADs have various physiological roles and are implicated in embryonic development, cell differentiation, cell death and gene regulation (Wang and Wang, 2013; Witalison et al., 2015). PADs are expressed in most body tissues; and while in mammals there are five genes designated PADI (PADI 1, 2, 3, 4, 6), in fish only one PADI has been identified (Vossenaar et al., 2003; Rebl et al., 2010). Mammalian PADI2 is the phylogenetically most conserved isozyme and shows the closest sequence alignment to fish PADI, which codes conserved amino acid residues involved in catalysis and binding of Ca^{2+} (Rebl et al., 2010). PADI has been identified in various fish species including rainbow trout (*Oncorhynchus mykiss*), stickleback (*Gasterosteus aculeatus*), Japanese pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*) and ricefish (*Oryzias latipes*) and in the Atlantic cod (*Gadus morhua* L) genome (Star et al., 2011; NEAC_001). In cod it is found to have a transcript length of 1785 bps, encoding a 594 aa protein (ENSGMOP00000008323).

PADs cause post-translational conversion of protein arginine to citrulline in target proteins in a Ca^{2+} - dependent manner. The conversion of each arginine to citrulline causes the loss of one positive charge and loss of 1 Da, thus changing intra- and intermolecular protein interactions, protein structure and function (Vossenaar et al., 2003; György et al., 2006). Structures most prone to deimination are beta-sheets and intrinsically disordered proteins, and identified deiminated targets include nuclear, cytoplasmic and mitochondrial proteins (György et al., 2006). Although protein deimination is linked to the generation of neo-epitopes and loss of function in many pathologies this post-translational modification can also facilitate novel interactions and contribute to protein moonlighting, an evolutionary acquired phenomenon allowing proteins to exhibit more than one physiologically relevant biochemical or biophysical function within one polypeptide

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chain (Henderson and Martin, 2014). While in mammals, PAD-isozyme specific preferences for target proteins have been described (Witalison et al., 2015), in teleosts protein deimination must be mediated by the one PAD enzyme present. Previously, PADI expression has been studied in early zebrafish development (Khajavi et al., 2017) and shown by qPCR in various organs in adult rainbow trout (Rebl et al., 2010), while studies on the presence of deiminated proteins have hitherto been limited in teleosts.

PAD-mediated histone deimination can affect gene regulation and also contribute to neutrophil extracellular trap formation (NETosis), which mediates extracellular pathogen killing and forms part of microbicidal innate immunity (Brinkmann et al., 2004; Li et al., 2010; Byrd et al., 2013; Yang et al., 2016). In teleosts, neutrophils display long-lasting contributions to host defence, including anti-microbial mechanisms, as well as in the maintenance of homeostasis (Neumann et al., 2001; Havixbeck and Barreda, 2015). NETs are composed of a backbone DNA, histones (H1, H2A, H2B, H3 and H4), cytoplasmic granule components, and antimicrobial peptides (Urban et al., 2009), and have been shown in mammals to be driven by PAD4, which is enriched in neutrophil nuclei (Nakashima et al., 2002; Neeli et al., 2008; Wang et al., 2009). PAD-induced NETs are for example crucial for effective bacterial killing in mouse models of infectious disease (Li et al., 2010) and the ability of NETs to kill parasites implies an important role in innate responses against a variety of pathogens (Guimarães-Costa et al., 2009). As this process involves the externalisation of deiminated histone H3, it is a commonly used NETosis marker (Li et al., 2010). NETs are evolutionarily conserved through phylogeny from fish to human (Brinkmann et al., 2004; Palić et al., 2007a, 2007b; Pijanowski et al., 2013). Bactericidal properties of histones have been known for a long time (Hirsch, 1958) and there is a considerable interest in using anti-microbial peptides in fish mucosal surfaces and immune barriers for immunostimulation (Magnadottir, 2010). Deiminated histones may thus offer novel mucosal immunotherapy approaches for immunostimulation and mucosal vaccines in aquaculture. Teleost mucosa-related epithelial tissues are a critical first barrier against infection, participate in trapping of pathogens (Ellis, 2001; Gomez et al., 2013) and contain amongst other complement proteins (Lange et al., 2004a, 2004b; Lovoll et al., 2006, 2007), lectins, (Jørndrup and Buchmann, 2005; Rajan et al., 2011), lysozyme (Fernandes et al., 2004a; Rajan et al., 2011), pentraxins (Audunsdottir et al., 2012) and IgT/IgZ (Zhang et al., 2010, 2017). Comparative studies on mucosal immunity in teleosts are translatable to human mucosal surfaces, as teleost mucosal surfaces have been found to share many characteristics with type I mucosal surfaces of mammals which are present in the respiratory tract, intestine and uterus (Zhang et al., 2010, 2017; Gomez et al., 2013; Xu et al., 2013). The present study verifies for the first time PAD-mediated protein deimination in cod ontogeny, indicating diverse roles in tissue remodelling and mucosal immune defences.

2. Materials and methods

2.1. PAD sequence alignment and phylogenetic reconstruction

PADI sequences were retrieved from Ensembl (http://www.ensembl.org/index.html) and NCBI (https://www.ncbi.nlm.nih.gov/), translated to protein and multiple sequence alignment was performed using the MUSCLE sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/muscle/). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Phylogenetic relationships of the PAD proteins were inferred using the Neighbour-Joining method under the conditions of the Poisson correction distance model. Bootstrap analysis with 1000 replicates was used to assess nodal support. The analysis involved 27 PAD protein sequences from species representing a range of taxa: teleost represented by three spined stickleback (*Gasterosteus aculeatus*, ENSGACP00000006356), medaka rice fish (*Oryzias latipes*, ENSORLP00000009419), rainbow trout (*Oncorhynchus mykiss*, CAX45844), zebrafish (*Danio rerio*, ENSDARP00000111628), pufferfish (*Takifugu rubripes*, ENSTRUP00000046360), Mexican tetra (*Astyanax mexicanus*), southern platyfish (*Xiphophorus maculatus*, NP_001080369), spotted gar (*Lepisosteus oculatus*, XP_006641986) and Atlantic cod (*Gadus morhua*, ENSGMOP00000008323); elasmobranch whaleshark (*Rhincodon typus*, XP_020374364), amphibian xenopus (*Xenopus laevis*, NP_001080369); reptilian python (*Python bivittatus*, XP_007438216) and alligator (*Alligator mississippiensis*, XP_019355591); avian red junglefowl (*Gallus gallus*, XP_425730); lancelet (*Branchiostoma floridae*, EEA40934); priapulid (*Priapulus caudatus*, XP_014670176); cyanobacterium (*Cyanothece* sp., YP_001804912); and man (*Homo sapiens*, PADI1, NP_037490; PADI2, NP_031391; PADI3, NP_057317; PADI4, NP_036519; PADI6, NP_997304). With the outgroup comprising of: flagellate protest (*Giardia intestinalis*, ESU43720); ciliate protist (*Tetrahymena thermophile*, XP_001010707); fungus (*Aspergillus fumigatus*, XP_748505); Gram-positive bacterium (*Clostridium botulinum*, YP_001253426); and Gram-negative bacterium (*Porphorymonas gingivalis*, YP_001928373).

2.2. Fish and sampling

2.2.1. Larval sampling

Experimentally farmed cod (*Gadus morhua* L) larvae were obtained from the Marine Institute's Experimental Fishfarm Stadur, Grindavik, Iceland; reared as described before (Lange et al., 2004a). Fertilized eggs from cultured cod were hatched in 25 L silos at 7 °C, larvae were kept in the dark for 3 days after hatching in 150 L silos at 7 °C, with flow-through water supplied from day 2. Nannochloropsis (1 mL per silo) was used twice a day for darkening and feeding. Larvae were fed with rotifers twice a day from day 3–15, when gradually replaced by *Artemia nauplii*. By day 24 *Artemia metanauplii* were used and dry food-pellets gradually introduced from day 40. The temperature control was as following: a gradual increase from 7 °C to 10 °C on day 3, 12–13 °C on day 24 and thereafter at 14 °C ± 2 °C. At age 20–30 days larvae were transferred to circular tanks (3 m diameter, water depth 0.8 m) and the water temperature was gradually reduced to 7 °C, which is the rearing temperature of adult cod (Steinarsson and Björnsson, 1999). Cod larvae were collected from 11 days post fertilisation (d.p.f.) until 70 days post hatching (d.p.h). The cod larvae were fixed in 4% formalin in phosphate buffered saline (PBS) at 4 °C for 24 h and thereafter embedded in paraffin.

2.2.2. LPS immunostimulated larvae

Five g cod were injected intraperitoneally (i.p.) with 250 µg lipopolysaccharide (LPS) isolated from the bacterium *Aeromonas salmonicida* (*Asa*) (Magnadottir et al., 2002; Gudmundsdottir et al., 2003). After 2 h, fish were sacrificed for immunohistological examination; while remaining fish were left for a further 23 h and then received an intramuscular injection (i.m.) with the same dose of LPS and were thereafter sacrificed 1 h later. Organ samples from liver, heart, spleen, gut, stomach, brain and muscle were collected, fixed in 4% formalin in phosphate buffered saline (PBS) at 4 °C for 24 h and thereafter embedded in paraffin.

2.2.3. Mucus sampling and preparation

Cod mucus was collected, gently using a glass slide, from the dorsal side of the body of adult fish (2–3 year old; 400–1000 g, reared at 4–9 °C). Samples were pooled from 10 individual fish, immediately frozen on dry ice and stored at −80 °C until used. Protein extraction was performed according to Al-Harbi and Austin (1993). Mucus (approximately 0.5 g), was homogenized in PBS and dialysed in PBS overnight at 4 °C. The preparation was then centrifuged, the pellet resuspended in PBS and protein extracted using 50% saturated ammonium sulphate for 1 h at room temperature. The resulting extract was resuspended in saline and dialysed in saline for 48 h at 4 °C.

Precipitated protein was collected and protein measured by Bradford assay (Bradford, 1976). The samples were reconstituted in 2 x Laemmli buffer for Western blotting analysis.

2.3. Immunoprecipitation and protein identification

Total deiminated proteins were isolated by immunoprecipitation from a pool of mucus protein extract (4.2 mg protein/ml) from 10 individual cod. Immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions, and the monoclonal F95 pan-deimination antibody (Nicholas and Whitaker, 2002) for isolation of total deiminated proteins. Bound proteins were eluted and analysed by liquid chromatography–mass spectrometry (LC-MS/MS) (Cambridge Centre for Proteomics, U.K.) and the peak list files submitted to MASCOT.

2.4. Western blotting

Pooled cod mucus protein extracts (n = 10) and pooled cod serum (n = 5) were analysed by Western blotting for detection of total deiminated proteins (F95), deiminated histone H3 (citH3; ab5103, Abcam, U.K.) and PAD2 (ab50257, Abcam). Approximately 5 µg of protein was loaded per lane, even load was assessed using Ponceau S staining (Sigma, U.K.), membranes were thereafter blocked in 5% bovine serum albumin (BSA) in Tris buffered saline with 0.01% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the primary antibodies (F95 1/3000; citH3 1/2000, PAD2 1/1000). Membranes were then washed 3 times in TBS-T, incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (anti-mouse IgM or anti-rabbit IgG; BioRad, U.K.), followed by 6 washes in TBS-T before visualisation with ECL (Amersham, U.K.). Membranes were imaged using a transilluminator (UVP BioDoc-IT™ System, U.K.).

2.5. Immunohistochemistry

Paraffin blocks were kept at room temperature and 5 µm serial tissue sections cut on a microtome. Samples used for immunostaining to detect the presence of deiminated proteins in ontogeny were at 11 d.p.f., 28 d.p.h., 43 d.p.h., 51 d.p.h., 57 d.p.h. and 70 d.p.h. Tissue sections were deparaffinised using xylene, taken to water (100%, 90%, 70% ethanol) and de-masked by heating (11 min in the microwave at power 6) in citric acid buffer (pH 6.0). Thereafter the sections were washed in 0.1% BSA in 100 mM phosphate buffer (PB) and washed two times in PB. Next the sections were incubated with 5% goat serum (Sigma, St. Louis, MO, USA) in PB for 1 h, followed by incubation in primary antibody at 4 °C overnight. Primary antibodies used were F95 (1/100; monoclonal mouse-IgM), for detection of pan-protein deimination, and cit-H3 (1/100; polyclonal rabbit IgG, ab5103) for the detection of deiminated histone H3. Following incubation the sections were washed for 2 min in serial washes of PB/BSA, PB, PB and PB/BSA, and thereafter incubated with the secondary antibodies (biotin-labelled anti-mouse IgM (1/200) and anti-rabbit IgG (1/200); Vector Laboratories, Inc., Burlingame, CA, USA). Visualisation was with Avidin-Biotinylated peroxidase Complex (ABC, Vector Laboratories, Inc.) and diaminobenzidine/hydrogen peroxide (DAB) stain. Sections were counterstained with Mayer's Haematoxylin (Sigma, U.K.), dehydrated in alcohol, immersed in xylene and mounted with DEPEX (Sigma). Three larvae for each developmental stage were analysed.

3. Results

3.1. Phylogenetic reconstruction of PAD sequences

Five well supported clades were formed within the Neighbour-joining phylogeny (Fig. 1), of which, clades 1–3 were formed by PAD2 representatives across several taxa. Vertebrate PAD2 sequences formed two closely related clades, including Clade 1 which contained the *Gadus morhua* and other teleost species, with the elasmobranch *Rhincodon typus* appearing basal to this clade. Clade 2 was formed only of tetrapod PAD2 representatives, comprising of amphibian, reptilian, avian and human sequences. Other human PAD sequences formed two unique paraphyletic clades, the first (clade 4) containing PAD4, PAD3 and PAD1 sequences and the second (clade 5) representing PAD6 which appeared to be the most derived. Clade 3 was formed of PAD2 sequences from invertebrate aquatic organisms and a cyanobacterium which appeared to be phylogenetically bracketed between the vertebrate PAD2 clades and clade 4 (Fig. 1).

3.2. Protein analysis of deiminated proteins in cod serum and mucosa

3.2.1. Western blotting of deiminated proteins and deiminated histone H3

Cod serum (two pools of 5 individual samples) and mucus (two pools of 10 individual samples) were analysed by Western blotting for total deiminated proteins (F95), deiminated histone H3 and PAD2 (anti-PAD2, ab50257). Bands in the size range of 100, 75, 37–50 and 20 kDa were revealed for total deiminated proteins (F95) in both mucus and serum, and an expected band of 17 kDa size was detected for deiminated histone H3. The presence of PAD2 was verified by detection of an expected band at 62 kDa (Fig. 2). Higher levels of deiminated protein product correlated with a fainter PAD2 band in serum, while in mucus PAD2 detection was stronger, indicative of less activated PAD2 and with correspondingly less, albeit clearly detectable, deiminated protein product than seen in serum.

3.2.2. Identification of deiminated proteins from cod mucosa by mass spectrophotometry

Immunoprecipitated proteins from cod mucosa, using the pan-deimination F95 antibody, were analysed by LC-MS/MS. Deiminated protein candidates included immunogenic, cytoskeletal, nuclear and metabolic proteins, and are listed in Table 1.

3.3. Histological analysis of deiminated proteins in cod ontogeny

Total deiminated proteins, as assessed by pan-deimination antibody F95, were detected in multiple organs as well as in mucosal tissues in cod larvae from age 28–70 d.p.h. (Table 2 and Fig. 3). Deiminated histone H3 showed high levels in mucosa and lower levels in other tissues at earlier larval stages (28 d.p.h.), and was then strongly detectable in multiple organs from 43 to 57 d.p.h. (Table 2 and Fig. 4). At 70 d.p.h. deiminated proteins were mainly detected in mucosal layers, brain, eye and liver. In fertilized eggs (11 d.p.f.) some positive staining was seen for both total deiminated proteins and deiminated histone H3 in eye, brain and muscle.

3.3.1. Detection of pan-deiminated proteins in cod organs and mucosa at 28–70 days post hatching

The presence of deiminated proteins (F95) was strong in the fin and in the skin at 28–70 d.p.h., where a clear positive was detected in the epidermis and loose connective tissue of the dermis. Deiminated proteins were particularly evident in sacciform cells in the ectoderm as well as in mucosal glands (Fig. 3A). The olfactory epithelium also showed positive for total deiminated proteins, particularly at 57 d.p.h. In the gills, there was a specific response in the mucosal layer, particularly in chloride cells (Fig. 3B), which are mitochondria-rich cells and principal site of trans-epithelial Ca^{2+} and Cl-influxes (Perry, 1997). Some positive chondrocytes were also detected in gills and maxilla (Fig. 3C), which were more strongly positive at 28 d.p.h. than at 43–70 d.p.h. Total deiminated proteins were detected in smooth muscle layer and submucosa of pharynx (Fig. 3D). Deiminated proteins were detected in liver at varying degrees from 28 to 70 d.p.h. (Fig. 3E), and prominent in the gall bladder at 57 d.p.h. (Fig. 3F). Deiminated proteins

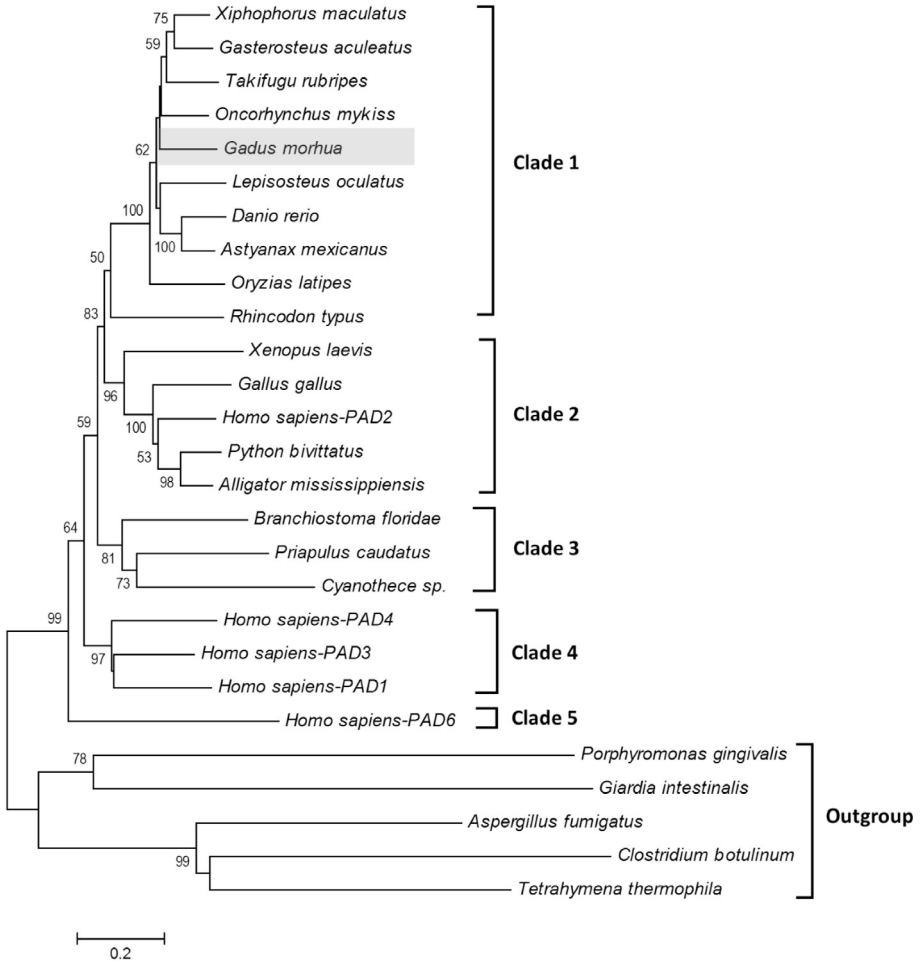

Fig. 1. Neighbour joining tree showing phylogenetic clustering of cod PAD. The evolutionary analysis was inferred using the Neighbour-Joining method under the conditions of the Poisson distance correction model in MEGA6 (Tamura et al., 2013). Bootstrap values > 50 based on 1000 replicates are shown as nodal support. Where Clade 1 contains fish sequences, Clade 2 contains tetrapod sequences, Clade 3 contains aquatic invertebrates and a cyanobacterium, Clade 4 contains human PAD1, PAD3 and PAD4 sequences, and Clade 5 contains Human PAD6.

were also found in kidney, strong in trunk kidney but lower in tubuli and glomeruli of head kidney (Fig. 3G). In the brain, main areas for total deiminated proteins were the medulla oblongata and the forebrain, particularly at 28 d.p.h. (Fig. 3H). The eye showed clear positive for deiminated proteins; at 28 d.p.h. nuclei of ganglion cells, internal plexiform layer and ellipsoids of cones all showed positive (Fig. 3I), while at 51–57 d.p.h. mainly the photoreceptor and plexiform layers showed positive. In spinal cord total deiminated proteins were strongest detected at 28 d.p.h. (Fig. 3J) and lower levels at 43–57 d.p.h. In muscle, high levels were seen in small peripheral and large muscle fibres at 28–43 d.p.h. (Fig. 3K), increased at 51 d.p.h. and then decreased again at 57 d.p.h. In the intestines, a strong response was seen in mucosal layers and most epithelial tissues, particularly at 43 d.p.h. (Fig. 3L). In pancreas, total deiminated proteins were detectable in Islet of Langerhans (endocrine tissue) at 57 d.p.h., but not in exocrine tissue. Spleen showed some positive, mainly at 57 d.p.h. The lining of the swimbladder showed positive at 28 d.p.h.

3.3.2. Detection of deiminated histone H3 in cod organs and mucosa at 28–70 days post hatching

Deiminated histone H3 was strongly detected at all stages tested 28–70 d.p.h. in the ectoderm and mucosal layer as well as in loose connective tissue under the ectoderm (Fig. 4A). A particularly strong response was detected in the sacciform cells in ectoderm (Fig. 4B), while at 57 d.p.h. a strong response was also seen in glandular structures in ectoderm (Fig. 4C), and in the neuroectoderm. The olfactory epithelium showed strong detection of histone H3 at all stages tested, and the epithelium on fin was also positive. In the gills, deiminated histone H3 detection was mainly confined to chondrocytes at 28 d.p.h., while at 43 d.p.h. the detection was very strong in lamellae and the mucosal layer in gills, as well as in chondrocytes (Fig. 4D). At 57 d.p.h. strong positive detection was confined to mucosal layer of the gills and in chloride cells, but reduced in chondrocytes. Chondrocytes in the head region varied with respect to deiminated histone H3 detection, low levels at 28 d.p.h., strong detection, especially in chondrocytes of gills at 43 d.p.h., and increased levels still in gill arch at 51 d.p.h.; while at 57 d.p.h. detection was lower with some positive in the maxilla. In the mouth and pharynx, deiminated histone H3 was positive at 28–57 d.p.h. and particularly strongly detected in the submucosa and underlying smooth muscle layer at 57 d.p.h. (Fig. 4E and F). Liver showed varying levels of deiminated histone H3; low levels at 28 d.p.h. but strong detection at 57 d.p.h. (Fig. 4G). In the pancreas, deiminated histone H3 was very strongly detected in the Island of Langerhans (endocrine tissue) at 57 d.p.h. (Fig. 4H) while exocrine tissue was not markedly stained. A strong positive was also seen in spleen at 57 d.p.h. (Fig. 4I). In the intestines, low levels of deiminated histone H3 were seen at 28 d.p.h., while strong positive was seen in intestinal mucosal layers at 43–57 d.p.h. (Fig. 4J). Trunk kidney showed strong positive, particularly at 57 d.p.h. while low levels were detected in tubuli of head kidney from 28 d.p.h. In brain, the levels of deiminated histone H3 varied in between the developmental stages, low detection at 28 d.p.h. which then increased and was very strong in forebrain, optic lobe,

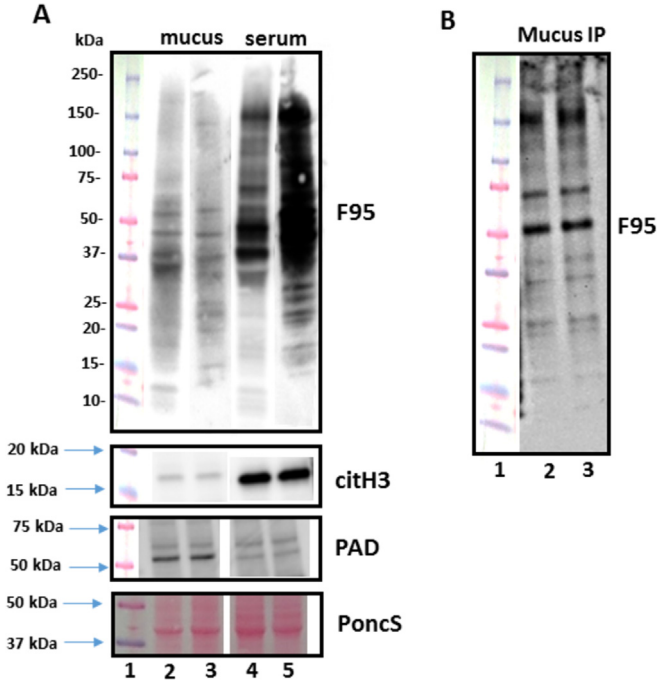

Fig. 2. Western blotting of deiminated proteins in cod mucosa and serum. **A)** Two pools of mucosa from 10 adult cod, and two pools of sera from 5 adult cod were analysed for total deiminated proteins (F95), deiminated histone H3 (CitH3) and PAD. Bands in the size range of 100, 75, 37–50 and 20 kDa were revealed for pan-deimination antibody (F95), and an expected band of 17 kDa size was detected for deiminated histone H3. PAD was detected at an expected band of 62 kDa. Ponceau S staining (PoncS) is shown for loading control. Lane 1 contains Precision Plus Protein Standard (161–0376, BioRad, U.K.), lanes 2–3 contain two mucosal pools (n = 10), lanes 4–5 contain two serum pools (n = 5). **B)** Immunoprecipitated deiminated proteins (F95) from cod mucosa (lanes 2–3). Bands are detected in the size range of 100, 75, 37–50 and 20 kDa and were identified by proteomic analysis (see Table 1). Lane 1 contains the protein standard.

pituitary gland, optic nerve, cerebellum and medulla oblongata at 57 d.p.h. (Fig. 4K). In eye, a similar increase in deiminated histone was observed from 28 to 57 d.p.h.; low levels at 28 d.p.h. but then strong positive in photoreceptors and neuronal bodies in layers of ganglion cells, in the external layer and nuclei of photoreceptors as well as in the lens at 57 d.p.h. (Fig. 4L). Spinal cord showed some positive for deiminated histone H3 at 28 d.p.h., very strong positive at 43 d.p.h. (Fig. 4M) and then lower levels at 57 d.p.h. Notochord showed positive for deiminated histone H3 in the peripheral layer and fibrous layer, particularly at 43 d.p.h. (Fig. 4M). In the lining of swim bladder, some positive was detected at 28 d.p.h. In heart, deiminated histone H3 was detected at low levels at 28 d.p.h. At 70 d.p.h. deiminated histone detection was very strong in epidermal mucosa, olfactory epidermis and mucosa of gills, but low in liver, heart, muscle and nervous tissue, except for high levels detected in the optical nerve.

3.3.3. Deiminated proteins in immunostimulated cod larvae

In larvae immunostimulated with LPS (*Asa*) a very strong response for deiminated histone H3 was specific to the gut and stomach mucosal tissue (Fig. 5A at 2 h post stimulation; B and C at 24 h post stimulation). Some positive was also found in parts of brain for deiminated histone H3 (24 h sample group), in spleen (2 h post stimulation), and low levels in kidney and heart. Total deiminated proteins (F95) were found particularly increased in gut mucosal tissue (Fig. 5D, 24 h sample group) as well as being increased in the liver (Fig. 5E, 24 h sample group), and some increased levels were detected in spleen (2 h post stimulation). In comparison, unstimulated controls showed very low presence of deiminated total proteins or histone H3 in mucosal layers of gut and levels were hardly detectable in liver and spleen (not shown). It had previously been established that in LPS stimulated cod larvae, LPS was detected in heart, kidney, stomach, intestines, liver, brain and spleen (Magnadottir et al., 2006 and Magnadottir et al., unpublished).

Table 1
Deiminated proteins identified in cod mucosa (*Gadus morhua* L.). Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody, analysed by LC-MS/MS and peak list files submitted to MASCOT.

Protein name	Symbol	Score ($p < 0.05$)[a]
Fast skeletal muscle alpha-actin	Q78AY8_GADMO	711
Serotransferrin	Q92079 TRFE_GADMO	631
Tubulin beta chain	Q9PUG4_GADMO	593
Tubulin beta-1 chain	TBB1_GADMO	586
Beta-actin	Q2PDJ0_GADMO	528
Elongation factor 1-alpha	A8CZC9_GADMO	524
Calpain small subunit 1	A0A067XL41_GADMO	479
Heat shock cognate 70 kDa protein	G8DZS1_GADMO	463
Profilin	A0A067XLH1_GADMO	415
Nucleoside diphosphate kinase	G8DZS2_GADMO	331
Betaine aldehyde dehydrogenase	BADH_GADMC	329
Cystatin B	G0XNX5_GADMO	280
S2 ribosomal protein	Q6WEU6_GADMO	261
Ribosomal protein	L15 Q8JHA8_GADMO	247
Peptidyl-prolyl cis-trans isomerase	G0XNX4_GADMO	245
Peptidylprolyl isomerase	G0XNX7_GADMO	238
Peroxiredoxin 6	A0A067XL90_GADMO	195
60S ribosomal protein	L22 RL22_GADMO	179
Alcohol dehydrogenase class-3 chain H	ADHH_GADMO	170
20-beta hydroxysteroid dehydrogenase	A8CZB9_GADMO	160
Galectin	G8ENP0_GADMO	155
Putative ribosomal protein	L8 D5LIQ8_GADMO	143
Preproapolipoprotein A-I	Q5XQS6_GADMO	123
Proliferating cell nuclear antigen	V9MA55_GADMO	103
Transglutaminase 2	V9I305_GADMO	98
Histone H3	A0A0G2QMS5_GADMO	90
Glyceraldehyde-3-phosphate dehydrogenase	Q8AWX8_GADMO	81
Fast skeletal muscle alpha-actinin	Q8JJ06_GADMO	80
Fast skeletal myosin heavy chain	Q8JIV4_GADMO	22
Creatine kinase muscle type A	A0A0E3TUY4_GADMO	78
Mannan-binding lectin	G0XNX6_GADMO	74
Beta2-microglobulin	Q9YGK6_GADMO	59
Creatine kinase	A7XA06_GADMO	55
Kininogen	KNG_GADMO	45
Putative ribosomal protein	L17b D5LIQ1_GADMO	42
Non-specific cytotoxic cell receptor protein-1	A4ZGE0_GADMO	32
Bloodthirsty	E3U9P6_GADMO	31
Interferon regulatory factor protein 7	A0A0D3RBU4_GADMO	29
Toll-like receptor 22d	K4FWD6_GADMO	22
Keratin Type II (Sheep)	K2M3_SHEEP	80
Keratin Type II (Human)	K2C1_HUMAN	2989
	K22E_HUMAN	2852
Keratin Type I (Human)	K1C10_HUMAN	2206
	K1C9_HUMAN	1989

[a] Ions score is $-10*\text{Log}(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 16 indicated identity or extensive homology ($p < 0.05$). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. Cut-off was set at Ions score 20.

4. Discussion

This study demonstrates for the first time the presence of deiminated proteins in various organs and mucosal tissues, in early teleost ontogeny. Given the range of novel functions that proteins can gain

Table 2
Deiminated proteins in organs and tissues during cod ontogeny. A schematic overview over immunohistochemical detection of total deiminated proteins (F95) and deiminated histone H3 (citH3) in various organs of cod larvae from 11 days post fertilisation (d.p.f.) to 70 days post hatching (d.p.h.).

	Pan-protein deimination (F95)	Deiminated histone H3 (citH3)
Skin	11 d.p.f. – 70 d.p.h.	11 d.p.f. – 70 d.p.h.
Olfactory epithelium	28–70 d.p.h.	28–70 d.p.h.
Fin	28–70 d.p.h.	28–70 d.p.h.
Gills	28–70 d.p.h.	28–70 d.p.h.
Pharynx, mouth	28–70 d.p.h.	28–70 d.p.h.
Intestines	28–70 d.p.h.	28–70 d.p.h.
Pancreas	57 d.p.h.	57 d.p.h.
Liver	28–70 d.p.h.	28–70 d.p.h.
Gall bladder	57 d.p.h.	57 d.p.h.
Kidney	28–57 d.p.h.	28–57 d.p.h.
Spleen	57 d.p.h.	57 d.p.h.
Heart	Negative	28 d.p.h.
Brain	11 d.p.f. – 70 d.p.h.	11 d.p.f. – 70 d.p.h.
Eye	11 d.p.f. – 70 d.p.h.	11 d.p.f. – 70 d.p.h.
Spinal cord	28–57 d.p.h.	28–57 d.p.h.
Notochord	28–43 d.p.h.	43 d.p.h.
Chondrocytes	28–57 d.p.h.	28–57 d.p.h.
Muscle	11 d.p.f. – 70 d.p.h.	11 d.p.f. – 70 d.p.h.

Fig. 3

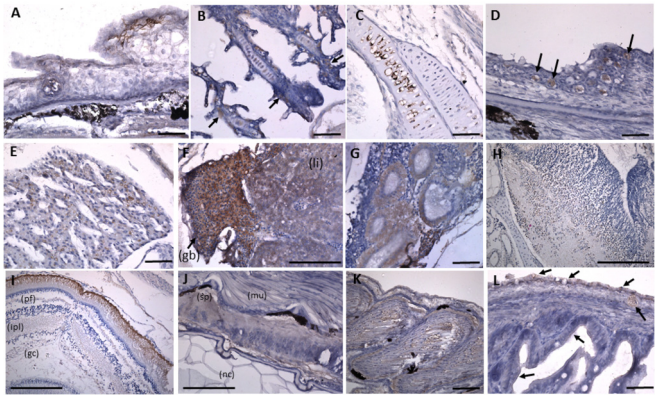

Fig. 3. Histological immunostaining showing total deiminated proteins (F95) in cod ontogeny. **A)** Ectoderm, sacciform cells in the ectoderm and mucosal cells; **B)** Gills, note positive chloride cells (arrows), (57 d.p.h.); **C)** Chondrocytes in maxilla (28 d.p.h.); **D)** Pharynx; mucosal cells in submucosa (57 d.p.h.); **E)** Hepatocytes of liver at 28 d.p.h.; **F)** Strong detection prominent in the gall bladder (gb) and lower in liver (li) at 57 d.p.h.; **G)** Tubuli of kidney (57 d.ph.h); **H)** Brain; strong positive in medulla oblongata (28 d.p.h).; **I)** Eye; positive in layer of ganglion cells (gc), internal plexiform layer (ipl), external plexiform layer (pf) (28 d.p.h.); **J)** Spinal cord (sp); positive neuronal cells (28 d.p.h.; nc = notochord; mu = muscle); **K)** Myofibrils are positive in myotome (51 d.p.h.); **L)** Intestines, strong response in epidermal mucosal layers and mucosa (arrows) (43 d.p.h.). Scale bars are 50 μM for A,B,C,D,E,G,K,L and 100 μM for F,H,I,J.

upon post-translational modifications, expanding the functional repertoire of the proteome, the effect of protein deimination in homeostasis and immune defences is of considerable interest. The strong presence of deiminated proteins in the mucosal surfaces of epidermis, gills and intestine correlates with previous studies detecting potent antibacterial activity at these sites in cod (Ruangsri et al., 2010). Proteins identified in cod mucosa included cytoskeletal, nuclear, metabolic and immune related proteins, as listed in Table 1, and are discussed below:

Histone H3 was here identified in deiminated form for the first time in cod mucus, while histones have been identified in mucus of various fish and histone H3 found in skin mucus of lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017). Besides affecting epigenetic

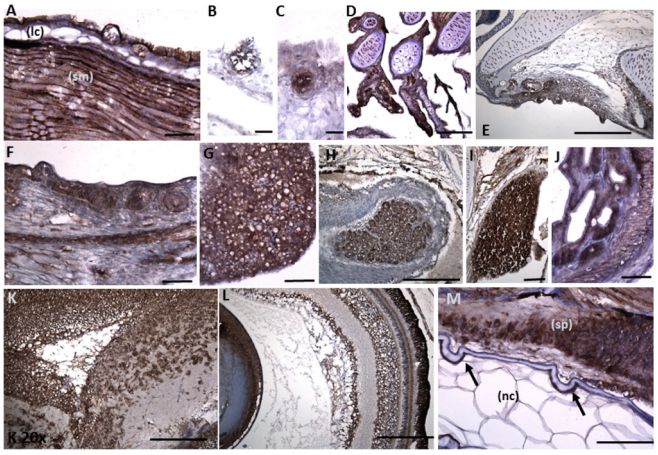

Fig. 4. Histological immunostaining showing deiminated histone H3 (citH3) in cod ontogeny. **A)** Positive detection in mucosal ectoderm, loose connective tissue (lc) under the ectoderm and small muscle cells (sm) (57 d.p.h.); **B)** Strong response was detected in the sacciform cells in ectoderm; **C)** Glandular structures in ectoderm; **D)** Strong positive mucosal layer in gills, chloride cells, chondrocytes and lamellae (43 d.p.h.); **E)** Mouth, mucosal layer; **F)** Pharynx, strong detection in the submucosa and underlying smooth muscle layer at 57 d.p.h.; **G)** Liver, positive hepatocytes at 57 d.p.h.; **H)** Pancreas, Island of Langerhans strongly positive (57 d.p.h.); **I)** Spleen (57 d.p.h.). **J)** Mucosal layers of intestines (43 d.ph.h); **K)** Brain: strong detection in medulla oblongata (57 d.p.h.); **L)** Eye, strong detection in photoreceptors and neuronal bodies in layers of ganglion cells, in the external layer and nuclei of photoreceptors and lens (57 d.p.h.); **M)** Spinal cord (sp) neurons show strong positive; and notochord (nc) shows positive staining in the peripheral layer and fibrous layer (arrows) (43 d.p.h.). Scale bars are 50 μM for A,B,C,D,F,G,I,J and 100 μM for E,H,K,L,M.

regulation, deiminated histone H3 is a marker for neutrophil extracellular trap formation (NETosis), an anti-pathogenic mechanism conserved between teleost and mammalian neutrophils. Deiminated histone H3 was here detected in cod larvae in all three main mucosal immune compartments described in teleost fish; the gut-associated lymphoid tissue (GALT), the skin-associated lymphoid tissue (SALT) and the gill-associated lymphoid tissue (GIALT) (Salinas et al., 2011). While NETosis has hitherto not been demonstrated in mucosal immunity in teleosts, it has previously been described in granulocytes of carp (*Cyprinus carpio*) (Pijanowski et al., 2013), kidney of zebrafish (*Danio rerio*) (Palić et al., 2007a) and neutrophils of fathead minnow (*Pimephales promelas*) (Palić et al., 2005). At high local concentration, NETs act as potent anti-microbial agents by binding and preventing spreading of microorganisms, including fungal hyphae, helminths and protozoans, which are too large for phagocytosis (Brinkmann et al., 2004; Urban et al., 2006; Papayannopoulos et al., 2009; Guimarães-Costa et al., 2009; Byrd et al., 2013; Branzk et al., 2014). The ability of viruses to induce NETosis has also been described (Schönrich and Raftery, 2016). In mammals, NETosis is associated with gut mucosal inflammation (Al-Ghoul et al., 2014) and antimicrobial defence in oral mucosa (Mohanty et al., 2015). PAD expression has been described in mammalian mucosal tissues including uterus, gastric and colon tissues (Akiyama et al., 1990; Xin and Song, 2016); where changes in deimination are associated with ulcerative colitis and cancer pathogenesis (Cantariño et al., 2016); and in bronchial and alveolar mucosa, where deiminated proteins are increased in response to harmful stimuli (Makrygiannakis et al., 2008).

Heatshock cognate 70 (Hsc70) is an ATP-dependent chaperone protein involved in cellular protein homeostasis, cell proliferation and promotes lysosomal degradation of cytosol proteins (Chiang et al., 1989; Nirdé et al., 2010). Hsc70 also participates in chaperone-mediated autophagy and microautophagy (Sato et al., 2016; Wang et al., 2017). In mandarin fish (*Siniperca chuatsi*), two different isoforms have

Fig. 5

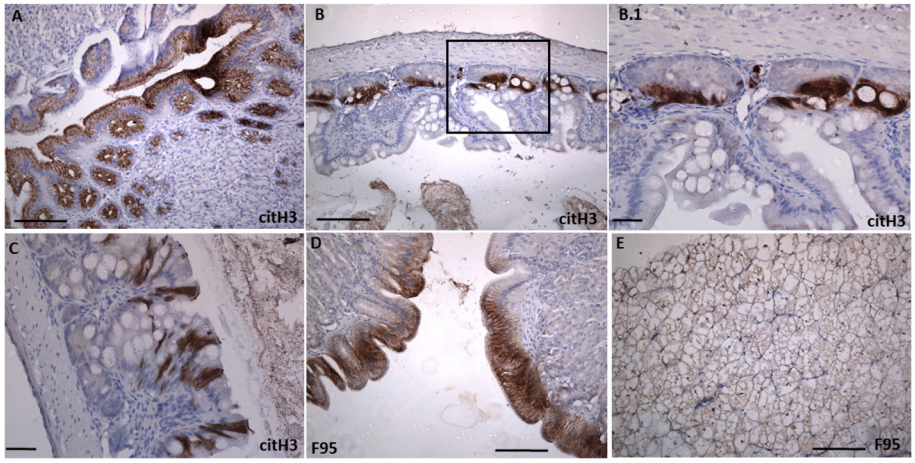

Fig. 5. Deiminated proteins are elevated in mucosal associated tissues and liver in LPS-immunostimulated cod larve. Larvae were injected (i.p.) with LPS and sacrificed either at 2 h or larvae were injected again (i.m.) at 23 h and sacrificed at 24 h and organs analysed by immunohistochemistry. **A)** Deiminated histone H3 is elevated in gut mucosal tissue 2 h post LPS i.p. injection; **B-C)** Deiminated histone H3 is increased in stomach and gut after 24 h LPS stimulation; Deiminated histone H3 in mucosal crypts of stomach (B; B.1 is boxed area in E, at 40× magnification); Deiminated histone H3 in columnar epithelium (C); **D-E)** Total deiminated proteins (F95) are increased in gut mucosal tissue (D) and in liver (E) of cod larvae 2 h after LPS i.p. injection; Scale bars are 100 μM for A,B,D,E and 50 μM for B.1 and C.

been described with tissue-specific distribution and differing functions during embryogenesis and in immune responses to bacterial infection, hypoxia and temperature challenge (Wang et al., 2015). The expression patterns of two Hsc70 genes in Chinese mitten crab (*Eriocheir sinensis*) differ during immune responses and larval development (Li et al., 2016), while it remains to be investigated whether these isozymes differ in levels of post-translational protein deimination.

Bloodthirsty is a tripartite motif (TRIM) protein, involved in cell proliferation and development. In cod, bloodthirsty has previously been shown to be involved in antiviral immune response (Furnes and Robertsen, 2010) and in antibacterial immune response in zebrafish (Zhang et al., 2015), where it has also been shown to be required for erythropoiesis (Yergeau et al., 2005).

Kininogen forms part of the acute phase response and has been previously described in cod and spotted wolffish (*Anarhichas minor*) (Ylönen et al., 2002). In mammals, elevated levels of kininogen are linked to sepsis (Hofman et al., 2018) and inflammatory and oxidative stress pathways in type I diabetes (Al Hariri et al., 2017).

Serotransferrin acts as an antimicrobial agent and is at the frontier in innate immune mechanisms in fish (Stafford and Belosevic, 2003; Mohd-Padil et al., 2013). Transferrin has been described in acute phase response of cod (Audunsdottir et al., 2012) and in cod mucus (Caipang et al., 2011; Easy et al., 2012). Serotransferrin is described in skin mucus of olive flounder (*Paralichthys olivaceus*) (Palaksha et al., 2008) and found to be upregulated in naturally infected channel catfish (*Ictalurus punctatus*) (Peatman et al., 2008) and vaccinated cod (Caipang et al., 2008).

Elongation factor 1-alpha has roles in cytoskeleton organisation and nuclear export of proteins (Khacho et al., 2008). It is involved in the regulation of cell growth, apoptosis and the immune response and has also been linked to degranulation of neutrophils (Talapatra et al., 2002; Hamrita et al., 2011; Vera et al., 2014).

Proliferating cell nuclear antigen (PCNA) controls DNA damage tolerance pathways (Kanao et al., 2017) and is related to both malignant and non-malignant skin diseases (Kawahira, 1999).

Non-specific cytotoxic cell receptor protein-1 has important roles in target cell recognition in various fish, including cod, and is found at high levels in lymphoid organs (Seppola et al., 2007). It has roles in calcium signalling and displays dual roles, both as an antigen recognition molecule for targeted cell lysis and as an initiator of cytokine release from non-specific cytotoxic cells (Jaso-Friedmann et al., 2001). Whether these multiple functions are in part facilitated by post-translational deimination remains to be further investigated. Non-specific cytotoxic cells are the teleost evolutionary precursor to natural killer cells, involved in acute stress response, and in mice, a non-specific cytotoxic cell receptor protein has been identified in gastric mucosa as a lectin-type protein (Kallio et al., 2011).

Toll-like receptor 22 has previously been described in the cod genome (Star et al., 2011). It is a pattern recognition receptor and a key immune response gene in teleost fish (Zhang et al., 2014). In heat-shock experiments in cod it is downregulated, suggesting that it forms part of heat-induced immunosuppression (Hori et al., 2010).

Calpain small subunit 1 belongs to a well-conserved family of calcium-dependent cysteine proteases. Calpain has been found in various fish tissues (Salem et al., 2004) and is downregulated in cod skin mucus upon natural *vibrio* infection due to elevated inflammatory cytokines (Rajan et al., 2013a). In higher vertebrates mucosal specific calpains participate in mucosal immune defences (Sorimachi et al., 2011). It may be possible that the deimination of calpain, detected here, regulates the activation of this protease, possibly balancing its functions as an antimicrobial while also protecting the skin from irreversible tissue damage in an inflammatory environment (Matsushita et al., 2005).

Nucleoside diphosphate kinase (NDPK) has previously been detected, while not in deiminated form, in cod mucosa upon natural infection with *Vibrio anguillarum* (Rajan et al., 2013a). Nucleoside diphosphate kinase proteins are multifunctional proteins conserved from bacteria to humans (Desvignes et al., 2013), with roles in regulation of cytoskeletal dynamics, cell growth, differentiation, migration and apoptosis (Snider et al., 2015). In teleosts, nine NDPK genes have been identified that display species-, organ- and tissue-specific differences and are expressed during embryonic development in Atlantic salmon (*Salmo salar*), Atlantic halibut (*Hippoglossus hippoglossus* L), wild seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), Persian sturgeon (*Acipenser persicus*), and zebrafish (*Danio rerio*) (Desvignes et al., 2013). NDPKs are also a marker of tumour metastasis and implicated in eye and cardiac pathologies (Hippe et al., 2009; Patil et al., 2011; Shu et al., 2011).

Preproapolipoprotein A-I, a high density lipoprotein has, in our previous studies, been detected in various organs in cod ontogeny, as well in mucosal layers, in close association with complement component C3 – a unique feature for Atlantic cod (Magnadottir and Lange, 2004; Lange et al., 2005). Preproapolipoprotein A-I is elevated in cod mucosa upon natural *vibrio* infection (Rajan et al., 2013a), has anti-bacterial effects in carp (*Cyprinus carpio*) (Concha et al., 2004), is upregulated in liver of *V. anguillarum* infected seabass (*Dicentrarchus labrax*) (Sarropoulou et al., 2009) and is also reported in skin mucus of lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017).

Mannan-binding lectin (MBL) is evolutionary conserved in bony fish (Dodds, 2002; Nakao et al., 2006; Kania et al., 2010) and has been described in cod mucus (Rajan et al., 2011). MBL has many biological roles and forms part of the innate immune mechanism, including through activation of the complement pathway (Kojima et al., 2003)

and by promoting opsonophagocytosis (Neth et al., 2000). MBL is linked to a range of pathologies, including cancer, and is also involved in the clearance of senescent cells (Scorza et al., 2015).

Galectins have a wide range of function in embryogenesis and innate immunity. The three major galectin types are present in teleost fish (Vasta et al., 2004; Zhou et al., 2016); multiple galectins are strongly expressed in mucosal tissues of skin, gill and intestines in channel catfish (*Ictalurus punctatus*) (Zhou et al., 2016) and two forms have been described in cod mucus (Rajan et al., 2013b). Galectins have been shown to reduce viral adhesion in zebrafish (*Danio rerio*) (Nita-Lazar et al., 2016) and to participate in phagocytosis of a wide range of bacteria in half-smooth tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2013). Galectins are involved in apoptosis and many pathological processes, including acute and chronic inflammatory diseases and autoimmunity (Sciacchitano et al., 2018). They are also important in tumour biology (Chou et al., 2018) and in the regulation of wound healing and fibrosis (McLeod et al., 2018).

Cystatin B is an endogenous cysteine protease inhibitor for papain and cathepsins, is localized in the cytosol, mitochondria and nucleus; it is linked to viral infections (Rivera et al., 2014) and innate immune responses, including in the brain (Kopitar-Jerala, 2015). Cystatin B has been previously identified in cod mucosa (Rajan et al., 2011) and in skin mucus of lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017), while deiminated forms were not reported. Cystatin B has been described in other fish including olive flounder (*Paralichthys olivaceus*) (Ahn et al., 2013) and rock bream (*Oplegnathus fasciatus*) (Premachandra et al., 2012). It enhances macrophage-mediated bacterial killing in head kidney of turbot (*Scophthalmus maximus*) (Xiao et al., 2010) and was downregulated in gill of Atlantic salmon (*Salmo salar*) affected with amoebic gill disease (Wynne et al., 2008). Cystatin B has also been identified as a hypoxia-induced gene in blastulae embryonic cells of goldfish (*Carassius auratus*), suggesting roles in hypoxia tolerance in fish (Zhong et al., 2009). Recently, various post-translational modifications of cystatin were studied, while deiminated cystatin B forms were not reported (Manconi et al., 2017). Epigenetic regulation of cystatin B has been implicated in various cancers, including brain, breast, lung and pancreatic cancer (Rivenbark and Coleman, 2009).

Tubulin beta-chain, tubulin beta-1 chain and *beta actin* participate in cytoskeletal rearrangement and their deimination has for example been linked to extracellular vesicle release, which participates in cell communication and immune defences (Kholia et al., 2015). Extracellular vesicles have been recently described in teleost fish; for example in rainbow trout, heat shock protein is released from target tissues via exosomes in response to stress (Faught et al., 2017). Cytoskeletal rearrangement is necessary also for successful phagocytosis, implying an important role for PAD-mediated protein deimination to facilitate phagocytic processes in mucosal cells. In a study on *vibrio* infected cod, tubulin-2 was shown to be increased in mucus after infection (Rajan et al., 2013a), while in a proteomic study of cod larvae three isoforms of beta-2 tubulin were identified and changes in the isoforms due to post-translational modifications were suggested (Sveinsdottir et al., 2008), albeit not mentioning deimination.

Profilin is a highly conserved protein with multiple functions in cytoskeletal actin dynamics. It is present in nearly all tissue and cell types, including lymphoid cells and glia (Alkam et al., 2017). Profilin-2 has been described in skin mucus of cod upon natural infection with *V. anguillarum* (Rajan et al., 2013a), and in sea urchin (*Echinoidea*) profilin has been shown to increase in response to systemic challenge or injury (Smith and Davidson, 1994). In mammals, profilin is linked to changes in vascular permeability and to various diseases, including cancer (Alkam et al., 2017).

Peroxiredoxin 6 belongs to the family of peroxiredoxins, a family of antioxidant enzymes that participate in the control of cellular redox potential and protect cells from oxidative damage (Fisher, 2017). They are modulators of inflammation, involved in tissue repair, and protect against cell death and tumour progression and in fish, show protective roles against various pathogenic infections (Valero et al., 2015a).

Interferon regulatory factor protein 7 regulates interferon genes and plays a critical role in the innate immune response against DNA and RNA viruses (Marsili et al., 2016). Various interferon regulatory factors have been identified in body scales of teleost fish (Qi et al., 2018) and also contribute to teleost gonad immunity (Valero et al., 2015b). Interferon regulatory factor protein also regulates adaptive immune responses and anti-tumour properties of primary macrophages (Ikushima et al., 2013). In human mucosal tissues, interferon regulatory factors have been shown in influenza-infected airway epithelia (Crotta et al., 2013) and to be protective against HIV in cervical tissues (Rollenhagen et al., 2015).

Ribosomal proteins S2, L15 and *putative L8, L17*b, were here identified as deiminated in cod mucosa. Ribosomal proteins have various roles in protein synthesis, have antimicrobial function in mucosal tissues (Nuding et al., 2013) and are implicated in modulation of cytokine production in mucosal inflammation and epithelial pathogenesis (Moon 2011, 2014).

Transglutaminase 2 is a multifunctional enzyme with transamidation, GTPase, protein disulfide isomerase and protein kinase activities (Min and Chung, 2018). In cod, transglutaminase expression has previously been described in reproductive and immunological organs and shown to be upregulated in head kidney in response to immunochallenge (Furnes and Robertsen, 2010). Transglutaminase has also been described in chum salmon (*Oncorhynchus keta*), Japanese flounder (*Paralichthys olivaceus*), Nile tilapia (*Oreochromis niloticus*), red sea bream (*Pagrus major*) and Alaska Pollock (*Gadus chalcogrammus*) (Furnes and Robertsen, 2010). In human intestinal mucosal tissue, transglutaminase 2 contributes to inflammatory responses in celiac disease (Cukrowska et al., 2017) and is a regulator of stem-ness and metastasis in colorectal cancer stem cells (Kang et al., 2018). Transglutaminase 2 also promotes invasion and migration of lung cancer cells (Lee et al., 2018) and regulates neural growth factor in neuroblastoma (Algarni et al., 2018) as well as contributing to amyloid aggregate generation in various neurodegenerative pathologies (Min and Chung, 2018). Transglutaminase 2 is related to liver fibrosis induced after *Schistosoma japonicum* infection (Wen et al., 2017) and stimulates re-differentiation of dedifferentiated chondrocytes (Ko et al., 2017).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway, conserved through evolution, and has roles in membrane fusion, DNA repair and nuclear RNA export (Baibai et al., 2010). GAPDH has been identified in epidermal mucus of discus fish (*Symphysodon* spp.) (Chong et al., 2005) and in skin mucus of lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017). In tilapia (*Oreochromis* spp.), GAPDH is upregulated in oral mucosa during mouthbrooding, when offspring is incubated in the parental mouth and parental oral mucus secretions protect offspring from pathogens and counteract hypoxia defences (Iq and Shu-Chien, 2011). In human, GAPDH is detected in olfactory, nasal, and lung mucus (Casado et al., 2005; Candiano et al., 2007; Débat et al., 2007), as well as in cervical fluid (Dasari et al., 2007). It also has roles in neuronal apoptosis (Saunders et al., 1999) and is implicated in neurodegenerative diseases (Mazzola et al., 2001) and cancer (Rondinelli et al., 1997).

Peptidylprolyl isomerase and *Peptidyl-prolyl cis-trans isomerase (PPIases)* have previously been identified in a proteome reference map of the skin mucus of cod (Rajan et al., 2011), albeit not in deiminated forms. PPIases are a chaperone superfamily forming part of the cellular protein folding homeostasis machinery (Vivoli et al., 2017). PPIases partake in immune responses and were for example shown to mediate chemotactic activity and leukocyte migration in skin mucus of yellow catfish (*Pelteobagrus fulvidraco*) (Dawar et al., 2016) and to partake in antimicrobial activity in skin mucus of Atlantic salmon (*Salmo salar*) (Lüders et al., 2005). In human, PPIases act as a regulatory switch during activation, folding and degradation of various proteins associated to cardiovascular disease, cancer and neurodegeneration (Dunyak and Gestwicki, 2016).

Alcohol dehydrogenase class-3 chain and *Betaine aldehyde dehydrogenase (ADHs)* belong to a complex metabolic enzyme family in vertebrates, are responsible for aldehyde detoxification in several organisms and cutaneous forms have been described in human skin (Cheung et al., 1999). Alcohol dehydrogenase function has been detected in liver, intestine, kidney and brain in a variety of fish (Nagai et al., 1997). Betaine has been described in cod liver (Hjelmqvist et al., 2003) and zebrafish (*Danio rerio*) liver in early development (Dasmahapatra et al., 2001), as well as in Japanese puffer fish (*Fugu rubripes*) (Aparicio et al., 2002). Betaine has protective function against hyperosmotic stress including in brain (Knight et al., 2017), liver, kidney (Kempson et al., 2013) and eye (Garrett et al., 2013).

20-beta hydroxysteroid dehydrogenase has previously been described in cod during maturation (Mittelholzer et al., 2007). In other teleost, including in rainbow trout (*Oncorhynchus mykiss*), air-breathing catfish (*Clarias gariepinus*) and zebrafish (*Danio rerio*), its involvement in final oocyte maturation and in steroid hormone metabolism has been described (Sreenivasulu et al., 2012), which is critical for inflammatory and antiviral responses (Niklasson et al., 2014) as well as in regulation of the mitochondria rich gill chloride cells (Shrimpton et al., 1999; Wong et al., 2001). In zebrafish, it is localized in the endoplasmic reticulum, expressed during embryonic development, and shows a ubiquitous expression pattern in adult fish (Tokarz et al., 2012), as well as being an important stress-response enzyme (Tokarz et al., 2013). In human, hydroxysteroid dehydrogenases are found at high levels in liver, lungs, adipose tissues, ovaries, and the central nervous system; and in the skin they are involved in cell proliferation, wound healing, inflammation, and ageing (Terao and Katayama, 2016).

Creatine kinase plays conserved and central roles in energy metabolism and has, in common carp (*Cyprinus carpio*), been shown to be adaptive to synchronized changes in intracellular pH and body temperature (Wu et al., 2008). Creatine kinase is regulated by hypoxic signalling and is implicated in human ischemic and inflammatory pathologies (Kitzenberg et al., 2016). It is also involved in the homeostasis of the intestinal tract mucosal barrier after injury (Turer et al., 2017).

Fast skeletal muscle alpha-actin and *myosin heavy chain* were identified here as deiminated in cod mucosa. Putative differences in post-translational modifications were previously suggested for four isoforms of fast skeletal muscle alpha-actin identified by proteomic analysis in early cod larval development, albeit protein deimination was not discussed (Sveinsdottir et al., 2008). Protein deimination of skeletal actin and of myosin has been described in mammalian muscle and heart (Terakawa et al., 1991; Fert-Bober, 2015).

Keratin has roles both as a cytoskeletal protein involved as first barrier to injury and anti-bacterial properties in skin mucus due to pore-forming abilities (Molle et al., 2011). In *vibrio* infected cod, down-regulation of Keratin II was reported in mucus following infection (Rajan et al., 2013a), and two forms of keratin have been identified in early development of cod larvae by 2D proteomic analysis where post-translational differences, albeit not deimination, were suggested (Sveinsdottir et al., 2008). In mammals, keratin deimination participates in skin physiology and cutaneous diseases (Chavanas et al., 2006; Ying et al., 2009).

In this study, an increase in deiminated proteins was strongly detected in mucosal surfaces of LPS-immunostimulated cod larvae, while elevated levels were also observed in liver, some in spleen and lower elevation in the brain. In mouse models, tolerance to LPS has been shown to enhance NET formation and result in more efficient bacterial clearance (Landoni et al., 2012). In a study on channel catfish challenged with *Aeromonas hydrophilia*, arginine deiminase 2 was one of the main 19 genes found to be significantly induced both after first infection and re-infection (Mu et al., 2013). PADI have been described in various bacteria, parasites and fungi, as well as in fish bacterial pathogens. While there is an increased interest in antimicrobial peptides from self and commensals for putative use in immunostimulation (Gomez et al., 2013), it remains to be elucidated to what extent the microbiota in cod mucosal surfaces may contribute to production of deiminated proteins. Arginine deiminases have been identified in *Vibrio anguillarum* (YP_004567339.1) and *Vibrio splendidus* (YP_002418462.1; YP_002418279.1), which are bacterial populations that have been particularly analysed in developing cod larvae as they are the main pathogens in larval rearing (Reid et al., 2009). Arginine deiminase has also been identified in *Aeromonas salmonicida* (YP_001140162.1), which causes furunculosis in a wide range of fish, posing a major problem in aquaculture (Bartkova et al., 2017), as well as in *Photobacterium damselae* (VDA_002926), which affects a variety of cultured fish including cod (Magnadottir, 2010). As in human oral mucosa, *Porphyromonas gingivalis* PAD is linked to generation of deiminated antigens (Stobernack et al., 2016), it is tempting to speculate whether in cod ontogeny the deiminating activity of both commensals and pathogens may affect surrounding tissue remodelling, including of the gastrointestinal tract, for example through generation of neo-epitopes and resulting damage associated molecular patterns (DAMPs). Indeed, there are speculations on the differences of microbiota in cultured versus wild fish (Gomez et al., 2013; Ringø et al., 2014). Furthering understanding of the bacterial flora and its activity in the digestive tract for improvement in survival in larval fish in hatcheries (Austin and Austin, 1999; Reid et al., 2009), where gut epithelial homeostasis is critical for survival and can for example be affected by crowding stress and hypoxia (Parra et al., 2015), is thus of great importance. Immunostimulatory approaches for increased larval survival using various immunostimulants of viral, bacterial, plant or parasitic origin for activation of the immune system through pattern recognition proteins/receptors, have indeed been employed to enhance resistance to diseases (Magnadottir et al., 2006; Magnadottir, 2010; Parra et al., 2015). The conservation of arginine deiminases throughout phylogeny, including their ability to deiminate proteins, puts them thus in an interesting position for prophylactic measures in aquaculture, where the diversity of the immune system of teleosts, and their variation in disease susceptibility, poses a continuing challenge (Magnadottir, 2010). Previous studies have reported isolation of histone H2B from epidermal mucus of Atlantic cod (Bergsson et al., 2005), and an anti-microbial histone H2A like protein from skin secretions of rainbow trout (*Oncorhynchus mykiss*) (Fernandes et al., 2002). A histone-like protein isolated from cod milt and fed to cod fry enhanced their resistance to *V. anguillarum* (Pedersen et al., 2004) and also stimulated leucocytes from Atlantic salmon (*Salmo salar*) (Pedersen et al., 2003). Short histone H1 and H6 derived oncorhyncins from rainbow trout are also suggested as potent anti-microbial agents (Fernandes et al., 2002, 2004b). These findings indicated that shorter histone fragments could stimulate non-specific immune responses and increase survivability in fry. The strong presence of deiminated histone H3 in cod larvae mucosa thus suggests that these may pose as candidate compounds for prophylactic measures in aquaculture.

Besides strong detection in mucosal surfaces, deiminated proteins and deiminated histone H3 were detected in most organs throughout early cod larval development, indicating roles in tissue remodelling. Previous zebrafish studies showed important developmental roles for PADI in angiogenesis (Khajavi et al., 2017), while histone deimination was shown to be crucial for gene regulation in early mouse embryo development (Kan et al., 2012; Zhang et al., 2016). In cod larvae, deiminated proteins were particularly prominent in all developmental stages tested in the brain and eye, while in muscle, in spleen, liver and pancreas the levels of deiminated proteins varied (Table 2). In comparison, a previous study on PADI expression in adult rainbow trout, using qPCR analysis, showed that PADI was most abundant in fin and skin, while moderate expression was detected in brain, gastrointestinal tract, gill and spleen, and low expression was detected in kidney, heart, liver and muscle, albeit the presence of deiminated proteins was not assessed (Rebl et al., 2010). In mammals, PAD2-mediated deimination is reported in brain, spinal cord, spleen, skeletal muscle and leukocytes,

while PAD4-mediated deimination is also seen in liver, lung, kidney and testis (van Beers et al., 2013). The strong detection of deiminated proteins and histone H3 in the islet of Langerhans in cod pancreas, which forms a considerable part of the digestive organs in developing larvae (Kamisaka and Ronnestad, 2011), as well as kidney and liver, may be of interest compared to mammalian studies, where protein deimination is described in pancreatic islets in relation to beta-cell stress and auto-antigen generation in diabetes (Crèvecoeur et al., 2017) and linked to immune defences and host tissue damage in liver and kidney (Kolaczkowska et al., 2015; Cedervall et al., 2017). In the head region, high levels of deiminated proteins and deiminated histone H3 were observed throughout cod ontogeny; high in the brain and eye, and at varying degrees in chondrocytes - indicative of putative roles in skull modelling. In zebrafish, PADI has been shown to be critical in head, intersegmental vessel, brain and eye development (Khajavi et al., 2017). Roles for PAD-mediated deimination of myelin basic protein are well described in the development of the central nervous system (Moscarello et al., 1994), in the regulation of CNS regeneration (Lange et al., 2011, 2014; Lange, 2016) and in neurodegeneration (Harauz and Musse, 2007; Musse et al., 2008). In the eye, deiminated proteins proceed wound healing mechanisms (Wizeman and Mohan, 2017), while differential expression of PAD isozymes and associated changes in deiminated proteins are linked to degenerative eye diseases (Bonilha et al., 2013; Wizeman et al., 2016). In mammalian eye, various deiminated target proteins, including histones, have been identified and age related reduction in protein deimination levels has been shown in the ganglion cell layer, inner plexiform layer and inner nuclear layer (Bhattacharya et al., 2008; Bhattacharya, 2009). The high levels of deiminated proteins observed here at similar sites in cod larvae eyes, point thus to important roles in early eye tissue remodelling, where the specific factors regulating timing of teleost retinal differentiation are yet not entirely understood (Ferreiro-Galve et al., 2010). Deimination may well contribute to retinal neurogenesis characteristic for the continuous retinal growth observed in fish throughout life (Raymond et al., 2006), for example through promoting phagocytosis of cell debris by Müller glia (Bejarano-Escobar et al., 2017) via exposure of deiminated neo-epitopes. Interestingly, in zebrafish, galectins, which were one of the identified deiminated proteins identified in cod mucosa in this study, were shown to be expressed by Müller glia and regulate the regeneration of rod photoreceptors (Craig et al., 2010).

As tissue undergoes constant remodelling during development, the spatial and temporal detection of deiminated proteins in various organs throughout cod ontogeny is of considerable interest. What the exact roles of deiminated proteins are, and how they facilitate protein moonlighting in tissue remodelling and homeostasis, remains to be further investigated. The observed immune-detection of deiminated proteins may for example follow similar patterns as neutrophil migration in zebrafish development, where roles are suggested in patrolling aseptic local tissue damage or developmental abnormalities (Le Guyader et al., 2008). The roles of PAD-mediated protein deimination may thus be multifactorial during ontogeny; both contributing to innate immune defences and tissue remodelling in homeostasis.

5. Conclusion

For the first time the presence of deiminated proteins is shown in a continuous series of samples from the early cod development from 11 d.p.f. to 70 d.p.h. These suggest important roles for PAD-mediated protein deimination in tissue remodelling and immune defences. During cod larval development, there seems to be a shift towards dominating functions in mucosal layers, likely forming part of the innate immune defences, as supported by a marked increase protein deimination upon LPS immunostimulation. Deiminated protein levels also remained relatively high in brain and eye, which in teleost undergo continuous neurogenesis throughout life. Deiminated protein targets identified in mucus included various critical components involved in acute immune responses, cytoskeletal organisation, metabolism, cell differentiation and regulation of apoptosis. The findings presented here are of relevance both for prophylactic measures in aquaculture, as well as for furthering understanding of conserved roles for protein deimination throughout phylogeny, particularly in the mammalian CNS and mucosal type I layers of the uterus, gut and the respiratory tract.

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