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Qualitative Shotgun Proteomics Strategy for Protein Expression Profiling of Fish Otoliths

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Abstract: Despite decades of research on fish otoliths and their capacity to serve as biochronological recorders, much remains unknown about their protein composition, the mechanisms by which proteins are incorporated into the otolith matrix, or the potential for using otolith proteins to provide insight into aspects of fish life history. We examined the protein composition of Atlantic cod (*Gadus morhua*) otoliths using a state-of-the-art shotgun proteomics approach with liquid chromatography coupled to an electrospray ionization-orbitrap tandem mass spectrometer. In addition to previously known otolith matrix proteins, we discovered over 2000 proteins not previously identified in cod otoliths and more than 1500 proteins not previously identified in any fish otoliths. These included three novel proteins (Somatolactin, F-actin-capping protein subunit beta, Annexin) primarily involved in binding calcium ions and likely mediating crystal nucleation. However, most of the otolith proteins were not necessarily related to otolith formation but rather to other aspects of fish physiology. For example, we identified sex-related biomarkers for males (SPATA6 protein) and females (Vitellogenin-2-like protein). We highlight some noteworthy classes of proteins having diverse functions; however, the primary goal here is not to discuss each protein separately. The number and diverse roles of the proteins discovered in the otoliths suggest that proteomics could reveal critical life history information from archived otolith collections that could be invaluable for understanding aspects of fish biology and population ecology. This proof-of-concept methodology paper provides a novel methodology whereby otolith proteomics can be further explored.

Keywords: otoliths; proteomics; biomineralization

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1. Introduction

Otoliths are dense structures, made primarily of calcium carbonate, whose movements in response to sound and motion play a critical role in the hearing and equilibrium of fishes [1]. The otoliths sit within endolymph-filled chambers, and their positioning and vibratory movements are picked up by the long ciliary bundles of sensory epithelial cells and relayed to the brain via the auditory nerve [1–6]. Among the three pairs of otoliths found in fishes, the sagittae are the largest and most widely studied and used in fisheries research.

For fisheries biologists, otoliths are regularly used to reveal critical fish life history information. Otoliths accrue daily and annual growth rings [7–9] and have long been considered invaluable in fish age determination. This information is essential for developing age-based population dynamics models used in the management of fisheries resources. Therefore most, if not all, fisheries research departments put extensive effort and

resources into collecting and ageing fish otoliths [10]. For example, Fisheries and Oceans Canada recently completed a partial internal survey of its fish otolith ageing programs and concluded that several hundred thousand otoliths are aged each year across more than 100 species/fish stocks at an aggregate cost of several million dollars [11].

In addition to aging fish, otoliths can be used to back-calculate growth rates based on the width of annual growth rings [12], marking the first use of otoliths as chronological recorders of fish life history information. Otoliths are composed of a crystalline calcium carbonate structure, usually in the form of aragonite, on a protein matrix [13,14]. Trace elements from a fish's environment are also incorporated into the otolith. Because fish otoliths are metabolically inert, materials accrued to the otolith in the form of new growth rings become permanent features of the otolith [15–18]. Understanding how the trace elements in otoliths correlate with environmental conditions, coupled with the availability of large libraries of stored otoliths, has resulted in an abundance of research using otoliths to reveal fishes' environmental history [14,19–21]. For example, the concentration of Strontium or Barium in relation to the amount of Calcium in the otoliths can reveal information about the salinity history of individuals, which has proven particularly useful for tracking the life history of diadromous fishes [14]. Many elements also have isotopes that are more or less prevalent under different environmental conditions, and these isotopes are also recorded in the otoliths. For example, the $\delta^{18}O$ content of otoliths is negatively correlated to water temperature, allowing a fish's temperature history to be gauged from its otoliths [14].

In comparison to trace elements, much less is known about the protein composition of fish otoliths. While proteins make up only a small portion (~2–3%) of the otolith, they are thought to play a critical role in otolith formation [14,22]. Otoliths grow via the daily formation of alternating mineral-rich and protein-rich bands [13,15,23]. The protein matrix not only provides a substrate for calcium carbonate deposition, but also appears to play an active role in determining the type of calcium carbonate that is incorporated into the otolith [14,22,24]. Proteins that appear to play important roles in otolith formation and maintenance include otolith matrix proteins (OC90, Otolin-1, OMP-1, Cochlin, etc.), otolith anchoring proteins (Otolin, Otoglin, Otoglin-like, Otancorin, α-Tectorin, β-Tectorin, KSPG, etc.), and otolith regulatory proteins (Plasma membrane calcium ATPase isomer 2 (PMCA2), Carbonic anhydrase, Otoptin-1, Otoptin-2, etc.) [7,21,24–29]. However, it has also been demonstrated that proteins with no functional role in biomineralization may also become trapped in the otoliths during the basic process of increment formation [20]. It has been proposed that these proteins could serve as biomarkers for aspects of development and physiological change and provide the potential to reconstruct the life histories of fishes to an unprecedented level, opening up new and exciting avenues of research in ecology and fisheries science [20].

The emergence of bottom-up shotgun proteomics approaches have greatly enhanced the capacity to identify proteins in complex mixtures [30,31]. Here, we performed a preliminary test of the use of shotgun proteomics using liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) to identify proteins in cod otoliths, and evaluated whether archived otoliths contain proteins that could potentially be used as biomarkers to reconstruct aspects of fish life history.

2. Materials and Methods

2.1. Otoliths

The archived otolith collection at the Northwest Atlantic Fisheries Centre (St. John's, Canada) contains thousands of Atlantic cod otoliths collected over several decades. The methods used to collect otoliths can vary slightly between individual technicians, but in general otoliths were exposed via a dorsal incision in the fish's skull and were removed using forceps. Otoliths were blotted dry with paper towel and stored in small paper envelopes. For this proof-of-concept study, we selected twelve otoliths (6 males, 6 females) collected in 2019 from the northeast coast of Newfoundland, Canada. Otoliths were washed with deionized water prior to protein extraction.

2.2. Chemicals and Standards

All standards, samples and buffers were prepared using MO; ultra-pure Milli-Q H_2O (18.2 Merck Millipore). All chemicals were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) and were of the highest available purity. Mass spectrometric grade trypsin was obtained from Promega (Madison, WI, USA). MS-grade solvents for chromatography were obtained from Canadian Life Science.

2.3. Otolith Protein Extraction

Otoliths were suspended in 20% w/v trichloroacetic acid (TCA) and incubated overnight at room temperature. Samples were then centrifuged at 10,000× g for 10 min, and the supernatant was discarded. The crystals were washed with 100 µL of ice-cold acetone and recentrifuged. The supernatant and any remaining undissolved otolith were discarded, and the vacuum-dried pellet was processed for in-solution digestion. Briefly, the pellet was resuspended in denaturing buffer containing 8 M urea and 0.4 M ammonium bicarbonate (NH_4HCO_3). Then, 10 µL of 0.5 M dithiothreitol (DTT) was added and it was incubated at 60 °C for 30 min. After cooling for 5 min at room temperature, 20 µL of 0.7 M iodoacetamide ($IAcNH_2$) was added and it was incubated for 30 min. Next, the sample was diluted with 1.2 mL of H_2O followed by 10 µL of 0.1 M $CaCl_2$. For enzymatic digestion, 100 µL of 0.02 µg/µL trypsin (Promega Trypsin Gold, Mass Spectrometry Grade, Promega) prepared in 50 mM NH_4HCO_3 was added to each sample. The samples were incubated overnight at 37 °C in a shaker. The trypsin activity was inhibited by adding 1 µL of trifluoroacetic acid (TFA), and the samples were acidified to a pH below 3 with formic acid.

The samples were then desalted using Oasis HLB 3cc Extraction Cartridges (Waters Corporation, Milford, MA, USA) connected to a SuperlcoVisiprep DL manifold (Sigma-Aldrich, Darmstadt, Germany). The column was conditioned using 0.5 mL methanol followed by 1 mL of an elution buffer containing 50% ACN and 0.1% TFA and then with 2 mL wash buffer prepared with 0.1% TFA. After loading the sample, the column was washed with 5 mL of wash buffer and eluted twice with 0.5 mL of 50% acetonitrile-0.1% TFA and once with 0.5 mL of 80% ACN-0.1% TFA. The eluant was dried using a vacuum concentrator (SpeedVac Concentrator, Thermo Electron Corp., Gormley, ON, Canada). The dried peptide was reconstituted in 12 µL of resuspension buffer containing 5% acetonitrile and 0.1% formic acid.

2.4. Shotgun Proteomics by LC-ESI-MS/MS

The LC separation was carried out using the Ultimate 3000RSLCnano system (Dionex/Thermo Fisher Scientific, San Jose, CA, USA). For analysis, 2 µL of the sample was injected onto an in-house packed capillary column (50 cm × 75 µm, pulled tip, ESI source solutions) packed with Jupiter C18 4 µm chromatographic media (Phenomenex, Torrance, CA, USA) at a flow rate of 300 nL/min. Chromatographic separation was performed with a 120 min method using solvent A (0.1% formic acid in MS-grade water) and solvent B (0.1% formic acid in MS-grade acetonitrile) from 5% to 30% for 90 min, then increasing to 55% for the next 12 min and then to 95% for 8 min before being reduced to 5% B for the remainder of the 120 min run. The column oven temperature was set at 40 °C.

The ESI-MS spectra were obtained using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (ThermoScientific Waltham, MA, USA) fitted with a Nanospray Flex Ion source and FAIMS Pro sources. The detailed acquisition parameters for mass spectrometry analysis were as follows: for ionization, the spray voltage was 1.8 kV with an ion transfer tube temperature of 300 °C. The data were acquired in data-dependent acquisition mode with full scan using Orbitrap at a resolution of 120,000 over a mass range of 400–2000 m/z. The FAIMS source was operated at three different compensation voltages (CV = 40, 60 and 80). The auto gain control (AGC) was set in standard mode and maximum injection time at auto mode. For each MS/MS, the acquisition of peaks with intensities above 5.0 e3 was performed using normalized HCD collision energy of 35%. The cycle time was set at 1 s.

The isolation window for MS/MS was set at 1.6 Da. The AGC target was set at standard mode, with the maximum injection time mode as auto. The precursor ions with positive charges 2 to 5 were selected for MS/MS analysis. After every single MS/MS acquisition, the dynamic exclusion time was set at 60 s with a mass tolerance of $\pm$10 ppm.

The MS/MS raw files were acquired using Thermo Scientific Xcalibur 4.5 and Tune 3.5 and searched against the *Gadus morhua* protein database (Taxon ID 8049) downloaded from Uniport using Proteome Discoverer 2.5 (ThermoFisher, Toronto, ON, Canada). The SequestHT search engine node was used for peptide and protein identification. Two missed cleavages for trypsin digestion were allowed with a 10 ppm precursor mass tolerance and fragment mass tolerance of 0.6 Da. The oxidization of methionine and N-terminal acetylation were set as dynamic modification, and carbamidomethyl cysteine was selected as a static modification.

3. Results and Discussion

The work presented here represents a preliminary experiment and the establishment of a proof-of-concept of a shotgun proteomics approach that could be used to evaluate the proteome of Atlantic Cod otoliths. Although otoliths from only twelve fish (six males, six females) were examined, the sheer number of proteins identified suggest that (1) the proof-of-concept was successful and that this method represents an advancement for further otolith proteomics work, and (2) archived otolith collections intended for ageing purposes are suitable for proteomics work.

A Sequest HT search comparing the MS/MS raw files for the 12 otoliths against the *Gadus morhua* protein database (Taxon ID 8049) downloaded from Uniport resulted in the identification of well over 2000 otolith proteins (Tables S1 and S2). In addition to previously known otolith matrix proteins, we discovered over 2000 proteins not previously identified in cod otoliths and more than 1500 proteins not previously identified in any fish otoliths.

It is important to state that at this stage our results are only qualitative in nature. The intent is to demonstrate the huge array of proteins identified by the shotgun proteomics approach that had not previously been recorded in cod otoliths. Here, we present detailed lists of proteins for only two representative fish (one male and one female), but note that the total number of otolith proteins identified per individual is variable. The two individuals highlighted here both contained more than 2000 otolith proteins, even after the exclusion of 27 uncharacterized proteins and 16 potential contaminant proteins (see below). This far exceeds the number of otolith proteins reported elsewhere [26]. The complete list of proteins identified via the shotgun approach is provided in the Supplementary Materials (Tables S1 and S2).

Listing and understanding the role of the thousands of proteins identified in cod otoliths is a daunting task and remains a future challenge. For now, the proteins were filtered according to sequence coverages to identify the most abundant proteins. While the sequence coverage was used as a guideline for protein abundance, we note that these values could also be affected by several factors, including how well a given protein is extracted from the otolith, the effectiveness of it undergoing trypsin digestion, and the readiness of individual peptides to ionize within the MS/MS [31]. Using this approach, we identified 302 proteins that could be considered to be abundant (defined here as having a sequence coverage greater than 20%) in the male otolith and 247 in the female otolith (Tables S3 and S4). The number of unique peptides in these proteins ranged from 2 to 107. Nearly half of the abundant proteins were common to both the male and female otoliths, whereas the others were exclusive to one sex or the other (Figure 1, Tables S5–S7).

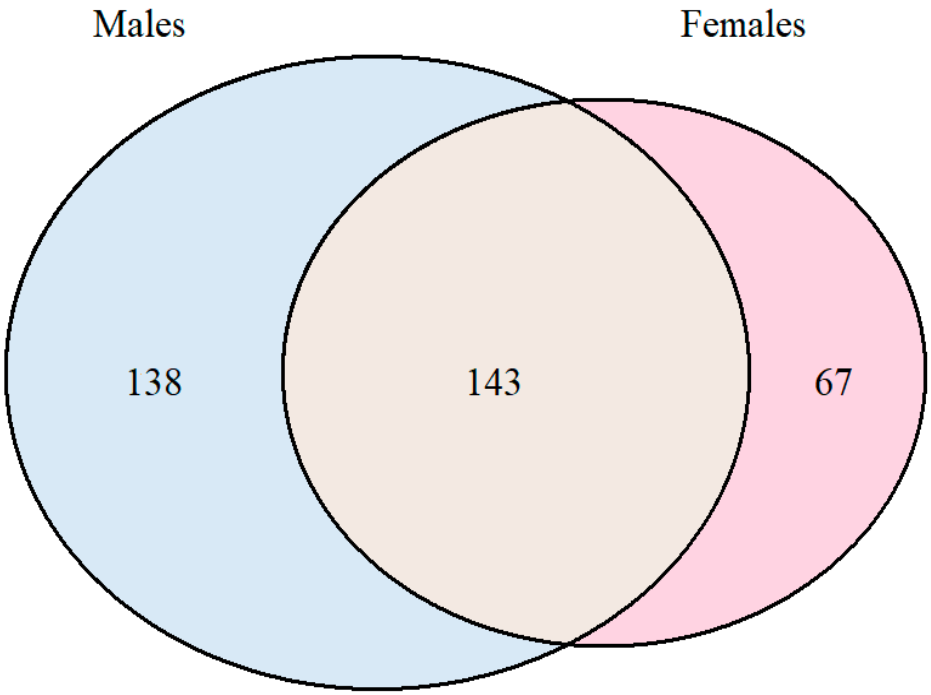

Figure 1. Venn diagram of protein expression: male vs. female. The diagram consists of two overlapping circles representing the male and female proteomes. The total number of unique proteins in the male sample is 138, while the female sample contains 67 unique proteins. Interestingly, 143 proteins are common to both genders, indicating shared protein expression. This figure was created in R using the package "VennDiagram" [32].

Previous studies on the otolith proteome have generally focused on identifying proteins important to biomineralization. It has been suggested that otolith biomineralization is produced by three main groups of proteins: (1) otolith matrix proteins (e.g., otoconin 90 (OC90), otolin-1, outer membrane protein-1 (OMP-1), cochlin), (2) otolith anchoring proteins of the membrane (e.g., otolin, five non-collagenous glycoproteins (Otoglin, Otoglin-like, Otancorin, α-Tectorin, and β-tectorin), and keratan sulfate proteoglycans (KSPGs)), and (3) otolith regulatory proteins (e.g., carbonic anhydrase (CA), plasma membrane calcium ATPase isomer 2 (PMCA2), otoptin1 and otoptin-2, NOx (nitrous oxides)) [7,21,24–29]. In addition to many of these proteins, we also identified more than 2000 proteins not previously reported in cod otoliths [29,33] and more than 1500 not previously documented in the otoliths of any fish species [26]. Three of these novel proteins (Somatolactin, F-actin-capping protein subunit beta, Annexin) are primarily involved in binding calcium ions and likely mediating crystal nucleation. However, most of the otolith proteins identified here were not necessarily related to otolith formation, but rather to other aspects of fish physiology, and may have been trapped during otolith formation rather than playing a necessary role in otolith construction.

Here, we noted both similarities and differences (Tables S3–S7) in male and female otolith proteomes, including the presence of sex-related biomarkers for males (SPATA6 protein) and females (Vitellogenin-2-like protein). Similar findings have been reported by Thomas et al. [26]. The inner ear of fish, which is enclosed in the brain case and has no direct link to the outside medium or other internal systems such as the circulatory system [2], is generally considered a closed system whereby the organic and inorganic materials necessary for otolith growth are secreted into the endolymph by the surrounding saccular epithelial cells [26]. The observations here and elsewhere [26] of large numbers of proteins in the otolith unrelated to biomineralization suggest that the endolymph and otolith proteomes may also reflect other physiological processes taking place in the fish (growth, maturation, etc.). Clearly, more work is needed to fully understand otolith

proteomics in relation to fish physiology, but our results suggest that the presence of these proteins in the otoliths could provide the potential to reconstruct the life histories of fishes to an unprecedented level [26].

The otoliths used in this study were white in colour, with no visible signs of contamination. However, because otoliths were not collected in a sterile environment and were also likely in contact with human hands on multiple occasions (e.g., as a part of our fish aging program), it was necessary to examine the protein data with respect to the potential for some proteins to be a result of otolith surface contamination. Otoliths were washed with deionized water prior to our analyses, yet we still detected 10 human keratin proteins, suggesting that some surface contamination still existed. We also identified six fish hemoglobin proteins. Although we treated these proteins as likely contaminants on the otolith surface, we could not definitively determine whether they were surface contaminants or proteins incorporated in the otoliths. Future work using a more thorough surface cleaning should help to answer these questions.

Our results demonstrate that the shotgun approach to proteomics is an effective way to examine the otolith proteome of fishes and is capable of producing much higher resolution results than typically reported. It is not the goal of this proof-of-concept paper to describe all of the observed proteins. However, we take the opportunity below to demonstrate the diverse types of proteins identified in cod otoliths, since these results provide a very different view of otolith proteomics than previous research, which has focused primarily on proteins actively involved in biomineralization. Cod otoliths contained several proteins implicated in protein synthesis. For example, we identified a series of seven subunit otolith proteins belonging to the regulatory 40S ribosomal proteome known to control an increasing number of essential biochemical mechanisms of the cellular lifecycle, including DNA synthesis, repair, transcription, translation and cell signal transduction [34]. In addition to the 40S ribosome proteins, we also identified four large 60S subunits [35]. Furthermore, we identified the presence of aminoacyl-tRNA hydrolase protein, which is responsible for the termination steps of protein biosynthesis [36]. Similarly, we identified the purine-rich element binding protein Ab (PURA), which is a single-stranded DNA-binding protein implicated in the control of DNA replication and transcription [37]. Further, we identified ribonucleoprotein (RNP), which is in charge of cellular processes such as transcription, translation and regulating gene expression and the metabolism of RNA [38].

Here we list (Table 1) and describe (below) some select noteworthy classes of cod otolith proteins. We chose a subset of proteins with diverse functions and grouped them based on whether their formation was driven by biochemical processes or physiological processes. A complete list of proteins is provided in the Supplementary Materials.

Table 1. Proteins obtained from biochemical and physiological processes involved in the formation of otoliths.

1. Biochemical Processes	
1.1. Protein Synthesis	
A0A8C4ZUE8	40S ribosomal protein S12
A0A8C5BT07	40S ribosomal protein S18
A0A8C5BHD6	40S ribosomal protein S19
A0A8C4ZDL6	40S ribosomal protein S28
A0A8C4YW06	40S ribosomal protein S3a
A0A8C5F524	40S ribosomal protein S9
A0A8C5B226	40S_SA_C domain-containing protein
A0A8C4ZT25	60S acidic ribosomal protein P2

Table 1. *Cont.*

A0A8C4Z3G5	60S ribosomal protein L27
A0A8C5BNH7	60S ribosomal protein L30
A0A8C4Z1D1	60S ribosomal protein L31
A0A8C4ZUJ5	Aminoacyl-tRNA hydrolase
A0A8C4ZR55	Purine-rich element binding protein Ab (PURA)
A0A8C5FJ16	Ribonucloprotein (RNP).
1.2. Biochemical Reaction Processes	
A0A8C5BPU1	Oxoisovalerate dehydrogenase subunit alpha
A0A8C5C4N4	Acetyl-CoA acetyltransferase 1 (ACAT1)
A0A8C5ADH8/ A0A8C5FBY3	Aconitate hydratase
A0A8C4ZEH5	Transketolase b (TKT)
A0A8C4YZP8	Malate dehydrogenase (MDH)
A0A8C4Z702	Peroxiredoxin-1 (PRX1)
P21919	Somatolactin
2. Physiological Processes	
2.1. Brain Function Processes	
A0A8C4ZJE1	14-3-3 protein beta/alpha-1-like protein
A0A8C5A8C5	Alpha-2-HS-glycoprotein-like
A0A8C5BJ01	Aspartate aminotransferase
2.2. Mediated Transport Processes	
A0A8C4Z7S3	AP-3 complex subunit mu-2 (PA3)
A0A8C5F5V1	Adaptor related protein complex 3 subunit sigma 1
2.3. Mitochondrial Processes	
A0A8C4YYC8	ADP/ATP translocase
A0A8C5AUM7	ATP-binding cassette sub-family B (MDR/TAP)
A0A8C4Z154	ATP synthase-coupling factor 6 mitochondria
A0A8C5AGK0/A0A8C4YZ60	ATP synthase subunit alpha and beta
A0A8C5FKS7	Voltage-Dependent Anion-selective Channel 2 (VDAC2)
2.4. Cytoskeleton and Extracellular Matrixes	
A0A8C5FJI3	Actin, beta protein
A0A8C4ZDZ9	Actin-related protein 2/3 complex subunit 5
A0A8C4ZHY1	Chondroadherin-like b (CHADL)
A0A8C5FEX0	Choclin: extracellular matrix (ECM)
A0A8C4ZF74	Decorin or pericellular matrix proteoglycan (SLRP)
A0A8C5AFR1	The Intermediate filament protein ON3-like (IFs) [39]
A0A8C5C226/A0A8C4ZFJ9/ A0A8C4YVX1/A0A8C5B373/ A0A8C4YSX2/A0A8C5B602/ A0A8C4ZH54	Keratins
A0A8C5FDF8	F-actin-capping protein subunit beta complex
A0A8C5C7G7	Smoothelin a
A0A8C5BEP0	Tubulin alpha-1A chain (TUBA 1A)

Table 1. *Cont.*

2.5. Adhesion and Binding Processes	
A0A8C5BRQ2	Annexin
A0A8C5AI73	Secreted acidic cysteine rich glycoprotein (Sparc)
A0A8C5B6I8	Myelin basic protein (MBP)
2.6. Cellular Signaling Processes	
A0A8C4ZK56	Arrestin-domain containing protein 1 (Arrdc1)
A0A8C5BSR3	Calmodulin 2a phosphorylase kinase (CaMKII)
A0A8C4Z128	Glypicans (Gpi)
A0A8C5BN57/A0A8C4Z2M4	Glycoprotein Tetraspanin
A0A8C4Z782/A0A8C5B4W1	Nucleobindin 1 (NUCB1)
2.7. Cellular Immunological-Processes	
A0A8C5F947	A20/AN1 zinc-finger domain-containing protein
A0A8C5BVB7	Pentraxin
2.8. Nuclear Physiological Processes	
A0A8C5FTZ2/A0A8C4ZQW9/A0A8C4ZTE5	High mobility group (HMG) protein
A0A8C5B042/A0A8C5FQN1/A0A8C5FAA0	Histone protein
2.9. Ions Transport Processes	
A0A8C5AKI2	Sodium/potassium-transporting ATPase subunit beta (NKA)
A0A8C4Z4K1/A0A8C5ASM5	Sodium/potassium-transporting ATPase subunit alpha
2.10. Sexual Differentiation Processes	
A0A8C5A412	SPATA6 protein
A0A8C5CHW7	Vitellogenin-2-like protein
3. Identifiecation of Know Otolith Proteins	
A0A8C4ZKX9	Carbonic anhydrase protein (CA)
A0A8C5CSC6	Cochlin
A0A8C5AZT6	Myosin
A0A8C5F796	Otogelin
A0A8C5CL43	Otolin-1
A0A8C5ASM5/A0A8C5AKI2	Na, K-ATPase proteins
A0A8C5AI73	Secreted acidic cysteine rich glycoprotein (Sparc)
A0A8C5BD06/A0A8C5D2V1/A0A8C5AT89	SERPIN domain-containing protein
A0A8C5AFL5/A0A8C4ZVQ3	α- and β-Tectorins

4. Proteins Linked to Biochemical Processes

4.1. Protein Synthesis

Cod otoliths contained several proteins implicated in protein synthesis. For example, we identified a series of seven subunit otolith proteins belonging to the regulatory 40S ribosomal proteome known to control an increasing number of essential biochemical mechanisms of the cellular lifecycle, including DNA synthesis, repair, transcription, translation and cell signal transduction [34]. In addition to the 40S ribosome proteins, we also identified four large 60S subunits [35]. We also identified the presence of aminoacyl-tRNA hydrolase protein, which is responsible for the termination steps of protein biosynthesis [36]. Similarly, we identified the purine-rich element binding protein Ab (PURA), which is a

single-stranded DNA-binding protein implicated in the control of DNA replication and transcription [37]. A final example of protein synthesis proteins identified was ribonucleoprotein (RNP), which is in charge of cellular processes such as transcription, translation and regulating gene expression and the metabolism of RNA [38].

4.2. Biochemical Reaction Processes

With respect to the biochemical reaction processes, we noted the presence of proteins responsible for the ketogenesis metabolic pathway, exemplified by the presence of oxoisovalerate dehydrogenase subunit alpha and acetyl-CoA acetyltransferase 1 (ACAT1) proteins [40]. Similarly, we identified the presence of aconitate hydratase, which is formed by the citric acid cycle [41], and the transketolase b (TKT) enzyme that links the pentose phosphate pathway with the glycolytic pathway [41]. Also present was malate dehydrogenase (MDH), a protein responsible for the central oxidative pathway [42] and peroxiredoxin-1 (PRX1), which plays an antioxidant protective role in cells [43]. Somatolactin, which is a pituitary gland glycoprotein growth hormone involved in regulating acid-base, calcium and phosphate levels [44], was also present.

5. Proteins Linked to Physiological Processes

5.1. Brain Function

We noted the presence of proteins within the otoliths that are linked to various brain functions. These included 14-3-3 beta/alpha-1-like protein, which is involved in different brain functions and the nervous system of fish (e.g., neural signaling, neuronal development and neuroprotection) [45], as well as the alpha-2-HS-glycoprotein-like, which plays a critical role in brain development [46]. Likewise, we identified aspartate aminotransferase, which is responsible for synthesizing neurotransmitters [47].

5.2. Mediated Transport Processes

Proteins identified in cod otoliths that are linked to mediated transport processes included AP-3 complex subunit mu-2 (PA3), which is responsible for protein trafficking to lysosomes and specialized organelles [39], and the adaptor-related protein complex 3 subunit sigma 1, which is involved in clathrin-mediated vesicular transport from the trans-Golgi network (TGN) [48].

5.3. Mitochondrial Processes

Proteins identified in the otoliths that are linked to mitochondrial processes included ADP/ATP translocase, which belongs to the mitochondrial carrier family [49]. We also found the ATP-binding cassette sub-family B (MDR/TAP), which regulates apoptotic and non-apoptotic cell death [50]; the ATP synthase-coupling factor 6, which was reported to be essential for energy transduction [51]; and the ATP synthase subunits alpha and beta that generate electron transport complexes for the respiratory chain [52]. Finally, we identified the voltage-dependent anion-selective channel 2 (VDAC2) protein, which is known to contribute to oxidative metabolism by facilitating solute transport across the outer mitochondrial membrane (OMM). VDAC2 also has a distinctive Ca^{2+} role in mediating the sarcoplasmic reticulum to mitochondria local transport in cardiomyocytes [53].

5.4. Cytoskeleton and Extracellular Matrices

Otolith proteins involved in the cytoskeleton and extracellular matrix formation included actin, beta protein and actin-related protein 2/3 complex subunit 5, all highly conserved major proteins involved in structure, the integrity of the contractile apparatus and non-muscle cytoskeletal actins that are ubiquitously expressed [54,55]. We also identified chondroadherin-like b (CHADL) protein, which is responsible for creating the extracellular matrices, and the assembly of collagen fibrils, which are composed by the collagen-associated small leucine-rich proteins (SLRPS) family [56]. Also observed were choclin, an extracellular matrix (ECM) protein, which constitutes the main acellular mi-

croenvironment of cells in almost all tissues and organs [57]; decorin, also known as the small cellular or pericellular matrix proteoglycan (SLRP), the component of the connective tissues and stabilizing inter-fibrillar organization [29,58]; and intermediate filament protein ON3-like (IFs) [59] and keratins [60], both proteins belonging to the cytoskeletal structural components. We also identified the F-actin-capping protein subunit beta complex, which binds in a calcium-independent way to the growing ends of actin filaments (also called barbed end), blocking the exchange of subunits at these ends [55]. Additionally, we observed the presence of smoothelin a, an actin-binding protein that directs the functional contractility of the intestinal smooth muscle [61]. Finally, we observed the presence of the tubulin alpha-1A chain (TUBA 1A). This protein provides structural support and the pathway for transport and force generation in cell division, neuronal development and maturation [62].

5.5. Adhesion and Binding Processes

We observed the presence of proteins involved in the physiological processes of adhesion and binding, including annexin, which is a Ca^{2+} dependent phospholipid binding protein. This protein is implicated in a variety of pathophysiological processes, including cell proliferation, apoptosis, differentiation, metastasis and inflammatory response [63]. We also identified myelin basic protein (MBP), which is an abundant protein in the central nervous system. MBP acts as a membrane actin-binding protein, and is responsible for the adhesion of the cytosolic surfaces of multilayered compact myelin [64]. It is involved in several other functions, such as interacting with polyanionic proteins, including actin, tubulin, Ca^{2+}-calmodulin, clathrin and negatively charged lipids [65].

5.6. Cellular Signaling Processes

We identified several proteins involved in cellular signal processing in the two cod otoliths. Arrestin-domain containing protein 1 (Arrdc1) was identified and is known to have the ability to arrest or turn off the coupling of G-protein-coupled-Receptors (GPCRs) and thereby inhibit signaling [66]. Similarly, we found calmodulin 2a phosphorylase kinase (CaMKII), which is involved in many signaling cascades and memory. It is well known that the divalent cation calcium (Ca^{2+}), which is vital to cellular physiology, is the most utilized second messenger in cellular signaling [67]. Due to its highly reactive nature, the low intracellular concentration of Ca^{2+} makes it a potent molecule for use in cellular signaling. Indeed, many of the secondary messenger effects of Ca^{2+} are mediated through the ubiquitous sensing protein calmodulin (CaM) [68]. We also identified the glypicans (Gpi) protein, which belongs to the heparan sulfate proteoglycans (HSPG) family. These are ubiquitous molecules playing essential functions in various biological processes. The Gpi-protein's main job is to anchor HSPGs directly to the cell surface and/or extracellular matrix, where they regulate growth factor signaling during development [69].

Similarly, we identified the presence of the glycoprotein tetraspanins, a membrane-spanning protein with a conserved structure that functions primarily as a tissue membrane protein organizer. All members of the tetraspanin family of proteins have four transmembrane domains, which contribute to creating a small (EC1) and large (EC2) extracellular loop. The large extracellular loop contains a conserved Cys-Cys-Gly amino acid motif (CCG motif). These four conserved cysteine residues within EC2 promote the formation of disulfide bridges [70]. Finally, we observed the presence of the Nucleobindin 1 (NUCB1) protein; it is a putative DNA- and calcium-binding protein which has a vital role in the central nervous system. NUCB1 was revealed to be present in the Golgi apparatus and can play an important role in the spatiotemporal calcium handling in signaling cells [71].

5.7. Cellular Immunological Processes

We identified two otolith proteins most commonly implicated in cellular immunological processes. The A20/AN1 zinc-finger protein's main role is to regulate the immune response. It also acts as a de-ubiquitinating enzyme, and is involved in controlling

fundamental cellular activities [72–74]. Pentraxin, which functions as a soluble pattern-recognition molecule, was also identified. Its main role is to induce host defense, primarily to induce the opsonization of the pathogens through activating the complement pathway and binding to Fc gamma receptors [75].

5.8. Physiological Nuclear Processes

For proteins associated with nuclear physiological processes, we found an otolith protein named the high mobility group (HMG) protein. The HMG is a superfamily of abundant and ubiquitous nuclear proteins that bind to DNA and nucleosomes and induce structural changes in the chromatin fiber. They are important in chromatin dynamics and influence the regulation of DNA-dependent processes such as transcription, replication, recombination, and DNA repair [76]. In addition, we found a histone protein that provides structural support for chromosomes containing an elongated molecule of DNA, which must acceptably fit into the cell nucleus. This allows DNA to wrap around complexes of histone proteins, allowing the chromosome to have a more compact shape [77].

5.9. Ion Transport Processes

We found two Na, K-ATPase proteins, the sodium/potassium-transporting ATPase subunit beta (NKA) and the sodium/potassium-transporting ATPase subunit alpha. Both proteins classically work as an ion pump that creates an electrochemical gradient across the plasma membrane [78]. This electrochemical gradient was created by pumping three molecules of Na^+ out of the cell in exchange for two molecules of K^+ entering the cell. This process is essential for transepithelial transport, nutrient uptake and membrane potential. In addition, Na, K-ATPase also functions as a receptor, a signal transducer and a cell adhesion molecule [78].

5.10. Sexual Differentiation

Our cod otoliths contained proteins related to sexual differentiation and gamete development. The SPATA6 protein identified in the male cod otolith is essential during the process of fertilization, during sperm–egg binding and fusion [79,80]. In zebrafish, this sperm membrane protein is required for the formation of the segmented columns and the capitulum, two major structures of the sperm-connecting piece essential for linking the developing flagellum to the head during late spermiogenesis [81].

We also identified Vitellogenin-2-like. Vitellogenin (Vtg) is a phosphoglycolipoprotein synthesized in the liver of oviparous animals in response to circulating estrogens [82]. The presence of the vitellogenin protein is specifically found in female blood serum during oocyte growth. It is generally accepted that vitellogenin protein, when hydrolyzed into a free amino acid pool, serves as the main nutritional source for the developing embryo [82,83]. These two identified otolith proteins could serve as major sexual differentiation biomarkers.

6. Identification of Known Otolith Proteins

Some of the proteins that we identified had been previously documented in fish otoliths, although not necessarily for Atlantic cod. For example, we identified carbonic anhydrase (CA), an enzyme critical in acid-base homeostasis and bone remodeling. CA is produced in the brain and drives the neuronally guided bio-mineralization process. The CA protein is an enzyme secreted in the inner ear epithelium of the fish, where it regulates the provision of carbonate for calcium carbonate incorporation into the otolith [84]. We identified cochlin, which is one of the main known otolith constituents and is responsible for calcium carbonate crystalline formation. This otolith protein also regulates the immune response against infection, specifically innate immunity [85]. In addition, we identified myosin, another known constituent of otoliths. Myosins are motor proteins linking the actin-cytoskeleton with membrane phospholipids. The main roles of these proteins are to promote connections between the cytoskeleton and the plasma membrane. They are essential in cellular processes such as cell migration, vesicular trafficking and cytokinesis.

Previous studies have implicated these molecules in cell functions, including endocytosis, exocytosis, the release of extracellular vesicles and the regulation of cell shape and membrane elasticity [86]. We also identified the important otogelin protein, a major constituent of the otoliths. The otogelin proteins are formed in the sensory maculae attached to the otolithic membrane (a gelatinous extracellular matrix) that provides a physical coupling (tethering) between the otolith and the underlying sensory epithelium [87].

Otolin-1, a collagen-like protein which plays an important role in the growth of otoliths, was also identified. It has been proposed that it may serve as a template for the calcification of the otoliths [23]. We also found Na and K-ATPase proteins in our cod otoliths. These proteins serve as an ion pump that creates the electrochemical gradient across the plasma membrane. This gradient is essential for transepithelial transport, nutrient uptake and membrane potential [78]. Na and K-ATPase proteins can also function as receptors, signal transducers and cell adhesion molecules. The Na and K-ATPase proteins are also responsible for developing various physiological needs [78]. We identified the known secreted acidic cysteine-rich glycoprotein (Sparc), which plays an important role in otolith morphogenesis [88]. This secreted acidic cysteine-rich glycoprotein, which binds collagen and Ca^{2+}, is a precerebellin-like protein. It was suggested that it could associate with both collagenous otolin-1 and neuroserpin during framework assembly [88]. Another identified known otolith SERPIN domain-containing protein belongs to the serpins family, which is a broadly distributed family of protease inhibitors that can induce conformational change to inhibit target enzymes that are known to be central in controlling many important proteolytic cascades [88]. Serpins are conformationally labile, and pathogens can misfold them into inactive protein polymers [89]. Likewise, α-tectorin and β-tectorin proteins were also identified. These are glycoprotein components of the otolithic membrane and are essential in developing the teleost otolithic membrane at embryonic stages [86]. The last previously identified otolith protein that we detected in cod ololiths was the V-type proton ATPase subunit G, which functions as a V-ATPase proton pump. This protein creates an acidic medium, which is necessary for the lysosome function and vesicular traffic. It is also essential for several developmental processes [90].

7. Conclusions and Future Directions

The qualitative preliminary shotgun proteomics approach used here revealed that cod otoliths contain thousands more proteins than previously documented. Most of these proteins are not implicated in the biomineralization of otoliths, raising the potential for the otolith proteome to help to recreate details of fish life history at previously unrealized levels. Much work is still required, however, to fully understand the otolith proteome, the mechanisms by which proteins are incorporated into the otolith matrix and the linkages between otolith proteins and fish physiology. Efforts are underway to accurately quantify the proteins in the cod otolith proteome for the twelve fish examined here, which may be a first step to addressing some of these questions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biochem3030008/s1. Table S1: Results of the shotgun proteomics analysis of male Atlantic Cod (*Gadus morhua*) proteins; Table S2: Results of the shotgun proteomics analysis of female Atlantic Cod (*Gadus morhua*) proteins; Table S3: The full list of the 302 most abundant proteins identified from male Atlantic Cod (*Gadus morhua*); Table S4: The full list of the 247 most abundant proteins identified from female Atlantic Cod (*Gadus morhua*); Table S5: According to the complete details of the protein description, there are 143 otolith core proteins, common between male and female Atlantic Cod (*Gadus morhua*) proteins; Table S6: According to the complete details of the protein description, the male Atlantic Cod (*Gadus morhua*) has 138 unique proteins; Table S7: According to the complete details of the protein description, the female Atlantic Cod (*Gadus morhua*) has 67 unique proteins.

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References

1. Popper, A.N.; Hawkins, A.D. The importance of particle motion to fishes and invertebrates. *J. Acoust. Soc. Am.* **2018**, *143*, 470. [CrossRef]
2. Lowenstein, O. 7 The Labyrinth. In *Fish Physiology*; Hoar, W.S., Randall, D.J., Eds.; Academic Press: Cambridge, MA, USA, 1971; Volume 5, pp. 207–240.
3. Manning, F.B. Hearing in the goldfish in relation to the structure of its ear. *J. Exp. Zool.* **1924**, *41*, 5–20. [CrossRef]
4. Schnetz, L.; Pfaff, C.; Libowitzky, E.; Johanson, Z.; Stepanek, R.; Kriwet, J. Morphology and evolutionary significance of phosphatic otoliths within the inner ears of cartilaginous fishes (Chondrichthyes). *BMC Evol. Biol.* **2019**, *19*, 238. [CrossRef]
5. Fay, R.R. The Goldfish Ear Codes the Axis of Acoustic Particle Motion in Three Dimensions. *Science* **1984**, *225*, 951–954. [CrossRef] [PubMed]
6. Popper, A.N.; Ramcharitar, J.; Campana, S.E. Why otoliths? Insights from inner ear physiology and fisheries biology. *Mar. Freshw. Res.* **2005**, *56*, 497–504. [CrossRef]
7. Pannella, G. Fish otoliths: Daily growth layers and periodical patterns. *Science* **1971**, *173*, 1124–1127. [CrossRef] [PubMed]
8. Brothers, E.B.; Mathews, C.P.; Lasker, R. Daily growth increments in otoliths from larval and adult fishes. *Fish Bull* **1976**, *74*, 1–8.
9. Allemand, D.; Mayer-Gostan, N.; De Pontual, H.; Boeuf, G.; Payan, P. Fish Otolith Calcification in Relation to Endolymph Chemistry. In *Handbook of Biomineralization*; Bäuerlein, E., Ed.; WILEY-VCH Verlag GmbH & Co. KgaA: Weinheim, Germany, 2007; pp. 291–308.
10. Campana, S.E.; Thorrold, S.R. Otoliths, increments, and elements: Key to a comprehensive understanding of fish populations. *Can. J. Fish. Aquat. Sci.* **2001**, *58*, 30–38. [CrossRef]
11. Ricard, D.; Adamack, A.; Burbank, J.; Comeau, P.; Daigle, A.; Debertin, A.; Emond, K.; Loewen, T.; Perreault, A.; Puncher, G.; et al. Canadian Technical Report of Fisheries and Aquatic Sciences. In Proceedings of the Technical Expertise in Stock Assessment (TESA) National Workshop on 'Best Practices in Age Estimation', Moncton, NB, Canada, 31 January–2 February 2023. in press.
12. Campana, S.E. How reliable are growth back-calculations based on otoliths? *Can. J. Fish. Aquat. Sci.* **1990**, *47*, 2219–2227. [CrossRef]
13. Degens, E.T.; Deuser, W.G.; Haedrich, R.L. Molecular structure and composition of fish otoliths. *Mar. Biol.* **1969**, *2*, 105–113. [CrossRef]
14. Campana, S.E. Chemistry and composition of fish otoliths: Pathways, mechanisms and applications. *Mar. Ecol. Prog. Ser.* **1999**, *188*, 263–297. [CrossRef]
15. Payan, P.; De Pontual, H.; Bœuf, G.; Mayer-Gostan, N. Endolymph chemistry and otolith growth in fish. *Comptes Rendus Palevol* **2004**, *3*, 535–547. [CrossRef]
16. Köppl, C.; Wilms, V.; Russell, I.J.; Nothwang, H.G. Evolution of Endolymph Secretion and Endolymphatic Potential Generation in the Vertebrate Inner Ear. *Brain Behav. Evol.* **2018**, *92*, 1–31. [CrossRef]
17. Romanek, C.S.; Gauldie, R.W. A predictive model of otolith growth in fish based on the chemistry of the endolymph. *Comp. Biochem. Physiol. Part A Physiol.* **1996**, *114*, 71–79. [CrossRef]
18. Disspain, M.C.F.; Ulm, S.; Gillanders, B.M. Otoliths in archaeology: Methods, applications and future prospects. *J. Archaeol. Sci. Rep.* **2016**, *6*, 623–632. [CrossRef]
19. Yasuo, M. Diurnal rhythm in otolith formation in the rainbow trout, Salmo gairdneri: Seasonal reversal of the rhythm in relation to plasma calcium concentrations. *Comp. Biochem. Physiol. Part A Physiol.* **1984**, *78*, 289–293. [CrossRef]

20. Thomas, O.R.B.; Swearer, S.E. Otolith Biochemistry—A Review. *Rev. Fish. Sci. Aquac.* **2019**, *27*, 458–489. [CrossRef]
21. Loewen, T.N.; Carriere, B.; Reist, J.D.; Halden, N.M.; Anderson, W.G. Linking physiology and biomineralization processes to ecological inferences on the life history of fishes. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2016**, *202*, 123–140. [CrossRef]
22. Falini, G.; Albeck, S.; Weiner, S.; Addadi, L. Control of Aragonite or Calcite Polymorphism by Mollusk Shell Macromolecules. *Science* **1996**, *271*, 67–69. [CrossRef]
23. Murayama, E.; Takagi, Y.; Ohira, T.; Davis, J.G.; Greene, M.I.; Nagasawa, H. Fish otolith contains a unique structural protein, otolin-1. *Eur. J. Biochem.* **2002**, *269*, 688–696. [CrossRef]
24. Huang, S.; Qian, S. Advances in otolith-related protein research. *Front. Neurosci.* **2022**, *16*, 956200. [CrossRef] [PubMed]
25. Lundberg, Y.W.; Xu, Y.; Thiessen, K.D.; Kramer, K.L. Mechanisms of otoconia and otolith development. *Dev. Dyn.* **2015**, *244*, 239–253. [CrossRef]
26. Thomas, O.R.B.; Swearer, S.E.; Kapp, E.A.; Peng, P.; Tonkin-Hill, G.Q.; Papenfuss, A.; Roberts, A.; Bernard, P.; Roberts, B.R. The inner ear proteome of fish. *FEBS J.* **2018**, *286*, 66–81. [CrossRef] [PubMed]
27. Mulry, E.; Parham, K. Inner Ear Proteins as Potential Biomarkers. *Otol. Neurotol.* **2020**, *41*, 145–152. [CrossRef]
28. Murayama, E.; Herbomel, P.; Kawakami, A.; Takeda, H.; Nagasawa, H. Otolith matrix proteins OMP-1 and Otolin-1 are necessary for normal otolith growth and their correct anchoring onto the sensory maculae. *Mech. Dev.* **2005**, *122*, 791–803. [CrossRef]
29. Miller, M.B.; Clough, A.M.; Batson, J.N.; Vachet, R.W. Transition metal binding to cod otolith proteins. *J. Exp. Mar. Biol. Ecol.* **2006**, *329*, 135–143. [CrossRef]
30. Fournier, M.L.; Gilmore, J.M.; Martin-Brown, S.A.; Washburn, M.P. Multidimensional separations-based shotgun proteomics. *Chem. Rev.* **2007**, *107*, 3654–3686. [CrossRef]
31. Meyer, J.G. Qualitative and Quantitative Shotgun Proteomics Data Analysis from Data-Dependent Acquisition Mass Spectrometry. *Methods Mol. Biol.* **2021**, *2259*, 297–308. [CrossRef]
32. Chen, H. "VennDiagram: Generate High-Resolution Venn and Euler Plots". R Package Version 1.7.3. 2022. Available online: https://CRAN.R-project.org/package=VennDiagram (accessed on 1 July 2022).
33. Dannevig, E.H. Chemical composition of the zones in cod otoliths. *ICES J. Mar. Sci.* **1956**, *21*, 156–159. [CrossRef]
34. Peters, J.-M.; Cejka, Z.; Harris, J.R.; Kleinschmidt, J.A.; Baumeister, W. Structural Features of the 26 S Proteasome Complex. *J. Mol. Biol.* **1993**, *234*, 932–937. [CrossRef] [PubMed]
35. Vanzi, F.; Vladimirov, S.; Knudsen, C.R.; Goldman, Y.E.; Cooperman, B.S. Protein synthesis by single ribosomes. *RNA* **2003**, *9*, 1174–1179. [CrossRef] [PubMed]
36. Das, G.; Varshney, U. Peptidyl-tRNA hydrolase and its critical role in protein biosynthesis. *Microbiology* **2006**, *152*, 2191–2195. [CrossRef]
37. Osugi, T.; Ding, Y.; Tanaka, H.; Kuo, C.-H.; Do, E.; Irie, Y.; Miki, N. Involvement of a single-stranded DNA binding protein, ssCRE-BP/Purα, in morphine dependence. *FEBS Lett.* **1996**, *391*, 11–16. [CrossRef] [PubMed]
38. Fey, E.G.; Krochmalnic, G.; Penman, S. The nonchromatin substructures of the nucleus: The ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* **1986**, *102*, 1654–1665. [CrossRef]
39. Kimpler, L.A.; Glosson, N.L.; Downs, D.; Gonyo, P.; May, N.A.; Hudson, A.W. Adaptor Protein Complexes AP-1 and AP-3 Are Required by the HHV-7 Immunoevasin U21 for Rerouting of Class I MHC Molecules to the Lysosomal Compartment. *PLoS ONE* **2014**, *9*, e99139. [CrossRef] [PubMed]
40. Chen, Q.; Du, J.; Cui, K.; Fang, W.; Zhao, Z.; Chen, Q.; Mai, K.; Ai, Q. Acetyl-CoA derived from hepatic mitochondrial fatty acid β-oxidation aggravates inflammation by enhancing p65 acetylation. *iScience* **2021**, *24*, 103244. [CrossRef]
41. Waagbø, R. Water-soluble vitamins in fish ontogeny. *Aquac. Res.* **2010**, *41*, 733–744. [CrossRef]
42. Panepucci, L.; Fernandes, M.N.; Sanches, J.R.; Rantin, F.T. Changes in lactate dehydrogenase and malate dehydrogenase activities during hypoxia and after temperature acclimation in the armored fish, *Rhinelepis strigosa* (Siluriformes, Loricariidae). *Rev. Bras. Biol.* **2000**, *60*, 353–360. [CrossRef]
43. Valero, Y.; Martínez-Morcillo, F.J.; Esteban, M.Á.; Chaves-Pozo, E.; Cuesta, A. Fish Peroxiredoxins and Their Role in Immunity. *Biology* **2015**, *4*, 860–880. [CrossRef]
44. Rand-Weaver, M.; Noso, T.; Muramoto, K.; Kawauchi, H. Isolation and characterization of somatolactin, a new protein related to growth hormone and prolactin from Atlantic cod (*Gadus morhua*) pituitary glands. *Biochemistry* **1991**, *30*, 1509–1515. [CrossRef] [PubMed]
45. Liu, Z.; Zhang, J.; Ma, A.; Wang, X.; Sun, Z.; Cui, W.; Yuan, C.; Zhu, C. Molecular characterization, expression analysis of 14-3-3 beta/alpha and the effect of RNA interference on ion transporter protein Na+-K+-ATPase, Na+–H+-exchanger and CFTR in turbot (*Scophthalmus maximus*). *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **2020**, *246–247*, 110458. [CrossRef] [PubMed]
46. Sower, S.A.; Decatur, W.A.; Hausken, K.N.; Marquis, T.J.; Barton, S.L.; Gargan, J.; Freamat, M.; Wilmot, M.; Hollander, L.; Hall, J.A.; et al. Emergence of an Ancestral Glycoprotein Hormone in the Pituitary of the Sea Lamprey, a Basal Vertebrate. *Endocrinology* **2015**, *156*, 3026–3037. [CrossRef] [PubMed]
47. Allah Abdulraheem, B. Aspartate Transaminase (AST) Activity in Selected Tissues & Organs of Clarias Gariepinus Exposed To Different Levels of Paraquat. *J. Environ. Anal. Toxicol.* **2014**, *4*, 71–73. [CrossRef]
48. Blumstein, J.; Faundez, V.; Nakatsu, F.; Saito, T.; Ohno, H.; Kelly, R.B. The Neuronal Form of Adaptor Protein-3 Is Required for Synaptic Vesicle Formation from Endosomes. *J. Neurosci.* **2001**, *21*, 8034. [CrossRef] [PubMed]

49. Sugahara, R.; Jouraku, A.; Nakakura, T.; Kusakabe, T.; Yamamoto, T.; Shinohara, Y.; Miyoshi, H.; Shiotsuki, T. Two Adenine Nucleotide Translocase Paralogues Involved in Cell Proliferation and Spermatogenesis in the Silkworm Bombyx mori. *PLoS ONE* **2015**, *10*, e0119429. [CrossRef]
50. Bieczynski, F.; Painefilú, J.C.; Venturino, A.; Luquet, C.M. Expression and Function of ABC Proteins in Fish Intestine. *Front. Physiol.* **2021**, *12*, 791834. [CrossRef] [PubMed]
51. Katyal, G.; Ebanks, B.; Lucassen, M.; Papetti, C.; Chakrabarti, L. Sequence and structure comparison of ATP synthase F0 subunits 6 and 8 in notothenioid fish. *PLoS ONE* **2021**, *16*, e0245822. [CrossRef] [PubMed]
52. Ni, S.; Zhou, Y.; Chen, Y.; Du, X.; Zhang, S. Identification of ATP synthase α subunit as a new maternal factor capable of protecting zebrafish embryos from bacterial infection. *FASEB J.* **2019**, *33*, 12983–13001. [CrossRef]
53. Naghdi, S.; Hajnóczky, G. VDAC2-specific cellular functions and the underlying structure. *Biochim. Biophys. Acta (BBA) Mol. Cell Res.* **2016**, *1863*, 2503–2514. [CrossRef] [PubMed]
54. Acharya, U.R.; Behera, S.; Swain, S.; Panda, M.K.; Mistri, A.R.; Sahoo, B. Sequence and structural analysis of β- actin protein of fishes, using bioinformatics tools and techniques. *Int. J. Biosci.* **2014**, *4*, 249–256. [CrossRef]
55. Dos Remedios, C.G.; Chhabra, D.; Kekic, M.; Dedova, I.V.; Tsubakihara, M.; Berry, D.A.; Nosworthy, N.J. Actin Binding Proteins: Regulation of Cytoskeletal Microfilaments. *Physiol. Rev.* **2003**, *83*, 433–473. [CrossRef] [PubMed]
56. Tillgren, V.; Ho, J.C.S.; Önnerfjord, P.; Kalamajski, S. The Novel Small Leucine-rich Protein Chondroadherin-like (CHADL) Is Expressed in Cartilage and Modulates Chondrocyte Differentiation. *J. Biol. Chem.* **2015**, *290*, 918–925. [CrossRef] [PubMed]
57. Hsueh, Y.-J.; Ma, D.H.-K.; Ma, K.S.-C.; Wang, T.-K.; Chou, C.-H.; Lin, C.-C.; Huang, M.-C.; Luo, L.-J.; Lai, J.-Y.; Chen, H.-C. Extracellular Matrix Protein Coating of Processed Fish Scales Improves Human Corneal Endothelial Cell Adhesion and Proliferation. *Transl. Vis. Sci. Technol.* **2019**, *8*, 27. [CrossRef] [PubMed]
58. Zoeller, J.J.; Pimtong, W.; Corby, H.; Goldoni, S.; Iozzo, A.E.; Owens, R.T.; Ho, S.-Y.; Iozzo, R.V. A Central Role for Decorin during Vertebrate Convergent Extension. *J. Biol. Chem.* **2009**, *284*, 11728–11737. [CrossRef] [PubMed]
59. Nishimura, Y.; Kasahara, K.; Inagaki, M. Intermediate filaments and IF-associated proteins: From cell architecture to cell proliferation. *Proc. Jpn. Acad. Ser. B* **2019**, *95*, 479–493. [CrossRef]
60. Schaffeld, M.; Markl, J. Fish Keratins. *Methods Cell Biol.* **2004**, *78*, 627–671. [CrossRef] [PubMed]
61. van der Loop, F.T.; Schaart, G.; Timmer, E.D.; Ramaekers, F.C.; van Eys, G.J. Smoothelin, a novel cytoskeletal protein specific for smooth muscle cells. *J. Cell Biol.* **1996**, *134*, 401–411. [CrossRef]
62. Detrich, H.W.; Fitzgerald, T.J.; Dinsmore, J.H.; Marchese-Ragona, S.P. Brain and egg tubulins from antarctic fishes are functionally and structurally distinct. *J. Biol. Chem.* **1992**, *267*, 18766–18775. [CrossRef] [PubMed]
63. Hwang Hyun, J.; Moon Chang, H.; Kim Han, G.; Kim Joo, Y.; Lee Jung, M.; Park Jeong, W.; Chung Dae, K. Identification and Functional Analysis of Salmon Annexin 1 Induced by a Virus Infection in a Fish Cell Line. *J. Virol.* **2007**, *81*, 13816–13824. [CrossRef] [PubMed]
64. Nawaz, S.; Schweitzer, J.; Jahn, O.; Werner, H.B. Molecular evolution of myelin basic protein, an abundant structural myelin component. *Glia* **2013**, *61*, 1364–1377. [CrossRef]
65. Boggs, J.M. Myelin basic protein: A multifunctional protein. *Cell. Mol. Life Sci. CMLS* **2006**, *63*, 1945–1961. [CrossRef] [PubMed]
66. Anand, S.; Foot, N.; Ang, C.-S.; Gembus, K.M.; Keerthikumar, S.; Adda, C.G.; Mathivanan, S.; Kumar, S. Arrestin-Domain Containing Protein 1 (Arrdc1) Regulates the Protein Cargo and Release of Extracellular Vesicles. *Proteomics* **2018**, *18*, 1800266. [CrossRef]
67. Kang, D.S.; Tian, X.; Benovic, J.L. Role of β-arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. *Curr. Opin. Cell Biol.* **2014**, *27*, 63–71. [CrossRef]
68. Swulius, M.T.; Waxham, M.N. Ca^{2+}/Calmodulin-dependent Protein Kinases. *Cell. Mol. Life Sci.* **2008**, *65*, 2637. [CrossRef]
69. Gupta, M.; Brand, M. Identification and Expression Analysis of Zebrafish Glypicans during Embryonic Development. *PLoS ONE* **2013**, *8*, e80824. [CrossRef]
70. Termini, C.M.; Gillette, J.M. Tetraspanins Function as Regulators of Cellular Signaling. *Front. Cell Dev. Biol.* **2017**, *5*, 34. [CrossRef] [PubMed]
71. Tulke, S.; Williams, P.; Hellysaz, A.; Ilegems, E.; Wendel, M.; Broberger, C. Nucleobindin 1 (NUCB1) is a Golgi-resident marker of neurons. *Neuroscience* **2016**, *314*, 179–188. [CrossRef]
72. Vij, S.; Tyagi, A.K. A20/AN1 zinc-finger domain-containing proteins in plants and animals represent common elements in stress response. *Funct. Integr. Genom.* **2008**, *8*, 301–307. [CrossRef]
73. Evans, P.C.; Ovaa, H.; Hamon, M.; Kilshaw, P.J.; Hamm, S.; Bauer, S.; Ploegh, H.L.; Smith, T.S. Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity. *Biochem. J.* **2004**, *378*, 727–734. [CrossRef]
74. Jang, J.H.; Kim, H.; Jung, I.Y.; Cho, J.H. A20 Inhibits LPS-Induced Inflammation by Regulating TRAF6 Polyubiquitination in Rainbow Trout. *Int. J. Mol. Sci.* **2021**, *22*, 9801. [CrossRef]
75. Du Clos, T.W. Pentraxins: Structure, Function, and Role in Inflammation. *ISRN Inflamm.* **2013**, *2013*, 379040. [CrossRef] [PubMed]
76. Bustin, M.; Reeves, R. High-mobility-group chromosomal proteins: Architectural components that facilitate chromatin function. *Prog. Nucleic Acid Res. Mol. Biol.* **1996**, *54*, 35–100. [PubMed]
77. DesJarlais, R.; Tummino, P.J. Role of Histone-Modifying Enzymes and Their Complexes in Regulation of Chromatin Biology. *Biochemistry* **2016**, *55*, 1584–1599. [CrossRef]
78. Li, Z.; Langhans, S.A. Transcriptional regulators of Na,K-ATPase subunits. *Front. Cell Dev. Biol.* **2015**, *3*, 66. [CrossRef] [PubMed]

79. Binner, M.I.; Kogan, A.; Panser, K.; Schleiffer, A.; Deneke, V.E.; Pauli, A. The sperm protein Spaca6 is essential for fertilization in zebrafish. *bioRxiv* **2021**, arXiv:2021.2011.2019.469324. [CrossRef]
80. Yuan, S.; Stratton, C.J.; Bao, J.; Zheng, H.; Bhetwal, B.P.; Yanagimachi, R.; Yan, W. Spata6 is required for normal assembly of the sperm connecting piece and tight head–tail conjunction. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E430–E439. [CrossRef]
81. Lehti, M.S.; Sironen, A. Formation and function of sperm tail structures in association with sperm motility defects. *Biol. Reprod.* **2017**, *97*, 522–536. [CrossRef] [PubMed]
82. Cohen, A.M.; Mansour, A.A.H.; Banoub, J.H. 'De novo' sequencing of Atlantic cod vitellogenin tryptic peptides by matrix-assisted laser desorption/ionization quadrupole time-of-flight tandem mass spectrometry: Similarities with haddock vitellogenin. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2454–2460. [CrossRef] [PubMed]
83. Hara, A.; Hiramatsu, N.; Fujita, T. Vitellogenesis and choriogenesis in fishes. *Fish. Sci.* **2016**, *82*, 187–202. [CrossRef]
84. Hamm, L.L.; Nakhoul, N.; Hering-Smith, K.S. Acid-Base Homeostasis. *Clin. J. Am. Soc. Nephrol.* **2015**, *10*, 2232. [CrossRef]
85. Rhyu, H.-J.; Bae, S.H.; Jung, J.; Hyun, Y.-M. Cochlin-cleaved LCCL is a dual-armed regulator of the innate immune response in the cochlea during inflammation. *BMB Rep.* **2020**, *53*, 449–452. [CrossRef] [PubMed]
86. Girón-Pérez, D.A.; Piedra-Quintero, Z.L.; Santos-Argumedo, L. Class I myosins: Highly versatile proteins with specific functions in the immune system. *J. Leukoc. Biol.* **2019**, *105*, 973–981. [CrossRef] [PubMed]
87. Stooke-Vaughan, G.A.; Obholzer, N.D.; Baxendale, S.; Megason, S.G.; Whitfield, T.T. Otolith tethering in the zebrafish otic vesicle requires Otogelin and α-Tectorin. *Development* **2015**, *142*, 1137–1145. [CrossRef] [PubMed]
88. Kang, Y.-J.; Stevenson, A.K.; Yau, P.M.; Kollmar, R. Sparc Protein Is Required for Normal Growth of Zebrafish Otoliths. *J. Assoc. Res. Otolaryngol.* **2008**, *9*, 436–451. [CrossRef]
89. Law, R.H.P.; Zhang, Q.; McGowan, S.; Buckle, A.M.; Silverman, G.A.; Wong, W.; Rosado, C.J.; Langendorf, C.G.; Pike, R.N.; Bird, P.I.; et al. An overview of the serpin superfamily. *Genome Biol.* **2006**, *7*, 216. [CrossRef]
90. Ramos-Balderas, J.L.; Carrillo-Rosas, S.; Guzman, A.; Navarro, R.E.; Maldonado, E. The zebrafish mutants for the V-ATPase subunits d, ac45, E, H and c and their variable pigment dilution phenotype. *BMC Res. Notes* **2013**, *6*, 39. [CrossRef] [PubMed]