An in-depth coho salmon (*Onchorhynchus kisutch)* ovarian follicle proteome reveals coordinated changes across diverse cellular processes during the transition from primary to secondary growth

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Abbreviations:

AGC: automatic gain control

ANOSIM: analysis of similarity

BCA: bicinchoninic acid

DIA: data independent acquisition

ECA: early cortical alveolus

EPN: early perinucleolar

FSH: follicle stimulating hormone

GPF: gas phase fractionated

HPG: hypothalamic-gonadal-pituitary

LCA: late cortical alvelolus

LDLR: low-density lipoprotein receptor

LH: luteinizing hormone

LPN: late perinucleolar

MS2: second mass spectrometry scan

NH4HCO3: ammonium bicarbonate

NMDS: nonmetric multidimensional scaling

PRTC: peptide retention time calibration

qPCR : quantitative polymerase chain reaction

TEAB: triethylammonium bicarbonate

TCA: tricarboxylic acid cycle

WGCNA: weighted gene correlation network analysis

VLDLR: very low-density lipoprotein receptor

ZP: zona pellucida

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# Abstract

Teleost fishes are a highly diverse and ecologically essential group of aquatic vertebrates and include coho salmon, *Onchorhynchus kisutch*. Coho are semelparous and all ovarian follicles develop synchronously. Owing to their ubiquitous distribution, teleost provide critical sources of food worldwide through subsistence, commercial fisheries, and aquaculture. Enhancement of commercial hatchery practices requires a detailed knowledge of teleost reproductive physiology. Despite decades of research on teleost reproductive processes, an in-depth proteome of teleost ovarian development has yet to be generated. We describe a coho salmon ovarian proteome of over 5700 proteins, generated with data independent acquisition, revealing the suite of detectable proteins that change through the transition from primary to secondary ovarian follicle development. This transition is critical for puberty onset, egg quality, and further embryonic development. Primary ovarian follicle development was marked by differential abundances of proteins involved in carbohydrate metabolism, protein turnover, and the complement pathway, suggesting elevated metabolism as the oocytes enter maturation. The greatest proteomic shift occurred during the transition from primary to secondary follicle growth, with increased abundance of proteins underlying cortical alveoli formation, extracellular matrix reorganization, iron binding, and cell-cell signaling. This work provides a foundation for identifying biomarkers of salmon oocyte stage and quality.

Significance of Study

This is the first ovarian proteome generated using DIA of an emerging vertebrate endocrine model, specifying timing of protein translation during key phases of coho ovarian maturation. Teleost fish are the basis of essential fisheries and aquaculture and our continued reliance on these fish as a food source depends upon understanding successful reproduction. This work identifies important protein abundance changes and putative interactions that will lay the foundation for a more in-depth exploration and understanding of teleost reproductive physiology. The conservation of multiple molecular pathways across diverse vertebrate taxa, now including fish, have also been confirmed.

# Introduction

Reproduction is the foundation of species propagation and diversification. Across the domains of life, there is a diversity of reproductive processes, but within vertebrates the essential mechanisms of oogenesis and spermatogenesis, followed by fertilization and development, are relatively conserved [1]. Transcriptomic and proteomic studies of developing gametes have led to advances in the fields of fertility assistance [2], conservation biology [3], and comparative physiology [4]. Expanding the variety of animal model systems employed to study reproductive endocrinology and physiology will have positive implications for both scientific research (such as understanding conserved mechanisms) and practical applications (including captive breeding, conservation, and management).

Coho salmon (*Oncorhynchus kisutch*), the model species used in this study, are semelparous, with all ovarian follicles developing synchronously. This reproductive history makes them an excellent model for the study of specific stages of oogenesis. Within the ovary, at any given point in time, all the follicles are homogeneous and physiological results are not confounded by the presence of other staged follicles. The majority of teleost fishes exhibit oviparity in which gametes develop internally until physiological and/or environmental cues signal spawning. In coho salmon, juveniles migrate to the ocean where they grow into adults for one or more years before migrating back to natal streams. Ovarian maturation and growth begin in the ocean, triggered by the activation of the hypothalamus- pituitary-gonad (HPG) axis which stimulates the production of sex hormones that promote gametogenesis [5]. A defining characteristic of an oviparous ovum are the large stores of maternal molecules laid down in the developing oocyte that are sufficient to support fertilization and early embryonic development. During the initial phase of ovarian development while parr are still in the natal stream, before hormonal activation of the HPG axis, somatic cells surrounding the oocyte differentiate into granulosa and theca cell layers associated with the ovarian follicle. Additionally, organelles proliferate and oocyte RNA synthesis substantially increases, resulting in the deposition of maternal RNAs and proteins. The oocyte’s non-cellular outer coat, the zona radiata, forms (reviewed in [6]).

Upon activation of the HPG axis (identified as the onset of puberty), the primary ovarian follicle undergoes transition into the secondary growth stage. This stage is characterized by substantial increases in oocyte volume, accumulation of cortical alveoli (similar to cortical granules of mammalian oocytes), and the uptake and processing of hepatically-derived phospholipoproteins (known as vitellogenins) into yolk proteins, a process known as vitellogenesis [6], [7]. In salmon, primary and secondary ovarian growth and their successful completion are closely tied to somatic growth rate [8],[9], emphasizing the complexity of cellular processes leading to reproductive success.

Pacific salmon populations are increasingly threatened by climate change and habitat destruction [10],[11],[12], resulting in a decline in wild populations in some regions and an increase in hatchery rearing efforts. Both conservation and commercial hatchery production rely on a detailed knowledge of reproductive processes and physiology to develop therapies to overcome a range of reproductive dysfunctions associated with captive rearing. One major problem common to breeding fish in captivity is poor “egg quality”, manifested in poor fertilization rates and failure of the embryo to develop. Although knowledge of the processes and control of later stages of oocyte maturation (i.e., vitellogenesis and the development of a haploid ovum also know as final oocyte maturation) is substantial (reviewed in [6],[13]), much of the maternal stores and mechanisms critical for fertilization and embryonic development are established earlier in development, when ovarian follicles transition into secondary growth. Therefore, gaining a comprehensive understanding of the diverse molecular components essential for successful gametogenesis involves investigating the molecular and cellular processes occurring within the ovarian follicle.

Much of the previous work on coho salmon to clarify the molecular mechanisms underlying the development of previtellogenic ovarian follicles (i.e., primary and secondary ovarian follicle growth) have leveraged qPCR of a limited number of targeted genes [14],[15] or characterization of the ovarian transcriptome [16]. A major gap in knowledge lies in the lack of data on how the ovarian proteome changes during ovarian follicle development. As the functional and structural molecules of cells, the expression of proteins is more closely aligned with phenotype than mRNA expression. There are some examples of strong links between changes in mRNA abundance and phenotypic or chemical change, for example changes in gene expression of reproductive pathway genes is strongly associated with certain reproductive hormones in coho salmon [17],[18]. Indeed, the activity of some proteins, such as aromatase, are well correlated to mRNA expression in fish [19], but the close association between mRNA and protein expression does not hold for most proteins. On a full proteome scale in ovaries of some other fish species, there is generally a very low correlation between mRNA and protein abundance [20],[21], complicating the interpretation of mRNA abundance changes in a functional phenotype context.

We collected coho salmon ovarian follicles over four stages of development that encompassed primary and early secondary previtellogenic stages (early and late perinucleolar, early and late cortical alveolus) to create an in-depth ovarian proteome using data independent acquisition (DIA) tandem mass spectrometry. We chose DIA proteomics over the more often utilized transcriptomics because of clear advantages: 1) Our interest was in the molecular and physiological changes specific to the developing ovary and we wanted to avoid potential confounding signals from maternal RNAs, which are allocated to developing oocytes during the time frame our study captures (6). 2) Proteins are highly responsive to change [22] and are often degraded/recycled when not needed (e.g., [23]) and are thus more likely to reveal stage-specific and physiologically-relevant changes in the ovary. 3) Proteins are more closely aligned with cellular physiology than mRNA. 4) DIA, as opposed to the commonly applied data dependent acquisition, more thoroughly samples the proteome analyzed, resulting in a better representation of the low abundance proteome. DIA allowed us to dig deeper into the relevant ovarian molecular physiology to uncover a diversity of pathways involved in oogenesis. This discovery dataset is a major step forward in establishing coho salmon as a model organism for comparative studies, as well as providing information needed to develop therapies to overcome reproductive dysfunction in captive fish populations.

# Materials and Methods

## 2.1 Experimental Design and Statistical Rationale

Fish were reared as previously described [24]. Briefly, juvenile coho salmon (Issaquah Hatchery stock, Issaquah, WA, which originated from wild stock in the Green River, WA) were reared at the hatchery facilities of the University of Washington, Seattle, WA under simulated natural photoperiod in re-circulated 10-11°C fresh water, untreated water pumped from nearby Portage Bay. Fish were fed twice daily with a commercial feed (BioDiet, Bio-Oregon, Longview, WA) according to the manufacturer’s guidelines. Welfare checks were performed twice daily for the duration of fish rearing. Fish were checked for abnormal swimming behavior or coloration and were sacrificed if any abnormalities were detected. Water quality was monitored daily for temperature, pH, total ammonia, nitrogen, nitrite, and nitrate.

Randomly selected fish were euthanized in buffered 0.05% tricaine methanesulfonate (IACUC approved anesthetic for fish) until movement of the gill operculum ceased. Fish were sacrified at approximately 12:00 pm at each sampling time point. Coho salmon ovarian tissue was sampled (April 2020-February 2021) to capture stages of the transition between primary growth and early secondary growth of ovarian follicles: early perinucleolar (EPN), late perinucleolar (LPN), early cortical alveolus (ECA), and late cortical alveolus (LCA) stages (e.g., [14],[16],[25]). Between 15-90 fish were sampled for histology per time point. Fork length (mm) and body weight (g) were recorded. Ovaries were removed and weighed. Ovarian tissues were preserved in Bouin’s fixative for histological analysis and snap frozen in liquid nitrogen for proteomic analysis. Staged ovarian follicles were selected for proteomics for each stage (EPN n = 6; LPN n = 8; ECA n = 5; LCA n = 6). Fish length, weight, and ovarian volume are provided in Tables S1 and S2. For length, weight, and ovarian volume, descriptive statistics and a one-way ANOVA followed by Tukey multiple comparisons test was performed using GraphPad Prism version 10.2.3 for macOS with a level of significance determined at p = 0.05 (GraphPad Software, Bost, MA). Fish were handled following an approved protocol established by the Institutional Animal Care and Use Committee at the University of Washington (4078-06) and California State University Fullerton (2022-1286).

Mass spectrometry proteomics was performed on an Oribtrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) in data independent acquisition mode (DIA). Our general analytical pipeline follows [26], leveraging a predicted spectral library generated by Prosit [27] and searching the DIA data with EncyclopeDIA [28], followed by Skyline [29] (Fig. 1). Complementary analyses using Limma [30] with a p-value cut-off of 0.05 and Weighted Gene Correlation Network Analysis (WGCNA) [31] with a correlation coefficient cut-off of |0.45| were applied to the dataset to identify differentially abundant proteins and protein groups strongly correlated with relevant physiological variables, respectively. Gene Ontology enrichment results were considered significant below a p-value of 0.1. All proteomics methods are MIAPE compliant.

2.2 Histology

Ovarian follicles were staged using paraffin wax histology. Briefly, ovarian tissues were fixed in Bouin’s fixative for 48 hours then stored in 70% ethanol until tissue processing. Tissues were processed in an automated tissue processor (Leica Biosystems TP 1020 tissue processor, Buffalo Grove, IL) in a series of graded ethanol solutions (i.e., 70, 95, 100%) for dehydration of the tissues, cleared with xylene, and infiltrated and embedded in molten paraffin wax. Tissues were sectioned at a thickness of 5 µm using a manual rotary microtome (Micron HM 325, Leica Biosystems, Buffalo Grove, IL), mounted on microscope slides and stained with hematoxylin and eosin. Tissues were examined using brightfield microscopy (Olympus BX60) and micrograph images captured uding a digital camera (QICAN QImaging Fast 1394) and imaging software (Q-Capture Pro 7, QImaging 2010). At least 15 ovarian follicles sectioned through the nucleus of the oocyte were measured for each ovarian tissue sample [25]. Ovarian follicles were staged based on the morphological characteristics previously established for salmoninds [32]. Ovarian follicle diameter (µm) and the volume (mm3) was calculated.

## 2.3 Protein extraction

Each sample corresponding to EPN and LPN (i.e., primary growth) stages was suspended in 250 µl of sonication buffer; ECA and LCA samples (i.e., secondary growth) were suspended in 500 µl since the tissue fragments were larger at these stages (5% SDS, 50 mM NH4HCO3, 2 mM MgCl2, 1X HALT protease and phosphatase inhibitors (Invitrogen)). Approximately 100 µl of 1.0 mm Zirconium oxide beads (Next Advance) were poured into each sample tube. Tissues were homogenized to a slurry in a Bullet Blender (Next Advance) using settings 4 - 6 until no tissue pieces remained. Tissue homogenates were subsampled (120 µl each) for sonication. Each sample was sonicated two times at 10 s each time, power setting of 1, with a Sonic Dismembrator Model 100 (Fisher Scientific). Total protein was quantified for each sample in triplicate using Pierce's bicinchoninic acid (BCA) assay following the manufacturer's protocol (ThermoFisher Scientific).

Proteins (100 µg per sample) were extracted following the S-trap mini protocol (ProtiFi; Supplemental Methods). Peptides were dried in a SpeedVac Vacuum Concentrator with Refrigerated Vapor Trap (Savant) with no heat applied and reconstituted in 2% acetonitrile with 0.1% formic acid. Before liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis, Pierce's peptide retention time calibration mix (PRTC) was added to each sample to a concentration of 25 fmol/µl. For the pooled sample to generate the spectral library, 5 µl from eight samples (two from each sampling time point) were mixed.

## 2.4 Proteomics LC-MS/MS

Liquid chromatography was run in-line with the mass spectrometer to separate peptides before MS/MS analysis. The fused silica pre-column, with an in-house made kasil frit (3.5 cm with 150 µm i.d.) and pulled fused silica analytical column (34 cm long, 75 µm i.d.). Both columns were packed in-house with Dr. Masich C18 3 µm beads. The Lumos mass spectrometer was run in data independent acquisition (DIA) mode. The pooled samples were analyzed with narrow window (2 *m/z*) DIA in gas-phase fractions (400-500 *m/z*, 500-600, 600-700, 700-800, 800-900, 900-1000) to generate an in-depth spectral library. The 130 minute instrument method included a gradient of 3-45% solvent B (80% aceotnitrile) over 90 minutes. Individual ovary peptide samples were analyzed with wider window DIA (8 *m/z*) across the massrange of 400-1000 *m/z* with the same solvent gradient. Technical replication of samples was not performed. Additional mass spectrometry methods can be found in Supplemental Methods

## 2.5 Proteomics data analysis

Raw mass spectrometry files were convered to .mzML format in MSConvert [33] with the following parameters: remove titleMaker filter, write index, TPP compatibility, use zlib compression, SIM as spectra, and added filters with default settings (peakPicking and demultiplex).

The database used for peptide detection was the coho proteome predicted from a whole genome sequencing project (BioProject PRJNA352719). We discovered that some proteins of interest for ovarian maturation were not included in this proteome database, so we added protein sequences for follicle stimulating hormone (Fsh), luteinizing hormone (Lh), the receptors for Fsh (Fshr) and Lh (Lhr), 3-oxo-5-alpha-steroid 4-dehydrogenases (Srda5a1 and Srda5a2), and an ovarian aromatase (Cyp19a1a) (see complete list of protein sequences in Table S3). All protein sequences were derived from coho salmon, except for the FSH receptor, which came from the Atlantic salmon proteome. The coho proteome was converted to a Prosit .csv in EnclycopeDIA v. 1.4.10 [28] and the .csv was subdivided into five sub-proteomes to comply with the file size limit in Prosit [27]. Prosit creates theoretical spectral libraries that frequently improve peptide detections in our pipeline [34]. Each Prosit .txt file was downloaded and converted to .dlib format in EncyclopeDIA v.1.4.10. The five .dlibs were combined into a single file for further analyses.

The combined .dlib was used as the library for chromatogram library construction in EnclycopeDIA v. 1.12.34. The fasta file with all the protein sequences used to create the Prosit library was used as the background library. The pooled GPF samples (in .mzML format) were used to generate an .elib chromatogram library file. This .elib was then used as the library to search the single injection, wide window .mzML files for each sample. The quant report resulting from these peptide detections becomes the library in the Skyline document. Search results and single injection .mzML files were imported into Skyline daily v. 22.2.1.256 [29]. The Skyline document, with all its settings and all files used to build it, can be found on Panorama Public [35] with the url <https://panoramaweb.org/yYtWfd.url>, or with the ProteomeXchange accession number PXD052361 and reviewer login ([panorama+reviewer261@proteinms.net](mailto:panorama+reviewer261@proteinms.net)) and password (AbVm0eL+H#&kvh). Abundance data was total ion current (TIC) normalized before export and further analysis.

The full proteomics dataset was analyzed using nonmetric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) using the vegan package [36] in R [37]. Three of the fish sampled in August had different proteomes than the other five fish from that time point, and were also smaller (by length and weight). These fish were excluded from the main analysis due to these differences, but are discussed in more detail in the Discussion section. Pairwise differential abundance between consecutive time points (April-August, August-December, December-February) was determined using Limma with a p-value cut-off of 0.05 [30]. Weighted gene (protein) correlation network analysis (WGCNA) was applied to the dataset to identify groups of proteins that co-vary in abundance, correlated to the variables of sampling time point, fish weight, or oocyte volume and excluding other variables that were strongly auto-correlated with the selected variables [31].

Functional Gene Ontology enrichment analysis was performed with a custom compGO portal (<https://meta.yeastrc.org/compgo_emma_dia_prots/pages/goAnalysisForm.jsp>

) (as described in [38]) on the groups of proteins that were either 1) differentially abundant in the Limma analysis or 2) included in a WGCNA protein module. A p-value cut-off of 0.1 was used for identifying enriched GO terms.

All R code used to generate results and figures is available on GitHub: <https://github.com/Nunn-Lab/Publication-Coho-DIA>

2.6 Discovery proteomics validation

A subset of the proteins detected in the DIA experiment described above were analyzed with selected reaction monitoring targeted proteomics on a Thermo TSQ Altis on coho ovarian tissue fragments. All .raw files and suppporting Skylines files can be found on Panorama Public: <https://panoramaweb.org/zrQjtj.url>. Additional details on these methods and results can be found in Supplementary Methods.

# Results

## 3.1 Ovarian stages and growth

Coho salmon ovaries at the primary growth stage were sampled in April at the early perinucleolar (EPN) stage and in August at the late perinucleolar (LPN) stage. By December, coho salmon ovaries had transitioned to the secondary growth stage with ovarian follicles at the early cortical alveolus (ECA) stage and the late cortical alveolus (LCA) stage in February. Fish weight significantly (p < 0.0001) increased from the EPN stage through LCA stage (Tables S1 and S2). There was a significant difference in body weight between progressive stages of development (EPN to LPN p = 0.0215, LPN to ECA p < 0.001, ECA to LCA p < 0.001). The largest increase in body weight occurred between the LPN stage to the ECA stage, which also represents the transition from primary to secondary ovarian follicle growth (Fig. 2A). Ovarian follicle volume also significantly (p < 0.0001) increased during the primary and secondary growth transition (Fig. 2B). The volume of ovarian follicles was not significantly different between EPN and LPN (p = 0.8973), nor between ECA and LCA (p = 0.1698). There was a significant difference (p < 0.0001) between the volumes of ovarian follicles at the LPN and ECA stages.

## 3.2 The coho ovarian proteome

**Global protein trends**

We identified 5773 proteins in the coho salmon ovarian proteome from four key stages of ovarian follicle development (Table S4). Proteomic profiles were distinct according to ovarian stage (ANOSIM R = 0.82, p = 0.001), with the largest proteomic shift occurring at the transition from primary to secondary ovarian follicle growth (i.e., LPN to ECA; Fig. 3).

**Differential abundance between ovarian stages**

Between the EPN and LPN ovarian follicle stages, there were 156 differentially abundant proteins (Table S5, Figure S1). Many of these proteins (n = 140) were at higher abundance in LPN fish. The 156 proteins were enriched for the GO terms related to protein turnover (e.g., glycoprotein cleavage, endoplasmic reticulum, protein folding) and immune function (complement activation) (Table S6). Several proteins that underwent changes during the transition from the EPN to LPN stages were also associated with carbohydrate metabolism. For instance, there were representative proteins that are implicated in glycolysis (phosphoglycerate mutase), the citric acid cycle (malate dehydrogenase, 2-oxoglutarate dehydrogenase, isocitrate dehydrogenase), and gluconeogenesis (fructose-1,6-bisphosphatase).

The largest set of differentially abundant proteins was detected between the LPN and ECA ovarian follicles; 821 proteins changed significantly during the transition from primary to secondary ovarian follicle growth (Table S5, Figure S1). 475 of these proteins were elevated in LPN-staged ovaries and 346 were elevated in ECA-staged ovaries. Proteins that decreased from LPN to ECA were enriched for GO terms associated with gene expression regulation during oogenesis and meiosis (regulation of transcription, DNA-templated; gene silencing by RNA) and with meiosis (meiotic cell cycle) (Table S6). Proteins that increased during this transition were enriched for GO terms that suggest ongoing ovarian follicle development (e.g., cortical granule), extracellular matrix reorganization (collagen trimer; extracellular matrix structural constituent), iron binding (metal ion transport), and cell-cell signaling (Notch signaling pathway) (Table S6). Many other proteins not captured by the GO enrichment analysis, but which are known to be associated with oogenesis also had significant abundance changes during the LPN to ECA transition: proteins in the sialic acid pathway, zona pellucida membrane proteins, oocyte-specific histone RNA stem-loop-binding protein 2, zygote arrest protein, high choriolytic enzyme, and sex-hormone binding globulin.

As mentioned in the Methods, three fish from the LPN stage group were excluded from the overall analysis because they were found to be smaller than the other fish in the group and their ovarian proteomes were distinctive (Figure S2). These three fish were smaller than the other five fish (38.2-41.9 g vs. 42.6-49.0 g). In a differential abundance analysis between these two groups of fish with ovaries at the LPN stage, >500 proteins were found to be differentially abundant, almost all of them at lower abundance in the three smaller fish (Table S7).

Following the transition into secondary ovarian follicle growth, during the period of increased lipid deposition, 255 proteins were observed to be differentially abundant between fish with ovaries at the ECA and LCA stages (Table S5). Most of these proteins (n = 232) were at decreased abundance in LCA fish ovaries. These proteins were enriched for GO terms that suggest changes to RNA binding and gene expression regulation (e.g., P-body; regulation of gene expression) (Table S6). The differentially abundant proteins between ECA and LCA also included vigilin, estradiol 17-beta-dehydrogenase 1, lipase maturation factor, and bone morphogenetic protein.

**Weighted correlation network analysis**

Weighted gene correlation network analysis (WGCNA) was applied to detect proteins that had strong correlations with different variables associated with the sampled fish (month of sampling, fish body weight, and ovarian follicle volume). WGCNA identifies groups, or modules, of proteins that are strongly correlated in their expression in response to a given variable [31]. Three modules (groups) of proteins had strong correlations with timing (month) of sampling: blue (correlation coefficient of 0.923), turquoise (-0.938), and brown (-0.603). Magenta module proteins were moderately correlated with month (0.478). Fish body weight was moderately correlated (correlation coefficient > 0.35) with three modules of proteins: blue (0.538), magenta (0.389), and yellow (0.409). Ovarian follicle volume was weakly correlated with protein abundance modules. WGCNA and GO enrichment results are summarized in Fig. 4 with detailed results in Tables S8 and S9.

# Discussion

Our research represents the pioneering application of DIA proteomics to teleost ovarian development, offering an unprecedented coverage of thousands of proteins spanning a wide dynamic abundance range. Over 5000 proteins were identified here as part of the coho salmon ovarian proteome. Weighted correlation network analysis of the ovarian proteome identified groups of proteins that have strongly correlated changes in abundance associated with either the month of sampling, fish body weight, or ovarian follicle volume. Changes in the co-varying abundances of these proteins across four ovarian follicle developmental stages (i.e., EPN, LPN, ECA, LCA), representing the transition from primary to secondary growth, highlight the metabolic, structural, and functional changes that occur in the ovary during this important developmental transition of ovarian follicle growth. This dataset can form the foundation for better understanding the physiology of successful reproductive maturation and for developing biomarkers of ovarian stage and and/or quality to support more efficient fish rearing and breeding and provide an additional model for comparative physiology studies.

4.1 Metabolic changes prepare the primary growth ovarian follicle for secondary growth

As development progressed during primary growth in the ovary, proteins related to protein turnover and carbohydrate metabolism increased between the EPN and LPN stages. Carbohydrate metabolism up-regulation has been previously observed in previtellogenic follicles of other fish species ([39],[40],[41]). This occurs during a time of dynamic changes in cellular and tissue structure and function, including an up-regulation of RNA synthesis and accumulation of maternal RNAs (reviewed in [6]). During the EPN to LPN transition, there is a rapid increase in ovarian follicle volume, likely facilitated by metabolic upregulation and structural changes. The increase in proteins involved in glycolysis, the TCA cycle, and gluconeogenesis during the EPN to LPN transition may reflect a breakdown of macromolecules to provide energy for the developing ovarian follicles.

Proteomic evidence also indicates an increase in RNA synthesis during primary ovarian growth in coho salmon. Many RNA binding proteins and helicases are at peak abundance in fish with ovaries at the LPN stage. Similar proteomic trends were detected in zebrafish (*Danio rerio*) and gilthead seabream (*Sparus aurata*) ovarian follicles [20]. During storage in the oocyte, maternal RNA poly(A) tails may be shortened for storage, and then rapidly polyadenylated when their associated protein is needed [6]. Proteins such as cleavage and polyadenylation factor and cytoplasmic polyadenylation element-binding protein are both significantly elevated in LPN-staged coho salmon ovaries. These proteins likely represent some of the cellular machinery necessary to synthesize RNAs during primary growth when heightened RNA synthesis occurs, as well as protect and stockpile maternal RNAs that are needed later in development.

Multiple lines of evidence from the ovarian proteome suggest that the complement pathway is essential during coho salmon primary ovarian follicle growth. Many complement pathway proteins increased in abundance from the EPN to LPN stages, in addition to several SERPIN-domain proteins. Serpins (serine proteinase inhibitors) may be involved in complement activation [42] and have previously been detected in teleost ovarian proteomes [20] and identified as important players in vertebrate oocyte maturation (e.g., [43]). In the coho salmon proteome, complement proteins B, C9, C1q, C3, factor H, factor I, and C4 were significantly differentially abundant, and many other proteins in the pathway were detected (Table S4). Complement proteins have been detected in human follicular fluid [2] and increase in human plasma during pregnancy [44], suggesting a conserved role in reproduction across vertebrates. Previous work across vertebrate taxa has also suggested that complement C3 plays a role in oocyte fertilization and maturation ([45],[46],[47]). Our findings in the coho salmon proteome bolster the hypothesis that C3, and possibly other complement proteins, play an important role in ovarian follicle development, and suggest these proteins are essential for successful vertebrate oogenesis.

4.2 Secondary ovarian follicle growth sets the stage for successful oocyte and embryonic development

The transition from primary to secondary growth in ovarian follicles is characterized by the shift of LPN ovarian follicles to the ECA stage. This transition is evident in the coho salmon proteome through alterations in proteins related to lipid deposition, cellular energy production, and preparation for embryonic development. In coho salmon, the ECA stage (i.e., first stage of secondary ovarian follicle growth) is characterized by a significant increase in ovarian follicle volume (Fig. 2; [7]). In large part, this is due to the increasing accumulation of cortical alveoli within the ooplasm. Cortical alveoli (similar to cortical granules in mammals) are subsequently released into the perivitelline space post-fertilization to prevent polyspermy [48]. L-rhamnose binding lectins are integral protein components of cortical alveoli [6] and play a role in ovarian development of salmonids [49]. The abundance of l-rhamnose binding lectins progressively increased during the ovarian follicle stages examined in this study. In the transcriptome of coho salmon ovaries, no difference in the abundance of l-rhamnose binding lectin mRNA was observed between primary and secondary growth stages [14]. This finding underscores the possibility of a disconnect between changes in mRNA levels and protein expression, and how they impact the functional cellular phenotype.

The transition from primary to secondary ovarian follicle growth involves substantial structural and functional changes that require increased cellular energy production. In fish with ECA-stage ovarian follicles, elevated proteins were linked to energy production pathways, which contribute to enriched GO terms within the blue module, including "transmembrane transport", "respiratory electron transport chain", and "nucleoside diphosphate metabolic process". This co-varying module of proteins also includes many involved in carbohydrate metabolism, which continues to be an important source of cellular energy during the vitellogenic phase of ovarian follicle development occurring later in secondary growth [39],[40],[41]. The co-varying abundance patterns of these proteins emphasize how diverse cellular processes are interconnected to achieve successful ovarian follicle development.

The increased deposition of lipids in coho salmon ovarian follicles is necessary for later provisioning of the embryo. Low-density and very low-density lipoprotein receptors (LDLR and VLDLR) increase in abundance between the transition from primary to secondary ovarian follicle growth (i.e., LPN, ECA stages), likely facilitating the uptake and processing of lipids during the later stages of developing ovarian follicles. Teleost genomes contain multiple LDLR, likely to specifically bind their respective forms of vitellogenin (e.g., [50],[51],[52]). In cutthroat trout (*Oncorhynchus clarkii*), the mRNA encoding LDLR13 exhibits higher expression in previtellogenic ovaries than in vitellogenic ovaries [52]. In support of Mushirobira's hypothesis, protein abundance of LDLRs is higher in vitellogenic ovaries, suggesting that genes are transcribed earlier so that translation of receptor proteins can occur at the appropriate time [52]. In the current study, different isoforms of LDLR follow two different trends of abundance, with some increasing in abundance between the EPN and LPN stages and others not increasing until later during the LPN and ECA stages. Differences in abundance may point to potential differences in the timing of uptake of different lipoproteins. The expression of VLDLR transcripts in coho salmon peaks during earlier stages of ovarian follicle development [14] and we similarly observed an increase in protein levels within ovarian follicles during early ovarian growth. None of the LDLRs detected in the coho salmon proteome contained conserved peptides that would indicate their specific roles as vitellogenin receptors [53], yet their abundance patterns suggested an important role during vitellogenesis, which requires further investigation.

The diversity of changes underlying the primary to secondary ovarian follicle transition is further highlighted by many other proteins that increased in abundance in coho salmon ovaries during the transition from LPN to ECA. These proteins span functions such as sperm recognition (sialic acid pathway; zona pellucida membrane proteins), transfer of maternal iron to the oocytes (ferritin), transition from oocyte to embryo (zygote arrest protein), hatching (high choriolytic enzyme), and sex-hormone binding globulin (testosterone binding and transport). Androgens are pivotal in establishing the molecular framework for responding to endocrine cues resulting from the initiation of the HPG axis (i.e, at the time of puberty). Our prior research has demonstrated their crucial involvement in the transition to secondary growth in coho salmon [16],[25], which has also been demonstrated in other species (e.g., [54],[55]). The increase in sex steroid-hormone binding globulin in ovaries at the ECA stage is likely another component in that process. The proteomic trends identified for the zona pellucida (ZP) proteins confirm Luckenbach et al.'s [14] hypothesis that the early (perinucleolar) transcription of ZP proteins they observed likely results in protein translation at a later stage of ovarian follicle development.

Underlying these physiological changes in the ovary are a network of proteins that are driving increased transcription and translation. These proteins are enriched for GO terms such as "mRNA splicing, via spliceosome" and "translation initiation". Our findings support earlier discoveries of co-varying protein synthesis in the striped bass (*Morone saxatilis*) ovary proteome [56]. Since our coho salmon proteome includes an order of magnitude more proteins than the striped bass ovary proteome (>5000 vs. 355) and thousands more than the zebrafish proteome (1379 proteins) [21], our network of protein interactions and associations may be more complete, increasing our understanding of the complex proteomic machinery underlying ovarian follicle development. These previous fish ovarian proteomes were generated with 2D gel electrophoresis followed by mass spectrometry (striped bass) and MudPIT DDA (zebrafish), both technologies that typically detect fewer proteins than DIA. In contrast to previous studies, DIA proteomics overcomes dynamic range challenges by allowing detection of less abundant proteins, including those expressed by the ovarian follicle, which were previously obscured by highly abundant proteins [20],[21].

In the coho salmon with ovarian follicles at the LPN stage, three fish were marked as potential outliers and may represent fish that would not have successfully undergone the transition into secondary ovarian growth. In a differential protein abundance analysis comparing the three outliers to the remaining LPN ovarian follicles, over 500 proteins were significantly differentially abundant between “same stage” cohorts. Notably, most of these proteins were at a lower abundance in the outliers and included proteins important for ovarian maturation and embryonic development (e.g., zygote arrest protein, estradiol 17B-dehydrogenase, progestin membrane receptor component). These lower abundance proteins could serve as biomarkers of ovarian follicle developmental failure. Fish growth rate and size are important for determining egg mass and fecundity [9], and it may be that the outlier fish did not achieve the growth trajectory necessary to transition into secondary ovarian follicle growth. In coho salmon, growth at specific stages of development dictate total investment in the ovary [9]. In our study, the onset of secondary growth coincided with Campbell et al.’s [8] critical growth period 1. If coho salmon do not attain a specific growth rate during this period, they are at risk of failing to accumulate lipid droplets within the ooplasm of ovarian follicles and are unable to further develop or ovulate [8]. The hundreds of differentially abundant proteins between the larger and smaller coho salmon with ovaries at the LPN stage may provide insight into the mechanism linking growth, lipid deposition, and maturation and shed light on reproductive energetic effort and limitations across vertebrates, providing more detailed guidance to hatchery managers.

4.3 Previtellogenic secondary follicle development

After the large-scale changes in the coho salmon ovarian proteome between the LPN and ECA stages, there was relatively little change to the proteome as the fish transitioned into the LCA stage. A similar proteomic "quiescence" was observed in the later stages of striped bass ovarian development [56]. During the LCA stage, lipid deposition and the increase in ovarian follicle volume persisted, likely accompanied by sustained upregulation of similar molecular pathways. The proteins that were differentially abundant decreased in coho salmon with LCA-staged ovaries, suggesting a down-regulation of some of the processes necessary to fuel the primary to secondary growth transition. The annotations of these proteins suggest changes in RNA binding and gene expression regulation, which corroborates previous findings in fish of higher RNA binding protein abundance in earlier ovarian stages [20], corresponding to the up-regulation of RNA synthesis in early development [6]. Other proteins that decreased at LCA stage are more directly related to ovarian vitellogenesis and maturation. For example, estradiol-17B, which promotes secondary ovarian follicle growth in teleosts [13]. The decrease in protein abundance of estradiol 17B-dehydrogenase, an enzyme that converts bioactive estradiol-17B in non-bioactive estrone, between the ECA and LCA-stage ovarian follicles suggests that metabolic clearance of estradiol-17B was reduced with the onset of secondary growth, contributing to the increase in plasma estradiol-17B levels as secondary growth progresses.

The current study represents the first DIA proteome of teleost ovarian development and significantly expands our knowledge of the diversity of proteins expressed during the critical transition from primary ovarian growth into secondary growth. Our comprehensive analysis of differentially abundant proteins and co-varying protein groups leads to testable hypotheses regarding the interconnected network of proteins essential for the transition from primary to secondary ovarian follicle growth. Comparing findings with previous transcriptomics studies highlights that mRNA and proteins serve distinct roles in cellular physiology, emphasizing the importance of considering their specific functions when designing studies linking molecular biology with physiology. Our dataset will be fundamental in advancing our understanding of comparative reproductive endocrinology and physiology by incorporating a multi-stage, DIA proteome into the existing collection of datasets.

1. Associated Data

The Skyline document for the DIA data, with all its settings and files used to build it, can be found on Panorama Public [35] with the url <https://panoramaweb.org/yYtWfd.url>, or with the ProteomeXchange accession number PXD052361 and reviewer login ([panorama+reviewer261@proteinms.net](mailto:panorama+reviewer261@proteinms.net)) and password (AbVm0eL+H#&kvh).

The Skyline document for the targeted proteomics validation can be found on Panorama Public: <https://panoramaweb.org/zrQjtj.url>.

All R code and associated files can be found on the project-specific GitHub repository: <https://github.com/Nunn-Lab/Publication-Coho-DIA>

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# The authors declare no conflict of interest.

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# Figure Legends

Figure 1. Schematic overview of proteomics samples and data processing pipeline.

Figure 2. Coho salmon (Oncorhynchus kisutch) A) body weight (g) and B) ovarian follicle volume (mm3) across sampling timepoints representing ovarian follicle development during primary growth C) early perinucleolar stage (EPN; April),D) late perinucleolar stage (LPN; August) and early secondary growth E) early cortical alveolus stage (ECA; December),and F) late cortical alveolus stage (LCA; February)]. Coho salmon ovarian follicle micrographs representative of fish with ovaries at each stage of development in the current study (paraffin histology, hematoxylin and eosin staining).

Figure 3. NMDS plot of coho salmon (Oncorhynchus kisutch) ovarian follicle proteome profiles representing the transition from primary to secondary ovarian follicle growth: early perinucleolar stage (EPN; April), late perinucleolar stage (LPN; August), early cortical alveolus stage (ECA; December), and late cortical alveolus stage (LCA; February).

Figure 4. Coho salmon (Oncorhynchus kisutch) ovarian follicle proteome WGCNA results with GO enrichments. GO enrichments (biological process) are presented for the modules with high correlation values with month (blue, turquoise, brown) or fish weight (blue, magenta). Pairwise comparisons of protein abundances for each significant WGCNA module (blue, magenta, turquoise, and brown) are shown for protein abundances that deviate from the dashed black 1:1 line, indicating a coordinated change in abundance at a specific time point.

# Supporting Information

This article contains supplemental data.

Table S1: Fish length, weight, and ovarian follicle stage for each ovarian tissue fragment analyzed with mass spectrometry proteomics. The .raw MS file name is provided with the fish ID, the month of sampling, and the described metrics.

Table S2: Ovarian follicle volume and stage for fish from the same experiment sampled at the same time points as those analyzed with proteomics mass spectrometry.

Table S3: Additional sequences not in the published coho genome added to fasta.

Table S4: For the 5,773 proteins identified in this dataset: protein accession, median normalized abundance for each mass spectrometry replicate, the annotation taken from the coho salmon genome fasta, the UniProt accession resulting from BLASTp against UniProt trembl, and the percent sequence coverage for each protein.

Table S5: Each tab of the workbook contains a list of differentially abundant proteins for the pairwise comparisons of EPN vs. LPN, LPN vs. ECA, and ECA vs. LCA. The protein accession number is listed, along with the statistical output of Limma, the annotation pulled from the coho salmon fasta, and the BLASTp annotation against UniProt trembl.

Table S6: GO enrichment results (from compGO) of the Limma differentially abundant proteins. Each workbook tab contains the GO enrichment output for GO biological process (BP), cellular component (CC), or molecular function (MF) for the three pairwise comparisons: EPN vs. LPN, LPN vs. ECA, and ECA vs. LCA.

Figure S1. Total proteins identified at each ovarian follicle stage/time point and volcano plots of pairwise differentially abundant proteins for EPN vs. LPN, LPN vs. ECA, and ECA vs. LCA.

Figure S2. Nonmetric multidimensional scaling plot of all coho salmon analyzed, including the three fish that were smaller than their cohort and outliers in this plot for the LPN (August) group. Color intensity of the dots increases with the progression of time from April (EPN fish), to August (LPN), to December (ECA), and February (LCA).

Table S7: Differentially abundant proteins for comparison of smaller and larger LPN fish. BLASTp annotations are provided.

Table S8: WGCNA module membership for proteins in modules with significant correlations to variables.

Table S9: GO enrichment (compGO) of WGCNA modules with significant correlations (blue, brown, green, magenta and turquoise). Enrichment results are presented for the GO categories of biological process (BP), cellular component (CC), and molecular function (MF).

Table S10: For each protein inferred in this dataset, this file contains the following: all peptide sequence detected, percent sequence coverage, precursor charge, precursor *m/z,* sequence modifications, missed cleavages, and detection Q value score. This report was exported directly from the Skyline document, available on PanoramaWeb.

# Author Contributions

**ETS**: Conceptualization; Data curation; Investigation; Formal analysis; Visualization; Writing – original draft. **JT**: Investigation; Writing – review & editing. **CF**: Investigation; Writing – review & editing. **CM**: Investigation; Writing – review & editing. **JMG**: Conceptualization; Funding acquisition; Writing – review & editing. **BLN**: Supervision; Formal analysis; Resources. **GY**: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing – review & editing. **KF**: Conceptualization; Formal analysis; Funding acquisition; Project administration; Supervision; Writing – review & editing.