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Peptidylarginine deiminase and deiminated proteins are detected throughout early halibut ontogeny - Complement components C3 and C4 are post-translationally deiminated in halibut (*Hippoglossus hippoglossus* L.)

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ABSTRACT

Post-translational protein deimination is mediated by peptidylarginine deiminases (PADs), which are calcium dependent enzymes conserved throughout phylogeny with physiological and pathophysiological roles. Protein deimination occurs via the conversion of protein arginine into citrulline, leading to structural and functional changes in target proteins. In a continuous series of early halibut development from 37 to 1050° d, PAD, total deiminated proteins and deiminated histone H3 showed variation in temporal and spatial detection in various organs including yolksac, muscle, skin, liver, brain, eye, spinal cord, chondrocytes, heart, intestines, kidney and pancreas throughout early ontogeny. For the first time in any species, deimination of complement components C3 and C4 is shown in halibut serum, indicating a novel mechanism of complement regulation in immune responses and homeostasis. Proteomic analysis of deiminated target proteins in halibut serum further identified complement components C5, C7, C8 C9 and C1 inhibitor, as well as various other immunogenic, metabolic, cytoskeletal and nuclear proteins. Post-translational deimination may facilitate protein moonlighting, an evolutionary conserved phenomenon, allowing one polypeptide chain to carry out various functions to meet functional requirements for diverse roles in immune defences and tissue remodelling.

1. Introduction

Peptidylarginine deiminases (PADs) are conserved throughout phylogeny and are a calcium-dependent enzyme family involved in physiological and pathophysiological processes (Vossenaar, 2003; György et al., 2006; Wang and Wang, 2013; Witalison et al., 2015). PADs cause irreversible post-translational protein deimination of protein arginine to citrulline, using oxygen from water and releasing nitrogen as ammonia (Fig. 1). Each conversion of an arginine into a citrulline causes the loss of one positive charge and increase of 1 Da, leading to structural and functional changes in target proteins (Vossenaar et al., 2003; György et al., 2006; Bicker and Thompson, 2013). Post-translational deimination can thus alter protein-protein interactions, modify protein structure and affect hydrogen bond formation, as well as in some cases cause denaturation (Tarsca et al., 1996; Witalison et al., 2015). Protein structures identified as being most prone to deimination are intrinsically disordered proteins and β-sheets, while the position of the arginine within the protein is also of importance (Nomura, 1992; Tarsca et al., 1996; Györgi et al., 2006). In mammals, five tissue-specific PAD isozymes have been identified, while only one PAD form is present in fish (Vossenaar et al., 2003; Rebl et al., 2010; Magnadottir et al., 2018a). While implications for PADs and post-

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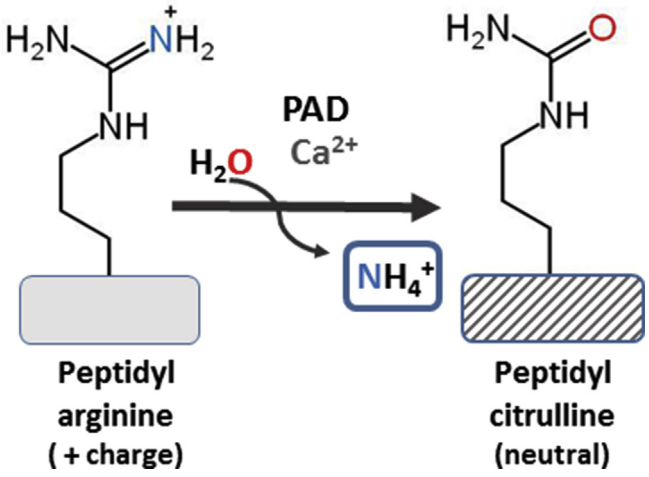

Fig. 1. Molecular scheme of post-translational protein deimination. Peptidylarginine deiminase (PAD) is activated via Ca^{2+} and catalyses the conversion of protein arginine into citrulline, using oxygen from water. Nitrogen is released as a by-product in ammonia (NH_4^+). Each conversion of a peptidyl arginine into a peptidyl citrulline results in the loss of a positive charge and an increased molecular mass of 1 Da.

translational deimination has been widely studied in the past few years in relation to various pathologies including cancer, autoimmune and neurodegenerative diseases (Vossenaar et al., 2003; Györgi et al., 2006; Wang and Wang, 2013; Witalison et al., 2015; Kholia et al., 2015; Lange et al., 2017), their physiological roles have received less attention. Roles for PAD-mediated deimination of myelin basic protein during development of the central nervous system have been described (Moscarello et al., 1994), and in zebrafish (*Danio rerio* F. Hamilton, 1822), PAD has been found to be critical for head, intersegmental vessel, brain and eye development (Khajavi et al., 2017). Our recent study on deiminated proteins in cod (*Gadus morhua* L.) ontogeny revealed putative novel roles in tissue remodelling and mucosal immune responses, showing that deiminated proteins and deiminated histone H3, also a marker of neutrophil extracellular trap formation, are expressed in various organs, including in the CNS and eye, throughout early larval ontogeny and that deiminated proteins are increased in mucosal tissues upon immunostimulation (Magnadottir et al., 2018a). While deiminated protein epitopes may contribute to autoimmune responses, via exposure of neo-epitopes and activation of the complement cascade, as well as to loss of protein function in various pathologies, such post-translational protein changes may also facilitate protein moonlighting, an evolutionary acquired phenomenon where proteins are allowed to exhibit more than one physiologically relevant function within one polypeptide chain (Henderson and Martin, 2014; Jeffrey, 2018). PAD-mediated deimination of histones has been shown to affect gene regulation and having roles in embryonic development (Kan et al., 2012; Zhang et al., 2016a), while also contributing to neutrophil extracellular trap formation (NETosis). NETosis is an extra-cellular anti-pathogenic mechanism which forms part of innate immunity and is conserved throughout phylogeny from fish to human (Brinkmann et al., 2004; Palic et al., 2007a; Li et al., 2010; Byrd et al., 2013; Yang et al., 2016). In mammals, NETosis has been shown to be driven by PAD4, which is enriched in neutrophil nuclei (Nakashima et al., 2002; Neeli et al., 2008; Wang et al., 2009). In teleosts, neutrophils play roles both in host defence as well as in the maintenance of homeostasis (Neumann et al., 2001; Havixbeck and Barreda, 2015), and the formation of NETs may, besides anti-pathogenic trapping, also be associated with clearance of apoptotic cells during tissue remodelling (Magnadottir et al., 2018a).

The complement system forms part of the first lines of immune defence against invading pathogens and in clearance of potentially damaging debris and necrotic or apoptotic cells (Dodds and Law, 1998; Fishelson et al., 2001; Reid et al., 2002; Hart et al., 2004; Carrol and Sim, 2011; Morgan et al., 2016). It is also implicated in diverse biological processes including regeneration (Del-Rio-Tsonis et al., 1998; Haynes et al., 2013) and tissue remodelling during development (Lange et al., 2004a, 2005, 2006, 2004b). The complement pathway can be activated via the classical, alternative or lectin pathways (Müller-Eberhard, 1988; Dodds and Law, 1998; Sunyer and Lambris, 1998), with all three pathways converging to forming the C3 convertase and the downstream lytic pathway and formation of the C5b-C9 membrane attack complex (MAC), leading to killing of the microorganism (Volanakis, 2002; Dodds, 2002). While the classical pathway is activated either via direct binding of C1 to acute phase proteins, such as CRP or proteins of bacterial and viral origin, the C1q subcomponent can also bind to the Fc region of immunoglobulins that are bound to antigen (Reid et al., 2002; Reid, 2018). Interestingly, an essential role for arginine in C1q has been suggested for C1q-IgG interaction (Kojouharova et al., 2004). As C1q also serves as a potent pattern recognition molecule which recognises self, non-self and altered self-signals (Nayak et al., 2012; Reid, 2018), it may also bind to deiminated neo-epitopes. Downstream of C1 activation, C4 is cleaved and participates in formation of the C3 convertase of the classical pathway. C3 plays a pivotal role in all pathways of complement activation and can, via the alternative pathway, be directly activated without a recognition molecule by subtle interactions of a range of proteases, inhibitors, activators and cofactors which are modulated differentially by self and non-self surfaces (Dodds and Law, 1998; Dodds, 2002). C3 is a glycoprotein, it contains a catalytic histidine (Isaac and Iseman, 1992; Law and Dodds, 1997) and is composed of two disulphide linked chains, α- and β-chains, which in halibut have previously been shown to be 115 and 68 kDa (Lange et al., 2004c), compared to in human being 115 and 74 kDa respectively (Law and Dodds, 1997). Complement component C4 is a thioester containing glycoprotein composed of three disulphide linked chains; α-, β- and γ-chains, which in human are 94, 72, and 30 kDa respectively, while in halibut C4 the three chains are 95, 68 and 30 kDa respectively (Supplementary Fig. 2). In humans, C4 is found in two isotypes which differ in the presence of a catalytic histidine (Dodds et al., 1996); a feature also observed in some teleost C3 isoforms (Nakao et al., 2000; Kuroda et al., 2000; Zarkadis et al., 2001). The presence of the two C4 isotypes has been verified in various jawed vertebrates including sharks, reptiles and birds (Nonaka et al., 2017). In teleost fish, a structural and functional diversification of complement components, via isoforms generated by several genes, has received considerable attention and is believed to contribute to expanding the innate immune repertoire as well as contributing to regulation in neutrophil migration and balancing inflammatory and homeostatic processes (Sunyer et al., 1996, 1997, 1998; Sunyer and Lambris, 1998; Kuroda et al., 2000; Zarkadis et al., 2001; Nakao et al., 2002, 2006, 2011; Kato et al., 2003; Boshra et al., 2004, 2006; Papanastiasiou and Zarkadis, 2005; Mauri et al., 2011; Forn-Cuni et al., 2014). In relation to previous studies of our laboratories on innate immune defences and tissue remodelling in early teleost development, including the complement system, pentraxins and deiminated proteins (Bricknell et al., 2000; Magnadottir et al., 2004; Magnadottir et al., 2005; Lange et al., 2004a; Lange et al., 2004b; Lange et al., 2005, Lange et al., 2006; Magnadottir et al., 2006; Magnadottir et al., 2018a and b), we set out to determine putative roles for PADs and their deiminated protein products in halibut ontogeny from 37 to 1050° days post hatching (° d). Halibut is a teleost belonging to the order Heteresomata (Pleuronectiformes) and undergoes metamorphosis of larval to flatfish shape at approximately 700° d with major obstacles in aquaculture being developmental abnormalities and viability in larval rearing (Russel, 1976; Mangor-Jensen et al., 1998). As fish larvae are exposed to micro-organisms immediately after hatching, an effective immune system is of vital importance. Here we show that PAD and deiminated protein products, as assessed by a pan-deimination antibody, as well as deiminated histone H3, are present in various

organs throughout early ontogeny and are prominently expressed in immune-related, neuronal and mucosal tissues in a temporal and spatial manner. We also describe, for the first time, deiminated forms of complement components C3 and C4 in halibut serum. Using proteomic analysis, further complement components, as well as other immunogenic, cytoskeletal and metabolic proteins were identified as being deiminated in halibut serum.

2. Materials and methods

2.1. Fish and sampling

2.1.1. Serum sampling

Experimentally farmed adult halibut (*Hippoglossus hippoglossus* L.; weight 4.5–5.0 kg), were obtained from the fish farm Fiskeldi Eyjafjardar hf, Thorlakshofn, Iceland. From each fish, 1–3 ml blood was collected from a gill vessel, the blood was allowed to clot overnight at 4 °C, and thereafter serum was collected by centrifugation at 750*g* for 10 min and aliquots stored at −20 °C until used. The Fish Disease Laboratory, Institute for Experimental Pathology, Keldur, Iceland, routinely examined the health status of the fish at 3 monthly intervals, declaring it disease free and healthy.

2.1.2. Larval sampling

Farmed Atlantic halibut larvae were obtained from Fiskey hf, Hjalteyri, Iceland, during routine heath checks by the Fish disease Laboratory, Institute for Experimental Pathology, University of Iceland. Farmed halibut larvae were also obtained from the Fisheries Research Services (FRS) Marine Laboratory in Aberdeen (now Marine Scotland), Scotland, U.K., under project license #60–3117, Animals Scientific Procedures Act 1986. Rearing of the larvae has been described before (Lange et al., 2004b) and the growth chart for halibut larval development upto 1050° d is further shown in Supplementary Fig. 1. In brief, first feeding with copepods started at the end of the yolksac stage (between 207 and 244° d) and continued until 670° d. From 280° d, *Artemia* was introduced and took over until 750° d when additional dried food pellets were introduced. The larvae were fed solely on dried food pellets from 940° d onwards. Cultivation temperature was at 5.5–7 °C until 207° d, thereafter at 9.8–12.2 °C until 750° d, whereafter larvae were kept at 9.3–9.5 °C. Halibut larval samples were collected at 37, 109, 206, 320, 408, 430, 440, 495, 655, 860 and 1000–1050° d (this range corresponds to approximately 5–99 days post hatching); three to five larvae were collected for each date. The larvae were fixed in 4% formalin in buffered PBS for 24 h and thereafter embedded in paraffin; paraffin embedded blocks were stored at room temperature until further use.

2.2. Immunoprecipitation and protein identification

Immunoprecipitation, using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck, U.K.) according to the manufacturer's instructions, was used to isolate total deiminated proteins from a pool (n = 3) of halibut sera. The pan-deimination specific F95 antibody, which has been developed against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002), was used to capture all deiminated proteins from the serum pool. The F95 bound proteins were then eluted under reducing conditions and substituted in 2 × Laemmli sample buffer for SDS-PAGE and Western blotting analysis. Total F95 bound protein eluate was also analysed by liquid chromatography–mass spectrometry (LC-MS/MS) (Cambridge Centre for Proteomics, U.K.) and the peak list files submitted to MASCOT.

The eluted deiminated protein pool was in addition tested specifically for halibut C3, C4 and pentraxin-like protein, using mono-specific antibodies prepared against these proteins (for C4 see Supplementary Figs. 2A–B; for C3 see Supplementary Fig. 2C and Lange et al., 2004c; for pentraxin-like protein see Supplementary Fig. 3). The mono-specific F95 antibody is predicted to react with all deiminated/citrullinated proteins based on 100% sequence homology and has for example been used to identify deiminated proteins in human, mouse, rat, and chicken tissue (MABN328 Merck; Nicholas and Whitaker, 2002; Lange et al., 2011; Lange et al., 2014), as well as in teleost (Magnadottir et al., 2018a,b).

2.3. Western blotting

Sera from adult halibut were pooled (n = 3 per pool), reconstituted 1:1 in 2 × Laemmli sample buffer, boiled at 100 °C for 5 min and separated by SDS-PAGE. Approximately 5 µg of protein was loaded per lane for SDS-PAGE, thereafter proteins were blotted onto 0.45 µm nitrocellulose membranes (BioRad, U.K.), even transfer was assessed by Ponceau S (Sigma, U.K.) staining, and membranes were blocked in 5% bovine serum albumin (BSA, Sigma) in tris-buffered saline containing Tween20 (TBS-T) for 1 h at room temperature. Membranes were next incubated in primary antibodies, diluted in TBS-T overnight at 4 °C on a shaking platform as follows: PAD2 (ab50257, Abcam, 1/1000), total deiminated proteins (F95, Nicholas and Whitaker, 2002; 1/5000), deiminated histone H3 (citH3; ab5103, Abcam, 1/2000). For deiminated protein eluates, 10 µl of protein eluate were separated by SDS-PAGE and immunoblotted with either F95 (1/5000), anti-halibut C3 (1/1000), anti-halibut C4 (1/1000) or anti-halibut pentraxin (1/1000). The membranes were thereafter washed three times in TBS-T, incubated in the corresponding anti-mouse IgM, anti-mouse IgG or anti-rabbit IgG HRP-conjugated secondary antibodies (1/4000; BioRad, U.K.), washed five times for 10 min in TBS-T and visualised using ECL (Amersham, U.K.) and the UVP BioDoc-IT™ System (U.K.).

2.4. Immunohistochemical analysis of halibut larvae

For immunohistochemical analysis of PAD, total deiminated proteins and deiminated histone H3, paraffin tissue sections were cut at 5 µm using a microtome (Leica RM2235 RTS, Leica Biosystems, U.K.). Sections of 3–5 larvae of each developmental stage at 37, 109, 206, 320, 408, 430, 440, 495, 655, 860 and 1000–1050° d, were used for each antibody. Staining was performed according to our previously published protocol (Magnadottir et al., 2018a). In brief, sections were deparaffinised using xylene, de-masked by heating in citric acid buffer (pH 6.0) and blocked in 5% goat serum (Sigma, St. Louis, MO, U.S.A.) in 100 mM phosphate buffer (PB) for 1 h, followed by incubation in primary antibody at 4 °C overnight. Primary antibody dilutions were as follows: PAD2 (1/100, ab50257), total deiminated proteins (F95 1/100, Nicholas and Whitaker, 2002) and deiminated histone H3 (citH3 1/100, ab5103). Following washing in PB, sections were incubated in secondary antibodies (biotin-labelled anti-mouse IgM (1/200) or anti-rabbit IgG (1/200); Vector Laboratories, Inc., Burlingame, CA, U.S.A.) and visualisation was carried out using Avidin-Biotinylated peroxidase Complex (ABC, Vector Laboratories, Inc.) and diaminobenzidine/hydrogen peroxide (DAB) stain. Sections were thereafter counterstained with Mayer's Haematoxylin (Sigma) and mounted using DEPEX (Sigma).

3. Results

3.1. Protein analysis of PAD and deiminated protein candidates in halibut serum

3.1.1. Western blotting analysis of PAD, total deiminated proteins and deiminated histone H3

Halibut serum was analysed for the presence of PAD, total deiminated proteins and deiminated histone H3, using Western blotting (Fig. 2). Total deiminated proteins were strongly detected in halibut serum (pool of 3 sera per lane), with immunopositive bands in the range

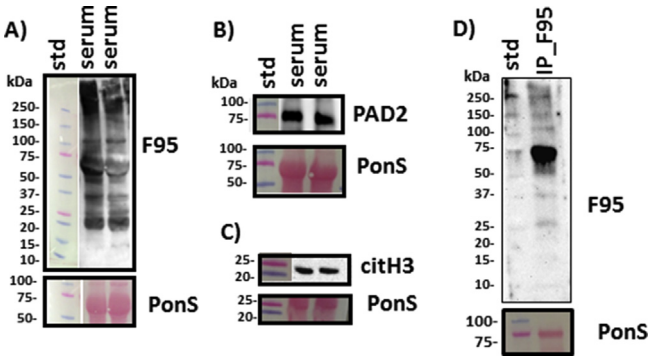

Fig. 2. PAD2, deiminated proteins and deiminated histone H3 in halibut serum. A) Total deiminated proteins were detected in halibut serum (pool of 3 sera per lane) using the pan-deimination F95 antibody (Nicholas and Whitaker, 2002). **B)** The presence of halibut PAD was verified in halibut sera (pool of 3 sera per lane), detecting a band of expected 75 kDa size using the PAD2 antibody (Abcam). **C)** Deiminated histone H3 was detected in halibut sera (pool of 3 sera per lane) using the citH3 antibody (Abcam), which is a marker for neutrophil extracellular traps. **D)** Total deiminated proteins were immunoprecipitated from a pool of halibut sera (n = 3) using the pan-deimination F95 antibody. Protein standard is indicated in the first lane of each blot and PonceauS staining is shown as a loading control for each blot.

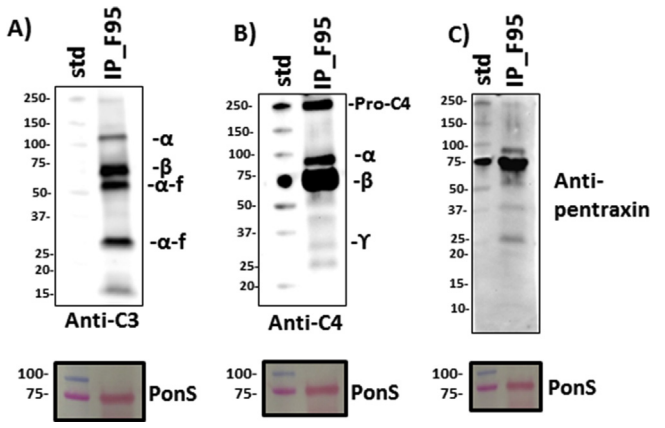

Fig. 3. Complement components C3, C4 and pentraxin-like protein are deiminated in halibut serum. Immunoprecipitated total deiminated protein eluate was immunoblotted against: **A)** Anti-halibut C3 antibody (Lange et al., 2004c); C3 was detected at 115 kDa (α-chain), 68 kDa (β-chain), 50 and 25 kDa (α-chain fragments; α-f), indicating that C3 is deiminated in halibut serum; **B)** Anti-halibut C4 antibody (see Supplementary Fig. 2). C4 positive bands were detected at 250 kDa (pro-C4), 95 kDa (α-chain) and 68 kDa (β-chain), and a faint positive band in the region of the γ-chain (30 kDa), indicating that C4 is deiminated in halibut serum; **C)** Anti-halibut pentraxin-like protein antibody (see Supplementary Fig. 3), reacted with bands in the deiminated protein eluate corresponding to the anticipated 22 kDa monomeric pentraxin-like band, while higher deimination positive bands at approximately 37, 50, 75 and 90 kDa indicate multimeric pentraxin forms.

of 20–250 kDa; particularly prominent bands were seen in regions at 20, 37, 50–70, 100 and above 150 kDa (Fig. 2A). The presence of halibut PAD was verified, detecting a band as expected at approximately 75 kDa (Fig. 2B), using the PAD2 antibody (ab50257), which previously was also shown to react with PAD from cod (*Gadus morhua* L.) (Magnadottir et al., 2018a). Deiminated histone H3 was clearly detected at the expected size range around 20 kDa (Fig. 2C). Deiminated proteins were further immunoprecipitated using the pan-deimination F95 antibody, revealing eluted deiminated proteins at 37, 50, 75, 100, 150 and 250 kDa (Fig. 2D). PonceauS staining of membranes was used to assess even protein load and transfer.

3.1.2. Complement components C3 and C4 and pentraxin-like protein are deiminated in halibut serum

The immunoprecipitated protein eluates isolated from halibut serum, using the pan-deimination F95 antibody, were further analysed for immunopositive reaction with mono-specific mouse antibodies generated against purified halibut C3, halibut C4 and halibut pentraxin-like protein, isolated from halibut serum, as previously described for C3 (Lange et al., 2004c). Purified halibut C4 protein and anti-halibut C4 antibody are shown in Supplementary Fig. 2; halibut pentraxin-like protein and the corresponding mono-specific antibody are shown in Supplementary Fig. 3. Both complement components C3 and C4 were present in the F95 eluate, and therefore contain deimination positive sites (Fig. 3A and B). Band sizes observed are consistent with those expected for C3, i.e. α-chain (115 kDa), the β-chain (68 kDa) and two smaller bands, likely to be α-fragments (α-f) at 52 and 26 kDa (Fig. 3A) (Seya and Nagasawa, 1981). For complement component C4, the prominent 80 and 68 kDa bands are consistent with the C4 α- and β-chains respectively, and a faint immunopositive band at 30 kDa may represent the γ-chain. The strong 250 kDa band is likely to correspond to Pro-C4, i.e. the form of C4 which has not been cleaved to form 3 chains. Other faint bands below the β-chain may represent α-chain fragments (Fig. 3B) (Davies and Sim, 1981). Anti-halibut pentraxin-like protein antibody (Supplementary Figs. 3B and 3C), generated against purified halibut pentraxin-like protein (Supplmentary Fig. 3A and D), reacted with bands in the deiminated protein eluate corresponding to the anticipated 22 kDa monomeric pentraxin-like band, while higher deimination positive bands at approximately 50, 75 and 90 kDa may indicate multimeric forms, with the 70–75 kDa band being most prominent, possibly indicating a trimeric form (Fig. 3C).

3.1.3. Mass spectrophotometry analysis of deiminated protein candidates in halibut serum

Deimination positive immunoprecipitated proteins, isolated from halibut serum using the pan-deimination F95 antibody, were further analysed by LC-MS/MS, with peak files submitted to Mascot, and are listed in Table 1.

[ł]Ions score is $-10*\mathrm{Log}(P)$, 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.

3.2. Immunohistochemical analysis of PAD and deiminated proteins in halibut ontogeny

3.2.1. Peptidylarginine deiminase is detected in organs throughout halibut larval ontogeny at 37–1050° d

The presence of PAD was detected in halibut at all larval stages tested, while the intensity of immunopositivity varied between organs and stages of development (Table 2). At 37° d PAD was detected in yolksac, muscle and skin, as well as spinal cord and notochord. At 72° d, PAD was detected in liver and low levels of PAD were also observed in brain and eye, while strong detection was observed in mucosal cells of the skin (Fig. 4A), which was also prominent at 109° d. At 206° d, brain eye and spinal cord showed strongly PAD positive, chondrocytes were also positive alongside liver, muscle and yolksac while low levels of PAD were observed in kidney. Island of Langerhans in the pancreas was also PAD positive. At 255° d a similar detection pattern was seen, and at this stage in eye both the photoreceptor layer as well as plexiform layer and inner and outer ganglion layers were strongly positive (Fig. 4B). PAD was also clearly detected in brain, spinal cord and skin, and in the head region particularly the olfactory region and chondrocytes of the maxilla were positive (Fig. 4C). At 320° d PAD detection stayed similar, staying high in eye and brain, while at 339° d skin and mucosal associated cells showed very strong, as well as olfactory surface, and liver was PAD positive (Fig. 4D) as well as intestines, which showed very strong PAD positive alongside pancreas, where the Island of Langerhans

Table 1
Deiminated proteins identified in halibut (*Hippoglossus hippoglossus* L.) serum by F95 enrichment. Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody, the F95 eluate was analysed by LC-MS/MS and peak list files were submitted to MASCOT. The identified peptide sequences and m/z values are indicated. Score with teleost proteins are highlighted in the species column with and asterix.

Protein name	m/z	Peptide sequence	Score ($p < 0.05$)‡	Species
A5JV31_HIPHI *Vitellogenin*	535.3462	*K.VEELIIILLK.V*	1264	*Hippoglossus hippoglossus**
	619.3463	*R.MVSAIVLFETK.L*		
	647.3977	*K.LVEGLAVNPIIR.E*		
	663.8446	*R.ICADGVLLSNHK.L*		
	682.3820	*K.IASAIVETYAVAR.N*		
	688.4119	*R.EIVLLGYGTLVAK.Y*		
	716.3398	*R.DQSQEQNEINVK.I*		
	743.3728	*K.FFGQEVAFANIDK.A*		
	748.9310	*K.LLPGFGSAAANLPLR.V*		
	749.8823	*R.FESIEALWTQFK.A*		
	754.4070	*K.MIQDIAIQLFMGK.A*		
	778.4607	*K.LPMGLVTTLADALLK.E*		
	538.6592	*K.VFAPAGISATVLNILK.G*		
	807.4852	*K.VFAPAGISATVLNILK.G*		
	819.9157	*K.QYETEITAETGLVGK.Q*		
	877.9629	*R.LQVIVANLAENDHYR.I*		
	981.9844	*K.TQNVYELQEPGVQGICK.T*		
Q4KVK3_HIPHI *Complement component C3 (Fragment)*	517.2432	*R.ESLDLGEDR.T*	694	*Hippoglossus hippoglossus**
	803.4123	*R.TLCAATVSSLPGSVDK.A*		
	877.0104	*R.IYTLEATAYALLALVK.V*		
	938.3368	*R.NDECTCAEENCSMQK.K*		
	945.9417	*R.LEEFTDGLSTDIYTMR.I*		
	946.3320	*R.NDECTCAEENCSMQK.K*		
	631.2254	*R.NDECTCAEENCSMQK.K*		
	953.9398	*R.LEEFTDGLSTDIYTMR.I*		
	1044.0238	*K.VGVLQPATVSVYEYYDQR.H*		
	1165.0738	*R.LDSLTNPYAVAMTSYALANEGK.L*		
	829.0848	*R.RLDSLTNPYAVAMTSYALANEGK.L*		
G4WAB7_EPICO *Complement component C3*	460.9660	*R.GQVLISLIVPITK.E*	357	*Epinephelus coioides**
	690.9464	*R.GQVLISLIVPITK.E*		
	704.3509	*K.FPESWLWSDIK.L*		
	877.0104	*R.IYTLEATAYALLALVK.A*		
	938.3368	*R.NDECTCAEENCSMQK.K*		
	946.3320	*R.NDECTCAEENCSMQK.K*		
	631.2254	*R.NDECTCAEENCSMQK.K*		
Q98TS6_ANAMI *Complement component C3*	803.4348	*K.YLILNAQQPDGVFK.D*	245	*Anarhichas minor**
	843.9054	*R.VDLLEEEHVCSAASK.R*		
	1071.0521	*K.SGIHSGDFQLAEIVSPGLWK.V*		
Q9PTY1_PAROL *Complement component C3*	533.2952	*K.AILHNYSPDVITVR.V*	232	*Paralichthys olivaceus**
	803.4348	*K.YLILNAQQPDGVFK.E*		
	941.9348	*K.FHSNPQESFSAEFEVK.E*		
	628.2924	*K.FHSNPQESFSAEFEVK.E*		
W0IAR6_PSEBE *Complement component pro-C3-2*	941.9348	*K.FHSNPQQSFSAEFEVK.E*	187	*Pseudotrematomus bernacchii**
	628.2924	*K.FHSNPQQSFSAEFEVK.*		
	995.4020	*R.SEEDDNSYMDSNEIVSR.T*		
	1003.4011	*R.SEEDDNSYMDSNEIVSR.T*		
	1003.8965	*R.SEEDDNSYMDSNEIVSR.T*		
A0A075E1K5_OPLFA *Complement component C3*	785.9202	*R.VTGDPEATVGLVAVDK.G*	114	*Oplegnathus fasciatus**
H2MT65_ORYLA *Complement component C5*	736.8266	*R.HIEQSDLGCGGGGGK.D*	96	*Oryzias latipes**
Q9PTY3_PAROL *Complement component C7*	877.4240	*K.ALSSLPFTYDYSAYR.Q*	74	*Paralichthys olivaceus**
A0A0E3GPE4_SINCH *Complement component C8 alpha*	549.2888	*R.LSTAADHVGAR.L*	75	*Siniperca chuatsi**
A0A1A7ZJC2_NOTFU *Complement component C8, beta*	736.8387	*R.ALSEYLAESSSCR.C*	75	*Nothobranchius furzeri**
Q9PVW6_PAROL *Complement component C9*	1065.4816	*R.TVEVFGQFGGESCQGSLGDR.E*	118	*Paralichthys olivaceus**
A0A0A7REJ5_LARCR *Complement component C9*	709.3743	*R.TAGYGINILGADPR.R*	73	*Larimichthys crocea**
J7FIH6_OPLFA *C1 inhibitor*	833.4657	*R.FALTGDSSLYILLPR.S*	77	*Oplegnathus fasciatus**
Q5DVG8_PLAFE *Apolipoprotein AI*	574.7939	*K.TSVAANVEETK.T*	80	*Platichthys flesus**

(*continued on next page*)

Table 1 (*continued*)

Protein name	m/z	Peptide sequence	Score ($p < 0.05$)‡	Species
A0A2R9AX99_PANPA *Keratin 9*	579.2984	*K.DQIVDLTVGNNK.T*	1024	*Pan paniscus*
	579.7908	*R.FSSSSGYGGGSSR.V 8251*		
	616.8025	*R.SGGGGGGGLGSGGSIR.S*		
	618.2694	*R.QGVDADINGLR.Q*		
	793.8864	*R.QGVDADINGLR.Q*		
	658.3470	*K.VQALEEANNDLENK.I*		
	794.3787	*K.VQALEEANNDLENK.I*		
	896.3692	*R.GGSGGSYGGGGSGGGYGGGSGSR.G*		
	613.3259	*R.HGVQELEIELQSQLSK.K*		
	919.4863	*R.HGVQELEIELQSQLSK.K*		
	1048.0216	*R.QEIECQNQEYSLLLSIK.M*		
	1086.0182	*K.SDLEMQYETLQEELMALK.K*		
	1094.0151	*K.SDLEMQYETLQEELMALK.K*		
	837.3832	*K.EIETYHNLLEGGQEDFESSGAGK.I*		
	1088.8442	*K.DIENQYETQITQIEHEVSSSGQEVQSSAK.E*		
A0A2J8KDE9_PANTR *KRT1 isoform 1*	546.7557	*R.GSGGGSSGGSIGGR.G*	858	*Pan troglodytes*
	590.3043	*K.YEELQITAGR.H*		
	633.3216	*R.TNAENEFVTIK.K*		
	633.8147	*R.TNAENEFVTIK.K*		
	650.7686	*K.NMQDMVEDYR.N*		
	651.8621	*R.SLDLDSIIAEVK.A*		
	670.8386	*K.SKAEAESLYQSK.Y*		
	679.3519	*K.LNDLEDALQQAK.E*		
	738.3783	*K.WELLQQVDTSTR.T*		
	738.3977	*R.FLEQQNQVLQTK.W*		
	858.9304	*K.QISNLQQSISDAEQR.G*		
	1104.7767	*R.GSYGSGGSSYGSGGGSYGSGGGGGGHGSYGSGSSSGGYR.G*		
A0A2I3S166_PANTR *Keratin 2*	604.8118	*R.FLEQQNQVLQTK.W*	739	*Pan troglodytes*
	627.8081	*K.VDLLNQEIEFLK.V*		
	632.3511	*K.LNDLEEALQQAK.E*		
	665.3218	*R.NLDLDSIIAEVK.A*		
	665.3671	*K.NVQDAIADAEQR.G*		
	686.3596	*K.LALDVEIATYR.K*		
	730.9031	*R.GFSSGSAVVSGGSR.R*		
	738.3977	*R.TAAENDFVTLK.K*		
	870.8568	*R.GSSSGGGYSSGSSSYGSGGR.Q*		
A0A146R063_FUNHE *Inter-alpha-trypsin inhibitor heavy chain H2*	506.2771	*R.YATTVITSR.V*	317	*Fundulus heteroclitus**
	559.3015	*R.GQSAGIVSSVGR.T 5758*		
	746.4125	*K.AGISFINVKGGLSTK.A*		
	762.8509	*K.EQAQQQYTDAVSR.G*		
Q546G4_MOUSE *Albumin 1*	716.3181	*K.YMCENQATISSK.L*	465	*Mus musculus*
	720.7900	*K.TCVADESAANCDK.S*		
	740.4023	*K.LGEYGFQNAILVR.Y*		
	740.4340	*K.GLVLIAFSQYLQK.C*		
	831.9312	*R.LPCVEDYLSAILNR.V*		
	875.3363	*K.ECCHGDLLECADDR.A*		
THIO_ECOLI *Thioredoxin 1*	634.3367	*K.LNIDQNPGTAPK.Y*	159	*Escherichia coli*
	903.4539	*K.MIAPILDEIADEYQGK.L*		
M4ANS3_XIPMA *Serotransferrin*	825.9031	*K.EADAVAVDGGQVYTAGK.C*	115	*Xiphophorus maculatus**
	826.3953	*K.EADAVAVDGGQVYTAGK.C*		
A0A287B5W2_PIG *Trypsinogen precursor*	1106.0550	*R.LGEHNIDVLEGNEQFINAAK.I*	110	*Sus scrofa*
	737.7067	*R.LGEHNIDVLEGNEQFINAAK.I*		
	738.0353	*R.LGEHNIDVLEGNEQFINAAK.I*		
D5A7I1_DICLA *Hemopexin*	812.3644	*R.CEGIEFDAITPDEK.G*	91	*Dicentrarchus labrax**
A0A161FU20_SCONI *Hemopexin*	677.8413	*K.GHDVHVYDIATK.T*	77	*Scomberomorus niphonius**
C8CEI3_PAROL *Hemopexin*	796.6914	*R.CSHVHLDAITSDHGGNMYAFR.G*	71	*Paralichthys olivaceus**
	597.7710	*R.CSHVHLDAITSDHGGNMYAFR.G*		
A0A0F8CD07_LARCR *Ig lambda-6 chain C region*	753.3769	*K.VSLGSQTSEKNINK.S*	89	*Larimichthys crocea**
	753.8692	*K.VSLGSQTSEKNINK.S*		
A0A0F8AH88_LARCR Ig heavy chain V region 5A	563.7837	*K.FSIELDTSSK.T*	69	*Larimichthys crocea**
G3Q4A0_GASAC *Fibrinogen beta chain*	556.7723	*R.GFGNTAFDVGK.G*	85	*Gasterosteus aculeatus**
Q6QZI3_PSEAM *Gamma fibrinogen*	1152.5293	*K.QGFGYLSPDDTTEFWLGNEK.I*	74	*Pseudopleuronectes americanus**
TRY1_RAT *Anionic trypsin-1*	737.7078	*R.LGEHNINVLEGDEQFINAAK.I*	74	*Rattus norvegicus*
	738.0348	*R.LGEHNINVLEGDEQFINAAK.I*		
	738.0353	*R.LGEHNINVLEGDEQFINAAK.I*		
A0A024TXY0_9STRA *Elongation factor 1-alpha*	522.7937	*K.EAGGKGKAAAGK.K*	72	*Aphanomyces invadans*
A0A151 × 8S7_9HYME Cathepsin L	821.3629	*K.NSWGETWGENGYIK.M*	71	*Trachymyrmex zeteki*
A0A087YMZ0_POEFO *Ceruloplasmin*	822.9576	*R.SPEEQHLGILGPVLR.A*	71	*Poecilia formosa**
A0A087XAX0_POEFO *Plasminogen*	697.4232	*K.LVLGPNGADIALLK.L*	69	*Poecilia formosa**
	697.9163	*K.LVLGPNGADIALLK.L*		

(*continued on next page*)

Table 1 (*continued*)

Protein name	m/z	Peptide sequence	Score ($p < 0.05$)‡	Species
H2U5S0_TAKRU *Uncharacterized protein*	819.4188	*K.EFEALSSGTQVELVK.K*	207	*Takifugu rubripes**
	1067.0118	*K.LGIWSIEASYSDDFTTTAR.T*		
M4A7F9_XIPMA *Uncharacterized protein*	559.3147	*K.AVSSGQTAGLVK.A*	174	*Xiphophorus maculatus**
	883.9081	*K.CPGCDGTLIDGDFVIK.Y*		
W5M7E6_LEPOC *Uncharacterized protein*	592.2538	*K.ASISVLGDILGR.A*	161	*Lepisosteus oculatus**
	600.8516	*K.HDDGSYSAFGK.S*		
H2L543_ORYLA *Uncharacterized protein*	920.4751	*R.VDVEEGYINIYLDGLK.K*	97	*Oryzias latipes**
A0A060YSX4_ONCMY *Uncharacterized protein*	855.9263	*R.YINNFQIVDNLSDR.G*	83	*Oncorhynchus mykiss**
A0A087XVJ8_POEFO *Uncharacterized protein*	813.9159	*K.FSIDIDSSSNTVTLK.G*	82	*Poecilia formosa**
E4XC47_OIKDI Uncharacterized protein	522.7752	*R.VQNLIAGATR.S*	69	*Oikopleura dioica*

was prominently PAD positive (Fig. 4E). At 408° d muscle, skin, liver, chondrocytes, notochord, eye and brain all showed PAD positive, with optic tectum and medulla oblongata of brain strongly positive (Fig. 4F). At 430–440° d eye brain and spinal cord showed prominent PAD detection and PAD was also detected in liver and chondrocytes of fin. Skin, mucosa of gills, muscle and intestines were also PAD positive. At 655° d PAD was detected in tubuli of kidney (Fig. 4G) and strong detection was observed in gills and mucosa of the mouth and in liver; intestines were positive, particularly goblet cells (Fig. 4H), as well as spinal cord and notochord but low detection was observed in brain and eye. PAD was also detected in chondrocytes. At 860° d PAD detection was prominent in the mucosa of gills; myeloma of kidney was positive and liver, brain, eye and spinal cord showed high PAD levels, as well as the intestines. In chondrocytes PAD was only detected at low levels. At 1000–1050° d PAD was detected in myofibrils of heart (Fig. 4I), prominent PAD detection was observed in mucosal tissue of intestines, in sacchiform cells of skin as well as in muscle (Fig. 4J); neurones of spinal cord were strongly positive (Fig. 4K) and eye also showed PAD positive. Mucosal surfaces of gills showed strongly positive while chondrocytes were negative (Fig. 4L). In kidney, myeloma and tubuli were PAD positive.

3.2.2. Pan-deiminated proteins are detected in organs throughout halibut larval ontogeny at 37–1050° d

The presence of total deiminated proteins was detected throughout halibut larval ontogeny both in organs as well as in mucosal surfaces of skin, gills, oesophagus and intestines (Table 3). At the earliest larval stages tested (37° d) skin, vacuoles of yolksac (Fig. 5A) and muscle were positive as well as neuronal tissue and notochord. At 72° d further organs were detected to contain deiminated proteins, including the liver and faint detection was also seen in the eye. At 109° d detection in eye and notochord was clearly present and skin was strongly positive. At 206° d brain showed strongly positive and clear detection was observed in eye, neuronal tissue and intestines, as well as liver (Fig. 5B) and some faint positive in kidney, mainly myeloma and tubuli, and some in glomerulus (Fig. 5C). At 255° d eye showed very strong positive (Fig. 5D), and kidney (myeloma and tubuli) were stronger positive at this stage, while heart was negative. At 320° d most organs tested were positive, except heart and chondrocytes. At 339° d heart showed faint positive, strong detection was observed in muscle (Fig. 5E) and otherwise deiminated proteins were detected at varying levels in the organs as listed in Table 3. At 408° d brain (Fig. 5F), spinal cord (Fig. 5G) and liver (Fig. 5H (li)) showed particularly strong positive; deiminated proteins were also seen in heart (Fig. 5H (he)), muscle, skin, eye, notochord, and in myeloma of kidney. In intestines prominent detection of deiminated proteins was seen in mucosal cells (Fig. 5I) and chondrocytes in the head region were also positive (Fig. 5J). At 430° d eye was particularly strong positive, faint detection was observed in liver, chondrocytes, spinal cord, notochord and kidney, and stronger detection in skin, brain, heart and intestines. At 495° d a similar detection was observed, although spinal cord and kidney showed some increase in F95 detection. At 655° d strong F95 detection was observed in liver and eye, faint in intestines, while kidney (myeloma and tubuli) and heart were positive, as well as spinal cord, chondrocytes (in gills and fins as well as head), brain, skin and muscle. At 860° d liver and eye remained strongly positive, low detection was observed in chondrocytes and spinal cord, but strong in skin and mucosal surfaces of gills and mouth. Brain showed positive as did muscle, notochord, intestines, while spinal cord was low. At 1000–1050° d, sacchiform cells of skin were strongly positive (Fig. 5K), chloride cells of gills were prominently F95 reactive and positive detection was also seen in chondrocytes of gill arch (Fig. 5L). Brain showed also strong positive along with intestines, and otherwise

Table 2
A summary of the presence of PAD in organs of halibut larvae from 37 until 1050° d. The positive detection in organs at each stage is indicated as V. Strong detection is indicated with S while low detection is indicated with L. An organ/tissue not found in the tissue sections of the sample are indicated with tissue not present (tnp). Zero (0) means that the organs were not positive for PAD tested at the relevant age. A blank means that the organ is absent at this stage of development.

	37° d	72° d	206° d	255° d	320° d	339° d	408° d	440° d	655° d	860° d	1050° d
Yolksac	V	V	V	tnp	V						
Muscle	V	V	V	V	V	V	V	V	V	V	V
Skin	V	S	V	S	V	V	V	V	V	V	V
Liver		tnp	S	tnp	tnp	V	V	tnp	L	V	V
Brain	V	V	V	S	S	S	V	V	L	V	V
Chondrocytes			L	V	0	V	V	L	V	L	0
Eye		V	S	S	S	V	V	V	L	V	V
Spinal cord	V	S	S	S	V	V	tnp	V	V	V	V
Notochord	V	S	V	tnp	V	V	V	L	V	V	V
Heart		tnp	tnp	tnp	tnp	tnp	tnp	tnp	V	tnp	V
Intestines			V	V	V	V	tnp	V	V	S	V
Kidney-myeloma/tubuli			V	tnp	tnp	V	tnp	tnp	V	V	V
Kidney-glomeruli			0	tnp	tnp	V	tnp	tnp	tnp	tnp	0
Pancreas			V	tnp	tnp	V	tnp	tnp	tnp	tnp	tnp

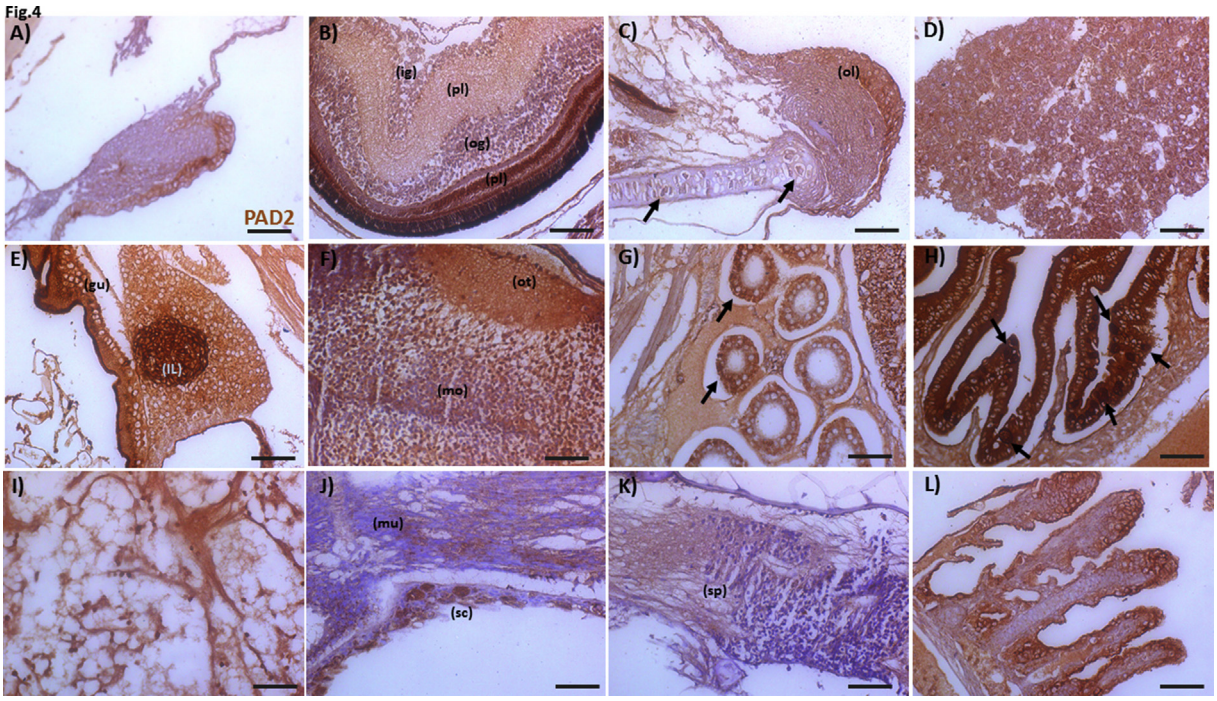

Fig. 4. PAD2 in halibut ontogeny. Halibut larvae from the age of 37–1050° d were analysed by immunohistochemistry for PAD2 protein. Representative figures for PAD2 detection throughout halibut ontogeny are presented as follows: **A)** PAD2 positive mucosal cells in skin at 72° d; **B)** PAD2 positive cells in eye at 255° d in photoreceptor layer (ph), plexiform layer (pl) and inner (ig) and outer ganglion layer (og); **C)** Chondrocytes of the maxilla (arrows) as well as mucosal surface and skin, particularly in the olfactory region (ol), are PAD2 positive (255° d); **D)** Hepatocytes of liver are PAD2 positive (339° d); **E)** Island of Langerhans (IL) in pancreas is prominently PAD2 positive as well as mucosal layers of gut (gu) at 339° d; **F)** PAD2 positive neurones in brain (408° d) in the optic tectum (ot) and medulla oblongata (mo); **G)** Tubuli of kidney (arrows) are strongly PAD2 positive (655° d); **H)** Mucosal layers of intestine, with prominent goblet cells (arrows) are PAD2 positive (655° d); **I)** Myofibrils in heart are PAD2 positive (1050° d), **J)** Muscle (mu) and sacchiform cells (sc) of skin are PAD2 positive (1050° d); **K)** PAD2 is detected in spinal cord (sp) (1050° d) and **L)** in mucosal surfaces surrounding gills (1050° d). PAD positive cells were visualised using DAB chromogen and counterstain was with haematoxylin blue. All pictures were photographed using a 40× lens; scale bars represent 50 μm.

F95 was observed in muscle, skin, liver eye, spinal cord, notochord, heart and myeloma of kidney as before. Pancreas was not present in the histological sections stained for F95 and detection could thus not be assessed.

3.2.3. Deiminated histone H3 is detected in organs throughout halibut larval ontogeny at 37–1050° d

Deiminated histone H3 was detected in a variety of organs between 37 and 1050° d (Table 4). Yolksac was most prominently positive at 37° d, and stayed positive until 320° d. Deiminated histone H3 was detected in muscle (Fig. 6A; (mu)) at low levels at 37–72° d, but stronger thereafter until 1050° d, when levels went somewhat down. Spinal cord (Fig. 6A; (sp)) showed positive from 37° d, albeit levels varied considerably between the developmental stages, sometimes not detectable such as at 109, 255, 339, 430 and 860° d. Intestines and stomach (Fig. 6B, C, J and K) showed positive at all stages between 109 and 1050° d. Pancreas (Fig. 6D) was detected in histological sections at 339 and 430° d and showed immunopositive for deiminated histone H3 in island of Langerhans at 339° d. Kidney (Fig. 6E) showed positive for citH3 at varying levels, but was positive from 206° d, particularly in myeloma and low positive was seen in glomeruli at some developmental stages. Liver (Fig. 6F) showed positive from 109° d, albeit levels

Table 3
A summary of the presence of total deiminated proteins (F95 detection) in organs of halibut larvae from 37 until 1050° d. The positive detection in organs at each stage is indicated as V. Strong detection is indicated with S while low detection is indicated with L. An organ/tissue not found in the tissue sections of the sample are indicated with tissue not present (tnp). Zero (0) means that the organs were not positive for F95 tested at the relevant age. A blank means that the organ is absent at this stage of development.

	37° d	72° d	109° d	206° d	255° d	320° d	339° d	408° d	430° d	440° d	495° d	655° d	860° d	1050° d
Yolksac	V	V	V	V	tnp	V								
Muscle	V	V	V	V	V	V	V	V	V	V	S	V	V	V
Skin	V	V	S	V	V	V	V	V	S	V	S	S	S	S
Liver		V	V	S	V	tnp	V	S	L	L	L	S	S	V
Brain	tnp	L	0	S	V	V	V	V	V	L	S	S	V	S
Chondrocytes			tnp	0	V	0	V	0	L	0	V	V	L	L
Eye		L	V	V	S	L	V	V	V	L	V	S	S	V
Spinal cord	V	tnp	tnp	V	V	V	V	V	L	0	S	V	L	V
Notochord	V	V	V	V	V	V	0	V	L	0	L	V	V	V
Heart		tnp	tnp	0	0	0	L	V	V	tnp	tnp	V	V	V
Intestines			tnp	V	V	V	V	V	V	V	S	L	V	S
Kidney-myeloma/tubuli				L	V	tnp	V	V	L	0	V	V	S	V
Kidney-glomeruli				L	0	tnp	L	L	0	0	tnp	tnp	tnp	tnp
Pancreas				tnp	tnp	tnp	tnp	tnp	tnp	tnp	tnp	tnp	tnp	tnp

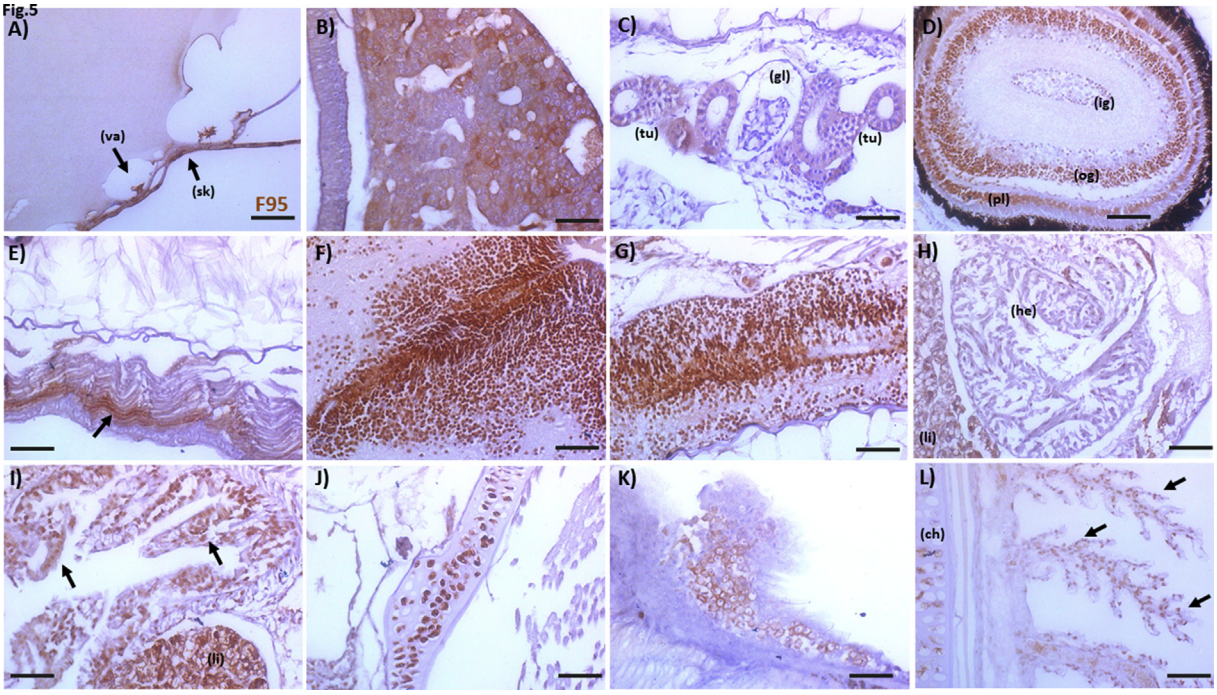

Fig. 5. Deiminated proteins in halibut ontogeny. Halibut larvae from the age of 37–1050° d were analysed by immunohistochemistry for deiminated proteins using the F95 pan-deimination antibody. Representative figures for deiminated proteins detected throughout halibut ontogeny are as follows: **A)** F95 positive skin (sk) and vacuoles (va) of yolksac (37° d); **B)** F95 positive hepatocytes of the liver (206° d); **C)** Tubuli of kidney are F95 positive while a faint response is seen in glomerulus (gl) (206° d); **D)** F95 positive cells in eye (255° d) are found in the plexiform layer (pl), inner (ig) and outer ganglion layer (og); **E)** Muscle (arrow) is F95 positive (339° d); **F)** Strong detection for deiminated proteins is found in brain (408° d); **G)** Spinal cord shows strong positive for deiminated proteins in neurones (408° d); **H)** Deiminated proteins are found in myofibrils of the heart (he) and liver is also seen F95 positive (li) (408° d); **I)** Mucosal cells in intestines are strongly F95 positive (arrows) as well as liver (li) (408° d); **J)** Chondrocytes in the head region are F95 positive (408° d); **K)** Sacchiform cells of skin show strong specificity for F95 (1050° d); **L)** Chloride cells of gills (arrows) are strongly F95 positive and chondrocytes (ch) in the gill arch are also F95 positive (1050° d). F95 positive cells were visualised using DAB chromogen and counterstain was with haematoxylin blue. All pictures were photographed using a 40× lens; scale bars represent 50 μm.

varied, being low at 255, 339, 430 and 655–1050° d. Deiminated histone was detected in eye (Fig. 6G) at low levels from 72° d, raised at 206° d but low at 408–430° d. Levels rose again at 495° d, low at 860° d but then higher at 100–1050° d. Brain (Fig. 6H and I) showed low positive between 37 and 72° d, with levels increasing at 206–339° d, lower at 408–430° d and rising again at 495° d, but thereafter low. Skin (Fig. 6L) was prominently positive at 37° d and stayed positive throughout all stages tested, albeit lower at 430° d. Chondrocytes were positive from 109 to 320° d, then low at 339° d, increased again at 408° d but were negative at 430° d and thereafter stayed low or negative. Notochord showed positive at low levels from 37° d, levels rose between 109 and 320° d and thereafter varied. Heart showed positive at varying levels, with deiminated histone H3 detectable at 206, 320, 408–495° d and at low levels at 1000–1050° d.

4. Discussion

Effects of post-translational protein modifications, which may allow for protein moonlighting in homeostasis and immune defences, are here revealed in early halibut ontogeny, showing deiminated proteins in various organs and mucosal tissues throughout a continuous series of early developmental stages. Post-translational deimination is a means

Table 4
A summary of the presence of deiminated histone H3 (citH3) in organs of halibut larvae from 37 until 1050° d. The positive detection in organs at each stage is indicated as V. Strong detection is indicated with S while low detection is indicated with L. An organ/tissue not found in the tissue sections of the sample are indicated with tissue not present (tnp). Zero (0) means that the organs were not positive for citH3 tested at the relevant age. A blank means that the organ is absent at this stage of development.

	37° d	72° d	109° d	206° d	255° d	320° d	339° d	408° d	430° d	495° d	655° d	860° d	1050° d
Yolksac	S	V	V	tnp	L	V							
Muscle	L	L	S	S	V	S	V	V	V	S	V	V	L
Skin	S	V	S	S	V	S	V	V	L	S	V	S	S
Liver			S	S	L	V	L	V	L	S	L	L	L
Brain	L	L	0	S	V	S	V	L	L	S	L	L	L
Chondrocytes			V	V	V	V	L	V	0	L	L	0	0
Eye		L	S	S	V	S	V	L	L	V	L	S	V
Spinal cord	L	tnp	0	S	0	tnp	0	V	0	V	L	0	L
Notochord	L	L	V	V	V	V	0	V	0	V	L	0	L
Heart	tnp	tnp	tnp	V	0	V	0	V	V	V	0	tnp	L
Intestines			S	V	V	S	S	V	S	S	S	S	S
Kidney-myeloma/tubuli			0	V	L	tnp	V	V	V	V	tnp	V	V
Kidney-glomeruli			0	V	0	tnp	L	V	0	L	tnp	0	0
Pancreas				V	tnp	tnp	S	tnp	tnp	tnp	tnp	tnp	tnp

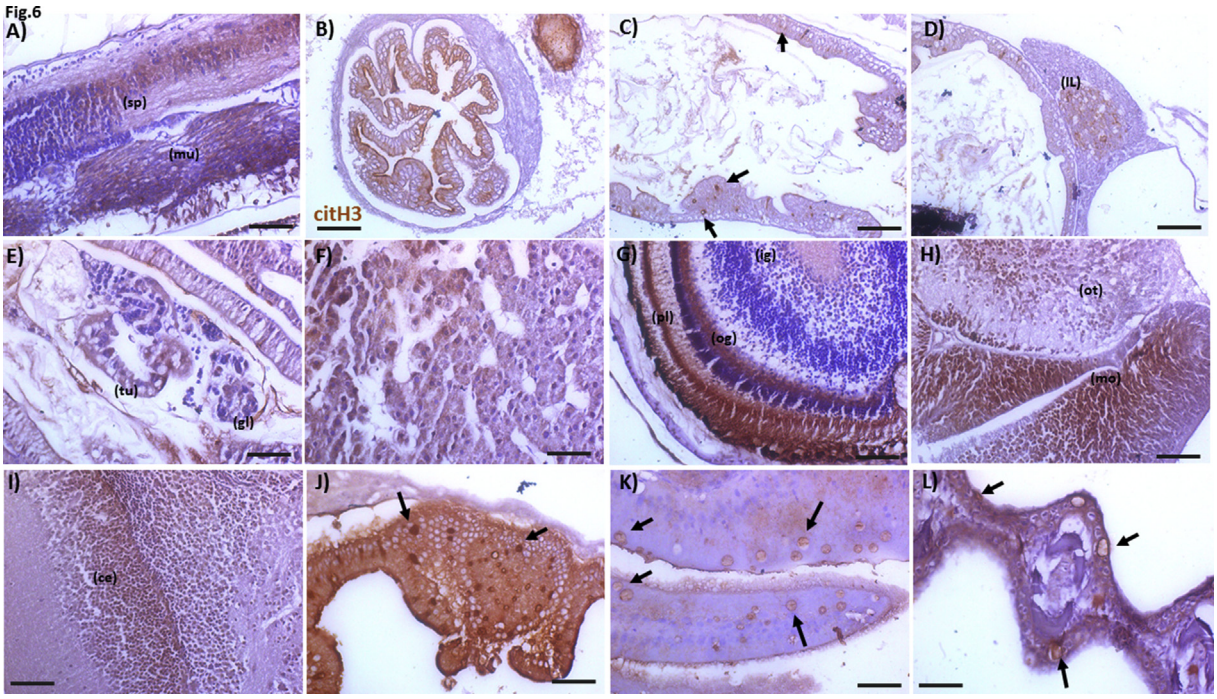

Fig. 6. Deiminated histone H3 in halibut ontogeny. Halibut larvae from 37 to 1050° d were analysed by immunohistochemistry for deiminated histone H3 (citH3), involved in gene regulation and also a marker for neutrophil extracellular trap formation. Representative examples showing deiminated histone H3 (citH3) throughout halibut larval ontogeny are as follows: **A)** Spinal cord (sp) and muscle (mu) at 206° d; **B)** Mucosal lining and mucosal cells of intestines at 255° d; **C)** mucosal goblet cells of the stomach are specific for citH3 (339° d); **D)** Island of Langerhans in pancreas is strongly specific for citH3 (339° d); **E)** In kidney, both tubuli (tu) and glomerulus (gl) stained positive for citH3 (408° d); **F)** Deiminated histone H3 is detected in liver (408° d); **G)** Plexiform layer (pl) and ganglion layers (ig; og) of eye are citH3 positive (495° d); **H)** Brain is strongly immunopositive for citH3, showing neurones in optic tectum (ot) and the medulla oblongata (mo) (495° d); **I)** CitH3 positive neurones in cerebellum (ce) (495° d); **J)** In mucosal cells of intestines (arrows), citH3 is strongly detected at 655° d; **K)** Goblet cells (arrows) of gut are specific for citH3 at 1050° d; **L)** Skin and sacchiform cells (arrows) are strongly positive for deiminated histone H3 (1050° d). CitH3 positive cells were visualised using DAB chromogen and counterstain was with haematoxylin blue. All pictures were photographed using a 40× lens; all scale bars represent 50 μm.

of increasing antigenic diversity via changes in the primary, secondary and tertiary structures of proteins, leading to altered antigen processing, antigen presentation and immune recognition (Doyle and Mamula, 2012). These changes can alter interaction with immune cells and affect signalling pathways (Nguyen and James, 2016). Peptidylarginine deiminase (PAD), and its deiminated protein products were detected in various halibut organs, as well as in mucosal surfaces of skin, gills and gut, indicating roles both in tissue remodelling and immune defences. PAD detection correlated with the detection of deiminated proteins, which were particularly prominent in all developmental stages tested in the brain, spinal cord, eye, muscle, skin and liver, while in kidney, heart and pancreas the levels of deiminated proteins varied (Tables 2–4). Compared to our previous study on protein deimination in cod ontogeny, deiminated proteins were detected at similar sites in halibut ontogeny, albeit differing in temporal and spatial distribution and also, in halibut larvae deiminated proteins and histone H3 were less prominent in chondrocytes compared to what was observed in cod (Magnadottir et al., 2018a). In comparison, a qPCR expression study on PADI in adult rainbow trout, showed PADI to be abundant in fin and skin, with moderate expression in brain, gastrointestinal tract, gill and spleen, and low expression in kidney, heart, liver and muscle (Rebl et al., 2010); however, the presence of deiminated protein products was not assessed. In mammals, PAD expression is well reported in serum and has also been described in mucosal tissues including bronchial and alveolar mucosa (Makrygiannakis et al., 2008) as well as the uterus, while in gastric and colon tissues PAD dysregulation is associated with ulcerative colitis and cancer pathogenesis (Akiyama et al., 1990; Xin and Song, 2016; Cantariño et al., 2016). In zebrafish, developmental roles for PAD in angiogenesis, heart, brain, eye and head formation have been shown (Khajavi et al., 2017), while in early mouse embryo development histone deimination was shown to be crucial for gene regulation (Kan et al., 2012; Zhang et al., 2016b). PAD2-mediated deimination has in mammals been reported in brain, spinal cord, spleen, skeletal muscle and leukocytes, while PAD4-mediated deimination was observed in liver, lung, kidney and testis (Van Beers et al., 2013). A strong detection of PAD and deiminated histone H3 in the islet of Langerhans in halibut pancreas, 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 β-cell stress and autoantigen 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). High levels of PAD and deiminated proteins were observed throughout halibut ontogeny in brain, spinal cord and eye, and correlates with previously observed roles for PAD-mediated deimination in the development of the central nervous system (Moscarello et al., 1994) and CNS regeneration (Lange et al., 2011, 2014; Lange, 2016) as well as in cod ontogeny (Magnadottir et al., 2018a). Deiminated proteins are also linked to retinal injury and have been shown to proceed wound healing mechanisms in the eye (Wizeman et al., 2016; Wizeman and Mohan, 2017), and detected in the ganglion cell layer, inner plexiform layer and inner nuclear layer (Bhattacharya et al., 2008; Bhattarchaya, 2009), similar as seen here in halibut eye and also previously detected in cod eye (Magnadottir et al., 2018a). This may indicate roles both in early eye tissue remodelling, where the specific factors regulating timing of teleost retinal differentiation remains to be fully understood (Ferreiro-Galve et al., 2010), as well as in retinal neurogenesis observed in fish throughout life (Raymond et al., 2006). It may be speculated that turnover of eye cells may for example happen through exposure of deiminated neo-epitopes and associated phagocytosis of cell debris by Müller glia (Bejarano-Escobar et al., 2017).

While Histone H3 affects epigenetic regulation, it is also a marker

for neutrophil extracellular trap formation (NETosis), which is an anti-pathogenic mechanism conserved between teleost and mammalian neutrophils. NETs can be induced by viruses (Schönrich and Raftery, 2016) and bacteria, while they can also bind and prevent 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). In halibut larvae, deiminated histone H3 was detected in various organs as well as the gut-associated lymphoid tissue (GALT), the skin-associated lymphoid tissue (SALT) and the gill-associated lymphoid tissue (GIALT). Deiminated histone H3 was previously found to be upregulated in cod intestinal mucosa upon immunostimulation with LPS (Magnadottir et al., 2018a), while NETosis has also been described in carp (*Cyprinus carpio* L.) granulocytes (Pijanowski et al., 2013), zebrafish (*Danio rerio*) kidney (Palic et al., 2007b) and in neutrophils of fathead minnow (*Pimephales promelas*) (Palic et al., 2005). In mammals, NETosis has been associated with gut mucosal inflammation (Al-Ghoul et al., 2014) and anti-microbial defence in oral mucosa (Mohanty et al., 2015). Importantly, teleost mucosal gut and skin surfaces represent human mucosal-I surfaces of the uterus, gut and respiratory tract (Gomez et al., 2013; Xu et al., 2013) and findings in teleost may thus be translatable to mucosal-associated human pathologies.

While halibut C3, as well as C3 from other fish and mammals, has previously been shown to be glycosylated to varying degrees, with carbohydrate moieties present in both chains (Zarkadis et al., 2001; Lange et al., 2004c), post-translational deimination of C3 is here described for the first time in any species. Similarly, post-translational deimination of C4 has not been shown before. It may be postulated that post-translational deimination of C3 and C4 may influence their function including their ability for cleavage, binding, deposition and in the generation of the convertase. Indeed, pan-PAD inhibitor Cl-amidine and PAD4-selective inhibitor GSK199 have been shown to decrease C3 deposition in synovium and cartilage and ameliorate collagen-induced arthritis in a model of rheumatoid arthritis (Willis et al., 2011, 2017). During the deimination process of proteins, as arginine is converted into citrulline, the by-product is ammonia (Vossenaar et al., 2003; and Fig. 1) and interestingly, it has previously been reported that ammonia can disrupt the thioester bond of C3, with this 'amidated' form of C3 serving as an alternative pathway convertase that can generate C5b-9 and stimulate phagocytic oxidative metabolism (Hostetter and Johnson, 1989). This same study showed that approximately 2.88 mM of NH_3 leads to statistically significant disruption of the C3 thioester *in vitro* (Hostetter and Johnson, 1989). LC-MS/MS analysis of deiminated proteins in halibut serum further confirmed the presence of deiminated halibut C3 in serum as well as various other complement components, including C5, C7, C8, C9, which form the membrane attack complex leading to lysis of the pathogen; C1 inhibitor was also identified as deiminated (Table 1). C1 inhibitor is a natural serpin and multi-functional serine protease inhibitor with regulatory roles in different plasmatic cascades including the complement system (Cooper, 1985; Sim and Tsiftsoglou, 2004; Wouters et al., 2008). While C1 is the defining component of the classical pathway, C1 is also implicated in multiple non-complement functions including binding of apoptotic cells, cleavage of nuclear antigens and cleavage of MHC class I molecule (Lu and Kishore, 2017). C1 inhibitor has been identified as the main regulator of the complement system in black rockfish (*Sebastes schlegelii*) (Nilojan et al., 2018), while in Nile tilapia (*Oreochromis niloticus*) C1-inibitor is found upregulated in head kidney upon immunostimulation (Ding et al., 2017). It has been suggested that activation of PAD through calcium influx during complement and perforin activity may contribute to deiminated autoantigen production in autoimmunity in rheumatoid arthritis (Romero et al., 2013; Darrah and Adrade, 2018). It has also been reported that in human oral mucosa, *Porphyromonas gingivalis* uses its PAD to evade killing by the complement system by disabling anaphylatoxin C5a protein function via deimination of a critical C-terminal arginine of C5a (Bielecka et al., 2014). PAD proteins have indeed been described in various bacterial fish pathogens including in *Vibrio anguillarum, Vibrio splendidus*, *Aeromonas salmonicida* and *Photobacterium damselae*, all of which affect a variety of culture fish and larval rearing (Reid et al., 2009; Magnadottir, 2010; Bartkova et al., 2017; Bowden et al., 2018). Based on the findings described above and the novel finding that C3 and C4 are deiminated in halibut serum, PADs and their protein deiminating activity, alongside the ammonia by-product released during the deimination of target proteins, may have diverse and multiple effects on complement activity, and add to flexible functions of diverse C3 and C4 isoforms, as well as other complement components, both in physiological and pathophysiological processes. Due to the fact that the halibut genome is not annotated, the hits identified here are against complement components from other teleost, besides halibut C3 which has previously been partially sequenced (Lange et al., 2006).

Further deiminated proteins verified using Western blotting in halibut sera were pentraxin-like protein (Fig. 2C), which previously was also shown to be deiminated in cod serum and mucus (Magnadottir et al., 2018b). Pentraxins participate in homeostatic regulation as well as forming part of the innate immune response and are ancient pattern recognition molecules that evolved alongside the complement system (Robey and Liu, 1981; Pepys et al., 1987; Martinez de la Torre et al., 2010). CRP activates the complement system via binding to C1q and has thus roles both in the clearance of bacteria as well as of altered and dying cells (Szalai et al., 1999; Mihlan et al., 2011; Thiele et al., 2015). In addition, C5a has been shown to promote up-regulation of acute-phase expression of CRP (Szalai et al., 2000). As changes in levels of CRP, observed in some pathologies, are not always consistently raised, there is an increased interest in putative effects of structural changes in CRP (Ji et al., 2007; Eisenhardt et al., 2009; Thiele et al., 2014; Bello-Perez et al., 2017; Braig et al., 2017). Interestingly, a variation was observed in the intensity of deimination positive reaction corresponding to band sizes expected for the 22 kDa monomeric pentraxin-like band, *versus* higher deimination positive bands at 50, 75 and 90 kDa respectively, possibly indicating a difference in deimination levels according to monomeric or multimeric forms. The most prominent deimination positive band was observed for pentraxin in the expected size for a trimeric pentraxin form at approximately 75 kDa (Fig. 2C), albeit it has to be considered that post-translational modifications and structural changes may also affect the migration of multimeric forms in the gel. The post-translational deimination of pentraxin, described here as well as recently in cod (Magnadottir et al., 2018b), may be of considerable importance in relation to CRP function due to structural changes, and elucidate novel functions in relation to CRP-associated pathologies in humans, such as autoimmune diseases, amyloidosis and cancer.

Proteomic analysis carried out on deiminated proteins in halibut sera revealed further target proteins associated to immunogenic and metabolic function (Table 1) and are discussed below:

Vitallogenin is a hepatic protein and a precursor of egg-yolk protein and is important for oocyte development and embryogenesis (Matsubara et al., 1999; Arukwe and Goksøyr, 2003). In fish, it is a major maternal immunocompetent protein and important for offspring (Magnadottir et al., 2004; Zhang et al., 2015) and also has anti-oxidant activities (Sun and Zhang, 2015). It is secreted into the bloodstream and its synthesis is under the control of estrogen and other hormones (Lubzens et al., 2010). Vitellogenin is also used as a biomarker of fish exposure to estrogenic compounds and other pollutants in aquatic environments (Marin and Matozzo, 2004; Leonardi et al., 2012; Jung et al., 2018). In male vertebrates, the hepatic expression of vitellogenin is to date the most validated biomarker of estrogenic exposure (Verderame and Scudiero, 2017). Deiminated forms of vitallogenin have not been described before.

Apolipoprotein A-I is a high density lipoprotein and shown to have anti-bacterial effects in carp (*Cyprinus carpio*) (Concha et al., 2004); while it has been shown to be upregulated in liver of *Vibrio anguillarum*

infected seabass (*Dicentrarchus labrax*) (Sarropoulou et al., 2009) and is described in skin mucus of lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017). It also changes as part of the acute phase and stress response in acclimation to changes in water temperatures (Dietrich et al., 2018). In cod serum, and throughout early ontogeny, Apo A-I is associated with complement component C3 (Magnadottir and Lange, 2004; Lange et al., 2005). Apo A-I was here found in deiminated forms in halibut serum but was also recently described in deiminated form in cod mucus (Magnadottir et al., 2018a). In human, Apo A-I is primarily involved in lipid metabolism where conformational plasticity and flexibility are regarded as key structural features (Arciello et al., 2016), which makes the current finding of deiminated forms in serum of quite some relevance. Apo A-I is also associated with regulation of mitochondrial function and bioenergetics (White et al., 2017). Furthermore, Apo A-I has been shown to have a regulatory role in the complement system by affecting MAC assembly and thus the final lytic pathway in two different ways: Firstly, Apo A-I can bind to C9 polymers and thus interfere with the assembly of the poly C9 tubule and its insertion into the cell membrane (Hamilton et al., 1993). Secondly, Apo A-I can form complexes with clusterin, interfering with the binding of C5b67 to cell membranes (Jenne et al., 1991; French et al., 1994).

Keratin has pore-forming abilities and is involved in anti-bacterial defences in skin mucus as well as serving as a first barrier to injury as a cytoskeletal protein involved as first barrier (Molle et al., 2008). Downregulation of keratin II has been observed in *vibrio* infected cod (Rajan et al., 2013), and post-translational differences (albeit not deimination) of two forms of keratin have been associated with cod larval development (Sveinsdottir et al., 2008), while deiminated keratin was recently identified in cod mucus (Magnadottir et al., 2018a). In mammals, deimination of keratin is important for example in skin physiology associated to cutaneous diseases (Chavanas et al., 2006; Ying et al., 2009).

Inter-alpha-trypsin inhibitor (heavy chain H2; ITIH2) belongs to the serpin family of proteins, which have protease-inhibitory functions and are involved in diverse physiological and pathophysiological processes including inflammation, coagulation, as well as tumorigenesis, metastasis and dementia (Weidle et al., 2018). Inter-alpha-trypsin inhibitor is synthesised in the liver, circulates in the blood and has two chains, a light and heavy chain, whereof the heavy-chain (ITIH) includes a von Willebrand domain and can interact with the extracellular matrix (Bost et al., 1998). The ITIH is associated to physiological functions including fertilisation, ovulation, inflammation, as well as to cancer (Zhuo and Kimata, 2008; Weidle et al., 2018). ITIH is downregulated in tumours via methylation and ITIH2 is strongly reduced in invasive cancers (Hamm et al., 2008). In teleosts, ITIH has been associated with responses to acute phase and stress responses in temperature acclimation of carp (*Cyprinus carpio*) (Dietrich et al., 2018) and related to changes in innate immune responses in growth hormone transgenic amago salmon (*Oncorhynchus masou*) (Mori et al., 2007).

Albumin is a major acidic plasma protein in vertebrates and serves as a transport molecule for fatty acids, bilirubin, steroids, amino acids and copper, as well as having roles in maintaining the colloid osmotic pressure of blood (Peters, 1996; Metcalf et al., 2007). Albumin has been characterised in Atlantic salmon (*Salmo salar* L.) (Byrnes and Gannon, 1990; Maillou and Nimmo, 1993a), in rainbow trout (*Salmo gairdneri* and *Oncorhynchus mykiss*) (Maillou and Nimmo, 1993b; Gong and Hew, 1998), as well as in Chinook salmon (*Oncorhynchus tshawytscha*) and brown trout (*Salmo trutta*) (Metcalf et al., 1998a). Albumin 1 was identified as deiminated in halibut serum and has previously been described as a glycoprotein in brown trout (*Salmo trutta*) (Metcalf et al., 1998b) while in Australian lungfish (*Neoceratodus forsteri*) it was reported not to be glycosylated, similar as in tetrapod (Metcalf et al., 2007). In Nile tilapia (*Oreochromis niloticus*) albumin levels have been reported to be raised in upon heavy metal exposure (Firat and Kargin, 2010).

Thioredoxin are small evolutionarily conserved proteins that are essential for the maintenance of cellular homeostasis and circulate in plasma as part of thiol/disulphide redox pools, which participate in various physiological processes; as well being implicated in pathological processes such as cardiovascular, cancer, neurodegenerative and autoimmune diseases (Oliveira and Laurindo, 2018; Smallwood et al., 2018). In teleost, two forms of thioredoxin has been described in black rockfish (*Sebastes schlegelii*), and show differing expression in peripheral blood leucocytes, liver and gills upon immunostimulation (Park et al., 2012); while in rock bream (*Oplegnathus fasciatus*) an antioxidant enzyme belonging to the peroxiredoxin subfamily was detected in eleven tissues with the highest level in the heart (Saranya Revathy et al., 2015) and shown to have a role in maintaining redox balance upon pathogen invasion (Godahewa et al., 2018). In Japanese flounder (*Paralichthys olivaceus*), thioredoxin mRNA expression levels showed roles in ontogeny and in anti-oxidation and immunoregulation (Yuan et al., 2016). Thioredoxin was also found upregulated in European flounder (*Platichthys flesus*) upon oxidative stress response to cadmium (Sheader et al., 2006).

Serotransferrin forms part of fish innate immunity and serves as an antimicrobial agent (Stafford and Belosevic, 2003; Audunsdottir et al., 2012; Mohd-Padil et al., 2013). Serotransferrin has previously been described in skin mucus of olive flounder (*Paralichthys olivaceus*) (Palaksha et al., 2008), while also found in cod mucus (Caipang et al., 2011; Easy et al., 2012), including in deiminated form (Magnadottir et al., 2018a). It is upregulated in infected channel catfish (Peatman et al., 2008), vaccinated cod (Caipang et al., 2008), and in pufferfish (*Takifugu rubripes*) exposed to tetrodotoxin (Kiriake et al., 2016); while it is downregulated in Japanese flounder (*Paralichthys olivaceus*) infected with *Edwardsiella tarda* (Wang et al., 2017).

Trypsinogen precursor is a pancreatic inactive precursor of trypsin that is secreted into, and activated in the intestine (Buettner et al., 2014). In teleost it has been described in pufferfish (*Takifugu rubripes*), in snakehead (*Channa argus*) (Zhou et al., 2012) and in medaka fish (*Oryzias latipes*), where it is implicated in testis function (Rajapakse et al., 2014), while in Antarctic fish (*Paranotothenia magellanica*) it is related to cold adaption (Genicot et al., 1996). In Senegalese sole (*Solea senegalensis Kaup*) six trypsinogens have been described in tissues and during larval development (Manchado et al., 2008). Changes in protein surface charge have been shown to change the activation of trypsinogen (Buettner et al., 2014) and it has been suggested as a biomarker for pancreatic cancer (Gao et al., 2010) and in pro-enzyme therapy of cancer (Novak and Trnka, 2005).

Anionic trypsin-1 is a trypsinogen and in human two anionic trypsin forms are found in pancreatic juice (Figarella et al., 1975; Lee et al., 2017). It has recently also been found to be modulated in glioma cells (Chen, 2013). Anionic trypsin has been described in eel (*Anguilla japonica*) (Yoshinaka et al., 1985), in intestine of the carnivorous fish smooth hound (*Mustelus mustelus*) (Bougatef et al., 2010), in hepatopancreas of Japanese sea bass (*Lateolabrax japonicus*) (Cai et al., 2011), as well as in pyloric caeca of mandarin fish (*Siniperca chuatsi*) (Lu et al., 2008), while three forms have been isolated from chum salmon (*Oncorhynchus keta*) (Toyota et al., 2009).

Hemopexin (Wap65) is a scavenger protein of haemoglobin and a predominant heme binding protein, which contributes to heme homeostasis (Smith and McCulloh, 2015; Immenschuh et al., 2017). Hemopexin also associates with high density lipoproteins (HDL), influencing their inflammatory properties (Mehta and Reddy, 2015). In fish, hemopexin is also named Warm temperature acclimation-associated 65-kDa protein (Wap65), which is a plasma glycoprotein, and is associated with physiological stresses, including increased water temperature, immune response and heavy metal exposure. Wap65 has been identified in two different forms in teleosts, which differ in response to stress-factors, in Kumgang fat minnow (*Rhynchocypris kumgangensis*) (Kwon and Ghil, 2017); as well as in turbot (*Scophthalmus maximus*), where they play important roles in the inflammatory response (Diaz-Rosales et al., 2014). Hemopexin has also been described in sea bass

(*Dicentrarchus labrax*) and sea bream (*Sparus aurata*), where it was found in various tissues while the main site of expression was the liver (Pierre et al., 2010). In channel catfish (*Ictalurus punctatus*) two forms identified differed in tissue expression, with one form specific to the liver (Sha et al., 2008); and in ayu (*Plecoglossus altivelis*), Wap65 was found to be upregulated in liver upon infection with *Listonella anguillarum* (Shi et al., 2010). In goldfish (*Carassius auratus* L.), hemopexin was detected in kidney, liver and spleen upon *Trypanosoma carassii* infection (Kovacevic et al., 2015). In Atlantic salmon (*Salmo salar*), hemopexin was identified as an immune related factor following infection with sea lice (Easy and Ross, 2009). While hemopexin is a known glycoprotein, post-translational deimination is here revealed for the first time.

Ig heavy chain V and Ig lambda-6 chain C region were identified here as being deiminated in halibut serum and scored with these Ig parts from large yellow croaker (*Larimichthys crocea*). Immunoglobulins (Ig) are key molecules in adaptive immunity and have been studied as part of the humoral immune response in adult halibut (Lange et al., 2001) and during early teleost ontogeny of several teleost species (Magnadottir et al., 2010). In recent years teleost Ig have received considerable attention for being much more diverse than previously thought with multiple Ig isotypes IgM, IgD, IgNAR and IgZ/T in bony fish (Wilson et al., 1997; Danilova et al., 2005; Zhang et al., 2010; Hikima et al., 2011; Fillatreau et al., 2013; Zhu et al., 2014; Basu et al., 2016; Patel et al., 2016; Zhang et al., 2016a,b; Zhang et al., 2017; Fu et al., 2018). While glycosylation of teleost Ig has been studied (Magnadottir et al., 2002; Nath et al., 2006) and activation-induced cytidine deaminase, a DNA-editing deaminase, is implicated as a crucial factor in Ig diversification, including in fish (Patel et al., 2018), post-translational deimination of Ig's has hitherto received little attention. A recent study reported deimination in the Fc region of IgGH in patients with bronchiectasis and RA (Hutchinson et al., 2017). Deimination of Ig's is thus a relatively novel concept that may add to furthering understanding of Ig diversity.

Fibrinogen is a glycoprotein, synthesised in liver (Tennent et al., 2007) and forms part of the acute phase response as part of the coagulation cascade (Tiscia and Margaglione, 2018). Impaired mechanism of fibrinogen formation and fibrin polymerization are implicated with various pathologies including coagulopathies and ischemic stroke (Weisel and Litvinov, 2013), while acquired fibrinogen disorders can be associated with cancer, liver disease or post-translational modifications (Besser and MacDonald, 2016). Fibrinogen is indeed a known deimination candidate and this post-translational modification contributes for example to its antigenicity in autoimmune diseases (Hida et al., 2004; Muller and Radic, 2015; Blachère et al., 2017). In teleost, fibrinogen has been associated with brain regeneration in *Apteronotus leptorhynchus* (Ilieş et al., 2012); and roles in the coagulation system in host defence against pathogens have been described in turbot (*Scophthalmus maximus* L.) (Blanco-Abad et al., 2018), as well as in acute phase and stress responses during temperature acclimation of carp (*Cyprinus carpio*) (Dietrich et al., 2018). Fibrinogen has been found to be increased in liver of African lungfish (*Protopterus annectens*) after aestivation (Hiong et al., 2015), and to be upregulated in pufferfish (*Takifugu rubripes*) exposed to tetrodotoxin (Kiriake et al., 2016). In addition, roles for fibrinogen-like protein have been associated with anti-bacterial immunity of whiteleg shrimp (*Litopenaeus vannamei*) (Tian et al., 2018) and red swamp crayfish (*Procambarus clarkia*) (Chen et al., 2016).

Elongation factor-1 alpha plays roles in the immune response as well as in cytoskeleton organisation and nuclear export of proteins (Khacho et al., 2008). It is involved in cell growth regulation and apoptosis and is linked to degranulation of neutrophils (Talapatra et al., 2002; Hamrita et al., 2011; Vera et al., 2014). It has been described in deiminated form in cod mucus (Magnadottir et al., 2018a). Deiminated Elongation factor-1 alpha identified here, scored with *Aphanomyces invadans*, which is a eukaryotic pathogen causing epizootic ulcerative syndrome, a global threat to wild and farmed fish, causing up to 100% mortalities in aquaculture (Iberahim et al., 2018).

Cathepsin L is an important cysteine protease found in lysosomes and found throughout vertebrate phylogeny (Zhou et al., 2015). Cathepsins are found in the extracellular space as well as in the cytosol and nucleus, and have physiological and pathophysiological roles, acting both as digestive and regulatory proteases and serve in host immune response (Reiser et al., 2010). Cathepsin has roles in epidermal differentiation (Brocklehurst and Philpot, 2013), while cathepsin L found in secretory vesicles is a key protease for proteolytic processing of proneuropeptides into active neuropeptides for neurotransmission in the nervous system (Hook et al., 2012). Cathepsin is also associated to angiogenesis, progression and metastasis in various cancers (Verissimo et al., 2011; Pranjol et al., 2015; Sudhan and Siemann, 2015) and found to promote pancreatic injury through anti-apoptotic effects (Thrower et al., 2010). In teleost, cathepsins have been described in eggs and larvae of sea bass, cod and salmonids and may have a bactericidal role in the skin of fish (Magnadottir et al., 2004, 2005). Cathepsin has also been described in red drum (*Sciaenops ocellatus*) and suggested to have anti-bacterial activity (Sun and Hu, 2015). In large yellow croaker (*Larimichthys crocea*) it is found expressed in various tissues and immune-related cells at varying levels (Li et al., 2015); while in Japanese flounder (*Paralichthys olivaceus*) it is upregulated upon bacterial infection (Wang and Sun, 2015). Cathepsin-L is also associated to vitellogenesis and oocyte maturation in mud minnow (*Fundulus heteroclitus*) (Fabra and Cerdà, 2004).

Ceruloplasmin is a serum ferroxidase with antioxidative function and roles in iron homeostasis and carries over 90% of the copper in plasma (Liu et al., 2011). In tilapia (*Oreochromis mossambicus*) it is upregulated as an acute phase protein in response to growth hormone (Yada, 2007) and in channel catfish (*Ictalurus punctatus*) it is upregulated in liver upon bacterial challenge (Liu et al., 2011) and related to bacterial resistance in rohu (*Labeo rohita*) (Sahoo et al., 2013). In goldfish (*Carassius auratus* L.), ceruloplasmin was found in kidney, liver and spleen upon *Trypanosoma carassii* infection (Kovacevic et al., 2015) and formes part of the immune response in gilthead sea bream (*Sparus aurata*) parasitized by *Sparicotyle chrysophrii* (Henry et al., 2015). Ceruloplasmin contributes to acute response of zebrafish (*Danio rerio*) skin (Lü et al., 2013) and ceruloplasmin levels were raised in Nile tilapia (*Oreochromis niloticus*) upon heavy metal exposure (Firat and Kargin, 2010). Fish can use iron deprivation as a nutritional immunity mechanism, withholding iron from iron-requiring pathogens, and thus hinder bacterial multiplication. Iron binding capacity of halibut has previously been reported to be high, suggesting it to be an effective mechanism in delaying or hindering bacterial infection (Lange et al., 2001). The Sub-Antarctic Notothenioid *Eleginops maclovinus* is also reported to show putative use for ceruloplasmin in withholding iron from pathogens (Martinez et al., 2017).

Plasminogen is a serine proteinase precursor and involved in embryogenesis, tissue regeneration and neoplasia (Cottage et al., 1999; Plow et al., 2012), as well as influencing inflammatory cell migration (Das et al., 2010). Plasminogen related growth factors have been identified in puffer fish (*Fugu rubripes*) (Cottage et al., 1999) and plasminogen is linked to ovulation in medaka (*Oryzias latipes*) (Ogiwara et al., 2012). Plasminogen-activating cascades have been identified in plasma proteomes of marine and freshwater of three-spined stickleback (*Gasterosteus aculeatus*) (Kültz et al., 2015). Serine protease function has previously been studied in halibut, where it showed sensitivity to heat and storage at −20 °C (Lange et al., 2001). Plasminogen has been suggested to play important roles in innate immunity by changing gene expression contributing to phagocytosis (Das et al., 2014). In Atlantic salmon (*Salmo salar*), plasminogen was identified as an immune related factor following infection with sea lice (Easy and Ross, 2009).

The temporal and spatial detection of peptidylarginine deiminase and deiminated protein products throughout early halibut ontogeny indicates relevant roles for tissue remodelling and immune defences. In

comparison to a previous analysis on deiminated proteins in cod mucus, some hits are the same, including serotransferrin, Apo A-I, keratin and Elongation factor-1 alpha (Magnadottir et al., 2018a), while in halibut serum critical proteins involved in the acute phase response, including multiple components of the complement system, were prominent hits. The identification of deiminated forms of pentraxin-like protein was here identified in halibut serum and correlates with previously deiminated forms of CRP identified in cod serum and mucus (Magnadottir et al., 2018b), while deimination of C3 and C4 are shown for the first time in any species. Implications for post-translational deimination in adaptive immunity are also of considerable importance in the light of recent discoveries of the diversity of Ig's in teleosts. The identification here of Ig's as deimination targets in halibut serum may thus provide novel insights into the generation of diversity and mechanistic functions of Ig's in the immune response. As the halibut genome is not sequenced, this may somewhat have influenced the identification of deiminated hits in the proteomic analysis and thus possibly underestimated deiminated proteins present in halibut serum. Nevertheless, it is clear that critical components of the immune defence, as well as molecules participating in cytoskeletal organisation, metabolic pathways and nuclear functions are found deiminated in serum. Throughout ontogeny, PAD and deiminated protein products, including histone H3 which forms part of bactericidal activity and affects gene regulation, may display multiple roles in tissue remodelling and contribute to immune defences during halibut larval growth.

5. Conclusion

During development, tissue undergoes constant remodelling. Throughout ontogeny, PAD-mediated protein deimination may facilitate protein moonlighting, allowing for multiple functions of one polypeptide chain in response to functional requirements. During halibut larval development, a temporal shift towards PAD expression and deiminated proteins in sites of mucosal layers was observed, indicating roles in immune defences, as well as the brain and eye, which are tissues undergoing ongoing neurogenesis. The post-translational deimination of halibut complement components C3 and C4, as well as components of the MAC, is revealed here for the first time in any species, indicating a hitherto overlooked mechanism possibly contributing to diverse functions of complement components and consequently modulation of the immune response. Our findings may further current understanding of post-translational protein deimination in tissue remodelling and host-pathogen interaction, including via the modulation of the complement system. This may be of relevance both for prophylactic measures in aquaculture, as well being translatable to immune and pathological related functions of PADs and their deiminated protein products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2018.10.016.

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