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Extracellular vesicles, deiminated protein cargo and microRNAs are novel serum biomarkers for environmental rearing temperature in Atlantic cod (*Gadus morhua* L.)

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ABSTRACT

Extracellular vesicles (EVs) are released from cells and carry protein and genetic cargo involved in cell communication. EVs isolated from bodyfluids, including plasma and serum, can also be used as markers of pathophysiological changes. Peptidylarginine deiminases (PADs) are phylogenetically conserved enzymes with physiological and pathophysiological roles and cause post-translational protein deimination. This can affect function of target proteins and deimination is also linked to EV release. Atlantic cod (*Gadus morhua* L.) reared at 4 °C and 9 °C respectively for 18 months, were here assessed for changes in serum derived EVs, including analysis of deiminated protein and micro-RNA cargo markers related to stress and growth. We found that cod reared at 9 °C showed significantly reduced numbers of EVs in serum, compared to cod reared at 4 °C. Some deiminated protein targets, including complement component C3, were found to be considerably higher in EVs of cod reared at 4 °C. Proteomic analysis revealed further differences in deiminated protein targets in EVs isolated from sera of the two temperature groups. Whole cod sera from the two temperature groups furthermore showed differences in deiminated protein targets, including C3, CRP and histone H3, which is a marker of neutrophil extracellular trap formation. MicroRNAs related to inflammation (miRNA-21) and stress (miRNA-155) were elevated in both total cod serum and serum-derived EVs from the 9 °C group, while the growth-related miRNA-206 was higher in the 4 °C group. Our findings highlight EVs as novel biomarkers to assess fish health in response to environmental rearing temperature.

1. Introduction

Extracellular vesicles (EVs) are 30–1000 nm lipid bilayer structures which are released from parent cells and participate in cell communication via transfer of cargo proteins, enzymes, DNA, lncRNA, RNA and microRNAs (Inal et al., 2013; Colombo et al., 2014; Kosgodage et al., 2018; Turchinovich et al., 2019; Vagner et al., 2019). EV release from cells is also linked to apoptotic processes and EVs play important roles in immunity and host-pathogen interactions (Inal et al., 2013; Gavinho et al., 2018, 2019). EVs can furthermore be used as biomarkers for assessment of health status and in response to therapeutic treatment (Hessvik and Llorente, 2018; Ramirez et al., 2018). While EVs are widely studied in human *in vitro* and *in vivo* models, particularly in relation to pathophysiology (Antwi-Baffour et al., 2010; Inal et al., 2013; Kosgodage et al., 2018; Wu et al., 2019), studies on EVs in teleost fish are scarce (Lagos et al., 2017; Iliev et al., 2018; Magnadottir et al., 2019a). This opens up a hugely understudied field of EV research in relation to teleost fish health, development and identification of novel biomarkers to assess fish health in aquaculture.

Peptidylarginine deiminases (PADs) mediate post-translational protein deimination in physiological and pathophysiological processes and are preserved throughout phylogeny from bacteria to mammals (Vossenaar et al., 2003; Rebl et al., 2010; Magnadottir et al., 2018a; Kosgodage et al., 2019; Magnadottir et al., 2019b). In fish only one PAD

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form has been identified (Rebl et al., 2010; Magnadottir et al., 2018a, b), while mammals have five PAD isozymes with tissue specific expression (Vossenaar et al., 2003; Wang and Wang, 2013; Witalison et al., 2015). Post-translational deimination can alter protein structure and protein-protein interactions, also facilitating protein moonlighting, an evolutionary acquired phenomenon where proteins are allowed to exhibit more than one physiologically relevant function within one polypeptide chain (Jeffrey, 2018). We have recently described novel roles for PADs and protein deimination in early ontogeny and innate immune defences in two teleost species, cod (*Gadus morhua* L.) and halibut (*Hippoglossus hippoglossus* L.), identifying a range of immunogenic, metabolic, cytoskeletal and nuclear proteins that are post-translationally deiminated in mucosa and serum (2018b; Magnadottir et al., 2019a, 2019b). Importantly, we further identified components of the acute phase response including C-reactive proteins (CRP/pentraxin) and complement component C3 to be deiminated in cod mucus and halibut serum respectively (Magnadottir et al., 2018b; Magnadottir et al., 2019a, 2019b), highlighting hitherto unknown immune-regulatory mechanisms via such post-translational modification.

A novel role for PADs in EV biogenesis and release was recently identified by our group (Kholia et al., 2015; Kosgodage et al., 2017), including effects on microRNA cargo (Kosgodage et al., 2018). EV research is a relatively new field in fish immunology. In rainbow trout (*Oncorhynchus mykiss*), EV-mediated export of heat shock protein has recently been described in response to stress (Faught et al., 2017) and extracellular DNA-mediated stimulation of EV release and export of MHCII has been shown in salmon (*Salmo salar* L.) leukocytes (Iliev et al., 2018). In cod, EVs have recently been described in mucus (Magnadottir et al., 2019a; Lange et al., 2019). Besides EV biogenesis and numbers of EVs released, EV cargo composition is of great importance for communication with the microenvironment. We have recently shown that both EV biogenesis and cargo composition are affected by PAD modulation (Kholia et al., 2015; Kosgodage et al., 2017; Lange et al., 2017; Kosgodage et al., 2018; Gavinho et al., 2019).

MicroRNAs (miRNAs) are gaining increased attention in teleost research as they are involved in post-transcriptional gene regulation by inhibiting translation, degradation and the regulation of mRNA (Pasquinelli, 2012; Bizuayehu and Babiak, 2014; Franchini et al., 2019; Leiva et al., 2019; Xiong et al., 2019). In fish immunity, various miRNAs have been identified both under normal conditions as well as part of stress responses and regulation of immunity. For example, miRNA-21 has been found to suppress cytokine production in teleost fish via targeting Toll-like receptor 28 (Bi et al., 2017) and to regulate inflammatory responses (Chu et al., 2019), including following bacterial infection (Tao et al., 2019), while miRNA-155 expression has been observed to be elevated in virus infected fish (Andreassen and Høyheim, 2017). MiRNA-155 has also been identified as a stress-marker in toxicology studies in silver carp (*Hypophthalmichthys molitrix*) (Ma et al., 2019) and zebrafish (*Danio rerio*) (Huang et al., 2016). MiRNA-155 has also been found to be upregulated in liver of common carp (*Cyprinus carpio*) in temperature tolerance studies (Sun et al., 2019). Furthermore, miRs related to growth, such as miRNA-206, have been implicated in regulation of tilapia growth (Yan et al., 2013), and have also been analysed in first feeding studies of cod (Bizuayehu et al., 2016). Functional roles of miRNAs in temperature adaptation is a growing research topic in fish biology (Sun et al., 2019), including in cod (Bizuayehu et al., 2015). As miRNAs are known to be exported via EVs, changes in such EV cargo in response to environmental changes may be of considerable interest.

Further understanding of the contribution of EV regulation and EV-mediated export of protein cargo and miRNAs, under normal culture conditions or environmental and immune-challenge, may inform the immunodiagnostic potential of EVs for optimal fish health.

2. Materials and methods

2.1. Fish and sampling

Experimentally farmed Atlantic cod (*Gadus morhua* L.), hatched and kept at the Marine Institute's Experimental Fishfarm Stadur, Grindavik, Iceland. The fish were reared for 12 months at 7 °C, which is the normal rearing temperature of adult cod, according to previously established conditions (Steinarsson and Björnsson, 1999; Magnadóttir et al., 2001). The fish (n = 45 per tank) were then moved into two circular fiberglass tanks and maintained at 4 °C or 9 °C, respectively. The tanks were 3 m in diameter and 0.8 m deep, supplied with sea water (32 ppm) connected with a flow-through system. The fish were fed commercial food pellets and natural photoperiod (64 N) was provided. The fish were kept in the two temperature controlled tanks (4 °C and 9 °C) for a period of 18 months and at the end of this period, blood samples were collected from a caudal vessel from four fish from each temperature tank (approximately 7 ml blood per fish). The blood was clotted at room temperature for 2 h and thereafter at 4 °C overnight, followed by collection of sera by centrifugation at 750 *g* for 10 min. The sera were divided into individual 250 µl aliquots, to avoid freeze-thawing cycles of individual serum samples, and stored at −80 °C until further use.

2.2. Extracellular vesicle isolation

Extracellular vesicles (EVs) were isolated from cod sera using stepwise centrifugation as follows: First sera were centrifuged at 4000 *g* for 30 min at 4 °C to remove cell debris and aggregates. Thereafter the supernatant was collected and diluted 1:4 in Dulbecco's phosphate buffered saline (DPBS; sterile filtered using 0.22 µm filters) and ultracentrifuged at 100,000 *g* for 1 h at 4 °C. The obtained EV pellets were then re-suspended and washed in DPBS (sterile filtered in 0.22 µM filters) and ultra-centrifuged again at 100,000 *g* for 1 h at 4 °C. The resulting pellets were solubilised in 50 µl DPBS and subjected to either nanoparticle tracking analysis (NTA) or protein extraction for immunoprecipitation and Western blotting analysis.

2.3. Transmission electron microscopy

Serum derived EVs were analysed for morphology using transmission electron microscopy (TEM). In brief, isolated EV pellets were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0) for 1 h at 4 °C and resuspended in 100 mM sodium cacodylate buffer (pH 7.0). The EVs were placed on to a grid with a glow discharged carbon support film, stained with 2% aqueous Uranyl Acetate (Sigma-Aldrich) and thereafter viewed in TEM. Imaging was performed using a JEOL JEM 1400 transmission electron microscope (JEOL, Japan) operated at 80 kV at a magnification of 80,000–100,000. Digital images were recorded using an AMT XR60 CCD camera (Deben, UK).

2.4. Nanoparticle tracking analysis (NTA) of EVs

For NTA analysis, the individual EV pellets derived from 250 µl of serum each, were resuspended in 50 µl DPBS and then diluted 1/200 in DPBS for NTA. EVs from 4 individual sera of each temperature group respectively, were counted using a NanoSight NS300 (Malvern, U.K.). Samples were applied to the NanoSight using a syringe pump to ensure even flow of the sample. Numbers of particles in the window were kept at 40–60 per frame. Videos were recorded for 5 × 60 s and the histograms generated from the repetitive reads were averaged per sample for each of the two temperature groups.

2.5. Western blotting

Atlantic cod sera (a pool of n = 4 per experimental temperature group) and serum-derived EVs (a pool of n = 4 per experimental

temperature group) were analysed by Western blotting for the detection of total deiminated proteins using the pan-deimination F95 antibody (MABN328 Merck, U.K), which is raised against a deca-citrullinated peptide and specifically detects proteins modified by citrullination (Nicholas and Whitaker, 2002). Cod sera and EVs were also assessed for deiminated histone H3 (citH3; ab5103, Abcam, U.K.) and PAD2 (ab50257, Abcam), anti-cod complement component C3 (characterised and described in Lange et al., 2004), as well as anti-cod CRP-I and CRP-II (characterised and described in Gisladottir et al., 2009 and Magnadottir et al., 2018a). In addition, serum-derived EVs were assessed for the EV-specific markers CD63 (ab68418, Abcam) and Flotillin-1 (Flot-1, ab41927, Abcam). Samples were reconstituted in 2 x Laemmli sample buffer (BioRad, U.K.) containing 5% beta-mercaptoethanol (Sigma, U.K.) and boiled for 5 min at 100 °C. Samples were separated on 4–20 % gradient TGX gels (BioRad). Even protein load, following protein measurement using the Bradford assay, was applied per each lane and proteins were transferred to 0.45 µm pore size nitrocellulose membranes (BioRad). Even protein transfer was assessed using Ponceau S staining (Sigma, U.K.). Membranes were blocked in 5% bovine serum albumin (BSA, Sigma) in Tris buffered saline with 0.1% Tween20 (TBS-T) for 1 h, followed by incubation at 4 °C overnight with the following primary antibodies diluted in TBS-T: F95 (1/3000); citH3 (1/2000); CRP-I and CRP-II (1/1000); C3 (1/1000); CD63 (1/1000) and Flot-1 (1/2000). Membranes were washed 3 times in TBS-T, incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (anti-mouse IgM, anti-mouse IgG or anti-rabbit IgG; BioRad, U.K.; diluted 1/4000 in TBS-T), followed by 6 washes in TBS-T before visualisation with ECL (Amersham, U.K.) using the UVP BioDoc-IT™ System (U.K.). Densitometry analysis of protein bands was carried out using ImageJ and normalisation was performed against the HRP-conjugated anti-beta-actin antibody (ab20272, Abcam, 1/5000 in TBS-T), which was used as the internal loading control (indicated by "R" which represents relative densitometry compared to β-actin).

2.6. Immunoprecipitation and protein identification in cod EVs

Total deiminated proteins were isolated by immunoprecipitation from a pool of 4 sera per experimental temperature group (100 µl serum pool constituted of 4 × 25 µl of individual sera) and a pool of 100 µl of isolated EVs (25 µl of reconstituted EVs per each of the 4 individual cod serum EV preparations) per experimental temperature group (4 °C or 9 °C), respectively. 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 (MABN328, Merck) for isolation of total deiminated proteins. F95-bound proteins were eluted under reducing conditions, run 1 cm into a 4–20 % gradient TGX gel (BioRad) and cut out as one band per sample and analysed by liquid-chromatography mass spectrometry (LC-MS/MS) (Cambridge Centre for Proteomics, U.K.). Peak list files were submitted to in-house Mascot (Cambridge Centre for Proteomics), using the following database: *Gadus_morhua*_20190405 (1283 sequences; 308,668 residues), and with setting set at significance threshold $p < 0.05$ and cut-off at Ions score 20.

2.7. Micro-RNA analysis

Serum pools from both temperature groups (4 °C and 9 °C; n = 4 per group and pooled), as well as individual EV isolates for each of the two temperature groups (n = 4 per group), were assessed for the expression of miRNA-21, miRNA-155 and miRNA-206, which are representative of immune and growth-related miRNAs, respectively. RNA was extracted from sera and EVs using Trizol (Sigma, U.K.). RNA concentration and purity were measured using the NanoDrop Spectrophotometer at 260 nm and 280 nm absorbance. The qScript micro-RNA cDNA Synthesis Kit (Quantabio, U.K.) was used to reverse-transcribe the RNA to cDNA, according to the manufacturer's instructions, and the resulting cDNA was used to assess the expression of miRNA-21 and miRNA-155 and miRNA-206. MiRNAs miR-25-3p and miR-210-5p, previously assessed as the most suitable normalizers for miRNA expression in Atlantic cod (Andreassen et al., 2016), were used as reference for normalization of miRNA expression levels. The PerfeCTa SYBR Green SuperMix (Quantabio, U.K.) was used together with MystiCq microRNA qPCR primers for the miRNA-21 (hsa-miR-21-5p), mirNA-155 (hsa-miR-155-5p) and miRNA-206 (hsa-miR-206-5p), obtained from Sigma (U.K.) and verified to be conserved. The following thermocycling conditions were used: denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 2 s, 60 °C for 15 s, and extension at 72 °C for 15 s. Normalization and relative expression level calculation was carried out using the 2ΔΔCT method according to Livak and Schmittgen (2001). Each reaction was repeated three times.

2.8. Statistical analysis

For preparation of graphs and histograms, as well as for statistical analysis, GraphPad Prism version 7 (GraphPad Software, San Diego, U.S.A.) was used. Significance differences were considered as $p \leq 0.05$, following Student's t-test, to assess differences between the two temperature groups.

3. Results

3.1. Extracellular vesicle release analysis in cod sera – effects of environmental rearing temperature

EVs from cod sera were characterised by size distribution based on Brownian motion of particles in suspension using nanoparticle tracking analysis (NTA), by morphological analysis using transmission electron microscopy (TEM) and by Western blotting using the EV-specific markers CD63 and Flot-1 (Fig. 1). A poly-dispersed population of mainly 30–500 nm sized vesicles was observed by NTA, with the majority of EVs being 40–300 nm and main peaks around 115–125 nm with smaller peaks at 300–400 nm for both temperature groups (Fig. 1). The total number of EVs present in serum of cod in the 9 °C group was found to be significantly lower ($p = 0.03$), as assessed by NTA analysis, than in serum of cod reared at 4 °C (Fig. 2A; n = 4 per group). Modal size of vesicles was not significantly affected (Fig. 2B; n = 4 per group).

3.2. Innate immune proteins in cod serum and serum-derived EVs

Total cod serum, as well as serum derived EVs, were analysed for the presence of the following immune proteins by Western blotting: CRP-I, CRP-II and complement component C3 (Fig. 3). Sera and serum-derived EVs were also assessed for total deiminated proteins, PAD and deiminated histone H3 (citH3) (Fig. 3). CRP-I and II were detected in EVs from both temperature groups (Fig. 3A-B, see arrows). Complement component C3 was also detected in EVs, with C3 α-chain, β-chain and bands representative of α –chain fragments (α-f) clearly visible (Fig. 3C). Protein levels of both C3 and CRP-I were found to be higher in EVs of the 4 °C group, compared to the 9 °C group. Total deiminated proteins (F95) were also detected at higher levels in EVs from the 4 °C group, compared to the 9 °C group, where deiminated proteins were of low abundance (Fig. 3D). In addition, the presence of PAD was verified at the expected 70–75 kDa size in EVs (Fig. 3E), with higher levels in the 4 °C temperature group, compared to the 9 °C group (Fig. 3E). Deiminated histone H3 was not detected in EVs by Western blotting (not shown).

In whole sera, CRP-I and II were detected, and both forms were found to be at somewhat higher levels in the 4 °C group, compared to the 9 °C group (4% and 12% respectively, Fig. 3F-G), also showing differences in oligomeric forms. Total C3 levels were higher in the 9 °C group, compared to the 4 °C group (9% increase; Fig. 3H). PAD protein

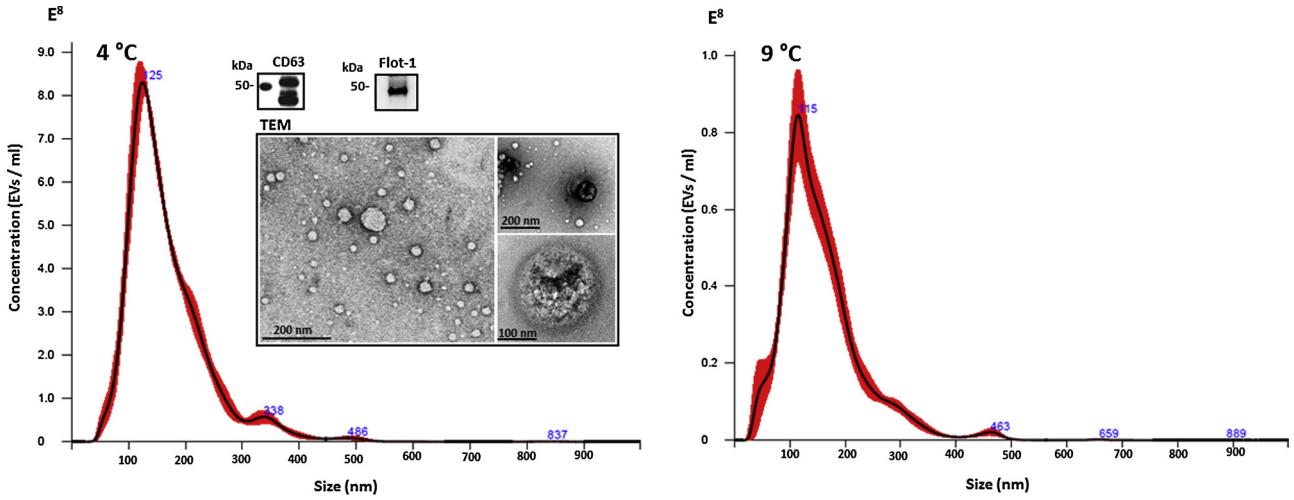

Fig. 1. Characterisation of EVs isolated from cod serum. EVs from cod sera were characterised by nanoparticle tracking analysis (NTA) for size distribution. A representative NTA histogram for a serum-EV sample for each temperature group is shown, with the black line representing the mean and the red lines (broad line) represent the standard error of the mean (SEM), based on five 60 s replicate video recordings of the same sample. Main EV-peaks are seen in the 115–125 nm range and smaller peaks of larger EVs are observed in the 200–400 nm size range. The Western blotting shows positive for the two EV-specific markers CD63 and Flot-1 (a pool of EVs isolated from 5 sera was used, mixed from both temperature groups). A composite image for representative morphological analysis of cod serum EVs by transmission electron microscopy (TEM) is shown, verifying EVs in the size ranges observed by NTA (scale bars are indicated at 100 and 200 nm, respectively) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

was detected in whole sera of both temperature groups (Fig. 3I) and deiminated histone H3 was found at higher levels in sera of the 9 °C group, compared to the 4 °C group (25% increase; Fig. 3J). Total deiminated proteins (F95), were detected in whole sera of both temperature groups, with higher levels in the 4 than the 9 °C group (Fig. 3K–L).

3.3. Deiminated forms of CRP-I, CRP-II and C3 in cod sera and serum-derived EVs

Deiminated F95 enriched protein eluates from serum derived EVs were analysed by Western blotting for C3, CRP-I and CRP-II to assess if these immune proteins were present in deiminated form. The F95 enriched eluates from EVs (Fig. 4A) showed positive for C3 in both temperature groups, with higher deiminated C3 levels in the 4 °C group, compared to the 9 °C group (Fig. 4B). Only the C3 β-chain showed positive for deimination in serum EVs (Fig. 4B). Both CRP-I and CRP-II showed negative for the F95 eluates from EVs of both temperature group (not shown). Deiminated F95 eluates from total sera (Fig. 4C) showed positive for C3 by immunoblotting (Fig. 4D), confirming higher levels of deiminated C3 in sera of the 4 °C group. Again, only a band representative for the expected size of the C3 β-chain showed F95 positive. For the F95 enriched eluates from total serum, both CRP-I and CRP-II showed positive in both temperature groups (Fig. 4E–F).

3.4. LC-MS/MS analysis of F95-enriched protein hits identified in serum-derived EVs

The F95-enriched eluates from serum-derived EVs, isolated from both temperature groups, were further analysed by LC-MS/MS and the peak files were submitted to Mascot (Tables 1 and 2). For both temperature groups, 13 proteins were identified to be in common, while 3 deiminated proteins were specific for EVs isolated from the 4 °C group, namely transglutaminase 2, ribosomal protein L8 and cathepsin K. For the 9 °C group, 2 deiminated protein candidates were specific: heath shock cognate 70 kDa protein and myosin heavy chain (Fig. 5 and Tables 1–2). Only hits scoring for cod protein sequences were included in this analysis.

3.5. Micro-RNA analysis of serum derived EVs

Total cod serum and serum-EVs from the two temperature groups were assessed for relative changes in the expression of three miRNAs related to inflammation (miRNA-21), stress-responses (miRNA-155) and growth (miRNA-206), respectively. In the 9 °C group, miRNA-21

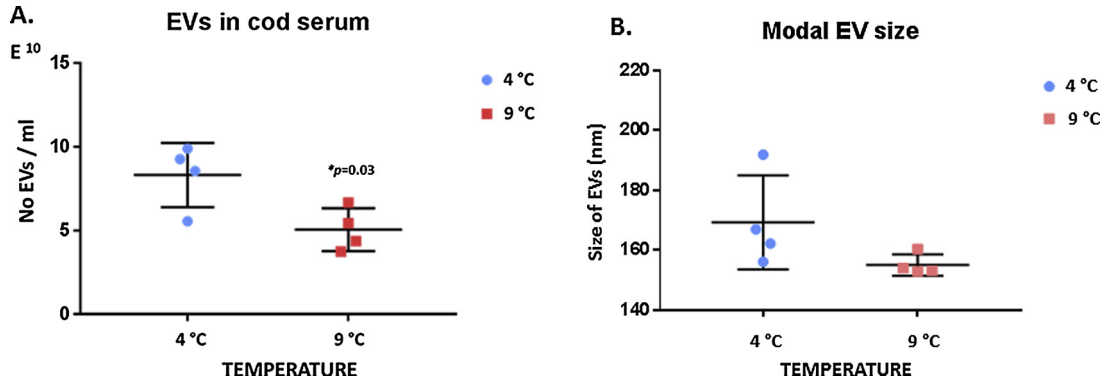

Fig. 2. EV numbers differ in serum of cod reared at 4 °C and 9 °C. A. The number of EVs present in serum of cod in the 9 °C group was found to be significantly lower compared to cod reared at 4 °C, as assessed by NTA analysis; exact *p*-value is indicated on the scatter plot, with each dot representing one individual serum-EV sample (n = 4). **B.** Modal size of EVs released did not show significant differences between the two temperature groups (n = 4). Error bars indicate standard deviation (SD).

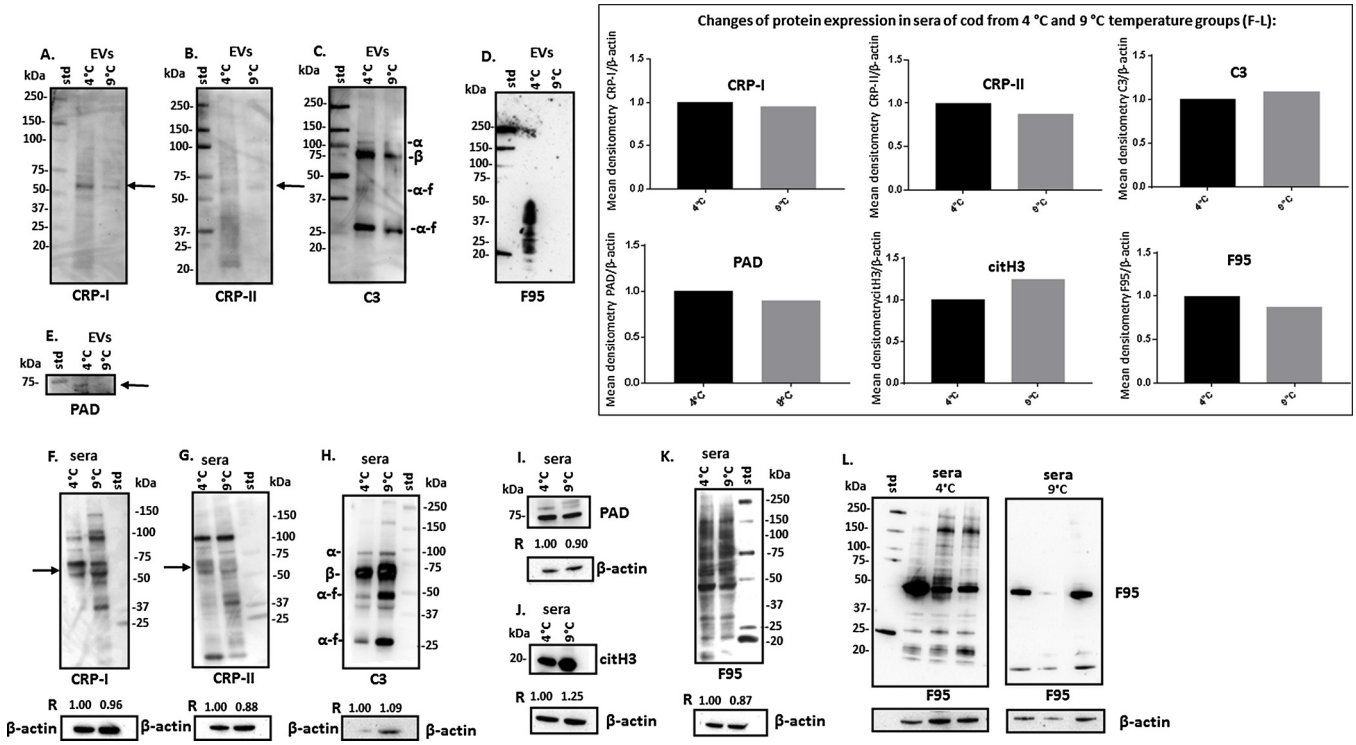

Fig. 3. Western blotting of cod EVs and cod sera for C3, CRP and deiminated proteins. A-E: Assessment of serum derived EVs from cod reared at 4 and 9 °C. A. CRP-I (arrow) is detected at higher levels in EVs from the 4 °C temperature group, compared to the 9 °C group (n = 4). B. CRP-II (arrow) is detected at higher levels in EVs from the 4 °C temperature group, compared to the 9 °C group (n = 4). C. Complement component C3 is detected at higher levels in EVs from the 4 °C temperature group, compared to the 9 °C group (C3 α-chain is detected at 115 kDa, β-chain at 74 kDa (β-chain) and α-chain fragments (α-f) at 50 and 25 kDa respectively (n = 4). D. Total deiminated proteins are detected as multiple bands between 20–150 kDa (as assessed by the pan-deimination F95 antibody) and found at higher levels in EVs from the 4 °C temperature group, compared to the 9 °C group. E. PAD (arrow) is detected at higher levels in serum-derived EVs from the 4 °C temperature group, compared to the 9 °C group (n = 4). F–J: Assessment of total serum from cod reared at 4 and 9 °C. F. CRP-I (arrow) is present at slightly higher protein levels in sera of the 4 °C group (4% based on relative densitometry compared to β-actin), compared to the 9 °C group (n = 4). G. CRP-II (arrow) is present at higher protein levels (12% increased) in sera of the 4 °C group, compared to the 9 °C group; some differences in oligomeric CRP forms (further bands present) are also observed (n = 4). H. Complement component C3 protein levels are higher (9% increased) in sera of the 9 °C temperature group, compared to the 4 °C group (n = 4). I. PAD protein is present at similar levels in serum of both groups (n = 4). J. Deiminated histone H3 levels are higher (25% increased) in sera from the 9 °C group (n = 4). K. Total deiminated proteins (F95) are lower in the 9 °C group (n = 4); individual sera are further shown in L (n = 3 per group). Levels of specific proteins assessed in total sera were normalised against beta-actin, which was used as the internal loading control (indicated by "R" which represents relative densitometry compared to β-actin) for assessment of relative differences. A pool of 4 serum or EV samples was used for each experimental group respectively and differences in protein expression in the sera of the two temperature groups are further represented in the histograms in Fig. 3.

was found to be significantly elevated both in total sera (189 fold) and in EVs (70 fold), compared to the 4 °C group. Similarly, miRNA-155 was also significantly elevated in both sera (122 fold) and EVs (47 fold) of the 9 °C group, compared to the 4 °C group. The growth-related miRNA-206 was found to be significantly reduced in both sera (2.7 fold) and EVs (2 fold) of the 9 °C group, compared to the 4 °C group (Fig. 6).

4. Discussion

This study assessed extracellular vesicles (EVs) and EV cargo as biomarkers for environmental rearing temperature in Atlantic cod (*Gadus morhua* L.), reared for 18 months at 4 °C and 9 °C, respectively. Specific deiminated protein cargo markers were identified in cod serum-EVs. Deiminated complement C3 was detected in whole cod serum and confirmed to be exported in EVs, both in native and deiminated form. Two cod pentraxins, CRP-I and CRP-II, were also detected in cod serum EVs, but not in deiminated form. To our knowledge, this is the first report of changes in miRNA-21, miRNA-155 and miRNA-206 in fish serum-EVs in response to environmental temperature differences.

EVs were isolated from cod serum and characterised according to the minimal information for studies of extracellular vesicles 2018 (MISEV2018) guidelines (Théry et al., 2018), using NTA, TEM and Western blotting for EV-specific markers. A poly-dispersed population of 30–600 nm, with the majority of EVs in the size range of 40–300 nm, was observed by nanoparticle tracking analysis and the EVs were positive for the EV-specific markers CD63 and Flot-1, which have previously been described to be conserved throughout phylogeny in bony fish (Iliev et al., 2018). EV numbers were significantly reduced in sera of fish reared at 9 °C for 18 months, compared to fish reared at 4 °C. As these fish were not exposed to immune-challenge, the difference in EV numbers can only directly be related to rearing temperature differences in this current study. Reduced EV numbers in serum may possibly be indicative of putative changes in cellular communication and possibly impact immune defences. Therefore, as there was a significant difference observed between the two temperature groups, EV count per se may be indicative of environmental temperature. Importantly, this can be linked also with changes in EV cargo, including specific immune proteins, post-translational protein deimination of key immune factors and inflammatory and growth related microRNAs, as identified here.

A range of immune related, metabolic, cytoskeletal and nuclear proteins have previously been described by our group to be deiminated in halibut serum and cod mucosa (Magnadottir et al., 2018a, 2018b, 20192018b, 2019a, 2019b). While some of these hits were identified here also in EVs of cod serum, new targets of deimination were further identified in response to temperature changes in the current study. Here

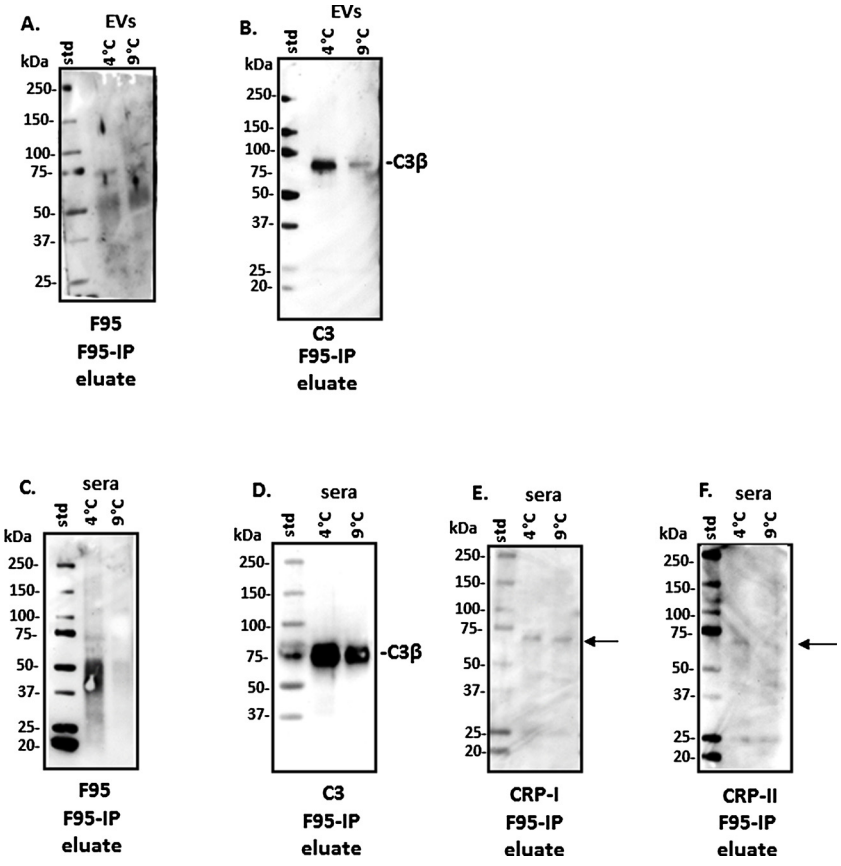

Fig. 4. Deiminated proteins in cod serum and serum-derived EVs. A-B. Deiminated proteins (F95 enriched eluates obtained after immunoprecipitation) are assessed in cod EVs: A. Total deiminated proteins were isolated from EVs by immunoprecipitation using the F95 antibody and the F95 enriched eluate was blotted against the F95 antibody. Multiple deimination positive protein bands were observed between 37–100 kDa mainly (n = 4). B. The F95 enriched eluates were blotted against complement component C3 antibody, showing positive for C3 β-chain and indicating that complement component C3 was more abundantly found in deiminated form in EVs from the 4 °C group, compared to EVs from the 9 °C group (n = 4). C-F: Deiminated proteins in cod total sera. C. The F95 enriched eluates from total sera were blotted against the F95 pan-deimination antibody, indicating higher abundance of deiminated proteins in sera of the 4 °C group, compared to sera from the 9 °C group (n = 4). D. The F95 enriched eluates, tested against the cod-C3 antibody, indicated higher abundance of deiminated C3 in sera of the 4 °C group, compared to sera from the 9 °C group (n = 4). E. The F95 enriched eluates, tested against the CRP-I antibody, indicated deiminated forms of CRP-I of similar abundance in serum of both temperature groups (n = 4). F. The F95 enriched eluates tested against the CRP-II antibody indicated the presence of deiminated forms of CRP-II in both temperature groups, albeit at somewhat higher level in the 4 °C group(n = 4). A pool of 4 EV or serum samples was used for each experimental group, respectively.

we found that while 13 proteins were in common as candidates of protein deimination in EVs for the two temperature groups (Tables 1 and 2), there were specific deimination candidates present in serum-derived EVs of cod reared at each of the two temperature groups respectively (Fig. 5). In EVs of the 4 °C group, transglutaminase 2, ribosomal protein L8 and cathepsin K were specific deimination candidates and not identified in EVs of the 9 °C group. Cathepsin K has previously been related to growth in cod (Lie and Moren, 2012), but has not been reported in deiminated form before. Transglutaminase 2 has been previously identified by our group to be deiminated in cod mucus (Magnadottir et al., 2018a), but is here identified as deiminated for the first time in serum-derived EVs. In cod, transglutaminase expression has previously been shown to be upregulated in head kidney in response to immunochallenge (Furnes and Robertsen, 2010). Ribosomal protein L8 was recently described as a deimination candidate in cod mucosa by our group (Magnadottir et al., 2018a) and was in the present study found to be exported in deiminated form in serum-derived EVs. Ribosomal proteins are involved in protein synthesis, antimicrobial function (Nuding et al., 2013) and modulation of cytokine production (Moon, 2011, 2014)

In EVs isolated from sera of cod reared at 9 °C, heatshock cognate 70 (Hsc70) and myosin heavy chain were unique deimination candidates. Our group has previously identified Hsc70 as a deimination candidate in cod mucosa (Magnadottir et al., 2018a). In mandarin fish (*Siniperca chuatsi*), Hsc70 has been linked to immune responses to bacterial infection, hypoxia and temperature challenge (Wang et al., 2015). In a study on rainbow trout (*Oncorhynchus mykiss*), enrichment of Hsp70 in EVs of plasma has been reported in response to heat shock treatment (Faught et al., 2017), but deiminated forms have not been previously described in EVs. As in the present study, Hsp70 was identified in deiminated form in serum-derived EVs of the 9 °C group only, this may possibly indicate a specific biomarker in relation to this higher environmental temperature. Myosin heavy chain has previously been identified by our group to be deiminated in cod mucosa (Magnadottir et al., 2018a) and was also identified here in deiminated form in serum-derived EVs. Myosin heavy chain is of importance as a fast skeletal myosin and has been assessed in salmon with respect to rearing under varying photoperiod regimes (Churova et al., 2019) and in thermal acclimation in rainbow smelt (Coughlin et al., 2019). Deimination of myosin heavy chain, observed here in the 9 °C temperature group, may therefore be of some interest.

A recent study assessed protein cargo in serum-derived EVs from Atlantic salmon (*Salmo salar* L.), including in response to infection, where amongst other glyceraldehyde-3-phosphatase dehydrogenase was identified as an infection-associated cargo (Lagos et al., 2017). Our present study was in particular focussed on identifying deiminated protein cargo, and glyceraldehyde-3-phosphatase dehydrogenase was indeed identified as deiminated in serum-derived EVs in both temperature groups tested. 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 related to a range mucosal immune responses (Patel and Brinchmann, 2017) and was previously identified by our group as deiminated in cod mucosa (Magnadottir et al., 2018a).

This is the first report on deiminated forms of complement component C3 in cod serum and serum-derived EVs. In the present study we found that in cod sera only the C3 β-chain showed as a deimination positive band, unlike what was seen in a previous study in halibut serum, where both C3 α- and β-chains were found to be positive for deimination (Magnadottir et al., 2019b). This also differs somewhat from cod mucus, where we recently identified that C3 is deiminated both on α- and β-chains in whole mucus, while in mucus-derived EVs only the β-chain was detected as deiminated (Magnadottir et al., 2019a). Interestingly, post-translationally deiminated cod C3 was here found at higher levels in serum-derived EVs from fish reared at 4 °C, indicating that deiminated C3 may be a specific EV-marker for cod

Table 1
Deiminated proteins identified by F95 enrichment in EVs of cod (*Gadus morhua* L.) reared at 4 °C. Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody from a pool of 4 sera per temperature group. The F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to Mascot. Only proteins scoring with *G. morhua* are included and the identified peptide sequences and *m/z*values are shown. Proteins unique to EVs in the 4 °C group are highlighted.with an asterix (*).

Protein name	m/z	Peptide sequence	Score ($p < 0.05$)[a]	Total score
Q9PUG4_GADMO *Tubulin beta chain*	514.7630	*K.TAVCDIPPR.G*	52	570
	565.8007	*R.FPGQLNADLR.K*	36	
	580.3174	*K.LAVNMVPFPR.L*	37	
	615.3023	*R.ISEQFTAMFR.R*	64	
	644.3138	*R.ISVYYNEASGGK.Y*	43	
	660.3552	*R.IMNTFSVVPSPK.V*	52	
	723.8495	*K.EVDEQMLNVQNK.N*	33	
	808.4211	*R.AILVDLEPGTMDSVR.S*	60	
	553.9698	*R.ALTVPELTQQVFDAK.N*	29	
	848.9198	*K.NSSYFVEWIPNNVK.T*	45	
	608.3130	*R.EIVHLQAGQCGNQIGAK.F*	74	
	653.6647	*K.GHYTEGAELVDSVLDVVR.K*	45	
Q9YHC3|TBB1_GADMO *Tubulin beta-1 chain*	514.7630	*K.TAVCDIPPR.G*	52	493
	527.3072	*R.YLTVAAIFR.G*	55	
	565.8007	*R.FPGQLNADLR.K*	36	
	580.3174	*K.LAVNMVPFPR.L*	37	
	615.3023	*R.ISEQFTAMFR.R*	64	
	660.3552	*R.IMNTFSVVPSPK.V*	52	
	723.8495	*K.EVDEQMLNVQNK.N*	33	
	848.9198	*K.NSSYFVEWIPNNVK.T*	45	
	608.3130	*R.EIVHLQAGQCGNQIGAK.F*	74	
	653.6647	*K.GHYTEGAELVDSVLDVVR.K*	45	
A8CZC9_GADMO *Elongation factor 1-alpha*	433.7345	*K.EVSQYIK.K*	31	357
	442.7814	*K.QLIVGINK.M*	31	
	457.7867	*R.QTVAVGVIK.S*	55	
	488.2801	*R.LPLQDVYK.I*	47	
	513.3085	*K.IGGIGTVPVGR.V*	25	
	433.5870	*R.EHALLAFTLGVK.Q*	56	
	468.9136	*K.YYVTIIDAPGHR.D*	53	
	853.8023	*R.VETGILKPNMVVTFAPANVTTEVK.S*	60	
Q78AY8_GADMO	398.2396	*K.IIAPPER.K*	39	282
Fast skeletal muscle alpha-actin	488.7277	*K.AGFAGDDAPR.A*	66	
	507.7439	*R.DLTDYLMK.I*	41	
	400.2403	*R.AVFPSIVGRPR.H*	28	
	895.9474	*K.SYELPDGQVITIGNER.F*	75	
	654.3076	*K.YPIEHGIITNWDDMEK.I*	34	
Q6WEU6_GADMO	380.2210	*K.EVATAIR.G*	35	249
S2 ribosomal protein (Fragment)	395.7151	*R.CGSVLVR.L*	59	
	426.7262	*K.ATFDAISK.T*	34	
	513.3028	*R.GTGIVSAPVPK.K*	33	
	693.8491	*K.TYSYLTPDLWK.E*	56	
	572.9734	*K.AFVAIGDYNGHVGLGVK.C*	32	
Q9PUG5_GADMO	493.7577	*K.VAVCDVAPR.G*	71	223
Tubulin beta chain	565.8007	*R.FPGQLNADLR.K*	36	
	580.3174	*K.LAVNMVPFPR.L*	37	
	723.8495	*K.EVDEQMLNVQNK.N*	33	
	848.9198	*K.NSSYFVEWIPNNVK.V*	45	
P52865|RL22_GADMO	603.8233	*K.ISVNSEVPFSK.R*	106	142
60S ribosomal protein L22 (Fragment)	651.3443	*K.SGNLGNGVVSIER.X*	36	
Q2PDJ0_GADMO	507.7439	*R.DLTDYLMK.I*	41	117
Beta-actin (Fragment)	566.7675	*R.GYSFTTTAER.E*	29	
	652.0261	*R.VAPEEHPVLLTEAPLNPK.A*	27	
	796.6588	*R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L*	20	
Q8AWX8_GADMO	398.2124	*K.ITGMAFR.V*	36	114
Glyceraldehyde-3-phosphate dehydrogenase	748.4271	*R.VPTPNVSVVDLTVR.L*	78	
G8ENP0_GADMO	419.2449	*K.FTVTLTR.E*	42	87
Galectin (Fragment)	696.6840	*R.EEFLVILSDGSEVHFPNR.L*	45	
D5LIQ2_GADMO	616.8176	*R.EELSNVLAAMR.K*	82	82
Putative ribosomal protein L36 (Fragment)				
Q8JHA8_GADMO *Ribosomal protein L15 (Fragment)*	509.7613	*R.SLQSVAEER.A*	48	73
	830.8975	*R.VLNSYWVGEDSTYK.F*	26	
A0A067XLH1_GADMO	489.2869	*R.VILDNLYK.E*	25	68
Profilin	734.3785	*R.STLFTDGLMLGGQK.C*	43	
V9I305_GADMO	407.7603	*K.GVATLVVR.K*	27	50*
Transglutaminase 2	493.2746	*R.ISGSIAPGQR.V*	24	
D5LIQ8_GADMO	752.3728	*R.TELFIAAEGIHTGQFIYCGK.K*	39	39*
Putative ribosomal protein L8				
F8SSA3_GADMO *Cathepsin K*	416.7684	*R.ALAVALFK.A*	34	34*
A0A0G2QMS5_GADMO *Histone H3 (Fragment)*	425.7188	*R.EIAQDFK.T*	21	21

[a] Ions score is -10*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.

Table 2
Deiminated proteins identified by F95 enrichment in EVs of cod (*Gadus morhua* L.) reared at 9 °C. Deiminated proteins were isolated by immunoprecipitation using the pan-deimination F95 antibody from a pool of EVs isolated from 4 sera per temperature group. The F95 enriched eluate was analysed by LC-MS/MS and peak list files were submitted to Mascot. Only proteins scoring with *G. morhua* are included and the identified peptide sequences and *m/z* values are shown. Proteins unique to EVs in the 9 °C group are highlighted with an asterix (*).

Protein name	m/z	Peptide sequence	Score ($p < 0.05$)[a]	Total score
Q78AY8_GADMO	400.7710	*K.RGILTLK.Y*	31	327
Fast skeletal muscle alpha-actin	488.7276	*K.AGFAGDDAPR.A*	60	
	499.7468	*R.DLTDYLMK.I*	38	
	589.3106	*K.EITALAPSTMK.I*	48	
	400.2402	*R.AVFPSIVGRPR.H*	30	
	895.9485	*K.SYELPDGQVITIGNER.F*	77	
	654.3088	*K.YPIEHGIITNWDDMEK.I*	43	
A8CZC9_GADMO	433.7337	*K.EVSQYIK.K*	31	295
Elongation factor 1-alpha	442.7815	*K.QLIVGINK.M*	39	
	457.7869	*R.QTVAVGVIK.S*	75	
	488.2791	*R.LPLQDVYK.I*	46	
	513.3086	*K.IGGIGTVPVGR.V*	52	
	468.9134	*K.YYVTIIDAPGHR.D*	51	
Q9PUG4_GADMO	565.8019	*R.FPGQLNADLR.K*	38	256
Tubulin beta chain	580.3191	*K.LAVNMVPFPR.L*	35	
	644.3137	*R.ISVYYNEASGGK.Y*	89	
	668.3530	*R.IMNTFSVVPSPK.V*	30	
	808.4219	*R.AILVDLEPGTMDSVR.S*	38	
	608.3119	*R.EIVHLQAGQCGNQIGAK.F*	27	
Q2PDJ0_GADMO	400.7710	*K.RGILTLK.Y*	31	188
Beta-actin (Fragment)	499.7468	*499.7468*	38	
	566.7681	*566.7681*	41	
	652.0261	*652.0261*	34	
	800.6565	*800.6565*	45	
Q9YHC3|TBB1_GADMO	527.3082	*R.YLTVAAIFR.G*	46	175
Tubulin beta-1 chain	565.8019	*R.FPGQLNADLR.K*	38	
	580.3191	*K.LAVNMVPFPR.L*	35	
	668.3530	*R.IMNTFSVVPSPK.V*	30	
	608.3119	*R.EIVHLQAGQCGNQIGAK.F*	27	
Q8AWX8_GADMO	406.2093	*K.ITGMAFR.V*	29	149
Glyceraldehyde-3-phosphate dehydrogenase	435.2581	*K.VIPELNGK.I*	22	
	748.4274	*R.VPTPNVSVVDLTVR.L*	98	
P52865|RL22_GADMO	603.8233	*K.ISVNSEVPFSK.R*	50	104
60S ribosomal protein L22 (Fragment)	651.3443	*K.SGNLGNGVVSIER.X*	55	
G8ENP0_GADMO *Galectin (Fragment)*	419.2449	*K.FTVTLTR.E*	37	97
	696.6844	*R.EEFLVILSDGSEVHFPNR.L*	60	
D5LIQ2_GADMO *Putative ribosomal protein L36 (Fragment)*	616.8184	*R.EELSNVLAAMR.K*	53	53
Q8JHA8_GADMO *Ribosomal protein L15 (Fragment)*	509.7617	*R.SLQSVAEER.A*	49	49
G8DZS1_GADMO	614.8176	*K.VEIIANDQGNR.T*	41*	41*
Heat shock cognate 70 kDa protein (Fragment)				
Q6WEU6_GADMO	513.3029	*R.GTGIVSAPVPK.K*	40	40
S2 ribosomal protein (Fragment)				
A0A0G2QMS5_GADMO	425.7186	*R.EIAQDFK.T*	27	27
Histone H3 (Fragment)				
A0A067XLH1_GADMO *Profilin*	489.2874	*R.VILDNLYK.E*	22	22
Q98SS9_GADMO *Myosin heavy chain (Fragment)*	658.8311	*R.QLNDMNAQRAR.L*	20*	20*

[a] Ions score is -10*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.

reared at this lower temperature. Such post-translational modification may possibly facilitate diverse functionality of C3. As C3 deimination was lower in the 9 °C group, it may be possible that C3 may how less diversity in its function due to lower deimination, and therefore possibly be less effective in the immune defence; such effects will need to be further validated in future studies. Overall levels of C3 were though found to be higher in the 9 °C group, indicative of elevated immune responses. Therefore it may be postulated that deiminated versus non-deiminated forms of C3 differ in their function. Here, CRPs were also found to form part of the serum EV cargo, but not in deiminated forms. Deiminated CRP was only detected in whole sera, and at similar levels in both temperature groups. The putative effects of deimination on complement C3 and CRP have previously been discussed in detail elsewhere (Magnadottir et al., 2018b, 2019b).

Comparing total sera from the two rearing temperature groups (4 °C and 9 °C), deiminated histone H3 (citH3) showed higher levels in sera of fish reared at 9 °C. PAD-mediated histone deimination can affect gene regulation and also contributes to neutrophil extracellular trap formation (NETosis). NETosis forms part of microbicidal innate immunity and is evolutionarily conserved through phylogeny from fish to human (Brinkmann et al., 2004; Li et al., 2010; Palić et al., 2007; Pijanowski et al., 2013). We have previously described citH3 in cod and halibut ontogeny, as well as in adult fish sera (Magnadottir et al., 2018a, 2019b), and found that citH3 levels are elevated in cod mucosa in response to bacterial infection (Magnadottir et al., 2018a). Higher levels of citH3 in sera of the 9 °C group observed here may therefore be indicative of a heightened stress response and possibly be also harmful to the fish. Elevated cithH3 and NETosis have indeed been linked to chronic disease conditions (Frangou et al., 2019). Using Western blotting, the citH3 antibody did not show positive for deiminated histone H3 in the EVs, while LC-MS/MS analysis did though identify histone H3 (fragment) as a deimination candidate in EVs from both temperature groups.

In addition, PAD was found to be exported in serum-EVs, indicative

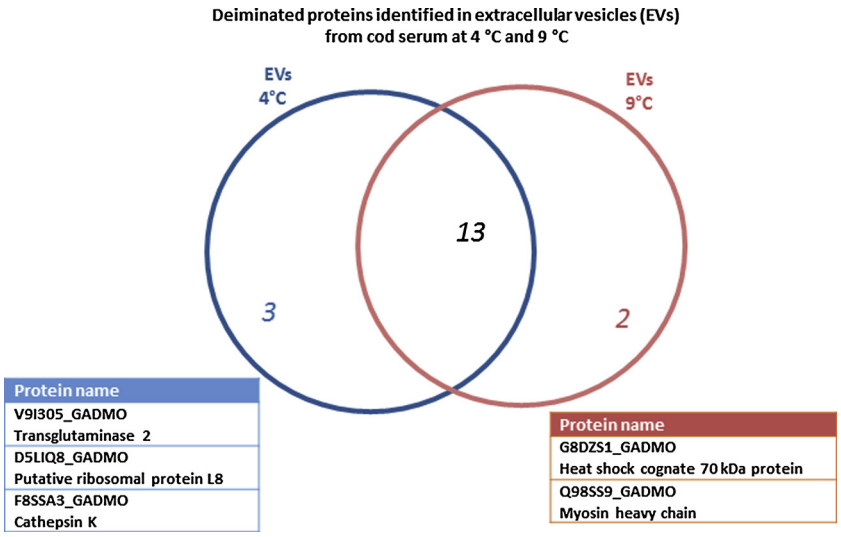

Fig. 5. Deiminated proteins identified by LC-MS/MS in cod serum EVs from 4 °C and 9 °C temperature groups. Common deiminated protein candidates identified in serum and EV pools (n = 4) in both temperature groups were overall 13, while 2 deimination candidates were specific to the 4 °C group and 3 to the 9 °C group respectively, as listed below the Venn diagram. For full details on protein hits see Tables 1 and 2. Note that only protein identity hits with *G. morhua* are included in this analysis.

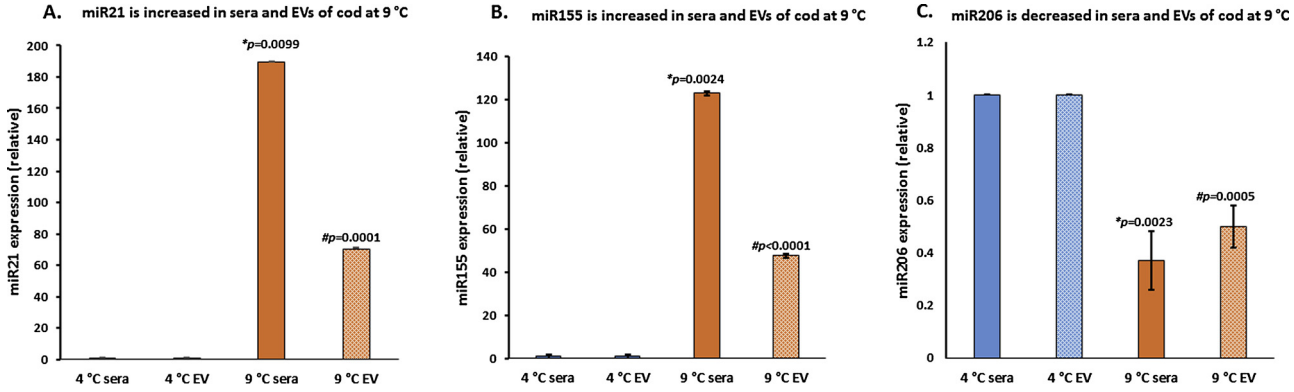

Fig. 6. MicroRNAs in cod serum and serum-derived EVs of fish reared at 4 °C and 9 °C. A. Analysis of miRNA-21 in cod serum and EVs. Inflammatory related miRNA-21 was elevated in the 9 °C group, both in sera (189 fold) (n is a pool of 4, with 3 technical replicates) and EVs (70 fold) (n is based on 4 individual samples), compared to the 4 °C group. B. Analysis of miRNA-155 in cod serum and EVs. The stress-related miRNA-155 was increased in the 9 °C group, both in sera (122 fold) (n is a pool of 4, with 3 technical replicates) and EVs (47 fold) (n is based on 4 individual samples), compared to the 4 °C group. C. Analysis of miRNA-206 in cod serum and EVs. The growth-related miRNA-206 was decreased in the 9 °C group, both in sera (2.7 fold) (n is a pool of 4, with 3 technical replicates) and in EVs (2 fold) (n is based on 4 individual samples), compared to the 4 °C group. For all miRNA analysis of total sera, a pool of n = 4 individual sera was assessed, while for serum-EVs individual EV isolates were assessed (n = 4 per group). The experiment was repeated 3 times and error bars indicate standard deviation (SD); exact *p*-values are shown (**p* indicates significant differences between sera of the two temperature groups, while *#p* indicates significant differences between EVs of the two temperature groups, respectively).

of lateral transfer of PAD enzyme via EVs. In other studies, PADs have been found to be exported in EVs released from various cancer cells (Hurwitz et al., 2016) and to deiminate target proteins in plasma (Chang and Han, 2006), possibly aiding spread of cancer indirectly (Lange et al., 2017). Modulation of immune proteins of the host, via deimination by membrane vesicle exported PADs, has also been shown in *P. gingivalis* (Bielecka et al., 2014). It can therefore be postulated that in cod, exported PADs in EVs may affect deimination of target proteins at sites of EV uptake and therefore play roles in immune function via such EV-mediated communication.

In teleost fish, miRNA-21 has been found to be up-regulated after immune stimulation and to regulate immune responses via various pathways, including via regulation of IRAK4 (Chu et al., 2019), by inhibiting the expression of cytokines via regulation of Toll-like receptor signalling (Bi et al., 2017) and by targeting jnk and ccr7 following bacterial infection (Tao et al., 2019). Both miRNA-21 and miRNA-155 have been associated to viral infections in fish (Andreassen and Høyheim, 2017) and both miRNAs were found to be upregulated in fish exposed to chronic [C_8mim]Br induced inflammation (Ma et al., 2019). Little is known about these miRs in relation to environmental temperature, while effects observed here in the two temperature groups showed significantly elevated levels of both miRNA-21 and miRNA-155 in the 9 °C group, compared to the 4 °C group, in both sera and EVs. This may possibly be related to elevated stress levels caused by this environmental rearing temperature. These two miRs therefore pose as novel biomarkers indicative of immune responses at higher environmental temperatures.

MiRNA-206 is a muscle-specific miRNA conserved in vertebrates (Tani et al., 2013) and importantly, it has been shown to be relevant for growth in tilapia (Yan et al., 2013). It has also been associated with the miRNA transcriptome during teleost development, and to form part of a complex interplay of miRNAs in temperature-induced phenotypic plasticity of growth in teleosts (Campos et al., 2014). Temperature mediated effects were indeed observed in the current study for miRNA-206, which showed significantly higher relative expression in both sera and EVs of the fish reared at 4 °C, compared to the 9 °C group. This may be indicative of better growth conditions for cod reared at the lower 4 °C temperature.

In cod, various previous studies have been carried out assessing environmental factors, including temperature, and their effects on immunological parameters and growth rate (Magnadottir et al., 1999; Björnsson et al., 2001; Arnason et al., 2013; Larsen et al., 2018).

Environmental temperature has been shown to modulate fish immunity (Bowden, 2008; Bowden et al., 2007). For example innate parameters, such as natural antibody activity and anti-trypsin activity, were in previous studies noted to be higher at lower temperatures, while parameters of specific immunity seemed more prominent in cod kept at higher temperatures (Magnadottir et al., 1999). In the current study we observed that both C3 and CRPs were present at higher levels in EVs from fish reared at lower temperatures, alongside elevated protein deimination. In total sera on the other hand, C3 levels were somewhat higher in the 9 °C group and both CRP forms were found to be somewhat lower in sera of the 9 °C group, as well as to differ in oligomeric forms present, compared to the 4 °C group.

In previous studies on cod, following challenge at high water temperature, immune relevant genes have been shown to be increased (Hori et al., 2013) and immune activation at higher temperatures has been suggested to be a possible trade-off between resistance and tolerance to survive infection (Larsen et al., 2018). We also propose that differences in post-translational changes may affect structure and function of a range of immune factors and affect their protein moonlighting functions in immunity and homeostasis. Furthermore, roles for immune related proteins in whole serum and those exported in EVs, including in deiminated form, will require further investigation.

EV research in teleost fish has hitherto been limited and to date no studies have been carried out to assess changes in EV release and cargo in response to environmental temperature. Recent studies on fish EVs have assessed EV release in response to extracellular DNA (Iliev et al., 2018) and miRNA cargo in EVs in response to infection in crayfish (*Procambarus clarkia*) (Yang et al., 2019). Analysis of deiminated proteins has hitherto only been performed in cod mucosa and halibut serum and no assessment of lateral transfer of deiminated proteins via EVs has been performed before in teleost fish sera. Regulatory roles for PADs in EV biogenesis and release have been described by our group (Kholia et al., 2015; Kosgodage et al., 2017, 2019), including effects on miRNA cargo (Kosgodage et al., 2018).

In the current study, changes in protein deimination and EV biogenesis is reported in sera of cod reared at 4 °C and 9 °C temperatures, respectively. The interest in studying EVs in teleost fish for identification of biomarkers of infection and environmental changes, such as temperature and toxicology, is rapidly increasing. Findings of the current study touch therefore upon a hugely understudied field of EV research in relation to teleost fish health and for the development of EV-related biomarkers to assess fish health, both in aquaculture, as well as in wild fish in response to changes in sea temperatures due to global warming. Further understanding of PAD-mediated mechanisms, including in the regulation of EV biogenesis EV cargo, including in response to environmental conditions remains subject to future studies.

5. Conclusion

Deiminated protein cargo was assessed in EVs of cod serum, identifying specific candidate biomarkers in relation to environmental rearing temperature. Reduced EV numbers in cod serum were found to be an indicator of higher environmental temperature, alongside changes in EV cargo proteins, including post-translationally deiminated proteins, and inflammatory microRNA cargo. For the first time, deiminated complement component C3 is reported to be exported in serum-derived EVs of teleost fish. Significant changes in inflammatory and growth related miRNA-21, miRNA-155 and miRNA-206 were detected in EVs of cod in response to environmental temperature differences. Based on our findings, serum-derived EVs may be developed as useful biomarkers for the assessment of fish health and environmental changes.

CRediT authorship contribution statement

Bergljót Magnadóttir: Methodology, Resources, Funding acquisition, Validation, Writing - review & editing. **Pinar Uysal-Onganer:** Methodology, Formal analysis, Resources, Visualization, Validation, Writing - review & editing. **Igor Kraev:** Formal analysis, Methodology, Resources, Visualization. **Alister W. Dodds:** Methodology, Resources, Writing - review & editing. **Sigríður Guðmundsdóttir:** Resources, Validation, Writing - review & editing. **Sigrun Lange:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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