Fish and Shellfish Immunology 154 (2024) 109974

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Extracellular vesicles and citrullination signatures are novel biomarkers in sturgeon (*Acipenser gueldenstaedtii*) during chronic stress due to seasonal temperature challenge

Ana María Ferreira [a,b], Valeria Silva-Álvarez [a,b], Igor Kraev [c], Pinar Uysal-Onganer [d], Sigrun Lange [e,*]

[a] *Laboratorio de Inmunología, Instituto de Química Biológica, Facultad de Ciencias, Instituto de Higiene, UdelaR, Uruguay*
[b] *Área de Inmunología, Departamento de Biociencias, Facultad de Química, Instituto de Higiene, UdelaR, Uruguay*
[c] *Electron Microscopy Suite, Faculty of Science, Technology, Engineering and Mathematics, Open University, Milton Keynes MK7 6AA, UK*
[d] *Cancer Mechanisms and Biomarkers Research Group, School of Life Sciences, University of Westminster, London W1W 6UW, UK*
[e] *Pathobiology and Extracellular Vesicles Research Group, School of Life Sciences, University of Westminster, London W1W 6UW, UK*

ARTICLE INFO

Keywords:
Sturgeon (*Acipenser gueldenstaedtii*)
Extracellular vesicles (EVs)
Citrullination/deimination
Peptidylarginine deiminase
Chronic stress
Environmental temperature
Micro-RNA
Complement system
Histone H3
Immune response

ABSTRACT

Acipenser gueldenstaedtii is one of the most cultured sturgeon species worldwide and of considerable economic value for caviar production. There are though considerable challenges around chronic stress responses due to increased summer temperatures, impacting sturgeons' immune responses and their susceptibility to opportunistic infections. The identification of molecular and cellular pathways involved in stress responses may contribute to identifying novel biomarkers reflective of fish health status, crucial for successful sturgeon aquaculture. Protein citrullination is a calcium-catalysed post-translational modification caused by peptidylarginine deiminases (PADs), altering target protein function and affecting protein interactions in physiological and pathobiological processes. PADs can also modulate extracellular vesicle (EVs) profiles, which play critical roles in cellular communication, via transport of their cargoes (proteins, including post-translationally modified proteins, genetic material and micro-RNAs).

This study identified differences in EV signatures, and citrullinated proteins in sera from winter and summer farmed sturegeons. EVs were significantly elevated in sera of the summer chronically stressed group. The citrullinated proteins and associated gene ontology (GO) pathways in sera and serum-EVs of chronically heat stressed *A. gueldenstaedtii*, showed some changes, with specific citrullinated serum protein targets including alpa-2-macroglobulin, alpha globin, calcium-dependent secretion activator, ceruloplasmin, chemokine XC receptor, complement C3 isoforms, complement C9, plectin, selenoprotein and vitellogenin. In serum-EVs, citrullinated protein cargoes identified only in the chronically stressed summer group included alpha-1-antiproteinase, apolipoprotein B-100, microtubule actin crosslinking factor and histone H3. Biological gene ontology (GO) pathways related to citrullinated serum proteins in the chronically stressed group were associated with innate and adaptive immune responses, stress responses and metabolic processes. In serum-EVs of the heat-stressed group the citrullinome associated with various metabolic GO pathways.

In addition to modified citrullinated protein content, Serum-EVs from the stressed summer group showed significantly increased levels of the inflammatory associated miR-155 and the hypoxia-associated miR-210, but significantly reduced levels of the growth-associated miR-206.

Our findings highlight roles for protein citrullination and EV signatures in response to chronic heat stress in *A. gueldenstaedtii*, indicating a trade-off in immunity versus growth and may be of value for sturgeon aquaculture.

* Corresponding author.
E-mail addresses: aferreira@fcien.edu.uy (A.M. Ferreira), mvsilva@fq.edu.uy (V. Silva-Álvarez), igor.kraev@open.ac.uk (I. Kraev), p.onganer@westminster.ac.uk (P. Uysal-Onganer), s.lange@westminster.ac.uk (S. Lange).

https://doi.org/10.1016/j.fsi.2024.109974
Received 19 June 2024; Received in revised form 3 October 2024; Accepted 16 October 2024
Available online 18 October 2024

1. Introduction

Sturgeons are an economically valuable chondrostean fish, critically endangered due to anthropogenic activities. Therefore, there is a considerable global interest in sturgeon aquaculture [1–6]. Russian sturgeon (*Acipenser gueldenstaedtii)*, the second most important sturgeon species reared for caviar, is successfully farmed in Uruguay and other subtropical countries. However, a major challenge for sturgeon aquaculture in these countries is the warmer summer temperatures, which induce chronic heat stress. This weakens sturgeons' immune defences and facilitates opportunistic infections, resulting in increased fish mortality and associated economic losses for fish farming [7–12]. Therefore, it is of pivotal importance to identify molecular and cellular mechanisms associated with chronic heat stress.

Peptidylarginine deiminases (PADs), a family of calcium catalysed enzymes, cause post-translational citrullination/deimination in proteins by converting arginine into citrulline, increasing hydrophobicity and affecting protein folding. Citrullination can alter intra- and/or intermolecular protein interactions, modify protein structure and function, or cause protein denaturation, depending on the extent of citrullination as the conversion of each arginine to citrulline leads to the loss of one positive charge and mass decrease of 1 Da [13]. PADs are phylogenetically conserved, with five isozymes (PAD1,2,3,4 and 6) in mammals, three isozymes (PAD1,2,3) in birds and reptiles and one PAD type (PAD2-like) in fish [13,14]. The mammalian PADs are structurally similar, with over 50 % sequence homology and highly conserved calcium binding sites, except PAD6, which is shorter and lacks some of the catalytic sites [15]. In mammals, the PAD isozymes differ in tissue specificity and preference for target proteins, with PAD2 considered to be the most ubiquitously expressed and phylogenetically most conserved isozyme [16]. As fish only contain one PAD-like protein (PAD2-like), this PAD must mediate citrullination in all fish tissues. PADs have been associated with various physiological, developmental and pathobiological processes [13,16–22]. PAD homologues (arginine deiminases, ADI) are furthermore found in bacteria and parasites and are believed to have undergone horizontal gene transfer from cyanobacteria to animals [23,24].

Structures most susceptible to citrullination are intrinsically disordered proteins and beta-sheets, while the position of the arginine is also relevant. Citrullination/deimination can cause structural and functional changes in a wide range of target proteins, including cytoskeletal, mitochondrial and nuclear ones, contributing to their multifaceted protein-moonlighting functions in health and disease [16,18,25]. Neo-epitope generation by citrullination can furthermore contribute to inflammatory responses [16,19,22]. Hence, the identification of modified citrullinated protein targets under stress conditions may provide important indications of changes in associated defence and metabolic pathways. Importantly, PADs have also been identified to have a role in extracellular vesicle (EV) biogenesis [26], which is important in cell communication in health and disease, and transport of citrullinated protein cargoes in EVs has been reported in various studies. Such EV mediated transport of citrullinated proteins may play important roles in cellular communication, in addition to EV mediated export of unmodified proteins. The PAD homologues (ADIs) reported in bacteria and parasites may, via their citrullinating activity, also play roles in host-pathogen interactions, via modification of host proteins and modulation of EVs [27–31].

PADs are gaining increasing interest in comparative immunology studies, including in aquatic species (Mollusca, Arthropoda, Crustacea, Echinodermata, Agnatha, Elasmobranchii, Teleostei, Cetacea and Pinnipedia) [23,32–39]. Roles for PADs in fish immunity have been highlighted in several teleost species, agnathans, and sharks [14,18,35,36,39–42]. EVs may be useful biomarkers as they carry a range of cargoes (proteins, non-coding RNAs, genetic material), and can be readily isolated from many body fluids, including serum and plasma. Previous research has indeed highlighted changes in EV signatures in fish in response to stress and immune stimulation, including with respect to citrullinated protein cargoes and micro-RNAs [36,43–45].

Micro-RNAs (miRNAs/miRs) are one of the cargoes contained in EVs and are enriched in EVs compared to plasma or serum. They play roles in mRNA translation and degradation and have also received attention as important epigenetic regulators in fish immunity [46–48]. In some sturgeon species, miRNAs have been associated to immune and metabolic roles [49,50], with a microRNA transcriptome analysis carried out in *A. schrenckii* [49] and the evolution of microRNA biogenesis genes studied in *A. ruthenus* [50]. In teleost fish, roles for miRNAs were reported in immune responses to environmental stressors [36,51,52], including in EVs, where changes were reported in the inflammation associated miR-155, while miR-206 was identified as a regulatory factor for fish growth [36,53–55].

In relation to our, and other groups', previous studies on citrullination and EVs in fish immunity, the current study focussed on assessing serum citrullinome and EV signatures in *A. gueldenstaedtii* from summer (chronically stressed) and winter conditions in an Uruguayan sturgeon farm. This might identify chronic stress related pathways caused by long exposure to high summer temperatures.

2. Materials and methods

2.1. Fish and sampling

Farmed *A. gueldenstaedtii*, 15 female 4-year-old fish per group, were utilised in this study. The fish were derived from the Black River Caviar fish farm, Baygorria, Uruguay. Freshwater from the Río Negro River was channelled via an open flow-through system of raceways rearing units with a 35 min exchange water rate. Total oxygen saturation was regularly measured, varying between 80 and 100 %. Adult fish were typically grown at a maximal stocking density of 30 kg/m^3 and fed daily with a standard pelleted diet at a feeding rate of 0.5 % of body weight. Blood was collected from fish during summer (March 2023) and winter (August 2023), respectively. Water temperature ranged between 24 °C and 30 °C and between 11 °C and 15 °C during the summer and winter months, respectively. Fish were fasted for 24 h before blood collection, which was undertaken according to a protocol approved by the Ethic Committee of the Honorary Commission of Animal Experimentation (Exp. Nº 111,900-000061-23, UdelaR, Uruguay). Blood samples (2.0 ml) were collected by caudal puncture within less than 1 min to minimize handling stress and were immediately transferred into conventional plastic tubes at 4 °C for 4 h to allow clotting. The serum was collected by centrifuging at 2800 *g* for 20 min at 4 °C, stored in 300 μL aliquots and kept frozen at −80 °C until further use.

2.2. EV isolation and profiling

EVs were isolated from 15 individual serum samples per experimental group (winter and summer conditions, respectively), using 100 μl serum aliquots per sample. Nanoparticle tracking analysis (NTA) was used for EV size profiling, including for changes in EV subgroups (small EVs ≤100 nm; medium EVs 101–200 nm; and large EVs >200 nm). Differential centrifugation was performed for EV isolation, adhering to the guidelines of MISEV (2023) [56] and previously published protocols [23,36]. In brief, sera were diluted 1/5 in DPBS and centrifuged at 4000 *g* for 20 min to remove any aggregates. The supernatant was then centrifuged at 100,000 *g* for 1h at 4 °C for enrichment of total EVs. Thereafter, the EV pellet was resuspended in 400 μL DPBS and centrifuged again at 4 °C for 1h at 100,000 *g*. The supernatant was discarded, and the final washed EV pellet was resuspended in 100 μL DPBS. For NTA analysis, 10 μL of resuspended EVs were diluted in 990 μL DPBS, for each sample, and applied to the NS300 Nanosight (Malvern Panalytical Ltd, Malvern, UK) with the syringe pump speed set at 50. Five 1-min videos were recorded using the Blue488 nm laser, with camera capture set at level 9 and post-processing analysis detection threshold set at

level 5. Using the NS300 software (Nanosight, Malvern Panalytical Ltd), replicate histograms were generated from the videos, and size profiling analysis and total EV counts were carried out. EVs were further characterised by western blotting for two key surface markers, CD63 and flotillin-1, and imaged by transmission electron microscopy (TEM) for morphology, according to previously published methods [36,39].

2.3. Western blotting

Serum samples were assessed from a pool of sera (n = 5 per pool of 3) for the presence of a PAD via cross-reaction with the anti-human PAD2 isozyme-specific antibody (ab50257, Abcam), as species specific antibodies are not available for sturgeon, and as fish PAD is PAD2-like; this antibody has also been verified to cross-react with several fish species [18,35,41,42]. For the detection of EV surface markers, CD63 (ab216130) and flotillin-1 (ab41927) antibodies were used on the EV isolates. Samples were reconstituted 1:1 in 2 × Laemmli sample buffer and boiled at 100 °C for 5 min before separation by SDS-PAGE, using 4–20 % TGX gels (BioRad, Watford, UK). Proteins were blotted onto 0.45 μm nitrocellulose membranes (BioRad, UK) using semi-dry transfer for 1 h at 15 V and even protein transfer was assessed using Ponceau S (Sigma, Gillingham, UK) staining. Membranes were blocked for 1 h at RT in 5 % bovine serum albumin (BSA, Sigma) in Tris-buffered saline containing Tween 20 (TBS-T). Primary antibody incubation was performed overnight at 4 °C on a shaking platform, diluting all primary antibodies 1/1000 in TBS-T. Membranes were washed three times in TBS-T and incubated at room temperature for 1h in secondary antibody (anti-rabbit IgG HRP-conjugated; BioRad) diluted 1/3000 in TBS-T. Membranes were washed five times in TBS-T and then visualised using a UVP BioDoc-ITTM System (Cambridge, UK) in conjunction with ECL (Amersham, Merck, UK).

2.4. Transmission electron microscopy

For imaging preparation, EV pellets were resuspended in 100 mM sodium cacodylate buffer (pH 7.4). Approximately 3–5 μL of the EV suspension was applied to a glow-discharged TEM grid with a carbon support film. The sample was partially air-dried for around 10 min before the grid was placed onto a drop of fixative solution (2.5 % glutaraldehyde (Agar Scientific Ltd, Stansted, UK) in 100 mM sodium cacodylate buffer, pH 7.4) for 1 min at room temperature. The grid was subsequently transferred across three drops of distilled water for washing, with excess water being removed using filter paper. Then the grid was placed onto a drop of staining solution of 2 % aqueous Uranyl Acetate (Agar Scientific Ltd, Stansted, UK) for 1 min, and any excess stain was removed with filter paper before air drying. Transmission electron microscopy (TEM) imaging of the EVs was conducted using a JEOL JEM 1400 microscope (JEOL, Tokyo, Japan) operated at 80 kV, with magnifications ranging from 30,000 × to 60,000 ×. Digital images were captured using an AMT XR60 CCD camera (Deben, Bury Saint Edmunds, UK).

2.5. Phylogram for the PAD (PAD2-like) protein from A. gueldenstaedtii

A phylogram was constructed for sturgeon PAD (where one PAD form, a PAD2-like protein, is reported similar as to in other fish) in ClustalOmega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo), following amino acid sequence alignment using the same programme. The PAD (PAD2-like) protein sequences obtained from *A. gueldenstaedtii* spleen and liver transcriptomes (GenBank Accession numbers PP911075 and PP911074, respectively) were used as well as PAD2-like protein sequences obtained from the *A. ruthenus* genome (isoform X1, XP_033897726.3 and isoform X2, XP_058849287.1). *Acipenser* PAD (PAD2-like) proteins were compared with the PADs reported in other Actinopterygii: American paddlefish (*Polyodon spathula*) PAD2-like protein (XP_041129502.1); European eel (*Anguilla anguilla*) PAD2-like protein (XP_035243851.1); salmon (*Salmo salar*) PAD2-like protein isoform X1 (XP_013988973.1) and isoform X2 (XP_013988974.1), and with the PAD2-like protein (CBN80708.1) from sea bass (*Dicentrarchus labrax*). In addition, the amphibian PAD2-like protein (NP_001080369.1) from *Xenopus laevis* was included, as well as the three PAD isozymes reported in chicken (*Gallus gallus* PAD1 NP_001305368.1; PAD2 NP_001305948.1; PAD3 NP_990374.2), and in alligator (*Alligator mississippiensis* PAD1 XP_006259278.4; PAD2 XP_059572240.1; PAD3 XP_059572875.1). The five human PAD isozymes were included (*Homo sapiens* PAD1 NP_037490.2; PAD2 NP_031391.2; PAD3 NP_057317.2; PAD4 NP_036519.2 and PAD6 NP_997304.3). In addition, ADI from a cyanobacterium (*Leptolyngbyaceae* sp. JSC-12 EKQ66906.1) and from *Giardia intestinalis* (AAC06116.1) were also included in the phylogram.

2.6. Immunoprecipitation of citrullinated/deiminated proteins

For the isolation of citrullinated/deiminated proteins from *A. gueldenstaedtii* serum and serum-EVs, pools of n = 15 samples per group (pooling 10 μl of each individual sample per group) were used to assess group representative citrullinome signatures for the winter and summer groups, respectively, for putative indication of changes in the chronically stressed summer group. Citrullinated/deiminated proteins were isolated using the pan-citrulline F95 antibody (MABN328, Merck), in conjunction with the Catch-and-Release immunoprecipitation kit (Merck) according to previously described methods [36,42]. Following overnight incubation at 4 °C on a rotating platform, immunoprecipitated citrullinated proteins were eluted from the sepharose columns using the elution buffer provided with the kit and citrullinated protein profiles were assessed by SDS-PAGE and silverstaining using the BioRad SilverStain kit. For identification of citrullinated protein hits, LC-MS/MS analysis was carried out.

2.7. LC-MS/MS and STRING protein-protein interaction network analysis

The eluates containing the F95 bound proteins from the serum and serum-EV sample groups (pools of n = 15 per group; winter and summer groups) were analysed by LC-MS/MS following in-gel digestion (carried out by Cambridge Proteomics, Cambridge, UK), according to previously described methods [36,42]. Citrullinated protein hits were analysed for each group against the species-specific database of Russian sturgeon (*A. gueldenstaedtii*_20240221; 266 sequences; 66,972 residues), using the Mascot search algorithm (Matrix Science, London, UK). Ions score for hit identification was set at −10*Log(P), where P was the probability that the observed match was a random event, with individual ions scores >14 indicating identity or extensive homology ($p < 0.05$). The fragment and peptide mass tolerances were set to 0.1 Da and 20 ppm, respectively. For a more comprehensive analysis, hits were furthermore assessed against the Actinopterygii database (CCP_Actinopterygii Actinopterygii_20240222 (6694388 sequences; 3820018598 residues) with individual ions scores >53 indicating identity or extensive homology ($p < 0.05$). To generate protein-protein interaction networks, protein hits were fed into the STRING database (https://string-db.org/; accessed February 8, 2024) and analysed based on the Actinopterygii database, using *Salmon salar* as a reference species as this provided the highest match with identified protein hits and as a species specific *A. gueldenstaedtii* database was not available in STRING. Settings were at medium confidence. Protein interaction networks were compared, by functional enrichment analysis in STRING, between the winter and summer groups for shared and distinct Gene ontology (GO), Reactome and STRING pathways relating to the citrullinated proteins identified in serum and serum-EVs per group, respectively.

2.8. Analysis of stress- and growth-related miRNAs miR-155, miR-210 and miR-206 in A. gueldenstaedtii serum-EVs

For the assessment of serum-EV miR signatures relating to stress

responses versus trade-off in growth, three key miRNAs relating to inflammation (miR-155), hypoxia (miR-210) and growth (miR-206), respectively, were assessed in serum-EVs from the winter and summer groups. For the analysis, 15 EV isolations per group (winter and summer) were pooled into three sub-groups, with n = 5 per pool, and changes in miRNA expressions were assessed in technical triplicates. RNA was extracted from the EVs using Trizol (Sigma, UK) according to standardised procedures and RNA purity and concentrations were assessed by NanoDrop Spectrophotometry (260 and 280 nm ab). RNA was reverse transcribed to cDNA using the qScript micro-RNA cDNA Synthesis Kit (Quantabio, U.K.). The PerfeCTa SYBR Green SuperMix (Quantabio, U.K.) was used together with MystiCq miRNA qPCR primers for miR-155 (hsa-miR-155–5p), miR-210 (hsa-miR-210–5p) and miR-206 (hsa-miR-206–5p). RNU6 was used as a reference gene for the normalization of miR expression levels, as it is evolutionarily conserved, has consistently high Ct values across all samples and narrow, reproducible SD [57,58]. miR primers were obtained from Sigma (U.K.) and verified to be conserved; the miRs have been reported in miRNA databases of fish species have been identified and annotated on miRBase (http://www.mirbase.org/index.shtml). Thermocycling conditions were as follows: denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 2 s, 60 °C for 15 s, and extension at 72 °C for 15 s. For normalization and relative expression level calculation the 2^(-ΔΔCt) method was used according to Livak and Schmittgen [59]. Each reaction was repeated three times.

2.9. Statistical analysis

For each group, winter (control) and summer (chronic heat stress group), individual sera and EV isolates were used (n = 15); for the group representative serum and serum-EV citrullinome LC-MS/MS analysis a pool of 15 individual samples was used per group. Graphs comparing EV numbers and miRNA expressions were generated in Graph Pad Prism (version 10), using *t*-test, with mean and error bars representing standard deviation; statistical significance was considered at $p \leq 0.05$. The NTA software was used to generate the NTA curves, showing replicates of 5 reads per sample, with the red line showing the standard error/deviation. STRING protein interaction networks were generated with a medium confidence setting. Ion scores in LC-MS/MS analysis were cut off for extensive homology considered at $p < 0.05$.

3. Results

3.1. A. gueldenstaedtii serum-EV characterisation and profiling in winter and summer groups

EVs were isolated from individual *A. gueldenstaedtii* sera of the summer and winter groups and assessed by NTA for size and number. EVs were distributed in the 30–500 nm range, with peaks around 100–200 nm for winter and 100–300 nm for summer samples (see representative NTA graphs in Fig. 1A). Western blotting (WB) for CD63 and flotillin-1 showed that these surface markers were detected on the serum-EVs (Fig. 1B). Assessment of serum-EVs by TEM confirmed the presence of EVs in sera, showing a poly-dispersed EV population (Fig. 1C).

Serum-EV profiles from the winter and summer groups were compared for differences in total EV numbers, including changes in EV-subpopulations (small, medium, and large EVs), as well as EV mean and modal size. Total serum-EV numbers were significantly increased in *A. gueldenstaedtii* from the summer group, compared with the winter group (Fig. 2A), and this was verified for all EV sub-populations: small EVs ≤100 nm; medium EVs 101–200 nm and large EVs >200 nm (Fig. 2B). EV mean size did not differ significantly between the two groups (Fig. 2C), nor did EV modal size (Fig. 2D).

3.2. A. gueldenstaedtii PAD phylogram and PAD detection in serum

To compare *A. gueldenstaedtii* PAD protein with other known PAD protein sequences across phylogeny, a phylogram was created, including representative PADs from fish (who have a PAD2 like protein only), amphibian (who have a PAD2-like protein), reptile and bird (who have 3 PAD isozymes), and human (who have 5 PAD isozymes), as presented in Fig. 3A. A PAD2 clade was observed, with the *A. gueldenstaedtii* PAD (PAD2-like) protein closest to the human PAD2 isozyme as expected, and human PAD6 next to the PAD2 clade, followed by PAD4, PAD1 and PAD3. The percentage identity matrix of *A. gueldenstaedtii* PAD with the

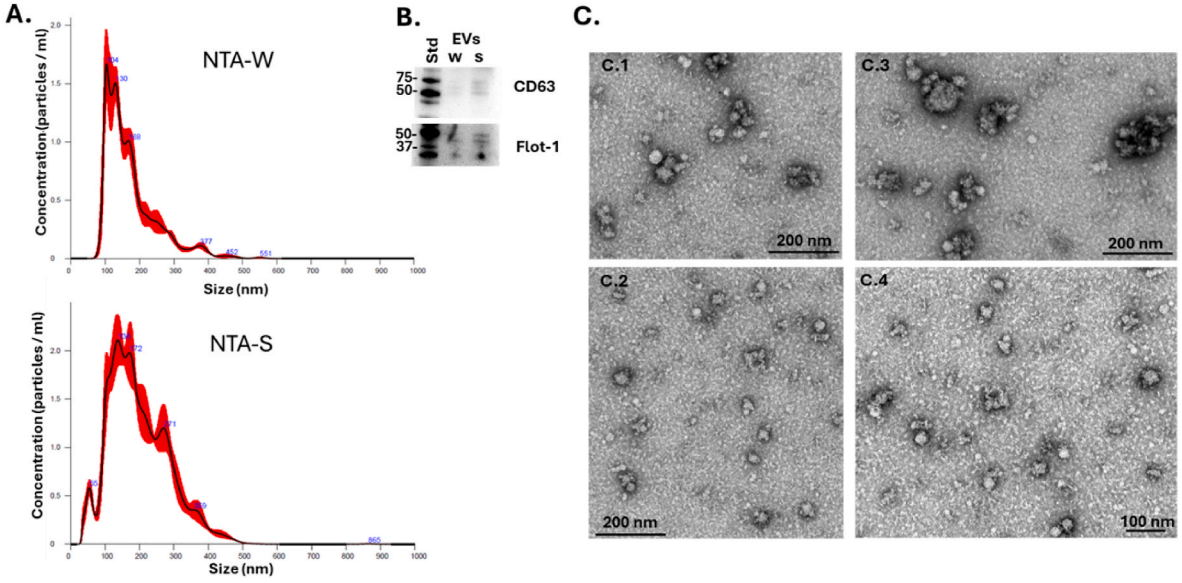


Fig. 1. Extracellular vesicle (EV) characterisation from *A. gueldenstaedtii* sera. **A)** Nanoparticle tracking analysis (NTA) with representative size profile curves for serum-EVs isolated from the winter (NTA-W) and summer (NTA-S) groups, with the black and red lines representing mean and confidence intervals of the five recordings for each sample; **B)** EV surface marker detection for CD63 and Flot-1 shown by western blotting, for representative EV-pools of winter (w) and summer (s) samples, with the size standard (std) indicated in kDa; **C)** EV morphology was verified by transmission electron microscopy (TEM) and representative images are shown from winter (C.1 & C.2) and summer (C.3 & C.4) serum-EV samples, with a scale bar indicated in nm in each image.

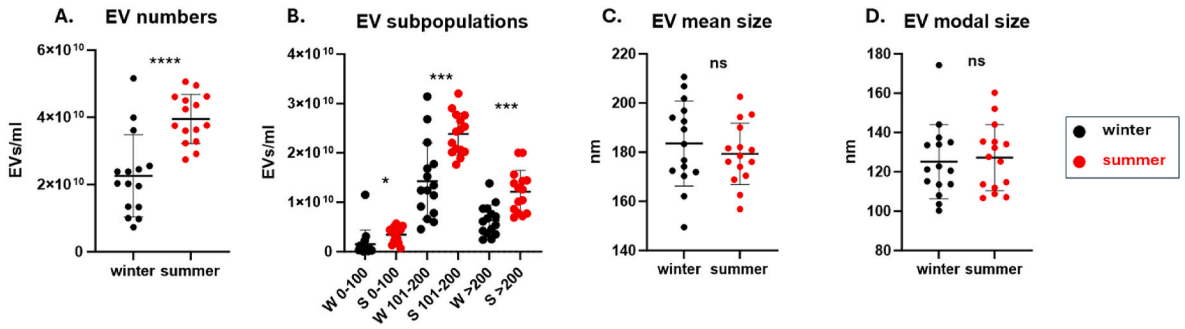
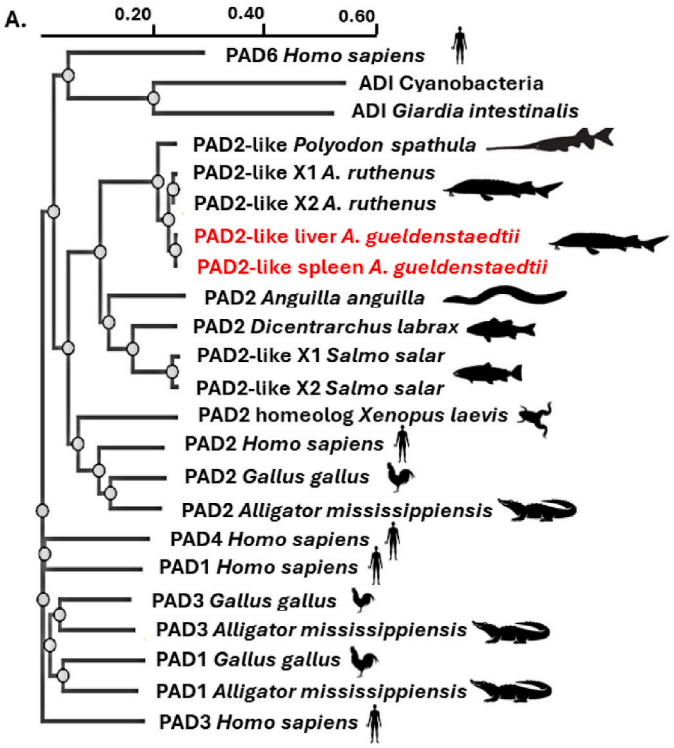


Fig. 2. Extracellular vesicle profiling by NTA analysis of *A. gueldenstaedtii* sera from winter (n = 15) and summer (n = 15) groups. **A)** Total serum-EV numbers; **B)** Numbers for serum-EV subpopulations, assessing small EVs (≤100 nm), medium EVs (101–200 nm) and large EVs (>200 nm). **C)** Serum-EV mean size; **D)** Serum-EV modal size. Statistical significance is indicated by *** ($p < 0.001$), and ****($p < 0.0001$).



B.

PAD6 *Homo sapiens*	100.00	44.86	40.21	40.37	46.44	45.36	44.48
PAD2 *Homo sapiens*	44.86	100.00	57.93	58.23	50.45	52.43	51.59
PAD2-like liver *A. gueldenstaedtii*	40.21	57.93	100.00	99.09	48.93	49.23	50.54
PAD2-like spleen *A. gueldenstaedtii*	40.37	58.23	99.09	100.00	49.08	49.23	50.38
PAD4 *Homo sapiens*	46.44	50.45	48.93	49.08	100.00	57.99	56.23
PAD1 *Homo sapiens*	45.36	52.43	49.23	49.23	57.99	100.00	58.05
PAD3 *Homo sapiens*	44.48	51.59	50.54	50.38	56.23	58.05	100.00

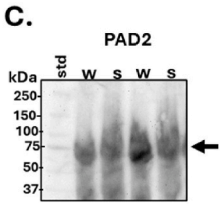


Fig. 3. PAD (PAD2-like) protein from *A. gueldenstaedtii*. **A)** A phylogram showing PAD (PAD2-like) protein sequences from *A. gueldenstaedtii* compared with PAD sequences from other fish, amphibian, reptile, bird and human, as well as cyanobacteria and *Giardia* ADI. A PAD2 clade is observed. **B)** Percent identity matrix for the amino acid sequence alignment for *A. gueldenstaedtii* PAD2-like protein (highlighted in red) and human PAD isozymes, using Clustal Omega. **C)** The anti-human PAD2 antibody cross reacted with an expected band in the 70–75 kDa range in *A. gueldenstaedtii* serum, as highlighted by the arrow (w-winter, s-summer samples, respectively; the size standard is indicated in kDa).

human PAD 1-6 isozymes is shown in Fig. 3B. For PAD protein detection in *A. gueldenstaedtii* serum by western blotting, a band of the expected 70–75 kDa size was observed by cross reaction with the human anti-PAD2 antibody, which represents the phylogenetically most conserved PAD isozyme (Fig. 3C).

3.3. The citrullinome of A. gueldenstaedtii serum and serum-EVs from winter and summer groups

Citrullinated proteins were isolated by immunoprecipitation with the F95 pan-citrullination antibody. Resulting immunoprecipitated fractions, containing citrullinated proteins, were analysed by silver

staining following SDS-PAGE (Fig. 4). Some differences in band intensity and patterns were observed between the winter and summer groups for the F95 bound proteins from sera (Fig. 4A) while less marked differences were observed for the serum-EVs (Fig. 4B). For analysis of protein ID differences of the citrullinated proteins, the fractions were analysed by LC-MS/MS. Venn diagram analysis of differential citrullination hits between winter and summer groups shows 24 and 9 unique citrullinated proteins in the serum and serum-EVs of the summer group, respectively (Fig. 4C). Citrullinated protein targets only identified in the sera of the chronically stressed summer group included alpa-2-macroglobulin, alpha globin, calcium-dependent secretion activator, ceruloplasmin, chemokine XC receptor, complement C3 isoforms, complement C9, plectin, selenoprotein and vitellogenin. Citrullinated proteins unique to the serum-EVs of the summer group included alpha-1-antiproteinase, apolipoprotein B-100, microtubule actin crosslinking factor and histone H3.

Tables 1 and 2, list the shared and specific citrullinated protein hits identified in sera of the winter and summer groups, based on ID hits with both the species-specific *A. gueldenstaedtii* database and the Actinopterygii database.

3.4. Protein-protein interaction network analysis of citrullinated proteins in A. gueldenstaedtii sera and serum-EVs, comparing winter and summer groups

Protein-protein interaction networks for citrullinated proteins identified in the winter and summer groups, for serum and serum-EVs respectively, were created in STRING. Gene ontology (GO), Reactome and STRING pathways associated to the serum citrullinomes of the winter and summer groups are shown in Fig. 5A and B, respectively. The Venn diagram in Fig. 5 represents shared and specific pathways related to citrullinated proteins detected in sera; the pathways are further listed in Table 3. Most of the identified GO, Reactome and STRING pathways in the serum citrullinome of the summer group were associated with innate immunity, as well as some metabolic pathways (Table 3).

In addition to whole sera, protein-protein interaction networks for citrullinated proteins identified in serum-EVs from both groups (winter and summer) were assessed. Protein-protein interaction networks are shown in Fig. 6 (Fig. 6A and B), and associated GO, Reactome and STRING pathways are listed in Table 4. For the serum-EVs citrullinome of the summer group, these pathways were mainly associated with lipid metabolism, but several immune pathways were also identified (Table 4).

3.5. miRNA analysis in A. gueldenstaedtii serum-EVs from winter and summer groups

Serum-EV miRNA cargoes were assessed for three miRNAs: the inflammatory associated miR-155, the hypoxia associated miR-210, and the growth-related miR-206. In serum-EVs of the heat-stressed summer group, miR-155 and miR-210 were significantly elevated compared with the winter group, by 396-fold and 63-fold, respectively (Fig. 7A and B). In the heat-stressed summer group, serum-EVs showed a significant decrease in the growth-related miR-206, by 56-fold, compared with the winter group (Fig. 7C) ($n = 3$, $p < 0.0001$ for all).

4. Discussion

In the light of increasing global warming and associated heat stress in fish populations, the identification of biomarkers to monitor fish health is of great importance. Previous studies in *A. gueldenstaedtii* showed that innate defences were weakened in response to chronic heat stress, also impacting fish growth and mortality [7,11]. Additional challenges during summer conditions include worse water quality in rivers, alongside increased phytoplankton and dominance of cyanobacteria [60,61]. Investigations of cellular and molecular mechanisms contributing to modified immune responses in chronic heat stress are therefore important. The current study assessed extracellular vesicles (EV) and protein citrullination/deimination signatures, alongside selected EV miRNA cargoes in chronic stress responses in *A. gueldenstaedtii* farmed in summer, compared with winter conditions.

4.1. Serum EV profiles

This is the first study to characterise EVs in *A. gueldenstaedtii*, showing serum-EVs as a poly-dispersed population in the size range of 40–500 nm, with the majority of EVs in the 100–200 nm range. This is similar to EV size profiles identified in other fish, with teleost serum-EVs reported at 50–300 nm and 50–600 nm in cod and halibut [36,39,62], while plasma EVs in shark and lamprey were reported at 20–200 nm and 40–500 nm, respectively [41,42]. Importantly, we found that in *A. gueldenstaedtii* the serum-EV numbers were significantly increased in the chronically stressed summer group, compared with the winter group. This was observed for all EV-subpopulations reflective of small (≤100 nm), medium (101–200 nm) and large (>200 nm) EVs. Increased circulatory EVs, with altered EV cargoes, are related to stress and pathological responses in many human diseases [63,64]. In several fish

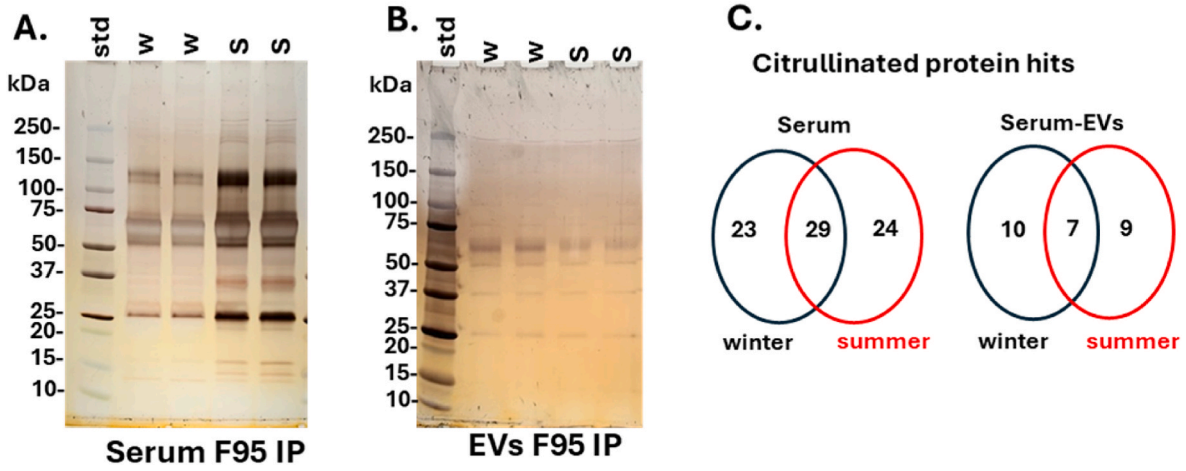


Fig. 4. Citrullinated proteins isolated by F95 immunoprecipitation from *A. gueldenstaedtii* serum and serum-EVs. **A)** Silver stained SDS-PAGE gel of citrullinated proteins isolated from sera of the winter (w) group and summer (s) groups, respectively. **B)** A silver stained SDS-PAGE gel showing citrullinated proteins from serum-EVs of the winter (w) and summer (s) groups, respectively. **C)** Venn diagrams for citrullinated protein hits identified in sera and serum-EVs by LC-MS/MS analysis, presenting unique and overlapping protein hits between winter and summer groups. IDs and names of protein hits are listed in Tables 1 and 2

Table 1
Citrullinated protein hits identified in sera of *A. gueldenstaedtii*, showing hits in winter and summer groups. Hits identified are indicated by a tick (V) per group. Hits with the *A. gueldenstaedtii* database are listed first in the table, followed by hits with the wider Actinopterygii database. Protein hits are ordered in alphabetical order of protein name.

Protein ID	Protein name	Winter	Summer	Score ($p < 0.05$)[a]
A. gueldenstaedtii				
A0A1B1CUR7	Alpha globin	V	V	179(w)/91(s)
A0A0P0QMC8	Beta-actin	V		29
A0A4Y5FCA0	C1GALT1-specific chaperone 1		V	20
A0A7S6IKW1	Hemopexin	V	V	1435(w)/1556(s)
A0A7S6IKM0	Hemopexin	V	V	1364(w)/1416(s)
A0A7S6IMQ3	Hemopexin	V	V	1293(w)/1333(s)
A0A7S6IMQ2	Intelectin	V	V	437(w)/198(s)
A0A976SHR3	Pentraxin family member	V		66
A0A976XHX7	Pentraxin family member		V	206
A0A7S6LAJ1	Transferrin a	V	V	1405(w)/1925(s)
A0A7S6IKM2	Transferrin a	V	V	975(w)/1444(s)
A0A7S6LE89	Transferrin a	V	V	922(w)/1340(s)
Actinopterygii				
A0A444UXV5	Adiponectin	V		246
A0A444UY21	Adiponectin	V	V	146(w)/267(s)
A0A5C6NST1	Alpha-1-antitrypsin-like protein	V	V	105(w)/100(s)
A0A662YNS4	Alpha-1-antiproteinase	V		1228
A0A1B1CUR1	Alpha globin		V	91
E1U341	Alpha-2-macroglobulin		V	73
A0A667YCP7	Alpha-2-macroglobulin-like protein 1	V		99
A0A8C7R6A5	Ankyrin 2a, neuronal	V		65
A0A444UVN0	Apolipoprotein A-I	V		370
A0A1X8XL86	Beta chain	V	V	113(w)/108(s)
A0A674AA58	Calcium-dependent secretion activator 2-like		V	68
A0A3Q3J933	Centromere protein H C-terminal domain-containing protein	V	V	85(w)/87(s)
A0A444U2S8	Ceruloplasmin		V	234
A0A444TWV8	Ceruloplasmin		V	78
A0A5A9PD67	Chemokine XC receptor 1 G-protein coupled receptor 5		V	63
A0A444UDE9	Complement C1q subcomponent subunit C	V	V	82(w)/62(s)
A0A8C4T8Y9	Complement C3	V	V	180(w)/184(s)
A0A8T2NBC2	Complement C3	V	V	151(w)/159(s)
A0A8C7GJC0	Complement C3	V	V	149(w)/146(s)
A0A7N6AKV7	Complement component c3a, duplicate 5	V		63
A0A6P7LXJ0	Complement C3-like isoform X1		V	101
0A3B5Q4H4	Complement C3-like		V	81
A0A444U2P1	Complement component C9		V	67
A0A3P8XPS4	Copper transport protein	V		55
A0A8T0BW81	DBF4-type domain-containing protein		V	65
A0A8C1RZL8	DDE Tnp4 domain-containing protein	V	V	60(w)/67(s)
A0A2D0RJ54	EF-hand calcium-binding domain-containing protein 6 isoform X1		V	69
A0A444TWV7	Fetuin-B	V	V	296(w)/326(s)
A0A662YSS8	Fetuin-B	V		126
A0A662YS68	Fibronectin	V	V	101(w)/115(s)
A0A444UCK2	Fibrinogen alpha chain	V	V	197(w)/249(s)
A0A444UVD8	Fibrinogen alpha chain		V	95
A0A3Q1F3U9	Fibrinogen alpha chain		V	68
A0A444UCL7	Fibrinogen beta chain	V		365
A0A444UCL0	Fibrinogen gamma chain	V	V	94(w)/194(s)
0A0A8M1KBC3	Fish-egg lectin-like	V	V	81(w)/81(s)
A0A8M9QGP4	Fish-egg lectin-like isoform X1	V		55
A0A9D3S7G7	GRIP domain-containing protein		V	57
A0A2D0PV63	Hemoglobin subunit alpha	V		65
A0A3B3URN6	Histone H4	V		62
A0A6F9BUK5	Homeobox-containing protein 1		V	61
A0A8J7NGI1	HV03 protein	V		95
A0A8J7NKS4	HV323 protein	V	V	142(w)/130(s)
A0A5N5KJN7	IF rod domain-containing protein	V		142
A0A9D3TER4	IF rod domain-containing protein	V		127
A0A3Q0R0M4	Ig-like domain-containing protein	V		156
A0A444V1B7	Ig kappa chain C region, A allele	V	V	145(w)/153(s)
A0A3Q3FM94	Ig-like domain-containing protein	V	V	131(w)/122(s)
A0A3Q3ILN3	Ig-like domain-containing protein	V		127
A0A3B4D751	Ig-like domain-containing protein	V	V	55(w)/81(s)
A0A3B4EHY6	Ig-like domain-containing protein	V	V	57(w)/73(s)
A0A665T2J4	Ig-like domain-containing protein		V	73
A0A3Q3FC42	Ig-like domain-containing protein		V	67
A0A444V3J6	Ig heavy chain V region G4	V	V	73(w)/108(s)
A0A444V3K3	Ig heavy chain V-III region 23	V		72
A0A444V141	Ig mu chain C region	V		534
A0A444V3L0	Ig mu chain C region membrane-bound form		V	55
A0A672LB90	Immunoglobulin heavy variable 9-4		V	116
A0A444UK46	Keratin, type I cytoskeletal 50 kDa	V		128
A0A8C4T3S0	Keratin, type I cytoskeletal 15-like	V		72
A0A8C4TM76	Keratin, type I cytoskeletal 47 kDa-like		V	64
A0A444V197	Major histocompatibility complex class I-related gene protein	V	V	61(w)/70(s)
A0A4Z2GBR5	Microtubule-actin cross-linking factor 1		V	53
A0A6P6LJY5	Multidrug resistance protein 1-like	V	V	66(w)/84(s)
A0A8C7D9M4	Nebulin	V	V	68(w)/68(s)
A0A662YMV0	Pentraxin family member	V		221
A0A444TZ64	Pinopsin	V		520
A0A662YZN9	Plasminogen	V	V	197(w)/60(s)

(*continued on next page*)

Table 1 (*continued*)

Protein ID	Protein name	Winter	Summer	Score ($p < 0.05$)[a]
A0A3B3XBJ8	Plectin b		V	59
A0A444UGT7	Prothrombin	V	V	159(w)/252(s)
A0A444UDM5	Prothrombin	V	V	148(w)/266(s)
A0A6G1QJS0	Retinitis pigmentosa 1-like 1 protein		V	62
A0A671V5Y5	Reverse transcriptase domain-containing protein	V		59
A0A6I9NSP4	Rho-related GTP-binding protein Rho6-like	V		59
A0A444UNQ6	RING-type E3 ubiquitin transferase	V	V	93(w)/75(s)
A0A444US59	Scavenger receptor cysteine-rich type 1 protein M130	V		60
A0A444V812	Selenoprotein P		V	149
A0A6B9VWG5	Serotransferrin isoform 3	V		1091
A0A444UYB6	Sex hormone-binding globulin	V	V	222(w)/198(s)
A0A3P8ZB53	Si:dkey-159a18.1	V		63
A0A7J5ZRR4	Symplekin		V	56
A0A662YXR3	Transferrin-like domain-containing protein	V		1121
A0A444U8B4	Transferrin-like domain-containing protein	V		950
A0A662YSD2	Transient receptor potential cation channel subfamily M member 6	V		194
A0A444UT93	Type-4 ice-structuring protein LS-12	V	V	121(w)/113(s)
A0A8M1KL24	Uncharacterized protein LOC122132339	V	V	162(w)/165(s)
W5MRN5	Uncharacterized protein	V	V	161(w)/169(s)
A0A1C8Y939	Vitellogenin AB2a		V	151
A0A8C6TNP3	WD repeat-containing protein 37		V	55

[a] Peptide sequence hits scores are shown; when a protein hit was shared the score is shown for winter (w) and summer (s) groups, within the same score column. Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores indicate 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 >14 for the *A. gueldenstaedtii* database and at >53 for the Actinopterygii database.

species, changes in serum EV numbers have been identified in response to stressors, for example in response to changed water temperatures [36], to environmental toxins [45] and other stress and immune challenges [43,44,65]. Therefore, quantification of serum-EVs might be a chronic stress biomarker for monitoring health in farmed *A. gueldenstaedtii*.

4.2. Serum citrullination/deimination signatures reveal a putative modulation of immune and metabolic pathways in summer-stressed *A. gueldenstaedtii*

The *A. gueldenstaedtii* PAD fell within the PAD2 clade on the phylogram, similar to PAD proteins reported in other fish. This is consistent with the detection of a PAD-like protein by western blotting in *A. gueldenstaedtii* sera by cross-reaction with the anti-human PAD2 antibody. Similar findings for PAD proteins, as most similar to human PAD2, have been reported in other fish, including teleosts, shark and lamprey [14,18,35,41,42], supporting that PAD2 is conserved across evolution. Interestingly, the PAD2-like protein seems to be active in *A. gueldenstaedtii* during both winter and summer farming conditions according to the citrullinated profiles obtained from the serum proteins. No upregulation of liver PAD2-like protein expression was found in

Table 2
Citrullinated protein hits identified in serum-EVs of *A. gueldenstaedtii*, showing hits in the winter and summer groups. Protein hits are indicated by a tick (V) per group. Hits are with the Actinopterygii database. Protein hits are ordered in alphabetical order of protein name.

Protein ID	Protein name	Winter	Summer	Score ($p < 0.05$)[a]
A0A0S7KFN5	ACTB (Fragment) beta-actin		V	62
A0A444UY21	Adiponectin	V		74
A0A662YNS4	Alpha-1-antiproteinase		V	63
A0A444UMP9	Apolipoprotein B-100		V	299
A0A3Q2PG63	Cyclin dependent kinase like 5	V		57
A0A4U5UIY0	Granzyme D	V	V	74(w)/72(s)
A0A7S6IKW1	Hemopexin	V	V	271(w)/385(s)
A0A7S6IMQ3	Hemopexin	V		178
Q5GB12	Histone H3		V	72
A0A3N0Y8D6	Histone H4	V		164
A0A5N5KJN7	IF rod domain-containing protein	V	V	197(w)/193(s)
A0A8C2W8G4	IF rod domain-containing protein		V	175
A0A444V141	Ig mu chain C region	V	V	111(w)/72(s)
A0A671KF31	Intermediate filament protein ON3-like	V		411
A0A498LWK8	Intermediate filament ON3-like isoform X2		V	253
Q9DGA7	Keratin	V		71
A0A8C3ZH57	Keratin, type I cytoskeletal 50 kDa-like	V		131
A0A6Q2XC09	Keratin 5	V		456
Q9DGA7	Keratin (fragment)		V	97
A0A3B3CLF1	Keratin, type I cytoskeletal 13-like		V	100
A0A668VX38	Keratin 15	V		71
A0A5C6N3H2	Keratin, type I cytoskeletal 18	V		88
A0A8C4ZJI8	Keratin 94	V	V	343(w)/351(s)
A0A8C7FG29	Low density lipoprotein receptor-related protein 1Ba		V	71
A0A087XDC5	lysozyme	V		71
A0A3B3QP35	Microtubule actin crosslinking factor 1		V	56
A0A8C2FJL3	Peptidyl-prolyl cis-trans isomerase	V		66
A0A662YVA5	Serum albumin 2	V	V	320(w)/370(s)
A0A6J2W3P0	Uncharacterized protein		V	227

[a] The peptide score is shown; when a protein hit was shared, the score is shown for winter (w) and summer (s) groups, within the same score column. Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores indicate 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 >53 (Actinopterygii database was used).

chronically heat stressed *A. gueldenstaedtii* [10]. Chronic stress conditions might upregulate PAD2-like protein expression in other tissues and/or induce its Ca^{2+} - catalysed activation and downstream protein citrullination. The elevation of Ca^{2+} in many pathological processes is well documented and importantly associated with PAD-mediated citrullination in many diseases [13,16,19,21]. Post-translational citrullination furthermore causes neo-epitope formation and can also increase antigenic diversity of proteins via changes in primary, secondary and tertiary protein structure, leading to altered antigen processing, presentation and immune recognition [66]. This can lead to altered interactions with immune cells and affect downstream signalling pathways [67]. This correlates with the identification of several immune related GO pathways associated with the serum citrullinome of the chronically stressed group.

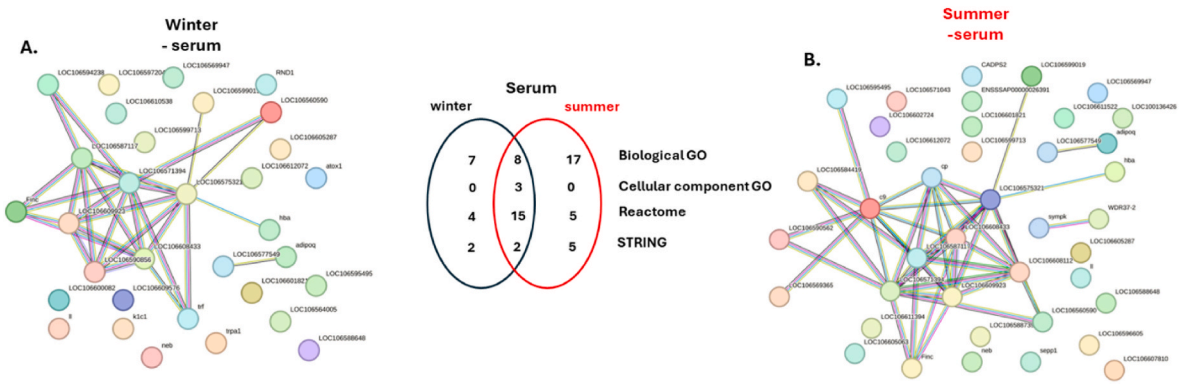


Fig. 5. Protein-protein interaction networks for citrullinated proteins identified in *A. gueldenstaedtii* sera, in winter (A) and summer groups (B), respectively. The Venn diagram summarises shared and unique Gene ontology (GO), Reactome and STRING pathways associated with the citrullinomes. Table 3 summarises the pathways.

Based on the proteomic analysis, which provided representative citrullinomes for the two groups, several serum proteins were targets of citrullination in both the summer and winter groups. This indicates that this post-translational modification might play a role in modulating baseline physiological processes that were preserved in summer conditions. Of note, some of these targets were key immune related proteins, such as the complement pathway-associated C3 component and pentraxin family members. These innate components have previously been identified as citrullinated protein targets in serum and mucosa, and/or serum/mucosa-EVs in teleost fish [18,35,36,39,40,62] and in shark [41]. Post-translational modifications may facilitate protein moonlighting in homeostasis and immune defences, and to what extent citrullination of complement proteins contributes to their function may be of considerable interest and will remain to be studied in further depth. Citrullination may affect C3 cleavage [18,35] and possibly affect binding specificity and pattern recognition capabilities of pentraxins [40]. In addition, two components linked to the activation of the classical pathway were found to be citrullinated: complement C1q, which is a potent pattern recognition molecule in the initial steps of the complement pathway [68], and the heavy and light chains of immunoglobulins.

Serum proteins associated with blood coagulation and fibrinolysis were also citrullinated in *A. gueldenstaedtii* farmed during winter and summer; they included plasminogen, prothrombin, fibronectin and fibrinogen. Plasminogen is involved in the removal of misfolded and aggregated proteins, the activation of complement and other immune and inflammatory processes [69]. It also has roles in sturgeon ovulation [70]. Prothrombin, has roles in blood coagulation and wound repair, including in sturgeon [71]. Fibronectin has multifaceted roles in cell signalling including in development and wound healing [72]. Fibrinogen, forms part of the acute phase response, including in stress responses during temperature acclimation or tetrodotoxin exposure [73,74]. It also has roles in the coagulation system in anti-pathogenic responses [75]. Other shared citrullinated protein targets were related to homeostasis pathways including nebulin, hemopexin and multidrug resistance protein 1-like. Nebulin has roles in calcium homeostasis and regulation of muscle contraction [76,77]. Hemopexin has roles in heme homeostasis and regulation of the physiological stress response to increased water temperature and exposure to heavy metals [78,79]. Hemopexin is also linked to inflammatory responses in fish and has been proposed as a minor positive acute-phase protein in *A. gueldenstaedtii* [8]. Multidrug resistance protein 1-like was also a shared citrullinated target. It is a permeability glycoprotein and ATP-dependent efflux transporter that excretes xenobiotics and other harmful substances across the plasma membrane and has critical roles in cellular defence mechanisms [80]. In addition, adiponectin was a shared citrullination candidate; it is a regulator of immune and metabolic functions, cytokine regulation and apoptosis [81–83]. In Siberian sturgeon (*A. baerii*), adiponectin has also been found to have roles in feeding behaviour regulation [84]. Collectively, these results highlight the relevance of this post-translational mechanism in influencing homeostasis in *A. gueldenstaedtii*.

Several protein targets were only identified as citrullinated in the sera of winter-farmed *A. gueldenstaedtii*. Their absence in the serum during the summer may result from protein expression regulatory mechanisms triggered by the chronic stress response. We have previously observed a relative decrease in the abundance of total serum proteins caused by the summer-induced chronic stress response [7]. Moreover, a reduction in total protein biosynthesis in the liver has indeed been observed in chronically heat-stressed sturgeons. This is part of a complex metabolic reprogramming to meet energy demands and provide glucose to peripheral tissues [10]. Citrullinated proteins only identified in winter-farmed sturgeons included some liver-derived components, whose serum levels could be reduced during the summer. These were a variety of proteins with different physiological functions, as follows: Alpha-1-antiproteinase, belonging to the serpin superfamily, plays multifaceted roles in innate immunity, with important roles in protecting tissues from enzymes released during inflammation [85]. Serpins have been linked to stress responses in sturgeon [86]. Serotransferrin forms part of innate immunity in fish, has an antimicrobial role [87] and was reported to be modulated in various sturgeon species by infection at different temperatures [8,88,89]. Apolipoprotein A-I, is involved in lipid metabolism [90], in mitochondrial function [91], in anti-bacterial and anti-viral function [92], and has roles in complement regulation, including C3 and MAC assembly [93–95]. Apolipoprotein A-I furthermore forms part of the acute phase and stress response during acclimatization to changed water temperatures [73]. Fetuin-B was identified as citrullinated in the winter group and is a cysteine proteinase inhibitor with roles in regulation of inflammatory responses, and in anti-bacterial responses in fish [96]. Further citrullinated protein targets in the winter group only included Ankyrin 2a, which plays roes in ion channel regulation, and has been reported in plasma profiles of fish during stress and infection [97,98]. Histone H4, was also citrullinated in the winter group and plays roles in inflammatory processes including lytic cell death [99] and early NETosis via citrullination [100]. Scavenger receptor cysteine-rich type 1 protein M130, plays roles in clearance and endocytosis, has roles in protection to oxidative damage and various immune responses [101,102]. Rho-related GTP-binding protein Rho6-like links to cytoskeletal regulation [103]. Copper transport protein plays roles in inflammatory and oxidative stress responses [104]. Furthermore, beta-actin was identified as citrullinated in the winter group. It is an important cytoskeletal protein with various roles in cell migration, in cellular protrusions for cell communication, in vesicle release, in nuclear function and mitochondrial function [26,105–108]. The identification of these proteins as citrullinated hits in winter-farmed

Table 3
Gene ontology (GO), Reactome and STRING pathways identified for citrullinated proteins in *A. gueldenstaedtii* sera. A tick (V) indicates that the pathway is present in the respective group.

Biological Process GO	Winter Sera	Summer Sera
Blood coagulation	V	V
Fibrinolysis	V	V
Negative regulation of blood coagulation	V	V
Negative regulation of proteolysis	V	V
Platelet activation	V	V
Positive regulation of glucose import	V	V
Regulation of body fluid levels	V	V
Response to other organism	V	V
Blood coagulation, fibrin clot formation	V	
Negative regulation of response to external stimulus	V	
Negative regulation of hydrolase activity	V	
Regulation of biological quality	V	
Regulation of heterotypic cell-cell adhesion	V	
Regulation of response to external stimulus	V	
Response to external stimulus	V	
Adaptive immune response		V
Complement activation, classical pathway		V
Defence response		V
Defence response to other organism		V
Humoral immune response		V
Immune response		V
Inflammatory response		V
Innate immune response		V
Leukocyte mediated immunity		V
Negative regulation of catalytic activity		V
Negative regulation of molecular function		V
Negative regulation of protein metabolic process		V
Positive regulation of immune response		V
Regulation of proteolysis		V
Regulation of protein metabolic process		V
Response to stress		V
Wound healing		V
Reactome Pathways	**Winter**	**Summer**
Common pathway of fibrin clot formation	V	V
Dissolution of fibrin clot	V	V
Formation of fibrin clot (clotting cascade)	V	V
GRB2:SOS provides linkage to MPK signalling for integrins	V	V
Haemostasis	V	V
Integrin cell surface interactions	V	V
Integrin signalling	V	V
MAP2K and MAPK activation	V	V
p130Cas linkage to MPK signalling for integrins	V	V
Platelet activation, signalling and aggregation	V	V
Platelet aggregation (plug formation)	V	V
Platelet degranulation	V	V
Post-translational protein phosphorylation	V	V
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	V	V
Response to elevated platelet cytosolic Ca2+	V	V
Extracellular matrix organisation	V	
MAPK1/MAPK3 signalling	V	
MAPK family signalling cascades	V	
RAF/MAP kinase cascade	V	
Activation of C3 and C5		V
Complement cascade		V
Innate immune system		V
Regulation of complement cascade		V
Regulation of TLR by endogenous ligand		V
Cellular component GO	**Winter**	**Summer**
Extracellular region	V	V
Extracellular space	V	V
Fibrinogen complex	V	V
Local network cluster (STRING)	**Winter**	**Summer**
Fibrinogen alpha/beta chain family and fibrinolysis	V	V
Mixed incl. protein activation cascade and common pathway	V	V
Mixed incl. fibrinogen and fibrinolysis	V	
Trypsin like serine protease and serin-type endopeptidase	V	
Activation of C3 and C5, and membrane attack complex		V
Complement activation, alternative pathway		V
Complement activation, and complement receptor activity		V
Complement B/C2 and complement C3/4/5. macroglobulin		V
Trypsin-like serine protease, and serine-type endopeptidase		V

sturgeons indicates a role for citrullination in their function under normal conditions.

Importantly, in sera of the summer-stressed group, numerous proteins associated with the innate immune system were identified as citrullinated. This included some complement components and ceruloplasmin. Two isoforms of complement C3 (C3-like isoform X1 and a complement C3-like protein) were citrullinated; these are likely abundant complement components in *A. gueldenstaedtii* serum, playing a central role in the activation of the three pathways of the complement system. It can be speculated that citrullination influences cleavage, binding and deposition of C3 and has possible roles in the generation of the convertase [18,35]. Complement component C9 was also citrullinated during summer conditions; this component forms part of the membrane attack complex (MAC), which is responsible for inducing the complement-mediated lysis of pathogens [109,110]. Ceruloplasmin is considered an acute-phase protein, although no evidence of liver upregulation has been found during *A. hydrophila* infection in *A. gueldenstaedtii* [10]. Interestingly, the activity of the complement alternative pathway (ACP) and ceruloplamin significantly dropped in summer-stressed *A. gueldenstaedtii* [7,9]. The hypothesis that citrullination of these innate components may be linked to this reduction in ACP and ceruloplasmin activities is of considerable interest and remains to be studied in further depth. Chemokine XC receptor 1 G-protein coupled receptor 5 has roles in immune responses [111]. Other immune-related proteins found to be citrullinated in the summer group included vitellogenin and selenoprotein. Vitellogenin is a maternal immune protein in fish, with antioxidant activities also an endocrine disruption associated biomarker in fish [112,113]. Selenoprotein P is a plasma glycoprotein with roles in selenium homeostasis, in regulation of inflammation and has antioxidant properties [114–116]. Further citrullinated protein targets in the summer group are involved in several physiological processes as discussed below. Alpha globin is part of the hemoglobin family and plays roles in aerobic metabolism and oxygen delivery [117]. EF-hand calcium-binding domain-containing protein 6 has multifaceted roles in cellular function [118,119]. Calcium-dependent secretion activator 2-like is of considerable interest as it is involved vesicle trafficking and exocytosis [120,121]. DBF4-type domain-containing protein is involved in cell cycle regulation [122]. Retinitis pigmentosa 1-like 1 protein is linked to photoreceptor biology but also has other unknown function and is associated with various diseases [123,124]. Homeobox-containing protein 1 has roles in transcriptional network regulation and cell movement processes [125]. Plectin b has key roles in cytoskeleton crosslinking and multifaceted roles in cellular organisation and signal transduction, as well as roles in mitochondrial function [126]. Microtubule-actin cross-linking factor 1 is critical in cytoskeletal dynamics, including cell migration and vesicular traffic [127,128]. GRIP domain-containing protein has roles in Golgi membrane binding [129] and Symplekin is associated with transcription of noncoding RNAs and RNA cleavage complexes [130]. A further citrullinated protein target specific for the summer group was WD repeat-containing protein 37. It is involved in critical neuronal and developmental processes and has been linked to poor growth and larval lethality in zebrafish [131].

Corresponding to the presence of immune proteins in the serum

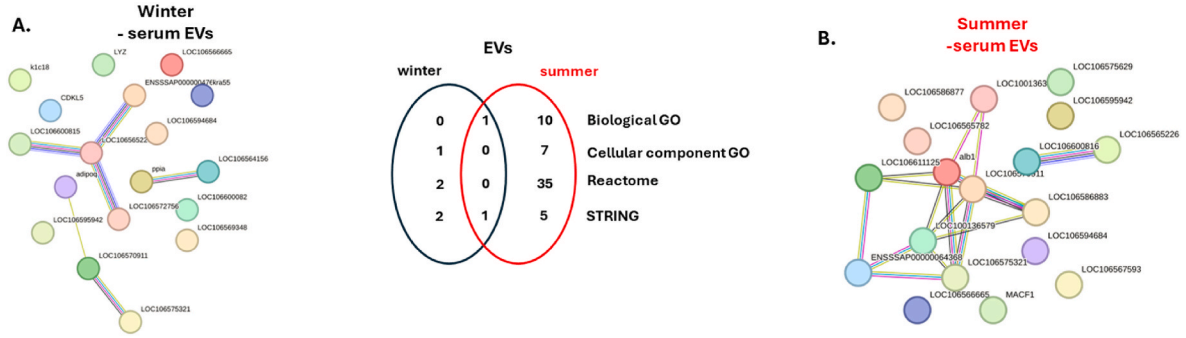


Fig. 6. Protein-protein interaction networks for citrullinated proteins identified in *A. gueldenstaedtii* serum-EVs, in winter (A) and summer groups (B), respectively. The Venn diagram summarises GO, Reactome and STRING pathways associated with the citrullinomes, showing shared and unique pathways for the winter and summer groups, respectively; Table 4 lists the pathways.

citrullinome of the summer group, several immune-associated Biological GO pathways were identified by protein-protein interaction network analysis. This included "Complement activation, classical pathway"; "Humoral immune response; "Leukocyte mediated immunity"; "Wound healing"; "Adaptive immune response"; "Inflammatory response"; "Positive regulation of immune response"; "Innate immune response"; "Immune response"; "Defence response to other organism"; and "Defence response". Proteins linked to innate immune mechanisms would be major citrullination targets during summer conditions according to the identified Reactome and STRING pathways. This suggests that citrullination of key serum immune proteins, particularly innate components, could be linked to the observed susceptibility of *A. gueldenstaedtii* to infection during the summer.

In addition, various key physiological and metabolic processes were identified in the serum citrullinome of the summer group, including: "Response to stress"; "Negative regulation of molecular function"; "Negative regulation of protein metabolic process"; "Regulation of protein metabolic process"; "Regulation of proteolysis"; and "Negative regulation of catalytic activity". The regulation of these metabolic processes via citrullination could possibly be related with the liver metabolism reprogramming caused by chronic-heat stress, affecting all biosynthesis pathways in favour of generating enough energy to sustain essential biological functions [10].

4.3. Serum-EV citrullination signatures reveal a putative modulation of lipid metabolism pathways in summer-stressed A. gueldenstaedtii and roles in NETosis

The citrullinome analysis of sturgeon serum-EVs identified several shared citrullinated protein targets between the winter and summer-farmed *A. gueldenstaedtii*. These proteins were: hemopexin, which was also identified in whole serum; Granzyme D, which is involved in the immune response and plays also roles in pyroptosis; an inflammatory mode of regulated cell death [132]. Shared serum-EV citrullinated proteins included also serum albumin, which forms part of defence mechanisms in response to stress [86,133]; as well as the cytoskeletal related proteins keratin 94 and IF rod domain-containing protein.

In addition, several EV cargo proteins were identified as citrullinated in the winter group only. These were cytoskeletal proteins including keratin 5, keratin 15 and intermediate filament protein ON3-like [134], as well as histone H4 and adiponectin, which were also identified as citrullinated in whole serum and discussed above. Furthermore, lysozyme, which plays roles in sturgeon antibacterial immune defences [135] and has been identified in stress responses in sturgeon [136] was citrullinated in serum-EVs of the winter group. Cyclin dependent kinase like 5, also a citrullination target in this group, has various roles in cellular signalling, cell migration and immunity [137,138].

Furthermore, summer-induced chronic stress led to citrullination of serum-EV proteins associated with lipid metabolism, which were absent in winter-farmed *A. gueldenstaedtii*. These included: Apolipoprotein B-100, which has critical roles in lipid metabolism [139]. Low density lipoprotein receptor-related protein 1Ba, an endocytic cell surface receptor which recognises a wide range of ligands and has roles in integrin trafficking [140]. Importantly, histone H3 was citrullinated in EV cargoes of the summer group only. This is of considerable significance as citrullinated histone H3 is a key factor in extracellular trap formation (ETosis and NETosis) which is a critical anti-microbial defence mechanism, which is well reported across phylogeny, including in fish [141–143]. Histone H3 citrullination is also involved in epigenetic mechanisms and was previously reported as elevated in serum-EVs of temperature challenged cod [36], and in fish mucosa in response to bacterial infection [18]. Our findings indicate modifications in metabolic and immune responses via changes in citrullinated EV protein cargoes in response to chronic stress.

Importantly also, increased citrullination is associated with cell death, which correlates to some of the stress and inflammatory responses in the summer group, and also for the serum EVs where both pyroptosis and necrosis were amongst the citrullinome associated GO pathways. Results from the protein-protein interaction network analysis suggested also significant roles for citrullination in modulating lipid and lipoprotein metabolic processes in chronically stressed *A. gueldenstaedtii*. Biological GO pathways for the serum-EV citrullinome of the summer group were: "Triglyceride mobilisation"; "Positive regulation of cholesterol storage"; "Plasma lipoprotein particle clearance"; "Lipoprotein transport"; "Cholesterol efflux"; "Regulation of cholesterol biosynthetic process"; "Heme transport"; "Plasma lipoprotein particle remodelling"; "Triglyceride catabolic process"; and "Lipoprotein catabolic process".

Similarly, the Reactome analysis showed that pathways associated only with the serum-EV citrullinome of the chronically stressed summer group related to metabolic changes and to anti-pathogenic responses, antimicrobial functions, immune responses and cellular communication. A similar correlation was found with the local network cluster STRING pathways, which highlighted further immune associated functions linked to the citrullinated proteins in the summer-stressed group.

4.4. Serum-EV associated miRNAs indicate trade-off in immunity and growth in chronically stressed A. gueldenstaedtii

When assessing EV cargoes for selected miRNA expressions, serum-EVs of *A. gueldenstaedtii* from the chronically stressed summer group showed a significant increase in inflammatory (miR-155) and hypoxia (miR-210) associated miRNAs. Both miRNAs have been related to inflammation and hypoxic stress in various pathologies [144–147]. In fish, miR-155 has previously been associated with heat stress [36,148], identified as a stress marker for fish toxicology studies [149,150] and been reported in virus infected fish [52]. The observed inflammatory status in the summer stressed group is in alignment with findings from a

Table 4
Gene ontology (GO), Reactome and STRING pathways identified for citrullinated proteins in *A. gueldenstaedtii* serum-EVs. A tick (V) indicates that the pathway is present in the respective group.

Biological Process GO	Winter Serum-EVs	Summer Serum-EVs
Intermediate filament cytoskeleton organisation	V	V
Cholesterol efflux		V
Heme transport		V
Lipoprotein catabolic process		V
Lipoprotein transport		V
Plasma lipoprotein particle clearance		V
Plasma lipoprotein particle remodelling		V
Positive regulation of cholesterol storage		V
Regulation of cholesterol biosynthetic process		V
Triglyceride catabolic process		V
Triglyceride mobilisation		V
Reactome Pathways	**Winter**	**Summer**
Formation of the cornified envelope	V	
Keratinisation	V	
Activation of matrix metalloproteinases		V
Activation, myristoylation of BID and translocation to mitochondria		V
Antimicrobial peptides		V
Binding and uptake of ligands by scavenger receptors		V
Chylomicron assembly		V
Chylomicron clearance		V
Chylomicron remodelling		V
Clathrin-mediated endocytosis		V
Degradation of extracellular matrix		V
Formation of the cornified envelope		V
Heme signalling		V
LDL clearance		V
LDL remodelling		V
Metabolism of angiotensinogen to angiotensins		V
Metabolism of fat-soluble vitamins		V
Metabolism of vitamins and cofactors		V
Platelet sensitisation by LDL		V
Plasma lipoprotein assembly		V
Plasma lipoprotein clearance		V
Plasma lipoprotein remodelling		V
Post-translational protein phosphorylation		V
Pyroptosis		V
Regulated necrosis		V
Regulation of Insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs)		V
Regulation of TLR by endogenous ligand		V
Retinoid metabolism and transport		V
Scavenging by Class A receptors		V
Scavenging by Class B receptors		V
Scavenging by Class H receptors		V
Scavenging of heme from plasma		V
Sensory perception		V
Signalling by SCF-KIT		V
Vesicle-mediated transport		V
Visual phototransduction		V
VLDL assembly		V
Cellular component GO	**Winter**	**Summer**
Intermediate filament	V	
Chylomicron		V
Endoplasmic reticulum exit site		V
Extracellular space		V
Intermediate-density lipoprotein particle		V
Low-density lipoprotein particle		V
Supramolecular fibre		V
Very-low density lipoprotein particle		V
Local network cluster (STRING)	**Winter**	**Summer**
Mixed, incl. Type I hemidesmosome assembly, and intracellular phosphatidylinositol-3,5-bisphosphate-sensitive cation channel activity	V	V
Mixed, incl. keratin type II head, and keratin filament	V	
Mixed, incl. nucleosome core, and linker histone H1/H5	V	
Mixed, incl. low-density lipoprotein receptor YWTD domain		V
Mixed, incl. uptake of dietary cobalamins into enterocytes		V
Serum albumin, N-terminal, and Peroxidasin, peroxidase		V
Trypsin-like serine protease, and serine-type endopeptidase		V
Trypsin-like serine protease, and signalling by MST1		V

previous study showing liver damage and inflammation in chronically heat-stressed sturgeons [10]. Furthermore, the significant increase observed here in miR-210 in *A. gueldenstaedtii* serum-EVs of the summer group suggests the induction of a hypoxic response in chronic stress. Roles for miR-210 in regulating hypoxia responses are well reported alongside roles in mitochondrial metabolism, DNA damage responses and apoptosis [146,151,152]. In fish, miR-210 has been shown to be elevated in response to viral and bacterial infection [153,154], and to be modulated in response to environmental hypoxic and nutritional stressors [155]. In zebrafish, miR-210 has been reported to promote immune-related genes and to suppress oocyte meiosis [156]. Changes in cellular aerobic capacity in response to elevated water temperature have been reported for farmed white sturgeon [157]. Furthermore, oxidative stress has been reported in response to starvation and refeeding in *A. stellatus* juveniles [158]. In addition, juvenile white sturgeons were shown to display changes in thermal and hypoxic stress markers and global DNA methylation in response to heatwaves [12]. In order to identify whether there may be a trade-off in immune responses and growth in summer-induced tress, the growth related miR-206 was also assessed in the serum-EVs. Interestingly, miR-206 was found to be significantly reduced in the heat-stressed summer group. This indicates that chronic heat stress impacts miRNAs regulating growth, which may result in smaller fish. This finding correlates with our previous study showing that *A. gueldenstaedtii* exposed to heat stress showed a progressive decrease in body weight, alongside increased fish mortality [7]. Likewise, a study on cod cultured in sub-optimal water temperatures showed a decrease in miR-206 in parallel to an increase in inflammatory markers [36]. Furthermore, miR-206 was previously reported as an important muscle enriched miRNA (myo-miR) during temperature challenge in early cod development [54]. Our results overall align with previously identified roles for these three miRs in fish immune responses and growth and highlight these as putative EV-associated biomarkers in response to chronic heat-stress in *A. gueldenstaedtii*.

The current study identified modifications in EV release profiles and EV cargoes, with significantly increased numbers of serum-EVs in chronically stressed *A. gueldenstaedtii.* The findings furthermore highlight putative roles for post-translational citrullination/deimination in modulating immune and metabolic responses in *A. gueldenstaedtii*. An increase in citrullinome associated pathways was identified in sera and serum-EVs of chronically stressed *A. gueldenstaedtii*. In addition, EV miRNA-cargoes relating to inflammation (miR-155) and hypoxic stress (miR-210) were significantly elevated, while the growth related miR-206 was downregulated in the chronically stressed group. Our findings highlight a trade-off in growth and inflammatory responses in *A. gueldenstaedtii* during chronic stress conditions associated to fish farmed in the summer. This supports our [10] and other studies, which reported that heat-challenged sturgeon species (*A. gueldenstaedtii* and *A. fulvescens*) have compromised metabolic, stress and immune responses [136]. It must be noted that for the purpose of this current study the citrullinome analysis carried out per group was based on a pool of 15 samples per group, therefore providing a representative list of citrullinated protein hits for the summer and winter groups, respectively. Further assessment on individual variability will need to be subject of future studies. As many key immune proteins were identified here as citrullinated targets, it will be of interest to assess some of these in further depth in future studies, including changes on protein function

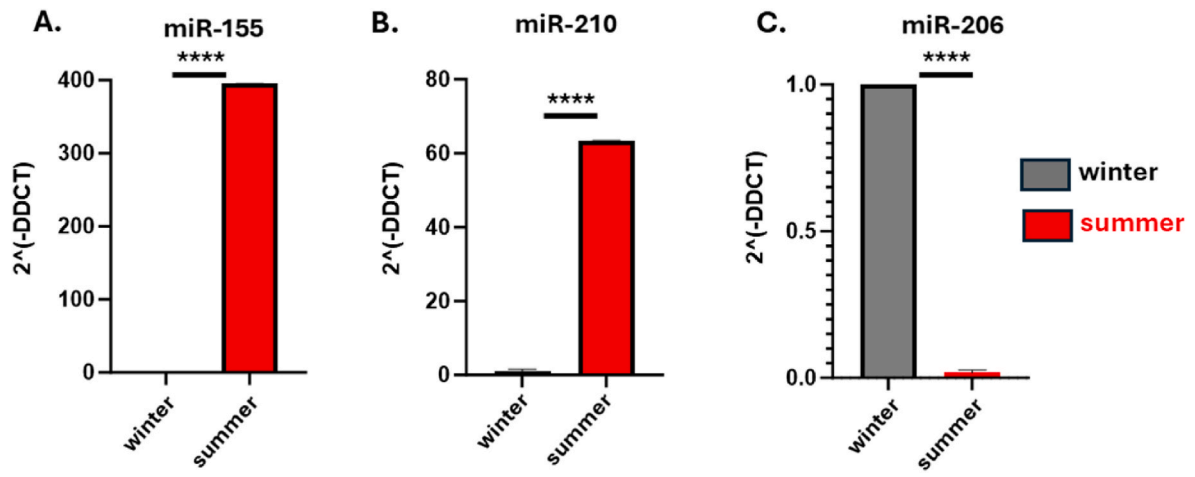


Fig. 7. Inflammatory, hypoxia, and growth-related micro-RNA expressions were modified in serum-EVs of *A. gueldenstaedtii* in response to summer conditions. **A)** miR-155, related to inflammatory responses, was significantly increased (396-fold) in serum-EVs of the summer group; **B)** miR-210, related to hypoxia responses, was significantly increased (63-fold) in serum-EVs of the summer group; **C)** miR-206, related to growth, was significantly reduced in serum-EVs (56-fold) of the summer group. Mean values with error bars representing standard deviation are presented. Normalised values 2 (-DDCT) are presented, and data was normalised according to RNU6 expression by fold analysis (n = 3, for each group where 3 × 5 samples were pooled per group; ****$p \leq 0.0001$).

caused by the post-translational conversion of arginine to citrullinine at different sites, also depending on numbers and positions of arginines in the chosen target protein. Our study paves the way for developing EV and citrullinome based biomarkers for sustainable sturgeon aquaculture. In addition, our findings may be translatable to other fish species both in the wild and in aquaculture.

5. Conclusion

The identification of chronic thermal stress biomarkers in *A. gueldenstaedtii* is of considerable interest for sustainable aquaculture in response to declining sturgeon populations and global climate change. This is the first study to assess circulatory extracellular vesicle (EV) signatures and post-translationally citrullinated proteins in sturgeon, a chondrostean fish. We reported elevated serum-EV numbers in chronically stressed *A. gueldenstaedtii*, with modified citrullinated proteins in serum and serum-EVs relating to immune-associated and metabolic pathways. The EVs of the stressed group furthermore showed changes in microRNA cargoes, with significantly increased pro-inflammatory (miR-155) and hypoxia-stress related (miR-210) markers, and reduced miR-206, which is associated with growth. Our findings indicated a trade off in immune responses versus growth in *A. gueldenstaedtii* exposed to chronic, including heat, stress. EVs and citrullinome signatures may be useful biomarkers in chronic stress in *A. gueldenstaedtii* and to monitor fish health.

CRediT authorship contribution statement

Ana María Ferreira: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Valeria Silva-Álvarez:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Igor Kraev:** Data curation, Methodology, Resources, Visualization, Writing – review & editing. **Pinar Uysal-Onganer:** Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing. **Sigrun Lange:** Conceptualisation, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of interest

The authors declare no competing interest.

Acknowledgements

The study was supported in parts by Quality-related Research (QR) funds from the University of Westminster to S.L. and by the Comisión Sectorial de Investigación Científica (UdelaR, CSIC grant # 2020–455, Uruguay), Agencia Nacional de Investigación e Innovación (ANII-Uruguay) and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA) to A.M.F and V.S.A. Thanks are due to the Guy Foundation for funding some of the equipment used in this study and to the Cambridge Centre for Proteomics for the LC-MS/MS analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109974.

Data availability

Supplementary Files contain additional supporting data for proteomics analysis.

References

[1] L. Long, H. Zhang, Q. Ni, H. Liu, F. Wu, X. Wang, Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 219 (2019) 25–34, https://doi.org/10.1016/j.cbpc.2019.02.002.

[2] M. Stachnik, M. Matras, E. Borzym, J. Maj-Paluch, M. Reichert, Emerging viral pathogens in sturgeon aquaculture in Poland: focus on herpesviruses and mimivirus detection, Viruses 13 (8) (2021) 1496, https://doi.org/10.3390/v13081496.

[3] N.K. Sergaliev, M.G. Kakishev, N.S. Ginayatov, F.K. Nurzhanova, E.E. Andronov, Microbiome structure in a recirculating aquaculture system and its connection to infections in sturgeon fish, Vet. World 14 (3) (2021) 661–668, https://doi.org/10.14202/vetworld.2021.661-668.

[4] A.L.T. Romney, D.M. Myers, F.R. Martin, T.N. Scanlan, S.A. Meyers, Germ cell recovery, cryopreservation and transplantation in the California white sturgeon, *Acipenser transmontanus*, Sci. Rep. 13 (1) (2023) 16905, https://doi.org/10.1038/s41598-023-44079-6.

[5] M. Minaz, İ.Z. Kurtoğlu, Long-term exposure of endangered Danube sturgeon (*Acipenser gueldenstaedtii*) to bisphenol A (BPA): growth, behavioral, histological,

genotoxic, and hematological evaluation, Environ. Sci. Pollut. Res. Int. 31 (21) (2024) 30836–30848, https://doi.org/10.1007/s11356-024-33168-2.
[6] A. Bodini, D. Pereira, M. Scotti, The decline of kilkas, sturgeons and seals in the Caspian Sea: the potential of qualitative loop analysis for the cumulative assessment of multiple drivers of stress, Mar. Pollut. Bull. 200 (2024) 116091, https://doi.org/10.1016/j.marpolbul.2024.116091.
[7] M. Castellano, V. Silva-Álvarez, E. Fernández-López, V. Mauris, D. Conijeski, A. Villarino, A.M. Ferreira, Russian sturgeon cultured in a subtropical climate shows weaken innate defences and a chronic stress response, Fish Shellfish Immunol. 68 (2017) 443–451, https://doi.org/10.1016/j.fsi.2017.07.048.
[8] M. Castellano, V. Silva-Álvarez, M. Aversa-Marnai, M. Lamas-Bervejillo, I. Quartiani, A. Perretta, A. Villarino, A.M. Ferreira, Serum amyloid A is a positive acute phase protein in Russian sturgeon challenged with *Aeromonas hydrophila*, Sci. Rep. 10 (1) (2020) 22162, https://doi.org/10.1038/s41598-020-79065-9.
[9] M. Aversa-Marnai, M. Castellano, I. Quartiani, D. Conijesky, A. Perretta, A. Villarino, V. Silva-Álvarez, A.M. Ferreira, Different response of *Acipenser gueldenstaedtii* CRP/SAP and SAA to bacterial challenge and chronic thermal stress sheds light on the innate immune system of sturgeons, Fish Shellfish Immunol. 121 (2022) 404–417, https://doi.org/10.1016/j.fsi.2021.12.029.
[10] A. Costábile, M. Castellano, M. Aversa-Marnai, I. Quartiani, D. Conijeski, A. Perretta, A. Villarino, V. Silva-Álvarez, A.M. Ferreira, A different transcriptional landscape sheds light on Russian sturgeon (*Acipenser gueldenstaedtii*) mechanisms to cope with bacterial infection and chronic heat stress, Fish Shellfish Immunol. 128 (2022) 505–522, https://doi.org/10.1016/j.fsi.2022.08.022.
[11] A.M. Ferreira, M. Aversa-Marnai, A. Villarino, V. Silva-Álvarez, Innate immune and chronic heat stress responses in sturgeons: advances and insights from studies on Russian sturgeons, Fish Shellfish Immunol. Rep 5 (2023) 100121, https://doi.org/10.1016/j.fsirep.2023.100121.
[12] M.L. Earhart, T.S. Blanchard, N. Strowbridge, R. Sheena, C. McMaster, B. Staples, C.J. Brauner, D.W. Baker, P.M. Schulte, Heatwave resilience of juvenile white sturgeon is associated with epigenetic and transcriptional alterations, Sci. Rep. 13 (1) (2023) 15451, https://doi.org/10.1038/s41598-023-42652-7.
[13] E.R. Vossenaar, A.J. Zendman, W.J. van Venrooij, G.J. Pruijn, PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease, Bioessays 25 (11) (2003) 1106–1118, https://doi.org/10.1002/bies.10357.
[14] A. Rebl, B. Köllner, E. Anders, K. Wimmers, T. Goldammer, Peptidylarginine deiminase gene is differentially expressed in freshwater and brackish water rainbow trout, Mol. Biol. Rep. 37 (5) (2010) 2333–2339, https://doi.org/10.1007/s11033-009-9738-5.
[15] J.P.C. Williams, L.J. Walport, PADI6: what we know about the elusive fifth member of the peptidyl arginine deiminase family, Philos. Trans. R. Soc. Lond. B Biol. Sci. 378 (1890) (2023) 20220242, https://doi.org/10.1098/rstb.2022.0242.
[16] E.E. Witalison, P.R. Thompson, L.J. Hofseth, Protein arginine deiminases and associated citrullination: physiological functions and diseases associated with dysregulation, Curr. Drug Targets 16 (7) (2015) 700–710, https://doi.org/10.2174/1389450116666150202160954.
[17] P. Yurttas, A.M. Vitale, R.J. Fitzhenry, L. Cohen-Gould, W. Wu, J.A. Gossen, S. A. Coonrod, Role for PADI6 and the cyto-plasmic lattices in ribosomal storage in oocytes and translational control in the early mouse embryo, Development 135 (2008) 2627–2636, https://doi.org/10.1242/dev.016329.
[18] B. Magnadóttir, P. Hayes, M. Hristova, B.T. Bragason, A.P. Nicholas, A.W. Dodds, S. Guðmundsdóttir, S. Lange, Post-translational protein deimination in cod (*Gadus morhua* L.) ontogeny novel roles in tissue remodelling and mucosal immune defences? Dev. Comp. Immunol. 87 (2018) 157–170, https://doi.org/10.1016/j.dci.2018.06.006.
[19] M. Alghamdi, D. Alasmari, A. Assiri, E. Mattar, A.A. Aljaddawi, S.G. Alattas, E. M. Redwan, An overview of the intrinsic role of citrullination in autoimmune disorders, J. Immunol. Res. (2019) 7592851, https://doi.org/10.1155/2019/7592851, 2019.
[20] M.C. Méchin, H. Takahara, M. Simon, M. Deimination and peptidylarginine deiminases in skin physiology and diseases, Int. J. Mol. Sci. 21 (2020) 566, https://doi.org/10.3390/ijms21020566.
[21] M.A. Christophorou, The virtues and vices of protein citrullination, R. Soc. Open Sci. 9 (6) (2022) 220125, https://doi.org/10.1098/rsos.220125.
[22] S. Lange, Peptidylarginine deiminases and extracellular vesicles: prospective drug targets and biomarkers in central nervous system diseases and repair, Neural Regen. Res. 16 (2021) 934–938, https://doi.org/10.4103/1673-5374.297058.
[23] S. D'Alessio, K.M. Buckley, I. Kraev, P. Hayes, S. Lange, Extracellular vesicle signatures and post-translational protein deimination in purple sea urchin (*Strongylocentrotus purpuratus*) coelomic fluid-novel insights into Echinodermata biology, Biology 10 (9) (2021) 866, https://doi.org/10.3390/biology10090866.
[24] T.F.M. Cummings, K. Gori, L. Sanchez-Pulido, G. Gavriilidis, D. Moi, A.R. Wilson, E. Murchison, C. Dessimoz, C.P. Ponting, M.A. Christophorou, Citrullination was introduced into animals by horizontal gene transfer from cyanobacteria, Mol. Biol. Evol. 39 (2) (2022) msab317, https://doi.org/10.1093/molbev/msab317.
[25] B. György, E. Tóth, E. Tarcsa, A. Falus, E.I. Buzás, Citrullination: a posttranslational modification in health and disease, Int. J. Biochem. Cell Biol. 38 (2006) 1662–1677, https://doi.org/10.1016/j.biocel.2006.03.008.
[26] S. Kholia, S. Jorfi, P. R, P.R. Thompson, C.P. Causey, A.P. Nicholas, J.M. Inal, S. Lange, A novel role for peptidylarginine deiminases in microvesicle release reveals therapeutic potential of PAD inhibition in sensitizing prostate cancer cells to chemotherapy, J. Extracell. Vesicles 4 (2015) 26192, https://doi.org/10.3402/jev.v4.26192.
[27] E. Bielecka, C. Scavenius, T. Kantyka, M. Jusko, D. Mizgalska, B. Szmigielski, B. Potempa, J.J. Enghild, E.R. Prossnitz, A.M. Blom, J. Potempa, Peptidyl arginine deiminase from *Porphyromonas gingivalis* abolishes anaphylatoxin C5a activity, J. Biol. Chem. 289 (2014) 32481–32487, https://doi.org/10.1074/jbc.C114.617142.
[28] U.S. Kosgodage, P. Matewele, G. Mastroianni, I. Kraev, D. Brotherton, B. Awamaria, A.P. Nicholas, S. Lange, J.M. Inal, Peptidylarginine deiminase inhibitors reduce bacterial membrane vesicle release and sensitize bacteria to antibiotic treatment, Front. Cell. Infect. Microbiol. 9 (2019) 227, https://doi.org/10.3389/fcimb.2019.00227.
[29] B. Gavinho, B. Sabatke, V. Feijoli, I.V. Rossi, J.M. da Silva, I. Evans-Osses, G. Palmisano, S. Lange, M.I. Ramirez, Peptidylarginine deiminase inhibition abolishes the production of large extracellular vesicles from *Giardia intestinalis*, affecting host-pathogen interactions by hindering adhesion to host cells, front, Cell Infect. Microbiol. 10 (2020) 417, https://doi.org/10.3389/fcimb.2020.00417.
[30] Á. Kristmundsson, Á. Erlingsdóttir, S. Lange, Peptidylarginine deiminase (PAD) and post-translational protein deimination-novel insights into alveolata metabolism, epigenetic regulation and host-pathogen interactions, Biology 10 (3) (2021) 177, https://doi.org/10.3390/biology10030177.
[31] C.J. Coates, I. Kraev, A.F. Rowley, S. Lange, Extracellular vesicle signatures and protein citrullination are modified in shore crabs (*Carcinus maenas*) infected with *Hematodinium* sp, Virulence 14 (1) (2023) 2180932, https://doi.org/10.1080/21505594.2023.2180932.
[32] T.J. Bowden, I. Kraev, S. Lange, Post-translational protein deimination signatures and extracellular vesicles (EVs) in the Atlantic horseshoe crab (*Limulus polyphemus*), Dev. Comp. Immunol. 110 (2020) 103714, https://doi.org/10.1016/j.dci.2020.103714.
[33] T.J. Bowden, I. Kraev, S. Lange, S, extracellular vesicles and post-translational protein deimination signatures in Mollusca-the blue mussel (*Mytilus edulis*), soft shell clam (*Mya arenaria*), eastern oyster (*Crassostrea virginica*) and atlantic jacknife clam (*Ensis leei*), Biology 9 (12) (2020) 416, https://doi.org/10.3390/biology9120416.
[34] T.J. Bowden, I. Kraev, S. Lange, Extracellular vesicles and post-translational protein deimination signatures in haemolymph of the American lobster (*Homarus americanus*), Fish Shellfish Immunol. 106 (2020) 79–102, https://doi.org/10.1016/j.fsi.2020.06.053.
[35] B. Magnadóttir, B.T. Bragason, I.R. Bricknell, T. Bowden, A.P. Nicholas, M. Hristova, S. Guðmundsdóttir, A.W. Dodds, S. Lange, 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.), Dev. Comp. Immunol. 92 (2019) 1–19, https://doi.org/10.1016/j.dci.2018.10.016.
[36] B. Magnadóttir, P. Uysal-Onganer, I. Kraev, A.W. Dodds, S. Guðmundsdóttir, S. Lange, Extracellular vesicles, deiminated protein cargo and microRNAs are novel serum biomarkers for environmental rearing temperature in Atlantic cod (*Gadus morhua* L.), Aquacult. Rep. 16 (2020) 100245, https://doi.org/10.1016/j.aqrep.2019.100245.
[37] B. Magnadóttir, P. Uysal-Onganer, I. Kraev, V. Svansson, P. Hayes, S. Lange, Deiminated proteins and extracellular vesicles - novel serum biomarkers in whales and orca, Comp. Biochem. Physiol., Part D: Genomics Proteomics 34 (2020) 100676, https://doi.org/10.1016/j.cbd.2020.100676.
[38] B. Magnadóttir, P. Uysal-Onganer, I. Kraev, V. Svansson, K. Skírnisson, S. Lange, Deiminated proteins and extracellular vesicles as novel biomarkers in pinnipeds: grey seal (*Halichoerus gryptus*) and harbour seal (*Phoca vitulina*), Biochimie 171–172 (2020) 79–90, https://doi.org/10.1016/j.biochi.2020.02.017.
[39] B. Magnadóttir, I. Kraev, A.W. Dodds, S. Lange, The proteome and citrullinome of *Hippoglossus hippoglossus* extracellular vesicles-novel insights into roles of the serum secretome in immune, gene regulatory and metabolic pathways, Int. J. Mol. Sci. 22 (2) (2021) 875, https://doi.org/10.3390/ijms22020875.
[40] B. Magnadóttir, P. Hayes, B. Gísladóttir, B.þ. Bragason, M. Hristova, A. P. Nicholas, S. Guðmundsdóttir, S. Lange, Pentraxins CRP-I and CRP-II are post-translationally deiminated and differ in tissue specificity in cod (*Gadus morhua* L.) ontogeny, Dev. Comp. Immunol. 87 (2018) 1–11, https://doi.org/10.1016/j.dci.2018.05.014.
[41] M.F. Criscitiello, I. Kraev, S. Lange, Deiminated proteins in extracellular vesicles and plasma of nurse shark (*Ginglymostoma cirratum*) - novel insights into shark immunity, Fish Shellfish Immunol. 92 (2019) 249–255, https://doi.org/10.1016/j.fsi.2019.06.012.
[42] J.P. Rast, S. D'Alessio, I. Kraev, S. Lange, Post-translational protein deimination signatures in sea lamprey (*Petromyzon marinus*) plasma and plasma-extracellular vesicles, Dev. Comp. Immunol. 125 (2021) 104225, https://doi.org/10.1016/j.dci.2021.104225.
[43] E. Faught, L. Henrickson, M.M. Vijayan, Plasma exosomes are enriched in Hsp 70 and modulated by stress and cortisol in rainbow trout, J. Endocrinol. 232 (2) (2017) 237–246, https://doi.org/10.1530/JOE-16-0427.
[44] D. Iliev, G. Strandskog, A. Nepal, A. Aspar, R. Olsen, J. Jørgensen, D. Wolfson, B. S. Ahluwalia, J. Handzhiyski, R. Mironova, Stimulation of exosome release by extracellular DNA is conserved across multiple cell types, FEBS J. 285 (16) (2018) 3114–3133, https://doi.org/10.1111/febs.14601.
[45] M. Tang, Y. Chen, H. Xian, S. Tan, Z. Lian, X. Peng, D. Hu, Circulating exosome level of indigenous fish may be a novel biomarker for the integrated ecotoxicity effect of water environment, Ecotoxicol. Environ. Saf. 229 (2022) 113084, https://doi.org/10.1016/j.ecoenv.2021.113084.
[46] A.E. Pasquinelli, MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship, Nat. Rev. Genet. 13 (4) (2012) 271–282, https://doi.org/10.1038/nrg3162.

[47] T.T. Bizuayehu, I. Babiak I, MicroRNA in teleost fish, Genome Biol. Evol. 6 (8) (2014) 1911–1937, https://doi.org/10.1093/gbe/evu151.

[48] C. Best, H. Ikert, D.J. Kostyniuk, P.M. Craig, L. Navarro-Martin, L. Marandel, J. A. Mennigen, Epigenetics in teleost fish: from molecular mechanisms to physiological phenotypes, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 224 (2018) 210–244, https://doi.org/10.1016/j.cbpb.2018.01.006.

[49] L. Yuan, X. Zhang, L. Li, H. Jiang, J. Chen, High-throughput sequencing of microRNA transcriptome and expression assay in the sturgeon, *Acipenser schrenckii*, PLoS One 9 (12) (2014) e115251, https://doi.org/10.1371/journal.pone.0115251.

[50] M.V. Fofanov, D.Y. Prokopov, H. Kuhl, M. Schartl, V.A. Trifonov, Evolution of MicroRNA biogenesis genes in the sterlet (Acipenser ruthenus) and other polyploid vertebrates, Int. J. Mol. Sci. 21 (24) (2020) 9562, https://doi.org/10.3390/ijms21249562.

[51] Q. Cao, H. Zhang, T. Li, L. He, J. Zong, H. Shan, L. Huang, Y. Zhang, H. Liu, J. Jiang, Profiling miRNAs of teleost fish in responses to environmental stress: a review, Biology 12 (3) (2023) 388, https://doi.org/10.3390/biology12030388.

[52] R. Andreassen, B. Høyheim, miRNAs associated with immune response in teleost fish, Dev. Comp. Immunol. 75 (2017) 77–85, https://doi.org/10.1016/j.dci.2017.02.023.

[53] B. Yan, C.D. Zhu, J.T. Guo, L.H. Zhao, J.L. Zhao, miR-206 regulates the growth of the teleost tilapia (*Oreochromis niloticus*) through the modulation of IGF-1 gene expression, J. Exp. Biol. 216 (Pt 7) (2013) 1265–1269, https://doi.org/10.1242/jeb.079590.

[54] T.T. Bizuayehu, S.D. Johansen, V. Puvanendran, H. Toften, I. Babiak, I, Temperature during early development has long-term effects on microRNA expression in Atlantic cod, BMC Genom. 16 (1) (2015) 305, https://doi.org/10.1186/s12864-015-1503-7.

[55] A. Ali, W.M. Shaalan, R. Al-Tobasei, M. Salem, Coding and noncoding genes involved in atrophy and compensatory muscle growth in nile Tilapia, Cells 11 (16) (2022) 2504, https://doi.org/10.3390/cells11162504.

[56] J.A. Welsh, D.C.I. Goberdhan, L. O'Driscoll, E.I. Buzas, C. Blenkiron, B. Bussolati, H. Cai, D. Di Vizio, T.A.P. Driedonks, U. Erdbrügger, J.M. Falcon-Perez, Q.L. Fu, A.F. Hill, M. Lenassi, S.K. Lim, M.G. Mahoney, S. Mohanty, A. Möller, R. Nieuwland, T. Ochiya, K.W. Witwer, Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches, J. Extracell. Vesicles 13 (2) (2024) e12404, https://doi.org/10.1002/jev2.12404.

[57] J. Rice, H. Roberts, S.N. Rai, S. Galandiuk, Housekeeping genes for studies of plasma microRNA: a need for more precise standardization, Surgery (St Louis) 158 (5) (2015) 1345–1351, https://doi.org/10.1016/j.surg.2015.04.025.

[58] R.E. Crossland, J. Norden, L.A. Bibby, J. Davis, A.M. Dickinson, Evaluation of optimal extracellular vesicle small RNA isolation and qRT-PCR normalisation for serum and urine, J. Immunol. Methods 429 (2016) 39–49, https://doi.org/10.1016/j.jim.2015.12.011.

[59] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (4) (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[60] G. Ferrari, M. del Carmen Pérez, M. Dabezies, D. Míguez, C. Saizar, Planktic cyanobacteria in the lower Uruguay river, south America, Fottea 11 (1) (2011) 225–234, https://doi.org/10.5507/fot.2011.021.

[61] S. Haakonsson, L. Rodríguez-Gallego, A. Somma, S. Bonilla, Temperature and precipitation shape the distribution of harmful cyanobacteria in subtropical lotic and lentic ecosystems, Sci. Total Environ. 609 (2017) 1132–1139, https://doi.org/10.1016/j.scitotenv.2017.07.067.

[62] B. Magnadóttir, I. Kraev, S. Guðmundsdóttir, A.W. Dodds, S. Lange, Extracellular vesicles from cod (*Gadus morhua* L.) mucus contain innate immune factors and deiminated protein cargo, Dev. Comp. Immunol. 99 (2019) 103397, https://doi.org/10.1016/j.dci.2019.103397.

[63] Q. Wu, H. Zhang, S. Sun, L. Wang, S. Sun, Extracellular vesicles and immunogenic stress in cancer, Cell Death Dis. 12 (10) (2021) 894, https://doi.org/10.1038/s41419-021-04171-z.

[64] A. Abramowicz, P. Widłak, M. Pietrowska, Different types of cellular stress affect the proteome composition of small extracellular vesicles: a mini review, Proteomes 7 (2) (2019) 23, https://doi.org/10.3390/proteomes7020023.

[65] L. Lagos, J. Tandberg, A. Kashulin-Bekkelund, D.J. Colquhoun, H. Sørum, H. C. Winther-Larsen, Isolation and characterization of serum extracellular vesicles (EVs) from atlantic salmon infected with *piscirickettsia salmonis*, Proteomes 5 (4) (2017) 34, https://doi.org/10.3390/proteomes5040034.

[66] H.A. Doyle, M.J. Mamula, Autoantigenesis: the evolution of protein modifications in autoimmune disease, Curr. Opin. Immunol. 24 (1) (2012) 112–118, https://doi.org/10.1016/j.coi.2011.12.003.

[67] H. Nguyen, E.A. James, Immune recognition of citrullinated epitopes, Immunology 149 (2) (2016) 131–138, https://doi.org/10.1111/imm.12640.

[68] K.B.M. Reid, Complement component C1q: historical perspective of a functionally versatile, and structurally unusual, serum protein, Front. Immunol. 9 (2018) 764, https://doi.org/10.3389/fimmu.2018.00764.

[69] C.B. Keragala, R.L. Medcalf, Plasminogen: an enigmatic zymogen, Blood 137 (21) (2021) 2881–2889, https://doi.org/10.1182/blood.2020008951.

[70] R. Surugaya, Y. Hasegawa, S. Adachi, S. Ijiri, Changes in ovulation-related gene expression during induced ovulation in the amur sturgeon (*Acipenser schrenckii*) ovarian follicles, Int. J. Mol. Sci. 23 (21) (2022) 13143, https://doi.org/10.3390/ijms232113143.

[71] D.K. Banfield, R.T. MacGillivray, Partial characterization of vertebrate prothrombin cDNAs: amplification and sequence analysis of the B chain of thrombin from nine different species, Proc. Natl. Acad. Sci. U.S.A. 89 (7) (1992) 2779–2783, https://doi.org/10.1073/pnas.89.7.2779.

[72] J. Patten, K. Wang, Fibronectin in development and wound healing, Adv. Drug Deliv. Rev. 170 (2021) 353–368, https://doi.org/10.1016/j.addr.2020.09.005.

[73] M.A. Dietrich, P. Hliwa, M. Adamek, D. Steinhagen, H. Karol, A. Ciereszko, Acclimation to cold and warm temperatures is associated with differential expression of male carp blood proteins involved in acute phase and stress responses, and lipid metabolism, Fish Shellfish Immunol. 76 (2018) 305–315, https://doi.org/10.1016/j.fsi.2018.03.018.

[74] A. Kiriake, A. Ohta, E. Suga, T. Matsumoto, S. Ishizaki, Y. Nagashima, Comparison of tetrodotoxin uptake and gene expression in the liver between juvenile and adult tiger pufferfish, *Takifugu rubripes*, Toxicon 111 (2016) 6–12, https://doi.org/10.1016/j.toxicon.2015.12.007.

[75] V. Blanco-Abad, M. Noia, A. Valle, F. Fontenla, I. Folgueira, A.P. De Felipe, P. Pereiro, J. Leiro, J. Lamas, The coagulation system helps control infection caused by the ciliate parasite *Philasterides dicentrarchi* in the turbot *Scophthalmus maximus* (L.), Dev. Comp. Immunol. 87 (2018) 147–156, https://doi.org/10.1016/j.dci.2018.06.001.

[76] M. Yuen, C.A.C. Ottenheijm, Nebulin: big protein with big responsibilities, J. Muscle Res. Cell Motil. 41 (1) (2020) 103–124, https://doi.org/10.1007/s10974-019-09565-3.

[77] U. Fock, H. Hinssen, Nebulin is a thin filament protein of the cardiac muscle of the agnathans, J. Muscle Res. Cell Motil. 23 (3) (2002) 205–213, https://doi.org/10.1023/a:1020909902462.

[78] G. Kwon, S. Ghil, Identification of warm temperature acclimation-associated 65-kDa protein-2 in Kumgang fat minnow *Rhynchocypris kumgangensis*, J. Exp. Zool. A Ecol. Integr. Physiol. 327 (10) (2017) 611–619, https://doi.org/10.1002/jez.2149.

[79] P. Diaz-Rosales, P. Pereiro, A. Figueras, B. Novoa, S. Dios, The warm temperature acclimation protein (Wap65) has an important role in the inflammatory response of turbot (*Scophthalmus maximus*), Fish Shellfish Immunol. 41 (1) (2014) 80–92, https://doi.org/10.1016/j.fsi.2014.04.012.

[80] V.O. Poku, S.H. Iram, A critical review on modulators of Multidrug Resistance Protein 1 in cancer cells, PeerJ 10 (2022) e12594, https://doi.org/10.7717/peerj.12594.

[81] T. Fiaschi, Mechanisms of adiponectin action, Int. J. Mol. Sci. 20 (12) (2019) 2894, https://doi.org/10.3390/ijms20122894.

[82] H. Liu, X.J. Lu, J. Chen, Full-length and a smaller globular fragment of adiponectin have opposite roles in regulating monocyte/macrophage functions in ayu, *Plecoglossus altivelis*, Fish Shellfish Immunol. 82 (2018) 319–329, https://doi.org/10.1016/j.fsi.2018.08.041.

[83] K. Zhang, Z. Tai, Q. Han, Y. Pang, Q. Li, Adiponectin as inducer of inflammatory and apoptosis involving in immune defense in lamprey, Fish Shellfish Immunol. 90 (2019) 446–455, https://doi.org/10.1016/j.fsi.2019.04.045.

[84] N. Tang, Y. Li, Y. Li, Y. Liu, S. Zhang, S. Xu, M. Wang, B. Wang, H. Chen, X. Zhang, B. Zhou, D. Chen, Z. Li, Molecular cloning, expression and appetite regulation function of adiponectin in Siberian sturgeon (*Acipenser baerii*), Int. J. Biol. Macromol. 214 (2022) 360–369, https://doi.org/10.1016/j.ijbiomac.2022.06.097.

[85] U. Lechowicz, S. Rudzinski, A. Jezela-Stanek, S. Janciauskiene, J. Chorostowska-Wynimko, Post-translational modifications of circulating alpha-1-antitrypsin protein, Int. J. Mol. Sci. 21 (23) (2020) 9187, https://doi.org/10.3390/ijms21239187.

[86] A.E. Murphy, M.J.W. Stokesbury, R.H. Easy, Exploring epidermal mucus protease activity as an indicator of stress in Atlantic sturgeon (*Acipenser oxyrinchus oxyrhinchus*), J. Fish. Biol. 97 (5) (2020) 1354–1362, https://doi.org/10.1111/jfb.14489.

[87] J.L. Stafford, M. Belosevic, Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation, Dev. Comp. Immunol. 27 (6–7) (2003) 539–554, https://doi.org/10.1016/s0145-305x(02)00138-6.

[88] J. Yang, T. Tian, K. Xiao, Q. Zeng, C. Tan, H. Du, Pathogenic infection and immune-related gene expression of Chinese sturgeon (*Acipenser sinensis*) challenged by *Citrobacter freundii*, Dev. Comp. Immunol. 114 (2021) 103872, https://doi.org/10.1016/j.dci.2020.103872.

[89] E. Soto, M.D. Fast, S.L. Purcell, D. Denver Coleman, Z. Yazdi, K. Kenelty, S. Yun, A. Camus, Expression of immune markers of white sturgeon (*Acipenser transmontanus*) during *Veronaea botryosa* infection at different temperatures, Comp. Biochem. Physiol., Part D: Genomics Proteomics 41 (2022) 100950, https://doi.org/10.1016/j.cbd.2021.100950.

[90] A. Arciello, R. Piccoli, D.M. Monti, Apolipoprotein A-I: the dual face of a protein, FEBS Lett. 590 (23) (2016) 4171–4179, https://doi.org/10.1002/1873-3468.12468.

[91] C.R. White, G. Datta, S. Giordano, High-density lipoprotein regulation of mitochondrial function, Adv. Exp. Med. Biol. 982 (2017) 407–429, https://doi.org/10.1007/978-3-319-55330-6_22.

[92] M.I. Concha, V.J. Smith, K. Castro, A. Bastías, A. Romero, R.J. Amthauer, Apolipoproteins A-I and A-II are potentially important effectors of innate immunity in the teleost fish *Cyprinus carpio*, Eur. J. Biochem. 271 (14) (2004) 2984–2990, https://doi.org/10.1111/j.1432-1033.2004.04228.x.

[93] D.E. Jenne, B. Lowin, M.C. Peitsch, A. Böttcher, G. Schmitz, J. Tschopp, Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma, J. Biol. Chem. 266 (17) (1991) 11030–11036.

[94] K.K. Hamilton, J. Zhao, P.J. Sims, Interaction between apolipoproteins A-I and A-II and the membrane attack complex of complement. Affinity of the apoproteins for polymeric C9, J. Biol. Chem. 268 (5) (1993) 3632–3638.

[95] B. Magnadóttir, S. Lange, Is Apolipoprotein A-I a regulating protein for the complement system of cod (*Gadus morhua* L.)? Fish Shellfish Immunol. 16 (2) (2004) 265–269, https://doi.org/10.1016/S1050-4648(03)00061-5.
[96] C. Li, C. Gao, Q. Fu, B. Su, J. Chen, Identification and expression analysis of fetuin B (FETUB) in turbot (*Scophthalmus maximus* L.) mucosal barriers following bacterial challenge, Fish Shellfish Immunol. 68 (2017) 386–394, https://doi.org/10.1016/j.fsi.2017.07.032.
[97] B. Morro, M.K. Doherty, P. Balseiro, S.O. Handeland, S. MacKenzie, H. Sveier, A. Albalat, Plasma proteome profiling of freshwater and seawater life stages of rainbow trout (*Oncorhynchus mykiss*), PLoS One 15 (1) (2020) e0227003, https://doi.org/10.1371/journal.pone.0227003.
[98] M.H. Jung, C. Nikapitiya, S.J. Kim, H.J. Han, M.S. Kim, H.S. Choi, S.J. Jung, Protective immunity induced by ankyrin repeat-containing protein-based DNA vaccine against rock bream iridovirus (RBIV) in rock bream (*Oplegnathus fasciatus*), Virus Res. 318 (2022) 198827, https://doi.org/10.1016/j.virusres.2022.198827.
[99] C. Silvestre-Roig, Q. Braster, K. Wichapong, E.Y. Lee, J.M. Teulon, N. Berrebeh, J. Winter, J.M. Adrover, G.S. Santos, A. Froese, P. Lemnitzer, A. Ortega-Gómez, R. Chevre, J. Marschner, A. Schumski, C. Winter, L. Perez-Olivares, C. Pan, N. Paulin, T. Schoufour, O. Soehnlein, Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death, Nature 569 (7755) (2019) 236–240, https://doi.org/10.1038/s41586-019-1167-6.
[100] E.A. Barbu, V.M. Dominical, L. Mendelsohn, S.L. Thein, Detection and quantification of histone H4 citrullination in early NETosis with image flow cytometry version 4, Front. Immunol. 11 (2020) 1335, https://doi.org/10.3389/fimmu.2020.01335.
[101] W.E. Mayer, H. Tichy, A cDNA clone from the sea lamprey Petromyzon marinus coding for a scavenger receptor Cys-rich (SRCR) domain protein, Gene 164 (2) (1995) 267–271, https://doi.org/10.1016/0378-1119(95)94092-z.
[102] C.T. Herzig, R.W. Waters, C.L. Baldwin, J.C. Telfer, Evolution of the CD163 family and its relationship to the bovine gamma delta T cell co-receptor WC1, BMC Evol. Biol. 10 (2010) 181, https://doi.org/10.1186/1471-2148-10-181.
[103] N. Lamarche, A. Hall, GAPs for rho-related GTPases, Trends Genet. 10 (12) (1994) 436–440, https://doi.org/10.1016/0168-9525(94)90114-7.
[104] L. Zeng, Y.H. Wang, C.X. Ai, J.S. Zhang, Differential effects of β-glucan on oxidative stress, inflammation and copper transport in two intestinal regions of large yellow croaker *Larimichthys crocea* under acute copper stress, Ecotoxicol. Environ. Saf. 165 (2018) 78–87, https://doi.org/10.1016/j.ecoenv.2018.08.098.
[105] L.K. Fritz-Laylin, M.A. Titus, The evolution and diversity of actin-dependent cell migration, Mol. Biol. Cell 34 (12) (2023) pe6, https://doi.org/10.1091/mbc.E22-08-0358.
[106] S. Belian, O. Korenkova, C. Zurzolo, Actin-based protrusions at a glance, J. Cell Sci. 136 (22) (2023) jcs261156, https://doi.org/10.1242/jcs.261156.
[107] M.K. Fernandez, M. Sinha, M. Zidan, M. Renz, Nuclear actin filaments - a historical perspective, Nucleus 15 (1) (2024) 2320656, https://doi.org/10.1080/19491034.2024.2320656.
[108] T.S. Fung, R. Chakrabarti, H.N. Higgs, The multiple links between actin and mitochondria, Nat. Rev. Mol. Cell Biol. 24 (9) (2023) 651–667, https://doi.org/10.1038/s41580-023-00613-y.
[109] J.E. Volanakis, The role of complement in innate and adaptive immunity, Curr. Top. Microbiol. Immunol. 266 (2002) 41–56, https://doi.org/10.1007/978-3-662-04700-2_4.
[110] A.W. Dodds, Which came first, the lectin/classical pathway or the alternative pathway of complement? Immunobiology 205 (4–5) (2002) 340–354, https://doi.org/10.1078/0171-2985-00137.
[111] L.D. Bennett, J.M. Fox, N. Signoret, Mechanisms regulating chemokine receptor activity, Immunology 134 (3) (2011) 246–256, https://doi.org/10.1111/j.1365-2567.2011.03485.x.
[112] C. Sun, S. Zhang, Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish, Nutrients 7 (10) (2015) 8818–8829, https://doi.org/10.3390/nu7105432.
[113] M.O. Leonardi, M. Puchi, P. Bustos, X. Romo, V. Morín, Vitellogenin induction and reproductive status in wild Chilean flounder *Paralichthys adspersus* (Steindachner, 1867) as biomarkers of endocrine disruption along the marine coast of the South Pacific, Arch. Environ. Contam. Toxicol. 62 (2) (2012) 314–322, https://doi.org/10.1007/s00244-011-9690-y.
[114] S. Hariharan, S. Dharmaraj, Selenium and selenoproteins: it's role in regulation of inflammation, Inflammopharmacology 28 (3) (2020) 667–695, https://doi.org/10.1007/s10787-020-00690-x.
[115] J. Chaudière, Biological and catalytic properties of selenoproteins, Int. J. Mol. Sci. 24 (12) (2023) 10109, https://doi.org/10.3390/ijms241210109.
[116] H. Zhang, L. Zhao, P. Zhang, Y. Xie, X. Yao, X. Pan, Y. Fu, J. Wei, H. Bai, X. Shao, J. Ye, C. Wu, Effects of selenoprotein extracts from *Cardamine hupingshanensis* on growth, selenium metabolism, antioxidant capacity, immunity and intestinal health in largemouth bass *Micropterus salmoides*, Front. Immunol. 15 (2024) 1342210, https://doi.org/10.3389/fimmu.2024.1342210.
[117] Y. Mao, T. Peng, F. Shao, Q. Zhao, Z. Peng, Molecular evolution of the hemoglobin gene family across vertebrates, Genetica 151 (3) (2023) 201–213, https://doi.org/10.1007/s10709-023-00187-9.
[118] A. Lewit-Bentley, S. Réty, EF-hand calcium-binding proteins, Curr. Opin. Struct. Biol. 10 (6) (2000) 637–643, https://doi.org/10.1016/s0959-440x(00)00142-1.
[119] D.E. Goll, V.F. Thompson, H. Li, W. Wei, J. Cong, The calpain system, Physiol. Rev. 83 (3) (2003) 731–801, https://doi.org/10.1152/physrev.00029.2002.
[120] F.A. Cisternas, J.B. Vincent, S.W. Scherer, P.N. Ray, Cloning and characterization of human CADPS and CADPS2, new members of the Ca2+-dependent activator for secretion protein family, Genomics 81 (3) (2003) 279–291, https://doi.org/10.1016/s0888-7543(02)00040-x.
[121] H. Yoo, S.H. Yang, J.Y. Kim, E. Yang, H.S. Park, S.J. Lee, I.J. Rhyu, G. Turecki, H. W. Lee, H. Kim, Down-regulation of habenular calcium-dependent secretion activator 2 induces despair-like behavior, Sci. Rep. 11 (1) (2021) 3700, https://doi.org/10.1038/s41598-021-83310-0.
[122] L.A. Matthews, A. Guarné, Dbf4: the whole is greater than the sum of its parts, Cell Cycle 12 (8) (2013) 1180–1188, https://doi.org/10.4161/cc.24416.
[123] R.K. Raghupathy, P. Gautier, D.C. Soares, A.F. Wright, X. Shu, Evolutionary characterization of the retinitis pigmentosa GTPase regulator gene, Invest. Ophthalmol. Vis. Sci. 56 (11) (2015) 6255–6264, https://doi.org/10.1167/iovs.15-17726.
[124] J. Liu, M.R. Hayden, Y. Yang, Research progress of RP1L1 gene in disease, Gene 912 (2024) 148367, https://doi.org/10.1016/j.gene.2024.148367.
[125] W.M. van den Akker, A.J. Durston, H.P. Spaink, Identification of hoxb1b downstream genes: hoxb1b as a regulatory factor controlling transcriptional networks and cell movement during zebrafish gastrulation, Int. J. Dev. Biol. 54 (1) (2010) 55–62, https://doi.org/10.1387/ijdb.082678wv.
[126] L. Winter, C. Abrahamsberg, G. Wiche, Plectin isoform 1b mediates mitochondrion-intermediate filament network linkage and controls organelle shape, J. Cell Biol. 181 (6) (2008) 903–911, https://doi.org/10.1083/jcb.200710151.
[127] D. Goryunov, R.K. Liem, Microtubule-actin cross-linking factor 1: domains, interaction partners, and tissue-specific functions, Methods Enzymol. 569 (2016) 331–353, https://doi.org/10.1016/bs.mie.2015.05.022.
[128] Z. Miao, A. Ali, L. Hu, F. Zhao, C. Yin, C. Chen, T. Yang, A. Qian, Microtubule actin cross-linking factor 1, a novel potential target in cancer, Cancer Sci. 108 (10) (2017) 1953–1958, https://doi.org/10.1111/cas.13344.
[129] M.C. Derby, C. van Vliet, D. Brown, M.R. Luke, L. Lu, W. Hong, J.L. Stow, P. A. Gleeson, Mammalian GRIP domain proteins differ in their membrane binding properties and are recruited to distinct domains of the TGN, J. Cell Sci. 117 (Pt 24) (2004) 5865–5874, https://doi.org/10.1242/jcs.01497.
[130] M. Russo, V. Piccolo, D. Polizzese, E. Prosperini, C. Borriero, S. Polletti, F. Bedin, M. Marenda, D. Michieletto, G.M. Mandana, S. Rodighiero, A. Cuomo, G. Natoli, Restrictor synergizes with Symplekin and PNUTS to terminate extragenic transcription, Genes Dev. 37 (21–24) (2023) 1017–1040, https://doi.org/10.1101/gad.351057.123.
[131] L.M. Reis, E.A. Sorokina, S. Thompson, S. Muheisen, M. Velinov, C. Zamora, A. S. Aylsworth, E.V. Semina, De novo missense variants in WDR37 cause a severe multisystemic syndrome, Am. J. Hum. Genet. 105 (2) (2019) 425–433, https://doi.org/10.1016/j.ajhg.2019.06.015.
[132] Y. Pan, W. Cai, J. Huang, A. Cheng, M. Wang, Z. Yin, R. Jia, Pyroptosis in development, inflammation and disease, Front. Immunol. 13 (2022) 991044, https://doi.org/10.3389/fimmu.2022.991044.
[133] Y. Zheng, Y. Zhang, Z. Xie, P.K.S. Shin, J. Xu, H. Fan, P. Zhuang, M. Hu, Y. Wang, Seasonal changes of growth, immune parameters and liver function in wild Chinese sturgeons under indoor conditions: implication for artificial rearing, Front. Physiol. 13 (2022) 894729, https://doi.org/10.3389/fphys.2022.894729.
[134] S.A. Eldirany, I.B. Lomakin, M. Ho, C.G. Bunick, Recent insight into intermediate filament structure, Curr. Opin. Cell Biol. 68 (2021) 132–143, https://doi.org/10.1016/j.ceb.2020.10.001.
[135] S. Zhang, Q. Xu, E. Boscari, H. Du, Z. Qi, Y. Li, J. Huang, J. Di, H. Yue, C. Li, L. Congiu, Q. Wei, Characterization and expression analysis of g- and c-type lysozymes in Dabry's sturgeon (*Acipenser dabryanus*), Fish Shellfish Immunol. 76 (2018) 260–265, https://doi.org/10.1016/j.fsi.2018.03.006.
[136] W.S. Bugg, G.R. Yoon, A.N. Schoen, A.M. Weinrauch, K.M. Jeffries, W. G. Anderson, Elevated temperatures dampen the innate immune capacity of developing lake sturgeon (*Acipenser fulvescens*), J. Exp. Biol. 226 (10) (2023) jeb245335, https://doi.org/10.1242/jeb.245335.
[137] E. Lampropoulou, I. Logoviti, M. Koutsioumpa, M. Hatziapostolou, C. Polytarchou, S.S. Skandalis, U. Hellman, M. Fousteris, S. Nikolaropoulos, E. Choleva, M. Lamprou, A. Skoura, V. Megalooikonomou, E. Papadimitriou, Cyclin-dependent kinase 5 mediates pleiotrophin-induced endothelial cell migration, Sci. Rep. 8 (1) (2018) 5893, https://doi.org/10.1038/s41598-018-24326-x.
[138] J.P. Ren, H.L. Cong, L.J. Gao, D.F. Jiang, X.T. Li, Y. Wang, J.Q. Wang, T.S. Tang, Cyclin-dependent kinase 5 negatively regulates antiviral immune response by disrupting myeloid differentiation primary response protein 88 self-association, Virulence 14 (1) (2023) 2223394, https://doi.org/10.1080/21505594.2023.2223394.
[139] L.H. Andersen, A.R. Miserez, Z. Ahmad, R.L. Andersen, Familial defective apolipoprotein B-100: a review, J. Clin. Lipidol. 10 (6) (2016) 1297–1302, https://doi.org/10.1016/j.jacl.2016.09.009.
[140] L. Wujak, R.T. Böttcher, O. Pak, H. Frey, E. El Agha, Y. Chen, S. Schmitt, S. Bellusci, L. Schaefer, N. Weissmann, R. Fässler, M. Wygrecka, Low density lipoprotein receptor-related protein 1 couples β1 integrin activation to degradation, Cell. Mol. Life Sci. 75 (9) (2018) 1671–1685, https://doi.org/10.1007/s00018-017-2707-6.
[141] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria, Science (New York, N.Y.) 303 (5663) (2004) 1532–1535, https://doi.org/10.1126/science.1092385.
[142] P. Li, M. Li, M.R. Lindberg, M.J. Kennett, N. Xiong, Y. Wang, PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps, J. Exp. Med. 207 (9) (2010) 1853–1862, https://doi.org/10.1084/jem.20100239.

[143] D. Palić, J. Ostojić, C.B. Andreasen, J.A. Roth, Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils, Dev. Comp. Immunol. 31 (8) (2007) 805–816, https://doi.org/10.1016/j.dci.2006.11.010.
[144] G. Mahesh, R. Biswas, MicroRNA-155: a master regulator of inflammation, J. Interferon Cytokine Res. 39 (6) (2019) 321–330, https://doi.org/10.1089/jir.2018.0155.
[145] J. Hu, S. Huang, X. Liu, Y. Zhang, S. Wei, X. Hu, miR-155: an important role in inflammation response, J. Immunol. Res. 2022 (2022) 7437281, https://doi.org/10.1155/2022/7437281.
[146] A. Bavelloni, G. Ramazzotti, A. Poli, M. Piazzi, E. Focaccia, W. Blalock, I. Faenza, MiRNA-210: a current overview, Anticancer Res. 37 (2017) 6511–6521, https://doi.org/10.21873/anticanres.12107.
[147] M. Lian, M. Mortoglou, P. Uysal-Onganer, P. Impact of Hypoxia-Induced miR-210 on Pancreatic Cancer, Curr. Issues Mol. Biol. 45 (12) (2023) 9778–9792, https://doi.org/10.3390/cimb45120611.
[148] J. Sun, L. Zhao, H. Wu, W. Lian, C. Cui, Z. Du, W. Luo, M. Li, S. Yang, Analysis of miRNA-seq in the liver of common carp (*Cyprinus carpio* L.) in response to different environmental temperatures, Funct. Integr, Genomics 19 (2) (2019) 265–280, https://doi.org/10.1007/s10142-018-0643-7.
[149] J. Ma, X. Chen, G. Xin, X. Li, Chronic exposure to the ionic liquid [C8mim]Br induces inflammation in silver carp spleen: involvement of oxidative stress-mediated p38MAPK/NF-κB signalling and microRNAs, Fish Shellfish Immunol. 84 (2019) 627–638, https://doi.org/10.1016/j.fsi.2018.09.052.
[150] H. Huang, K. Zhang, Y. Zhou, X. Ding, L. Yu, G. Zhu, J. Guo, MicroRNA-155 targets cyb561d2 in zebrafish in response to fipronil exposure, Environ. Toxicol. 31 (7) (2016) 877–886, https://doi.org/10.1002/tox.22099.
[151] X. Huang, Q.- Le, A.J. Giaccia, MiR-210—micromanager of the hypoxia pathway, Trends Mol. Med. 16 (2010) 230–237, https://doi.org/10.1016/j.molmed.2010.03.004.
[152] S. Khalilian, A. Bijanvand, H. Abedinlou, S. Ghafouri-Fard, A review on the role of miR-210 in human disorders, Pathol. Res. Pract. 241 (2023) 154244, https://doi.org/10.1016/j.prp.2022.154244.
[153] T. Xu, Q. Chu, J. Cui, Rhabdovirus-inducible MicroRNA-210 modulates antiviral innate immune response via targeting STING/MITA in fish, J. Immunol. 201 (3) (2018) 982–994, https://doi.org/10.4049/jimmunol.1800377.
[154] H. Su, R. Chang, W. Zheng, Y. Sun, T. Xu, microRNA-210 and microRNA-3570 negatively regulate NF-κB-Mediated inflammatory responses by targeting RIPK2 in teleost fish, Front. Immunol. 12 (2021) 617753, https://doi.org/10.3389/fimmu.2021.617753.
[155] E. Cardona, L. Milhade, A. Pourtau, S. Panserat, F. Terrier, A. Lanuque, J. Roy, L. Marandel, J. Bobe, S. Skiba-Cassy, Tissue origin of circulating microRNAs and their response to nutritional and environmental stress in rainbow trout (*Oncorhynchus mykiss*), Sci. Total Environ. 853 (2022) 158584, https://doi.org/10.1016/j.scitotenv.2022.158584.
[156] T.A. van Gelderen, L. Ribas, miR-210 promotes immune- and suppresses oocyte meiosis-related genes in the zebrafish ovarian cells, Genomics 116 (2) (2024) 110820, https://doi.org/10.1016/j.ygeno.2024.110820.
[157] M.J. Leal, B.E. Clark, J.P. Van Eenennaam, A.D. Schreier, A.E. Todgham, The effects of warm temperature acclimation on constitutive stress, immunity, and metabolism in white sturgeon (*Acipenser transmontanus*) of different ploidies, Comp. Biochem. Physiol. Mol. Integr. Physiol. 224 (2018) 23–34, https://doi.org/10.1016/j.cbpa.2018.05.021.
[158] I.E. Florescu Gune, S.E. Georgescu, A. Dudu, M. Balaș, S. Voicu, I. Grecu, L. Dediu, A. Dinischiotu, M. Costache, Oxidative stress and antioxidant defense mechanisms in response to starvation and refeeding in the intestine of stellate sturgeon (*Acipenser stellatus*) juveniles from aquaculture, Animals 11 (1) (2021) 76, https://doi.org/10.3390/ani11010076.