

Article

Reference Intervals for Blood Biomarkers in Farmed Atlantic Salmon, Coho Salmon and Rainbow Trout in Chile: Promoting a Preventive Approach in Aquamedicine

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Simple Summary: We report the integrated reference intervals (RIs) of 44 blood biomarkers for psmolts, smolts, postsmolts and adults of intensively farmed Atlantic salmon, coho salmon and rainbow trout species in Chile. Overall, RIs were obtained from 3.059 healthy salmon and trout from 78 different culture centers. Our results indicate that the variability of most blood biomarkers depends on the salmonid species, age range and/or interaction between them, but they are often biologically related to each other. Finally, we provide a standardized pre-analytical protocol to improve preventive vision in aquamedicine. RIs for blood biomarkers specific to salmonid species and age ranges are essential to help improve clinical, zootechnical and nutritional management for the health and welfare of farmed fish.



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Abstract: The mission of veterinary clinical pathology is to support the diagnostic process by using tests to measure different blood biomarkers to support decision making about farmed fish health and welfare. The objective of this study is to provide reference intervals (RIs) for 44 key hematological, blood biochemistry, blood gasometry and hormones biomarkers for the three most economically important farmed salmonid species in Chile (Atlantic salmon, coho salmon and rainbow trout) during the freshwater (presmolt and smolt age range) and seawater stages (post-smolt and adult age range). Our results confirmed that the concentration or activity of most blood biomarkers depend on the salmonid species, age range and/or the interaction between them, and they are often biologically related to each other. Erythrogram and leukogram profiles revealed a similar distribution in rainbow trout and coho salmon, but those in Atlantic salmon were significantly different. While the activity of the most clinically important plasma enzymes demonstrated a similar profile in Atlantic salmon and rainbow trout, coho salmon demonstrated a significantly different distribution. Plasma electrolyte and mineral profiles showed significant differences between salmonid species, especially for rainbow trout, while Atlantic salmon and coho salmon demonstrated a high degree of similarity. Furthermore, electrolytes, minerals and blood gasometry biomarkers were significantly different between age ranges, suggesting a considerably different distribution between freshwater and seawater-farmed fish. The RIs of clinically healthy fish described in this study take into account the high biological variation of farmed fish in Chile, as the 3.059 individuals came from 78 different fish farms, both freshwater and seawater, and blood samples were collected using the same pre-analytical protocol. Likewise, our study provides the Chilean salmon farming industry with standardized protocols that can be used routinely and provides valuable information to improve the preventive vision of aquamedicine through the application of blood biomarkers to support and optimize health, welfare and husbandry management in the salmon farming industry.

Keywords: Atlantic salmon; coho salmon; rainbow trout; blood biomarkers; reference intervals; fish health

1. Introduction

Chile is the world's second largest producer of salmonids due to the 978.328 metric tons harvested in 2021, which consisted of 74.9% Atlantic salmon (*Salmo salar*), 19.3% coho salmon (*Oncorhynchus kisutch*) and 5.8% rainbow trout (*Oncorhynchus mykiss*) [1]. Consequently, it is essential to generate strategies to maximize the health and welfare of these farmed salmonid species in order to optimize their productivity and sustainability. Clinical pathology is a specialty of veterinary medicine that supports the diagnosis of diseases through laboratory tests using fish blood and providing necessary tools for decision making in the field of health, welfare, nutrition and, indirectly, the husbandry and environment where animals are farmed [2–6]. For practical reasons, in this paper we will refer to the set of hematological parameters, biochemistry and blood gases and hormones as “blood biomarkers”.

Blood is distributed to all tissues and organs of the body by the vascular system, so that the presence or absence of tissue-specific components in the blood makes it possible to determine any alterations in the tissues. Total blood volume in various species of salmonid fish has been estimated at 4.5 ± 1.5 to 7.2 ± 0.1 mL/100 g body weight using the re-injected Evan's blue technique (4.5 to 7.2%), while total plasma volume in the same fish was measured at 3.0 ± 0.5 to 4.8 ± 1.3 mL/100 g [7]. Alterations in the number of leukocytes and their differential count (lymphocytes, neutrophils, eosinophils and monocytes) represent important clinical indicators as they point to possible infectious causes, acute and/or chronic stress, among others [6,8]. Fish neutrophils show myeloperoxidase activity in cytoplasmic granules, which makes them more similar to mammalian neutrophils than to heterophils of birds and reptiles [9]. Furthermore, basophils have only been identified in 4 out of 121 species of cartilaginous and teleost fish [10], and in 14 out of 20 species of freshwater fish [11].

Erythrocytes are the predominant blood cells in the vast majority of fish species and, unlike in mammals, fish erythrocytes and thrombocytes are nucleated cells [9]. This is the main biological explanation why clinical and research laboratories continue to use manual hematological methods. There are some reports on the use of automated methods in fish [12,13], but they have not been readily adopted. In addition, it is important to emphasize that the use of automated hematological methods must be preceded by technical validation using traditional manual analysis in each laboratory [6]. With the development of the machine learning, there are some attempts to computerize the hematological study and some guidelines for designing and evaluating them are provided [14]. Using machine learning, Gültepe and Gültepe [15] described hematological parameters of sea bream (*Sparus aurata*) and, Mani et al. [16] evaluated the use of probiotics on hematological parameters in common carp (*Cyprinus carpio*).

Blood biochemistry is based on the detection and quantification of elements such as enzymes, substrates, minerals, among others, in plasma or serum. The methods used in mammals have been adapted for fish analysis, but the results interpretation may be different as they are directly or indirectly influenced by different intrinsic factors such as fish species, productive stage, sex, nutritional, metabolic and reproductive status [3,16–31] and extrinsic factors such as environmental conditions, water type, stocking density, capture and sampling method, health status or disease [3,4,25,30–34].

Similarly, some parameters associated with ion-regulation, acid/base balance and hormones can be quantified in farmed salmonids, which represent important biomarkers or proxies to assess key pathophysiological processes such as smoltification, stress and reproduction [35,36]. These applications are especially relevant for salmon farmed in recirculating aquaculture systems (RAS) that use both freshwater and seawater. As a result of RAS production, CO₂ in solution is acidic and will have an impact on both the pH and alkalinity of the system [37]. Operational recommendations for CO₂ levels in RAS systems are typically below 15 mg/L; however, it has been demonstrated that salmonids can tolerate CO₂ levels of 20–25 mg/L in high alkalinity freshwater without adverse effects [38,39]. Chronically elevated CO₂ levels have often been associated with the development of

nephrocalcinosis in RAS farmed fish [40,41]; therefore, blood gases biomarkers can be correlated with CO₂ concentrations in the water where fish are cultured and ultimately with their welfare and productive performance.

Overall, all pre-analytical and analytical factors can affect the results of blood biomarkers in farmed fish, so not only is experience and care necessary to obtain reliable results, but it is critical to estimate reference intervals (RIs) representative of the distribution of each blood biomarker under normal or healthy conditions. There are some studies describing the RIs for several biochemical and hematological parameters in farmed salmonid species [29,30,42–46], but there is no published information on RIs for these biomarkers analyzed comprehensively and comparatively by salmonid species on a sufficiently representative sample size of fish for different age ranges and water types, and using the same pre-analytical, analytical and post-analytical procedure. Hence, the objective of this work is to provide RIs for 44 key hematological, blood biochemistry, blood gasometry and hormones biomarkers for the three most economically important farmed salmonid species during the freshwater (presmolt and smolt age range) and seawater stages (post-smolt and adult age range) in Chile, contributing to improve the interpretation and application of clinical laboratory test results in aquamedicine.

2. Material and Methods

2.1. Pre-Analytical Stage

2.1.1. Fish Selection and Catching

A total of 3059 healthy fish of the three salmonid species from 78 fish farms in Chile were collected during December 2014 and May 2015, and January and June 2017 to establish RIs for the most important blood biomarkers applied to assess fish health and welfare in salmon aquaculture (Table 1). Of the fish sampled, 50.7% (1550 fish) were Atlantic salmon, 26.0% (794 fish) were rainbow trout and 23.4% (715 fish) were coho salmon. The 14.1% (432 specimens) and 24.8% (759 specimens) of the sampled fish were presmolts (<50 g) and smolts (50 to 150 g) reared in freshwater, respectively (Table 1). Presmolt and smolt individuals were collected from 14 and 20 hatcheries, respectively, located in the Araucanía, Los Ríos, Los Lagos, Aysén and Magallanes regions. Likewise, the 31.1% (952 specimens) and 29.9% (916 specimens) of the sampled fish were postsmolts (150 to 800 g) and adult fish (>800 g) reared in marine cage farms, respectively (Table 1), located in Los Lagos, Aysén and Magallanes regions. Prior to selecting each fish group, the absence of clinical disease or asymptomatic carrier status of enzootic pathogens such as *Flavobacterium psychrophilum*, *Renibacterium salmoninarum*, *Piscirickettsia salmonis*, Infectious Salmon Anemia Virus (ISAV), Infectious Pancreatic Necrosis Virus (IPNV) and Piscine Orthoreovirus (PRV) was confirmed by RT-PCR. Furthermore, the health status of each farm was certified by the veterinarian in charge by means of health, welfare, husbandry management and environmental conditions. Farms with positive or diseased fish were immediately discarded. All freshwater-reared fish (presmolt and smolts) were captured using the same protocol, regardless of salmon producer and hatchery. Briefly, no more than five fish were collected at the same time directly from each selected tank using a small fishing net with handle and then quickly deposited into the bucket with anesthesia. In addition, all sea-reared fish (postsmolt and adults) were also captured using the same protocol between seawater farms, but fish were captured by the crowd and net method.

Table 1. Number of samples and farms to determinate RIs for blood biomarkers by salmonid species (Atlantic salmon, coho salmon, and rainbow trout) and age ranges (presmolt and smolt in freshwater and postsmolt and adult in seawater).

| Water Type | Age Range | Atlantic Salmon | | Coho Salmon | | Rainbow Trout | | Total of Fish | Total (%) | Total of Farms | Total (%) |
|------------|--------------------------|------------------------|-----------------|------------------------|-----------------|------------------------|-----------------|---------------|-----------|----------------|-----------|
| | | Number of Healthy Fish | Number of Farms | Number of Healthy Fish | Number of Farms | Number of Healthy Fish | Number of Farms | | | | |
| Freshwater | Presmolt (<50 g) | 230 | 9 | | | 202 | 5 | 432 | 14.1% | 14 | 17.9% |
| | Smolt (50 to 150 g) | 380 | 9 | 259 | 7 | 120 | 4 | 759 | 24.8% | 20 | 25.6% |
| Seawater | Postsmolt (150 to 800 g) | 560 | 12 | 150 | 5 | 242 | 6 | 952 | 31.1% | 23 | 29.5% |
| | Adult (>800 g) | 380 | 8 | 306 | 7 | 230 | 6 | 916 | 29.9% | 21 | 26.9% |
| Total (N) | | 1550 | 38 | 715 | 19 | 794 | 21 | 3059 | 100.0% | 78 | 100.0% |
| Total (%) | | 50.7% | 48.7% | 23.4% | 24.4% | 26.0% | 26.9% | 100.0% | | 100.0% | |

2.1.2. Anesthetic Procedure

Prior to anesthesia, a minimum of 12 h of fasting of the specimens were checked and the water conditions in the containers were controlled. Fish were exposed to a solution of 15 to 20 mL of 20% benzocaine per 100 L of water for 2 to 5 min depending on whether deep sedation (non-lethal sampling) or euthanasia (lethal sampling) was to be induced. For non-lethal sampling (>50 g), fish were immediately returned to a container with fresh water after sampling and the recovery process was monitored. Smaller animals (<50 g) were euthanized by benzocaine overdose according to animal welfare standards and, once blood samples were obtained, the fish were discarded according to the General Sanitary Program for Mortality Management of the National Fisheries and Aquaculture Service (Sernapesca).

2.1.3. Blood Sampling Procedure

Whole blood samples for gasometry were collected in a volume that varied from 1 to 3 mL from the caudal vein of each fish using 1 mL capacity heparinized syringe with Pulset™ technology and Crickett™ Needle Protection (SunMed, Grand Rapids, MI, USA). Each heparinized syringe was filled to the maximum because the incomplete filling could alter the results. Air was quickly removed from the syringes and the blood was thoroughly mixed by inversion before injecting the sample into the portable equipment. Whole blood samples for hematological and blood biochemistry tests were collected in a volume that varied from 1 to 3 mL from the caudal vein of each fish using a non-vacuum sealed blood collection tube containing lithium heparin (BD, Franklin Lakes, NJ, USA). The needle was disassembled from the syringe and the blood was carefully emptied using the inner wall of the respective tube. Each tube was filled to the mark indicated by the manufacturer because the volume of blood should be commensurate with the amount of anticoagulant. The tubes were shaken gently by inversion between 10 to 15 times until the correct homogenization with the anticoagulant was achieved.

2.1.4. Procedure for Transport, Preparation and Storage of Samples at the Laboratory

The tubes were labeled and placed in expanded polystyrene boxes with gelpack or ice and datalogger to transport the samples to the laboratory under a carefully maintained cold chain. Part of the whole blood volume was used for hematological analysis and the rest was centrifuged at $10,000 \times g$ for 3 min to separate the plasma, which was transferred to a labeled tube and placed on wet ice at 4 °C until analysis. All samples were analyzed 24–48 h after blood collection.

2.2. Analytical Stage

2.2.1. Hemotological and Blood Gasometry Biomarkers

The blood gas biomarkers analyzed were bicarbonate ion concentration (HCO_3), partial pressure of carbon dioxide (pCO_2) and hydrogen potential (pH). Heparinized whole blood samples were analyzed for blood gasometry using the IRMA TRUPOINT[®] System single-use Point-of-Care (POC) analyzer (Lifehealth, Roseville, MN, USA). The cartridges were removed from their packaging, their protective tape was removed, and they were fully inserted into the analyzer. Once the cartridge was inserted, the equipment was automatically calibrated and the whole blood sample was injected directly from the heparinized syringe. Once the analysis of each sample was completed, the cartridge and syringe were removed and discarded in a biological material container. The results were automatically displayed on the touch screen of the equipment when the analysis was finished and the voucher with the printed results was obtained.

The blood count biomarkers analyzed were hematocrit (Htc), red blood cell count (RBC), hemoglobin (Hgb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), lymphocytes (LYM), neutrophils (NEU), monocytes (MON) and thrombocyte count (TCC). Hemoglobin concentration was estimated by the cyanomethemoglobin method using the HumaMeterTM Hb Plus (HUMAN, Wiesbaden, Germany), while Hct was determined by centrifuging the microcapillary-loaded blood at $10,000 \times g$ for 10 min at room temperature in a FrontierTM 5515 microcentrifuge (Ohaus, Parsippany, NJ, USA). A Neubauer hemocytometer was used to determine the total RBC, total WBC, and TCC in blood mixed with Natt–Herrick staining solution. Calculation of RBC was performed using the number of counted cells, number of squares in which they were counted, square volume and blood dilution ($\text{RBC (mm}^3) = \text{cells counted} \times 5 \times 10 \times \text{dilution factor}$). Similarly, the total WBC count per mm^3 was determined using the number of counted cells, blood dilution, area in which they were counted and depth. The differential leukocyte count was calculated from the analysis of Giemsa-stained blood smears in which the number of various types of leukocytes per 100 cells was counted in several fields of a smear [11]. Finally, MCV and MCHC were calculated accordingly [47].

2.2.2. Blood Biochemistry and Hormones Biomarkers

Plasma samples were analyzed for plasma substrates [Total protein (TPO), Albumins (ALB), Globulins (GLO), Total bilirubin (TBI), Direct bilirubin (DBI), Creatinine (CRE), Glucose (GLU), Lactate (LAC), Urea (URE), Uric acid (UAC), Ammonia (NH_3), Total Cholesterol (TCH), Triglycerides (TRG), High-density lipoprotein cholesterol (HDL), and Low-density lipoprotein cholesterol (LDL)], enzymes [Alkaline Phosphatase (ALP), Alanine transaminase (ALT), Aspartate aminotransferase (AST), Total amylase (TAM), Lipase (LIP), Creatine Kinase total (CKT), Cardiac Creatine Kinase isoenzyme (CK-MB), and Lactate dehydrogenase (LDH)], electrolytes and minerals [Sodium (Na), Potassium (K), Chloride (Cl), Calcium (Ca), Magnesium (Mg), Iron (Fe) and Phosphorus (P)] using a cobas c311 autoanalyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland), while plasma cortisol (COR) concentration was determined using a cobas e411 automatic endocrinology analyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland). A standard kit developed by the manufacturer (Roche Diagnostics, Mannheim, Germany) was used in each assay.

2.3. Post-Analytical Stage

2.3.1. Differences between Salmon Species and Age Ranges

Significant differences were evaluated for the 44 biomarkers between age range ($n_i = 4$) and salmonid species ($n_j = 3$). For this, linear models were constructed for each blood biomarker to evaluate the salmonid species and age ranges interaction. To evaluate the assumption of normality of the residuals, we used Q-Q plots and the Shapiro–Wilks test ($p > 0.05$), and for homoscedasticity we evaluated the trend of the standardized Pearson residuals and the predicted values and Leven Test ($p > 0.05$). In case the assumptions of normality and homoscedasticity of the residuals were not met, a normality adjustment with

Box-Cox, Power Box-Cox, log with offset ($\log[Y + z]$), square root (\sqrt{Y}) transformations were performed. Tukey's multiple comparisons ($\alpha = 0.05$) were performed between the salmonid species and age range interaction. In case a normal distribution could not be fitted, a Kruskal–Wallis test was performed between salmonid species and age ranges using Dunn's test (Bonferroni) multiple comparison. The package "multcompView", "Car" and "MASS" implemented in the R program (v2021.09.2) (Core Team, 2019, Vienna, Austria) were used.

2.3.2. Reference Intervals (RIs) and Confidence Intervals (CIs)

The RIs and CIs for the salmonid species and age ranges interaction for all 44 blood biomarkers were estimated accordingly, as previously described by [48]. The distribution of each blood biomarker was determined by the Shapiro–Wilks test ($p > 0.05$) and the representation of the observations by Boxplot and histograms with the density distribution. Horn and Dixon method was used to determine and eliminate outliers from parametric and nonparametric distributions, respectively [49]. The package "referenceIntervals" was used to calculate the confidence intervals of the RIs according to the type of normal or non-normal distribution and bootstrap methods for small samples ($n < 120$) [49]. All statistical analyses were performed with the "referenceIntervals" package in the R program (R Development Core Team).

2.3.3. Multivariate Analysis

To simplify the multivariate interactions between blood biomarkers and salmonid species and age ranges, principal component analysis (PCA) was performed grouped by blood biomarker types (erythrogram, leukogram, plasma substrates, plasma enzymes, plasma electrolytes, minerals and gases). Furthermore, the multivariate similarity of blood biomarkers between salmonid species and age ranges was evaluated by analysis of similarities (ANOSIM) using a Bray Curtis matrix and the groups were plotted with a non-metric multidimensional scaling (nMDS) ordination. The Vegan package implemented in R was used.

3. Results

3.1. Erythrogram

Some erythrogram biomarkers exhibited a normal distribution (e.g., Htc) or were transformed to a normal distribution (e.g., RBC, Hgb, MCV, MCHC) over the age range and/or salmonid species (Table 2, Figure S1). While MCHC demonstrated an association with age range independently of salmonid species ($r < 0.20$; $p < 0.05$) (Figure S2), and Htc and RBC had an association with species regardless of age range ($r < 0.20$; $p < 0.05$) (Figure S3), Hgb and MCV showed association with the interaction between salmonid species and age range. A cluster between RBC and Hgb was observed (Figure 1). The 56.3% of the total variability of the erythrogram profile was captured by two-dimensional analysis both between salmonid species and age range (Figure 1), mainly driven by Htc, Hgb and RBC. Furthermore, the erythrogram profile showed significant differences between species ($R_{ANOSIM} = 0.3360$; $p = 0.0110$), mainly contributed by Atlantic salmon, since rainbow trout and coho salmon demonstrated a high degree of similarity (Figure 1). On the other hand, erythrogram biomarkers demonstrated a high similarity between age ranges ($R_{ANOSIM} = 0.1611$; $p = 0.0001$), but although a homogeneous distribution was observed between smolts and post-smolt, a greater distance was detected between presmolt and adult (Figure 1). Taken together, these results reveal that the outcome of most erythrogram parameters depends on the salmonid species, age range and/or the interaction between them, and they are often biologically related to each other.

Table 2. Reference intervals (RIs) for erythrogram biomarkers in presmolt and smolt (freshwater) and postsmolt and adult (seawater) of Atlantic salmon, coho salmon, and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|----------------------|--------------|----------------------|-----------------|-----------|-----------|-----------|---------|---------|--------------------------|------------------------|---------------------------|--------------|-----------------|
| Hematocrit | Htc | % | Coho salmon | Smolt | 53.00 | 53.20 | 7.46 | 0.97 | a | 38.31–68.49 | 35.58–71.19 | ANOVA, Tukey | Normal |
| | | | | Postsmolt | 51.00 | 51.17 | 9.94 | 0.95 | a | 31.14–70.79 | 28.65–73.38 | ANOVA, Tukey | Normal |
| | | | | Adult | 55.00 | 53.42 | 10.21 | 1.83 | a | 32.43–75.49 | 26.16–79.85 | ANOVA, Tukey | Normal |
| | | | Atlantic salmon | Presmolt | 37.50 | 40.46 | 7.02 | 1.43 | b | 22.41–54.60 | 17.27–60.64 | ANOVA, Tukey | Normal |
| | | | | Smolt | 39.00 | 41.23 | 7.43 | 1.46 | b | 23.97–56.60 | 19.80–62.83 | ANOVA, Tukey | Normal |
| | | | | Postsmolt | 47.00 | 46.59 | 6.99 | 0.91 | a | 32.62–60.94 | 29.72–63.28 | ANOVA, Tukey | Normal |
| | | | Rainbow trout | Adult | 40.00 | 41.24 | 5.25 | 1.27 | b | 29.15–52.80 | 25.27–57.75 | ANOVA, Tukey | Normal |
| | | | | Presmolt | 46.00 | 46.83 | 4.39 | 0.80 | a | 36.76–55.31 | 34.05–58.02 | ANOVA, Tukey | Normal |
| | | | | Smolt | 47.00 | 47.42 | 6.90 | 0.73 | a | 33.04–60.67 | 30.57–63.30 | ANOVA, Tukey | Normal |
| Red Blood Cell Count | RBC | $10^7 / \mu\text{L}$ | Coho salmon | Postsmolt | 49.00 | 48.72 | 7.27 | 1.29 | a | 34.25–64.35 | 29.65–69.06 | ANOVA, Tukey | Normal |
| | | | | Smolt | 9832.40 | 9389.25 | 3388.90 | 441.19 | c | 2225.89–16,017.98 | 962.34–17,318.70 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Postsmolt | 10,762.60 | 11,354.00 | 4526.60 | 414.95 | b | 1641.65–19,781.52 | 1012.74–21,512.40 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | Atlantic salmon | Adult | 15,384.60 | 16,389.70 | 6885.50 | 1236.70 | a | 4995.0–32,447.52 | 4995.0–32,447.52 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Presmolt | 3629.70 | 4479.40 | 1663.50 | 339.56 | a | 2419.8–8436.0 | 2419.80–8436.0 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Smolt | 6371.40 | 6840.67 | 3620.80 | 710.11 | a | 2131.2–13,066.92 | 131.20–13,066.92 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | Rainbow trout | Postsmolt | 5423.50 | 6344.49 | 3309.90 | 434.61 | a | 1968.25–16,083.4 | 1740.48–16,643.34 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Adult | 1953.60 | 2181.08 | 1543.90 | 374.44 | b | 319.68–6233.76 | 319.68–6233.76 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Presmolt | 3747.40 | 4620.12 | 2412.60 | 440.48 | a | 1678.32–10,966.80 | 1678.32–10,966.8 | ANOVA, Tukey | $\log(x + 1)$ |
| Hemoglobin | Hgb | g/L | Coho salmon | Smolt | 4955.00 | 5078.64 | 1900.10 | 201.41 | ab | 1071.69–8683.46 | 508.05–9387.03 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Postsmolt | 3636.40 | 3768.45 | 1483.80 | 262.30 | b | 952.38–7965.36 | 952.38–7965.36 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Adult | 509.30 | 525.51 | 110.50 | 14.39 | b | 299.15–749.86 | 254.73–801.51 | ANOVA, Tukey | Normal |
| | | | Atlantic salmon | Postsmolt | 607.50 | 599.16 | 107.07 | 10.26 | a | 391.02–819.18 | 358.59–843.14 | ANOVA, Tukey | Normal |
| | | | | Adult | 640.80 | 628.51 | 87.76 | 15.76 | a | 454.66–823.04 | 394.08–861.15 | ANOVA, Tukey | Normal |
| | | | | Presmolt | 430.90 | 477.24 | 132.27 | 27.00 | bc | 144.71–738.62 | 56.0–844.63 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | Rainbow trout | Smolt | 383.60 | 409.10 | 111.94 | 21.95 | c | 154.32–636.29 | 84.93–723.91 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Postsmolt | 545.50 | 566.49 | 110.73 | 14.42 | a | 326.56–780.92 | 268.60–844.32 | ANOVA, Tukey | $\log(x + 1)$ |
| | | | | Adult | 555.60 | 545.63 | 62.70 | 15.21 | ab | 407.50–687.30 | 355.78–728.24 | ANOVA, Tukey | $\log(x + 1)$ |

Table 2. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | |
|-------------------------|--------------|-----------------|---|-----------|--------|-------------|-----------|--------|--------------------------|------------------------|---------------------------|--------------|-----------------|--------------|--------------|--------|
| Mean Corpuscular Volume | MCV | fL | Coho salmon | Smolt | 110.9 | 119.74 | 33.12 | 4.31 | b | 40.12–177.76 | 22.08–196.44 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 145.70 | 146.24 | 33.77 | 3.23 | a | 77.89–212.55 | 69.63–222.13 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Adult | 133.30 | 131.62 | 22.40 | 4.02 | ab | 84.83–178.64 | 73.21–189.43 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Atlantic salmon | Presmolt | 165.90 | 169.84 | 24.46 | 4.99 | a | 115.81–220.94 | 102.93–240.33 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Smolt | 158.60 | 156.26 | 22.58 | 4.43 | ab | 110.72–205.97 | 96.41–220.43 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 138.10 | 145.97 | 24.91 | 3.24 | b | 88.55–194.02 | 78.10–207.21 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Rainbow trout | Adult | 155.60 | 159.71 | 18.37 | 4.45 | ab | 116.36–198.56 | 102.10–216.07 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Presmolt | 118.10 | 119.58 | 15.27 | 2.79 | c | 86.37–149.92 | 75.23–160.49 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Smolt | 137.20 | 140.97 | 26.41 | 2.78 | b | 86.01–192.72 | 79.36–202.20 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | | | Postsmolt | 158.50 | 158.52 | 16.68 | 2.95 | a | 122.4–191.51 | 112.81–201.41 | ANOVA, Tukey | log(x + 1) | |
| | | | Mean Corpuscular Hemoglobin Concentration | MCHC | g/L | Coho salmon | Smolt | 1.00 | 1.02 | 0.17 | 0.02 | a | 0.69–1.36 | 0.62–1.42 | ANOVA, Tukey | Normal |
| | | | | | | | Postsmolt | 0.90 | 0.87 | 0.14 | 0.01 | b | 0.59–1.13 | 0.55–1.18 | ANOVA, Tukey | Normal |
| Adult | 0.90 | 0.86 | | | | | 0.16 | 0.03 | b | 0.53–1.19 | 0.45–1.28 | ANOVA, Tukey | Normal | | | |
| Atlantic salmon | Presmolt | 0.90 | | | | 0.89 | 0.22 | 0.05 | b | 0.42–1.36 | 0.28–1.50 | ANOVA, Tukey | Normal | | | |
| | Smolt | 1.00 | | | | 1.04 | 0.18 | 0.03 | a | 0.68–1.42 | 0.56–1.49 | ANOVA, Tukey | Normal | | | |
| | Postsmolt | 0.80 | | | | 0.84 | 0.15 | 0.02 | b | 0.52–1.14 | 0.46–1.21 | ANOVA, Tukey | Normal | | | |
| Rainbow trout | Adult | 0.80 | | | | 0.76 | 0.10 | 0.03 | b | 0.52–0.98 | 0.44–1.08 | ANOVA, Tukey | Normal | | | |
| | Presmolt | 0.90 | | | | 0.99 | 0.19 | 0.04 | a | 0.68–1.15 | 0.60–1.23 | ANOVA, Tukey | log(x + 1) | | | |
| | Smolt | 0.90 | | | | 0.89 | 0.15 | 0.02 | b | 0.65–1.13 | 0.61–1.17 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | | | Postsmolt | 0.80 | 0.81 | 0.16 | 0.03 | b | 0.62–1.06 | 0.55–1.13 | ANOVA, Tukey | log(x + 1) | |

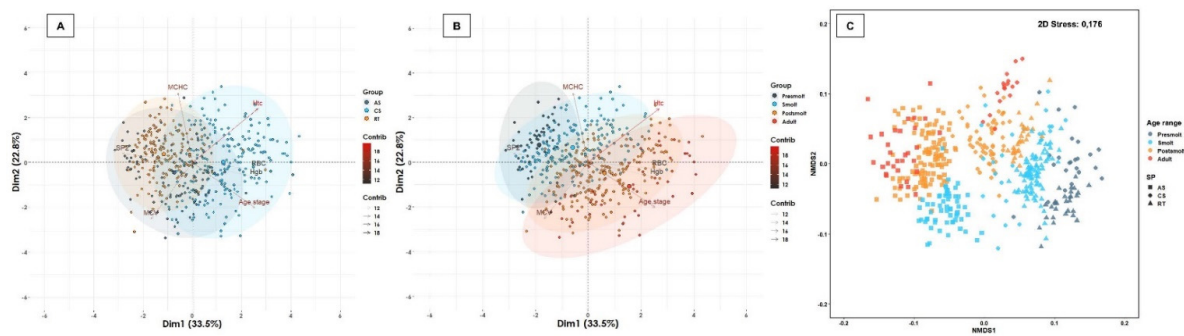


Figure 1. Multivariate analysis of erythrogram biomarkers. Salmonid species and range contribute significantly to the total variation of the erythrogram profile. (A) Spatial sorting of the erythrogram biomarkers according to salmonid species and (B) age ranges. The two-dimensional analysis captures 56.3% of the total variance of the erythrogram biomarker profile. Htc, Hgb and RBC contribute significantly to the total variance of the erythrogram in dimension 1, while the MCV, MCHC and Htc are biomarkers that contribute the most to dimension 2. A cluster between Hgb and RBC, MCHC and MCV, and Htc was observed. (C) The multivariate analysis of interdependence of the erythrogram profile demonstrates significant differences between salmonid species ($R_{ANOSIM} = 0.3360$; $p = 0.0110$), suggesting a uniform distribution between rainbow trout and Atlantic salmon, but another distribution for coho salmon. Similarly, significant differences were observed between age ranges ($R_{ANOSIM} = 0.1611$; $p = 0.0001$), suggesting differences in erythrogram biomarkers between freshwater and seawater productive stages. R_{ANOSIM} close to 0 suggests a uniform distribution of high and low ranges within and between groups, and an R_{ANOSIM} close to 1 suggests dissimilarity between groups.

3.2. Leukogram

Several leukogram biomarkers exhibited a normal distribution or were transformed to a normal distribution (e.g., WBC, LYM, NEU) (Table 3, Figure S4). In addition, the MON count showed a normal distribution in coho salmon and Atlantic salmon, but not in rainbow trout, whereas the TC count demonstrated a normal distribution in Atlantic salmon and rainbow trout, but not in coho salmon (Table 3, Figure S4). The RI for the eosinophils count could not be calculated due to the lack of variability in the data. In the post-smolt and adult age range (seawater), higher levels of NEU were observed in coho salmon as well as lower counts of LYM and WBC were observed in presmolt and smolt (freshwater) of coho salmon and rainbow trout, respectively (Table 3). The WBC count had a positive association with the MON count in all salmonid species (Figure S5), but the LYM count demonstrated a positive association with the NEU count only in Atlantic salmon and with the MON count in both Atlantic and coho salmon (Figure S5). The LYM count presented a significant negative association with the NEU and MON count in rainbow trout ($r < 0.20$; $p < 0.05$) (Figure S5), but only demonstrated a significant positive association in post-smolt with the WBC, MON and NEU (Figure S6). Interestingly, each of the leukogram biomarkers demonstrated a significant positive association with each other only in the post-smolt stage (Figure S6). The 62.1% of the total variability of the leukogram profile was captured by two-dimensional analysis, both considering salmonid species and age range, mainly driven by WBC and LYM counts (Figure 2). A cluster between MON and WBC was found (Figure 2). Leukogram biomarkers were significantly different between salmonid species (Figure 2), as while we found a similar distribution between rainbow trout and coho salmon, an unrelated distribution in Atlantic salmon was detected ($R_{ANOSIM} = 0.3703$; $p = 0.0001$) (Figure 2). On the other hand, leukogram biomarkers profile demonstrated a high similarity between age ranges ($R_{ANOSIM} = 0.3138$; $p = 0.0001$), but although a homogeneous distribution was observed between smolts and post-smolt, a greater distance was detected between presmolt and adult (Figure 2). Taken together, our results denote that the level of most leukogram biomarkers depends on the salmonid species, age range and/or the interaction between them, and they are often biologically related to each other.

Table 3. Reference intervals (RIs) for leukogram biomarkers in presmolt and smolt (freshwater) and postsmolt and adult (seawater) of Atlantic salmon, coho salmon and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | |
|------------------------|--------------|-----------------|-----------------|-------------|-----------|-----------|-------------------|-------------------|--------------------------|------------------------|---------------------------|-------------------|-----------------|---------------|
| White Blood Cell Count | WBC | N° / μ L | Coho salmon | Smolt | 13,906.08 | 14,026.11 | 5075.93 | 660.83 | ab | 3662.20–24,199.37 | 2007.55–25,976.80 | ANOVA, Tukey | Normal | |
| | | | | Postsmolt | 14,385.60 | 14,517.42 | 4396.45 | 403.02 | ab | 5820.81–23,316.12 | 4626.99–24,548.04 | ANOVA, Tukey | Normal | |
| | | | | Adult | 12,387.60 | 11,976.18 | 3658.25 | 657.04 | b | 4325.58–19,625.58 | 2457.66–21,488.70 | ANOVA, Tukey | Normal | |
| | | | Atlantic salmon | Presmolt | 15,631.02 | 14,927.09 | 3543.82 | 723.38 | a | 7426.32–22,727.96 | 5492.03–24,359.88 | ANOVA, Tukey | log(x + 1) | |
| | | | | Smolt | 8531.46 | 9510.48 | 3604.02 | 706.81 | b | 4528.80–16,472.40 | 4528.80–16,472.40 | ANOVA, Tukey | log(x + 1) | |
| | | | | Postsmolt | 12,170.04 | 12,797.27 | 3794.08 | 498.19 | a | 4816.03–20,387.69 | 3378.23–21,844.69 | ANOVA, Tukey | log(x + 1) | |
| | | | Rainbow trout | Adult | 7192.80 | 8670.80 | 4206.19 | 1020.15 | b | 3247.86–18,115.20 | 3247.86–18,115.20 | ANOVA, Tukey | log(x + 1) | |
| | | | | Presmolt | 15,717.60 | 15,130.48 | 6015.99 | 1098.36 | a | 6493.50–29,989.98 | 6493.50–29,989.98 | ANOVA, Tukey | Normal | |
| | | | | Smolt | 15,984.0 | 15,834.26 | 4328.83 | 458.85 | a | 7172.65–24,508.47 | 5998.39–25,667.40 | ANOVA, Tukey | Normal | |
| | | | | Postsmolt | 14,635.35 | 15,087.67 | 4069.13 | 719.33 | a | 10,657.80–18,645.87 | 9740.24–19,738.03 | ANOVA, Tukey | Normal | |
| | | | | Coho salmon | Smolt | 10,842.48 | 11,034.92 | 4160.62 | 541.67 | a | 2417.39–19,259.23 | 1149.24–20,820.34 | ANOVA, Tukey | Normal |
| | | | | | Postsmolt | 11,487.17 | 11,272.84 | 3470.47 | 318.14 | a | 4472.30–18,300.84 | 3498.96–19,228.08 | ANOVA, Tukey | Normal |
| Adult | 2511.93 | 2543.75 | 1176.67 | | 211.34 | b | 426.20–5388.60 | 426.20–5388.60 | ANOVA, Tukey | Normal | | | | |
| Lymphocytes | LYM | N° / μ L | Atlantic salmon | Presmolt | 11,979.79 | 12,302.29 | 2736.12 | 583.34 | a | 6543.81–18,283.54 | 4835.86–19,814.92 | ANOVA, Tukey | Normal | |
| | | | | Smolt | 6852.27 | 7427.00 | 2756.76 | 540.64 | c | 3849.50–13,013.20 | 3849.50–13,013.20 | ANOVA, Tukey | Normal | |
| | | | | Postsmolt | 9131.13 | 9394.85 | 3050.20 | 404.01 | b | 3076.28–15,484.43 | 2041.53–16,610.05 | ANOVA, Tukey | Normal | |
| | | | Rainbow trout | Adult | 5665.26 | 6035.71 | 2542.45 | 616.63 | c | 2435.90–12,774.40 | 2435.90–12,774.40 | ANOVA, Tukey | Normal | |
| | | | | Presmolt | 13,661.82 | 12,758.34 | 4590.63 | 838.13 | a | 9704.06–17,824.74 | 8145.22–19,054.68 | ANOVA, Tukey | BOX COX | |
| | | | | Smolt | 12,221.97 | 12,004.36 | 1903.40 | 200.64 | b | 9113.07–15,677.21 | 8434.18–16,138.72 | ANOVA, Tukey | BOX COX | |
| | | | | Postsmolt | 11,489.03 | 11,889.42 | 3299.63 | 623.57 | b | 8021.64–15,157.85 | 7146.13–16,124.68 | ANOVA, Tukey | BOX COX | |
| | | | | Coho salmon | Smolt | 2850.48 | 2924.59 | 1180.06 | 153.63 | b | 512.42–5301.31 | 181.04–5723.28 | ANOVA, Tukey | POWER BOX COX |
| | | | | | Postsmolt | 2815.28 | 3023.69 | 1217.21 | 112.05 | b | 442.96–5350.66 | 32.73–5766.80 | ANOVA, Tukey | POWER BOX COX |
| Adult | 9292.92 | 9214.03 | 3073.02 | | 551.93 | a | 2618.39–15,383.08 | 1111.62–17,089.10 | ANOVA, Tukey | POWER BOX COX | | | | |
| Neutrophils | NEU | N° / μ L | Atlantic salmon | Presmolt | 3201.53 | 3039.47 | 930.72 | 198.43 | a | 1071.70–5108.78 | 554.10–5569.84 | ANOVA, Tukey | BOX COX | |
| | | | | Smolt | 1657.21 | 1844.93 | 699.71 | 137.22 | b | 679.32–3459.20 | 679.32–3459.20 | ANOVA, Tukey | BOX COX | |
| | | | Rainbow trout | Postsmolt | 3020.98 | 3366.32 | 1100.68 | 145.79 | a | 1612.89–6397.74 | 1551.51–6764.83 | ANOVA, Tukey | BOX COX | |
| | | | | Adult | 1948.05 | 2068.82 | 1057.98 | 256.60 | b | 772.03–4967.83 | 772.03–4967.83 | ANOVA, Tukey | BOX COX | |

Table 3. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | |
|-----------------|--------------|-----------------|-------------------|-----------|---------|-------------|-----------|---------|--------------------------|------------------------|---------------------------|----------------------|-----------------|------------------|----------------------|---------|
| Monocytes | MON | N°/μL | Rainbow trout | Presmolt | 1627.37 | 1781.97 | 1047.13 | 191.18 | b | 417.72–4523.81 | 417.72–4523.81 | ANOVA, Tukey | BOX COX | | | |
| | | | | Smolt | 1914.28 | 2215.56 | 1438.91 | 154.27 | b | 323.96–5860.65 | 294.51–6184.61 | ANOVA, Tukey | BOX COX | | | |
| | | | | Postsmolt | 2826.50 | 3028.72 | 1364.58 | 257.88 | a | 956.04–7970.69 | 956.04–7970.69 | ANOVA, Tukey | BOX COX | | | |
| | | | Coho salmon | Smolt | 174.58 | 280.68 | 247.41 | 66.12 | a | 59.19–978.22 | 59.19–978.22 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 251.75 | 304.65 | 187.17 | 23.96 | a | 84.95–834.96 | 78.59–991.01 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Adult | 245.75 | 307.76 | 204.13 | 43.52 | a | 83.12–796.54 | 83.12–796.54 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Atlantic salmon | Presmolt | 350.76 | 351.87 | 148.66 | 49.55 | a | 163.61–600.07 | 163.61–600.07 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Smolt | 144.52 | 182.02 | 123.89 | 34.36 | b | 51.77–445.24 | 51.77–445.24 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 256.41 | 258.11 | 110.56 | 24.13 | ab | 86.58–496.30 | 86.58–496.30 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Rainbow trout | Smolt | 84.18 | 162.21 | 173.82 | 86.91 | b | 58.50–421.98 | 58.50–421.98 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 139.24 | 188.44 | 145.54 | 48.51 | b | 65.36–447.55 | 65.36–447.55 | Kruskal-Wallis, Dunn | | | | |
| | | | | Adult | 294.51 | 446.15 | 387.88 | 47.39 | a | 147.25–1472.53 | 250.05–1472.53 | Kruskal-Wallis, Dunn | | | | |
| | | | Thrombocyte count | TCC | N°/μL | Coho salmon | Presmolt | 208.86 | 269.41 | 190.49 | 54.99 | ab | 121.57–703.30 | 121.57–703.30 | Kruskal-Wallis, Dunn | |
| | | | | | | | Smolt | 5688.88 | 5550.22 | 1898.31 | 247.14 | a | 1632.27–9319.54 | 930.44–10,048.76 | ANOVA, Tukey | BOX COX |
| | | | | | | | Postsmolt | 5967.36 | 6003.87 | 1623.96 | 148.87 | a | 2705.02–9166.50 | 2275.34–9644.43 | ANOVA, Tukey | BOX COX |
| Atlantic salmon | Adult | 6433.56 | | | | 6252.88 | 1364.83 | 245.13 | a | 3479.77–9212.12 | 2739.57–9871.98 | ANOVA, Tukey | BOX COX | | | |
| | Presmolt | 3184.88 | | | | 3468.78 | 1386.72 | 283.06 | a | 1606.39–6600.73 | 1606.39–6600.73 | ANOVA, Tukey | Normal | | | |
| | Smolt | 3491.17 | | | | 3854.07 | 1852.63 | 363.33 | a | 1594.14–7948.71 | 1594.14–7948.71 | ANOVA, Tukey | Normal | | | |
| Rainbow trout | Postsmolt | 3562.63 | | | | 4173.17 | 1757.30 | 234.83 | a | 1763.70–8826.51 | 1759.04–9224.77 | ANOVA, Tukey | Normal | | | |
| | Adult | 1566.52 | | | | 1660.49 | 963.82 | 233.76 | b | 357.26–3985.34 | 357.26–3985.34 | ANOVA, Tukey | Normal | | | |
| | Postsmolt | 3449.88 | | | | 3533.37 | 1629.58 | 302.61 | a | 1438.56–7384.61 | 1438.56–7384.61 | ANOVA, Tukey | log(x + 1) | | | |
| Rainbow trout | Adult | 3866.66 | | | | 3779.87 | 1198.54 | 127.05 | ab | 1308.01–6113.98 | 961.13–6498.79 | ANOVA, Tukey | log(x + 1) | | | |
| | Presmolt | 2781.35 | | | | 2935.75 | 971.37 | 177.35 | b | 797.91–4886.41 | 213.08–5488.10 | ANOVA, Tukey | log(x + 1) | | | |

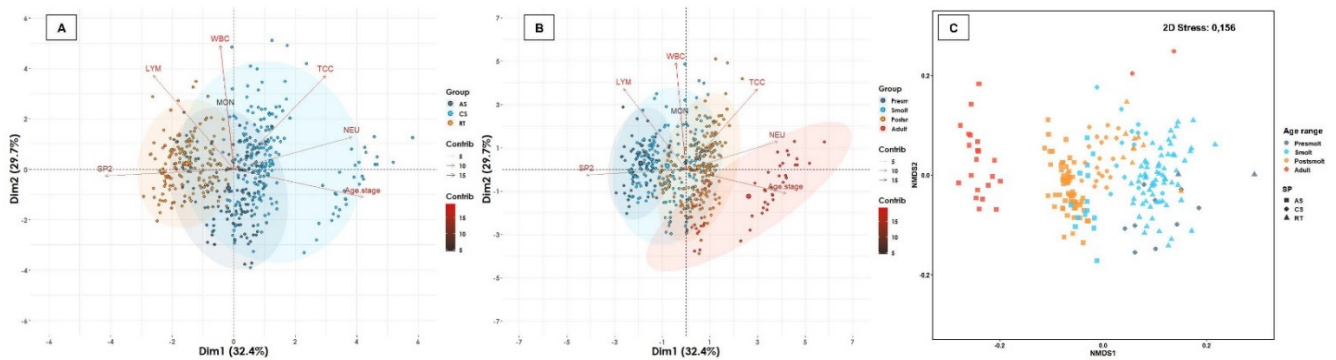


Figure 2. Multivariate analysis of leukogram biomarkers. Salmonid species and range contribute significantly to the total variation of the leukogram profile. (A) Spatial sorting of the leukogram biomarkers according to salmonid species and (B) age ranges. The two-dimensional analysis captures 62.1% of the total variance of the leukogram biomarker profile. NEU count contributes significantly to the total variance of the leukogram in dimension 1, while the WBC, TCC and LYM are biomarkers that contribute the most to dimension 2. A cluster between Hgb and RBC, MCHC and MCV, and Htc was observed. (C) The multivariate analysis of interdependence of the leukogram profile demonstrates significant differences between salmonid species ($R_{ANOSIM} = 0.3703$; $p = 0.0001$), suggesting a uniform distribution between rainbow trout and Atlantic salmon, but another distribution for coho salmon. Similarly, significant differences were observed between age ranges ($R_{ANOSIM} = 0.3138$; $p = 0.0001$), suggesting differences in leukogram biomarkers between freshwater and seawater-reared fish.

3.3. Plasma Substrates

Plasma substrates such as TPO, ALB, DBI, URE and LDL demonstrated a normal distribution or were transformed to a normal distribution (Table 4, Figure S7), while GLO, TBI, CRE, GLU, LAC, UAC, NH₃, TCH, TRG and HDL did not demonstrate a normal distribution (Table 4, Figure S7). Higher levels of TPO, ALB, GLU, HDL were observed in the postsmolt and adult age range of Atlantic salmon, while in presmolt and smolt higher levels of HCT were found in Atlantic salmon and lower levels of CRE in rainbow trout. (Table 4, Figure S7). While LDL had a positive association with species independent of age range, DBI demonstrated no association with the interaction between species and age range. The plasma concentration of TPO demonstrated a significant positive association with the concentration of ALB and GLO in all three species and age ranges, as did ALB quantities with those of GLO and LDL ($r < 0.20$; $p < 0.05$) (Figures S8 and S9). In coho salmon, a significant positive association was observed between TCH with TPO, LDL, ALB and GLO, as well as HDL with TRG (Figures S8 and S9). In rainbow trout, TCH had a significant positive association with ALB, GLO, LDL, UAC and CRE; while CRE demonstrated a positive association with TRG, LDL, HDL and UAC (Figures S8 and S9). In Atlantic salmon, a positive association of TCH with TPO, GLO and URE was observed, but a negative association with HDL (Figures S8 and S9). LAC demonstrated a significant negative association with TPO, ALB, GLO, CRE and GLU in all age ranges of the three salmonid species, as well as NH₃ with TPO, ALB, GLO and CRE, and HDL with TCH ($r > -0.20$; $p < 0.05$) (Figures S8 and S9). The 32.5% of the total variability of the substrates profile was captured by two-dimensional analysis, mainly driven by the TCH, HDL, TBI, TRG, URE and NH₃ (Figure 3). Clusters between TPO, GLO and ALB; between TCH, LDL, LAC and TRG; and between NH₃ and URE were distinguished (Figure 3). The substrate biomarker profile demonstrated significant differences between species ($R_{ANOSIM} = 0.1580$, $p = 0.011$) and age range ($R_{ANOSIM} = 0.0910$; $p = 0.0001$), although the distribution of observations was homogeneous among the different growth stages in the three salmonid species (Figure 3). These results indicate that the concentration of plasma substrates depends on salmonid species, age range and/or the interaction between them, but the variability of the distribution of the same biomarkers is more significantly associated with age range than with salmonid species.

Table 4. Reference intervals (RIs) for plasma substrates biomarkers in presmolt, smolt, postsmolt and adult of Atlantic salmon, coho salmon and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | |
|-----------------|--------------|-----------------|-----------------|-----------|--------|---------------|-----------|-------|--------------------------|------------------------|---------------------------|----------------------|-----------------|-------------|--------------|------------|
| Total protein | TPO | g/L | Coho salmon | Smolt | 40.00 | 39.33 | 10.00 | 0.64 | b | 20.03–59.61 | 18.21–61.17 | ANOVA, Tukey | Normal | | | |
| | | | | Postsmolt | 43.90 | 43.42 | 6.46 | 0.60 | a | 30.69–56.50 | 28.88–58.06 | ANOVA, Tukey | Normal | | | |
| | | | | Adult | 41.20 | 42.24 | 7.63 | 0.41 | a | 26.58–56.92 | 25.32–58.30 | ANOVA, Tukey | Normal | | | |
| | | | Atlantic salmon | Presmolt | 43.80 | 44.97 | 8.61 | 0.64 | b | 27.38–61.79 | 25.34–63.69 | ANOVA, Tukey | Normal | | | |
| | | | | Smolt | 45.50 | 45.39 | 9.96 | 0.52 | b | 25.67–64.90 | 24.35–66.29 | ANOVA, Tukey | Normal | | | |
| | | | | Postsmolt | 44.50 | 44.60 | 7.61 | 0.33 | a | 29.60–59.54 | 28.67–60.44 | ANOVA, Tukey | Normal | | | |
| | | | Rainbow trout | Adult | 49.80 | 48.29 | 7.90 | 0.41 | b | 33.75–65.39 | 32.27–66.60 | ANOVA, Tukey | Normal | | | |
| | | | | Presmolt | 33.00 | 34.42 | 10.09 | 1.84 | c | 13.05–55.80 | 7.44–63.16 | ANOVA, Tukey | Normal | | | |
| | | | | Smolt | 41.00 | 40.15 | 7.63 | 0.45 | b | 25.11–55.38 | 23.86–56.61 | ANOVA, Tukey | Normal | | | |
| | | | | | | Rainbow trout | Postsmolt | 41.00 | 41.53 | 6.56 | 0.42 | a | 28.17–54.17 | 27.06–55.39 | ANOVA, Tukey | Normal |
| | | | | | | | Adult | 46.90 | 46.52 | 8.93 | 0.60 | b | 28.79–64.13 | 27.06–65.80 | ANOVA, Tukey | Normal |
| | | | | | | | Smolt | 15.70 | 15.77 | 2.73 | 0.18 | b | 10.21–21.0 | 9.70–21.56 | ANOVA, Tukey | log(x + 1) |
| Albumins | ALB | g/L | Coho salmon | Postsmolt | 16.60 | 16.73 | 2.75 | 0.26 | a | 11.28–22.26 | 10.58–22.91 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Adult | 16.20 | 16.35 | 2.38 | 0.13 | a | 11.40–20.80 | 11.00–21.30 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Presmolt | 16.60 | 16.81 | 2.26 | 0.17 | b | 12.31–21.32 | 11.87–21.75 | ANOVA, Tukey | Normal | | | |
| | | | Atlantic salmon | Smolt | 18.15 | 18.23 | 3.29 | 0.18 | a | 11.66–24.65 | 11.21–25.16 | ANOVA, Tukey | Normal | | | |
| | | | | Postsmolt | 17.90 | 18.03 | 2.44 | 0.11 | a | 13.09–22.70 | 12.77–23.03 | ANOVA, Tukey | Normal | | | |
| | | | | Adult | 18.60 | 18.47 | 2.67 | 0.14 | a | 13.45–23.97 | 13.01–24.33 | ANOVA, Tukey | Normal | | | |
| | | | Rainbow trout | Presmolt | 15.45 | 15.49 | 2.87 | 0.17 | c | 9.81–21.11 | 9.34–21.60 | ANOVA, Tukey | Normal | | | |
| | | | | Smolt | 16.50 | 16.49 | 2.28 | 0.15 | b | 11.91–20.91 | 11.50–21.35 | ANOVA, Tukey | Normal | | | |
| | | | | Postsmolt | 18.40 | 18.62 | 3.43 | 0.23 | a | 11.68–25.27 | 11.08–25.96 | ANOVA, Tukey | Normal | | | |
| | | | Globulins | GLO | g/L | Coho salmon | Smolt | 26.20 | 25.61 | 7.00 | 0.46 | a | 11.83–39.57 | 10.61–40.69 | ANOVA, Tukey | Normal |
| | | | | | | | Postsmolt | 26.70 | 26.68 | 4.43 | 0.41 | a | 17.71–35.36 | 16.64–36.54 | ANOVA, Tukey | Normal |
| | | | | | | | Adult | 25.50 | 25.91 | 6.08 | 0.33 | a | 13.60–37.60 | 12.70–38.59 | ANOVA, Tukey | Normal |
| Atlantic salmon | Presmolt | 27.20 | | | | 27.74 | 6.50 | 0.49 | b | 14.59–40.42 | 13.40–41.79 | Kruskal-Wallis, Dunn | | | | |
| | Smolt | 27.90 | | | | 27.31 | 8.53 | 0.47 | b | 10.88–44.06 | 9.53–45.06 | Kruskal-Wallis, Dunn | | | | |
| | Postsmolt | 26.50 | | | | 26.64 | 6.32 | 0.28 | b | 14.37–39.11 | 13.58–39.88 | Kruskal-Wallis, Dunn | | | | |
| | Adult | 30.75 | | | | 29.83 | 6.02 | 0.31 | a | 18.78–42.34 | 17.75–43.21 | Kruskal-Wallis, Dunn | | | | |

Table 4. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-----------------|--------------|-----------------|-----------------|-----------|--------|-----------|-----------|--------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Total bilirubin | TBI | µmol/L | Rainbow trout | Smolt | 25.00 | 24.77 | 5.56 | 0.33 | b | 13.71–35.68 | 12.80–36.59 | ANOVA, Tukey | Normal |
| | | | | Postsmolt | 24.60 | 25.04 | 5.58 | 0.36 | b | 13.67–35.77 | 12.69–36.91 | ANOVA, Tukey | Normal |
| | | | | Adult | 28.00 | 27.95 | 6.61 | 0.45 | a | 14.95–41.06 | 13.74–42.24 | ANOVA, Tukey | Normal |
| | | | Coho salmon | Smolt | 2.48 | 2.79 | 0.55 | 0.08 | b | 1.7–3.87 | 1.60–3.97 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 3.55 | 3.58 | 0.60 | 0.15 | a | 2.23–4.90 | 1.79–5.39 | Kruskal-Wallis, Dunn | |
| | | | Atlantic salmon | Presmolt | 2.90 | 2.97 | 0.12 | 0.07 | a | 2.74–3.19 | 2.72–3.22 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 2.90 | 2.96 | 0.32 | 0.06 | a | 2.22–3.58 | 1.90–3.88 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 2.95 | 3.03 | 0.40 | 0.08 | a | 2.16–3.88 | 1.93–4.08 | ANOVA, Tukey | POWER BOX COX |
| | | | Rainbow trout | Adult | 2.80 | 3.06 | 0.72 | 0.18 | a | 1.11–4.37 | 0.19–5.46 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 3.00 | 3.18 | 0.58 | 0.16 | a | 2.11–3.92 | 1.76–4.33 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 3.20 | 3.30 | 0.17 | 0.10 | a | 2.96–3.64 | 2.93–3.67 | ANOVA, Tukey | POWER BOX COX |
| | | | Coho salmon | Adult | 2.90 | 3.06 | 0.34 | 0.11 | a | 2.09–3.95 | 1.71–4.41 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 1.88 | 2.05 | 0.40 | 0.10 | a | 1.03–2.95 | 0.82–3.23 | ANOVA, Tukey | BOX COX |
| | | | | Adult | 1.90 | 1.98 | 0.40 | 0.07 | a | 1.10–2.80 | 0.78–3.04 | ANOVA, Tukey | BOX COX |
| | | | Atlantic salmon | Presmolt | 1.80 | 1.83 | 0.26 | 0.13 | ab | 1.31–2.34 | 1.26–2.39 | ANOVA, Tukey | POWER BOX COX |
| Smolt | 1.80 | 1.88 | | 0.32 | 0.05 | b | 1.31–2.29 | 1.20–2.40 | ANOVA, Tukey | POWER BOX COX | | | |
| Postsmolt | 2.10 | 2.16 | | 0.36 | 0.06 | a | 1.47–2.76 | 1.31–2.90 | ANOVA, Tukey | POWER BOX COX | | | |
| Rainbow trout | Adult | 1.80 | 1.87 | 0.27 | 0.05 | b | 1.26–2.43 | 1.12–2.60 | ANOVA, Tukey | POWER BOX COX | | | |
| | Smolt | 1.70 | 1.78 | 0.28 | 0.06 | a | 1.09–2.29 | 0.89–2.56 | ANOVA, Tukey | POWER BOX COX | | | |
| | Postsmolt | 1.60 | 1.77 | 0.29 | 0.17 | a | 1.20–2.33 | 1.15–2.38 | ANOVA, Tukey | POWER BOX COX | | | |
| Adult | 1.60 | 1.57 | 0.09 | 0.03 | a | 1.39–1.75 | 1.37–1.77 | ANOVA, Tukey | POWER BOX COX | | | | |

Table 4. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | |
|------------|--------------|-----------------|-----------------|-----------|--------|-------|-----------|-------|--------------------------|------------------------|---------------------------|----------------------|-----------------|------------|----------------------|---------------|
| Creatinine | CRE | µmol/L | Coho salmon | Smolt | 24.64 | 26.67 | 9.15 | 1.08 | a | 6.63–44.29 | 3.61–48.26 | Kruskal-Wallis, Dunn | | | | |
| | | | | Postsmolt | 23.87 | 21.51 | 4.87 | 2.81 | a | 15.91–24.75 | 15.91–24.75 | Kruskal-Wallis, Dunn | | | | |
| | | | | Adult | 22.10 | 24.21 | 8.04 | 1.02 | a | 5.56–39.03 | 2.30–43.24 | Kruskal-Wallis, Dunn | | | | |
| | | | Atlantic salmon | Presmolt | 23.90 | 26.91 | 10.27 | 1.00 | b | 6.79–47.04 | 4.78–49.05 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Smolt | 25.64 | 26.72 | 7.67 | 0.51 | b | 11.09–41.70 | 9.67–43.21 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 31.90 | 31.28 | 8.80 | 0.85 | a | 13.94–49.13 | 11.50–51.25 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Rainbow trout | Adult | 23.90 | 24.32 | 5.57 | 0.92 | b | 11.90–34.83 | 9.09–38.17 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Presmolt | 61.44 | 59.63 | 12.95 | 2.90 | a | 33.25–89.47 | 22.86–101.49 | ANOVA, Tukey | POWER BOX COX | | | |
| | | | | Smolt | 28.68 | 32.01 | 13.77 | 1.44 | b | 5.03–58.99 | 2.77–61.26 | ANOVA, Tukey | POWER BOX COX | | | |
| | | | | | | | Postsmolt | 26.52 | 27.77 | 8.18 | 0.99 | b | 10.60–43.93 | 8.00–46.86 | ANOVA, Tukey | POWER BOX COX |
| | | | | | | | Adult | 26.08 | 27.35 | 8.74 | 1.15 | b | 8.60–44.26 | 4.00–48.23 | ANOVA, Tukey | POWER BOX COX |
| | | | | | | | | | | | | | | | | |
| Glucose | GLU | mmol/L | Coho salmon | Smolt | 4.29 | 4.65 | 1.51 | 0.13 | a | 1.34–7.51 | 0.94–7.98 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Postsmolt | 4.58 | 4.61 | 0.76 | 0.07 | a | 3.11–6.12 | 2.92–6.31 | ANOVA, Tukey | log(x + 1) | | | |
| | | | | Adult | 2.85 | 3.07 | 1.47 | 0.15 | b | 1.10–5.96 | 1.02–5.99 | ANOVA, Tukey | log(x + 1) | | | |
| | | | Atlantic salmon | Presmolt | 5.40 | 5.40 | 1.85 | 0.13 | b | 1.77–9.02 | 1.40–9.39 | Kruskal-Wallis, Dunn | | | | |
| | | | | Smolt | 6.10 | 6.33 | 1.68 | 0.09 | a | 2.91–9.60 | 2.68–9.87 | Kruskal-Wallis, Dunn | | | | |
| | | | | Postsmolt | 4.10 | 4.06 | 1.65 | 0.08 | c | 0.76–7.06 | 0.57–7.29 | Kruskal-Wallis, Dunn | | | | |
| | | | Rainbow trout | Adult | 5.50 | 5.70 | 0.81 | 0.05 | b | 4.0–7.30 | 3.86–7.43 | Kruskal-Wallis, Dunn | | | | |
| | | | | Presmolt | 2.79 | 2.76 | 0.56 | 0.10 | c | 1.60–3.93 | 1.36–4.16 | Kruskal-Wallis, Dunn | | | | |
| | | | | Smolt | 2.76 | 3.13 | 1.82 | 0.12 | c | 1.08–7.48 | 1.06–8.46 | Kruskal-Wallis, Dunn | | | | |
| | | | | | | | Postsmolt | 4.08 | 4.32 | 2.13 | 0.18 | b | 0.15–8.49 | 8.11–8.88 | Kruskal-Wallis, Dunn | |
| | | | | | | | Adult | 5.24 | 5.04 | 2.05 | 0.17 | a | 0.92–9.12 | 0.51–9.60 | Kruskal-Wallis, Dunn | |
| | | | | | | | | | | | | | | | | |
| Lactate | LAC | mmol/L | Coho salmon | Smolt | 4.69 | 5.44 | 3.20 | 0.23 | b | 1.25–13.17 | 1.12–13.81 | Kruskal-Wallis, Dunn | | | | |
| | | | | Postsmolt | 5.15 | 5.47 | 1.78 | 0.16 | a | 1.67–8.84 | 1.21–9.43 | Kruskal-Wallis, Dunn | | | | |
| | | | | Adult | 5.63 | 5.81 | 1.85 | 0.11 | b | 1.90–9.22 | 1.59–9.59 | Kruskal-Wallis, Dunn | | | | |
| | | | Atlantic salmon | Presmolt | 4.98 | 5.60 | 2.21 | 0.17 | a | 3.03–11.44 | 3.0–12.59 | Kruskal-Wallis, Dunn | | | | |
| | | | | Smolt | 5.10 | 5.77 | 2.16 | 0.13 | a | 3.10–11.29 | 3.0–13.18 | Kruskal-Wallis, Dunn | | | | |
| | | | | Postsmolt | 4.80 | 5.25 | 2.33 | 0.11 | b | 1.56–11.35 | 1.40–11.74 | Kruskal-Wallis, Dunn | | | | |

Table 4. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|---------------|-----------------|-----------------|-----------------|-----------|--------|-------|-----------|-----------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Urea | URE | mmol/L | Rainbow trout | Adult | 3.80 | 3.84 | 0.94 | 0.06 | a | 1.97–5.69 | 1.82–5.85 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 11.77 | 12.27 | 2.84 | 0.52 | c | 8.05–19.26 | 8.05–19.26 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 6.55 | 7.00 | 3.54 | 0.22 | c | 2.27–14.18 | 2.0–14.97 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 6.00 | 6.23 | 2.38 | 0.15 | b | 1.38–10.84 | 0.96–11.29 | Kruskal-Wallis, Dunn | |
| | | | Coho salmon | Adult | 4.72 | 4.88 | 1.99 | 0.14 | a | 0.77–8.65 | 0.41–9.11 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 1.80 | 1.76 | 0.27 | 0.03 | a | 1.21–2.30 | 1.13–2.39 | ANOVA, Tukey | Normal |
| | | | Atlantic salmon | Postsmolt | 0.90 | 0.91 | 0.20 | 0.02 | b | 0.52–1.31 | 0.45–1.39 | ANOVA, Tukey | Normal |
| | | | | Adult | 1.60 | 1.60 | 0.35 | 0.06 | c | 0.89–2.33 | 0.70–2.51 | ANOVA, Tukey | Normal |
| | | | | Presmolt | 1.45 | 1.40 | 0.26 | 0.03 | a | 0.87–1.94 | 0.78–2.03 | ANOVA, Tukey | BOX COX |
| | | | | Smolt | 1.10 | 1.14 | 0.30 | 0.04 | b | 0.51–1.76 | 0.37–1.88 | ANOVA, Tukey | BOX COX |
| | | | | Postsmolt | 1.50 | 1.50 | 0.19 | 0.03 | a | 1.09–1.86 | 0.97–1.98 | ANOVA, Tukey | BOX COX |
| | | | | Adult | 1.10 | 1.09 | 0.30 | 0.05 | b | 0.46–1.70 | 0.32–1.86 | ANOVA, Tukey | BOX COX |
| Rainbow trout | Presmolt | 1.20 | 1.20 | 0.09 | 0.02 | a | 1.0–1.38 | 0.94–1.45 | ANOVA, Tukey | BOX COX | | | |
| | Smolt | 1.00 | 1.07 | 0.19 | 0.02 | b | 0.65–1.42 | 0.59–1.48 | ANOVA, Tukey | BOX COX | | | |
| | Postsmolt | 1.30 | 1.27 | 0.16 | 0.03 | a | 0.92–1.58 | 0.83–1.69 | ANOVA, Tukey | BOX COX | | | |
| | Smolt | 1.80 | 1.76 | 0.27 | 0.03 | a | 1.21–2.30 | 1.12–2.39 | ANOVA, Tukey | Normal | | | |
| Uric acid | UAC | µmol/L | Coho salmon | Postsmolt | 0.90 | 0.91 | 0.20 | 0.02 | c | 0.52–1.31 | 0.45–1.38 | ANOVA, Tukey | Normal |
| | | | | Adult | 1.60 | 1.60 | 0.35 | 0.06 | b | 0.89–2.33 | 0.71–2.51 | ANOVA, Tukey | Normal |
| | | | Atlantic salmon | Presmolt | 1.45 | 1.40 | 0.26 | 0.03 | a | 0.87–1.94 | 0.78–2.33 | ANOVA, Tukey | log(x + 1) |
| | | | | Smolt | 1.10 | 1.13 | 0.28 | 0.04 | b | 0.55–1.70 | 0.44–1.8 | ANOVA, Tukey | log(x + 1) |
| | | | | Postsmolt | 1.50 | 1.49 | 0.16 | 0.02 | a | 1.15–1.80 | 1.08–1.87 | ANOVA, Tukey | log(x + 1) |
| | | | Rainbow trout | Adult | 1.10 | 1.09 | 0.30 | 0.05 | b | 0.46–1.70 | 0.32–1.86 | ANOVA, Tukey | log(x + 1) |
| | | | | Presmolt | 1.20 | 1.20 | 0.09 | 0.02 | a | 1.0–1.38 | 0.94–1.46 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 1.00 | 1.06 | 0.17 | 0.02 | b | 0.68–1.39 | 0.63–1.44 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 1.20 | 1.24 | 0.12 | 0.02 | a | 0.97–1.51 | 0.93–1.56 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 2.92 | 2.75 | 0.60 | 0.08 | a | 1.58–4.09 | 1.29–4.32 | Kruskal-Wallis, Dunn | |
| Ammonia | NH ₃ | mmol/L | Coho salmon | Postsmolt | 1.07 | 0.98 | 0.34 | 0.03 | b | 0.36–1.76 | 0.24–1.83 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 2.62 | 2.51 | 0.50 | 0.10 | a | 1.53–3.66 | 1.18–4.06 | Kruskal-Wallis, Dunn | |
| | | | Atlantic salmon | Presmolt | 1.92 | 1.83 | 0.34 | 0.05 | b | 1.22–2.63 | 1.02–2.75 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 1.85 | 1.51 | 0.68 | 0.09 | b | 0.19–2.84 | 0.09–2.94 | Kruskal-Wallis, Dunn | |

Table 4. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-------------------|--------------|-----------------|-----------------|-----------|--------|-------------|------------|------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Total Cholesterol | TCH | mmol/L | Rainbow trout | Postsmolt | 1.83 | 1.88 | 0.22 | 0.03 | b | 1.38–2.30 | 1.25–2.44 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 1.24 | 1.13 | 0.36 | 0.07 | a | 0.36–1.95 | 0.16–2.11 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 1.71 | 1.73 | 0.13 | 0.02 | a | 1.46–1.99 | 1.40–2.05 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 1.63 | 1.62 | 0.32 | 0.03 | a | 0.97–2.27 | 0.83–2.41 | Kruskal-Wallis, Dunn | |
| | | | Coho salmon | Postsmolt | 1.69 | 1.72 | 0.24 | 0.04 | a | 1.21–2.20 | 1.10–2.34 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 7.14 | 7.76 | 2.95 | 0.19 | c | 1.41–13.30 | 0.99–13.94 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 9.04 | 8.86 | 1.67 | 0.16 | a | 5.62–12.29 | 5.11–12.77 | Kruskal-Wallis, Dunn | |
| | | | Atlantic salmon | Adult | 8.09 | 8.13 | 2.84 | 0.16 | b | 2.46–13.65 | 1.96–14.11 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 12.32 | 12.78 | 2.74 | 0.20 | a | 7.17–18.17 | 6.67–18.72 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 11.10 | 11.44 | 3.66 | 0.20 | b | 4.08–18.56 | 3.62–19.09 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 7.50 | 7.69 | 2.63 | 0.11 | d | 2.39–12.78 | 2.05–13.11 | Kruskal-Wallis, Dunn | |
| | | | Rainbow trout | Adult | 8.80 | 9.06 | 1.90 | 0.10 | c | 5.05–12.59 | 4.69–12.96 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 5.34 | 6.08 | 2.44 | 0.45 | b | 2.07–12.07 | 2.07–12.07 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 8.94 | 9.08 | 3.19 | 0.19 | a | 2.54–15.14 | 1.96–15.73 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 6.78 | 6.87 | 2.56 | 0.17 | b | 1.65–11.79 | 1.21–12.29 | ANOVA, Tukey | POWER BOX COX |
| | | | Triglycerides | TRG | mmol/L | Coho salmon | Adult | 5.91 | 6.34 | 2.85 | 0.20 | b | 0.74–11.93 |
| Smolt | 2.84 | 2.90 | | | | | 1.31 | 0.09 | a | 0.34–5.47 | 0.11–5.70 | ANOVA, Tukey | POWER BOX COX |
| Postsmolt | 2.73 | 3.12 | | | | | 1.26 | 0.12 | a | 1.57–6.94 | 1.48–7.58 | ANOVA, Tukey | POWER BOX COX |
| Atlantic salmon | Adult | 2.77 | | | | 2.91 | 1.24 | 0.07 | a | 0.34–5.24 | 0.13–5.45 | ANOVA, Tukey | POWER BOX COX |
| | Presmolt | 4.25 | | | | 4.54 | 1.42 | 0.11 | c | 1.4–7.15 | 0.98–7.60 | Kruskal-Wallis, Dunn | |
| | Smolt | 4.10 | | | | 4.19 | 1.71 | 0.09 | a | 0.54–7.29 | 0.29–7.60 | Kruskal-Wallis, Dunn | |
| | Postsmolt | 2.80 | | | | 3.01 | 1.39 | 0.06 | b | 0.27–5.74 | 0.10–5.91 | Kruskal-Wallis, Dunn | |
| Rainbow trout | Adult | 2.75 | 3.20 | 1.59 | 0.09 | b | 0.31–6.19 | 0.60–6.46 | Kruskal-Wallis, Dunn | | | | |
| Rainbow trout | Presmolt | 6.73 | 7.40 | 2.62 | 0.50 | a | 1.63–12.97 | 0.56–14.27 | ANOVA, Tukey | POWER BOX COX | | | |

Table 4. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|--------------------------------------|--------------|-----------------|-----------------|-----------|--------|------|------|------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| High-density lipoprotein cholesterol | HDL | mmol/L | | Smolt | 5.00 | 4.99 | 1.85 | 0.11 | b | 1.26–8.55 | 1.0–8.89 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 4.20 | 4.70 | 2.09 | 0.14 | b | 0.59–8.80 | 0.22–9.18 | ANOVA, Tukey | POWER BOX COX |
| | | | | Adult | 4.33 | 4.73 | 2.11 | 0.15 | b | 0.6–8.86 | 0.20–9.26 | ANOVA, Tukey | POWER BOX COX |
| | | | Coho salmon | Smolt | 3.39 | 3.49 | 1.49 | 0.20 | b | 0.57–6.40 | 0.31–6.66 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 5.65 | 5.45 | 1.62 | 0.16 | a | 2.26–8.76 | 1.82–9.20 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 0.28 | 0.32 | 0.11 | 0.02 | c | 0.10–0.54 | 0.09–0.56 | Kruskal-Wallis, Dunn | |
| | | | Atlantic salmon | Presmolt | 1.86 | 1.98 | 0.67 | 0.10 | c | 0.45–3.19 | 0.14–3.56 | ANOVA, Tukey | Normal |
| | | | | Smolt | 5.41 | 5.60 | 1.27 | 0.17 | b | 2.94–8.14 | 2.49–8.61 | ANOVA, Tukey | Normal |
| | | | | Postsmolt | 6.61 | 6.23 | 1.54 | 0.21 | a | 3.18–9.61 | 2.47–10.11 | ANOVA, Tukey | Normal |
| | | | Rainbow trout | Adult | 6.88 | 6.90 | 1.21 | 0.27 | a | 4.36–9.59 | 3.48–10.39 | ANOVA, Tukey | Normal |
| | | | | Presmolt | 1.63 | 1.62 | 0.44 | 0.08 | c | 0.69–2.53 | 0.48–2.74 | ANOVA, Tukey | Normal |
| | | | | Smolt | 4.46 | 4.26 | 1.64 | 0.18 | b | 0.99–7.61 | 0.54–8.04 | ANOVA, Tukey | Normal |
| Low-density lipoprotein cholesterol | LDL | mmol/L | | Postsmolt | 2.53 | 2.50 | 0.90 | 0.17 | a | 0.65–4.40 | 0.15–4.86 | ANOVA, Tukey | Normal |
| | | | | Smolt | 1.20 | 1.34 | 0.95 | 0.12 | c | 0.15–4.25 | 0.30–5.30 | ANOVA, Tukey | BOX COX |
| | | | | Postsmolt | 1.57 | 1.60 | 0.51 | 0.05 | b | 0.57–2.59 | 0.40–2.75 | ANOVA, Tukey | BOX COX |
| | | | Coho salmon | Adult | 2.10 | 2.20 | 1.02 | 0.19 | a | 0.19–4.20 | 0.04–4.36 | ANOVA, Tukey | BOX COX |
| | | | | Presmolt | 2.35 | 2.52 | 0.90 | 0.16 | a | 0.75–4.29 | 0.57–4.47 | ANOVA, Tukey | BOX COX |
| | | | | Smolt | 2.42 | 2.40 | 1.00 | 0.13 | a | 0.33–4.40 | 0.04–4.77 | ANOVA, Tukey | BOX COX |
| | | | Atlantic salmon | Postsmolt | 1.55 | 1.62 | 0.56 | 0.07 | b | 0.39–2.66 | 0.16–2.93 | ANOVA, Tukey | BOX COX |
| | | | | Adult | 0.90 | 0.97 | 0.33 | 0.06 | c | 0.22–1.63 | 0.04–1.87 | ANOVA, Tukey | BOX COX |
| | | | | Presmolt | 1.45 | 1.46 | 0.60 | 0.11 | b | 0.50–3.10 | 0.50–3.10 | ANOVA, Tukey | BOX COX |
| | | | Rainbow trout | Smolt | 1.89 | 1.98 | 0.96 | 0.12 | a | 0.41–4.74 | 0.60–4.88 | ANOVA, Tukey | BOX COX |
| | | | | Postsmolt | 2.40 | 2.50 | 1.20 | 0.22 | a | 0.36–4.79 | 0.94–4.79 | ANOVA, Tukey | BOX COX |

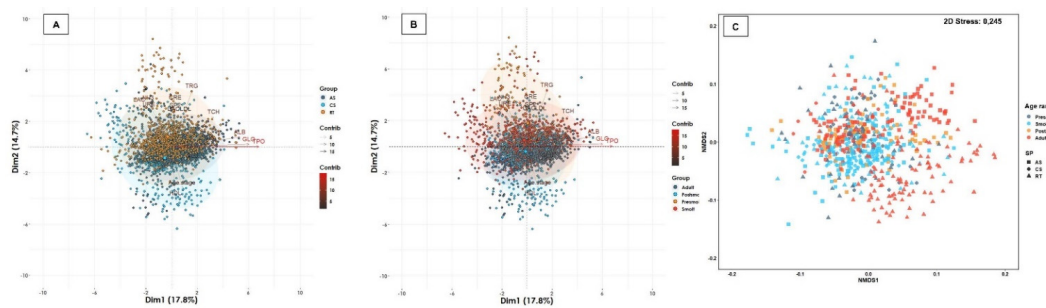


Figure 3. Multivariate analysis of plasma substrates biomarkers. Salmonid species and range contribute significantly to the total variation of the plasma substrates profile. (A) Spatial sorting of the plasma substrates biomarkers according to salmonid species and (B) age ranges. The two-dimensional analysis captures 54.2% of the total variance of the plasma substrate biomarkers profile. TPO, URE, NH_3 , GLO, ALB, TCH and HDL contribute significantly to the total variance of the plasma substrates in dimension 1, while the TRG is the biomarker that contributes the most to dimension 2. (C) The multivariate analysis of interdependence of the plasma substrates profile demonstrates significant differences between salmonid species ($R_{\text{ANOSIM}} = 0.1580$; $p = 0.0110$), suggesting a uniform distribution between all three salmonid species. At the same time, significant differences were observed between age ranges ($R_{\text{ANOSIM}} = 0.0910$; $p = 0.0001$), suggesting differences in plasma substrate biomarkers between freshwater and seawater-reared fish.

3.4. Plasma Enzymes

As for plasma enzymes, only ALT, AST and CKT demonstrated a normal distribution or were transformed to a normal distribution (Table 5, Figure S10), while ALP, TAM, LIP, CK-MB and LDH were not normally distributed (Table 5, Figure S10). The highest TAM activity was observed in postsmolt and adult of coho salmon, whereas the highest CK-MB activity was recorded in presmolt and smolt of Atlantic salmon. The lowest ALP activity was observed in coho salmon independent of age range, while a similar CKT activity between species and age range was observed. Three salmonid species demonstrated the same positive association profile of ALT activity with AST, AST with LDH, LIP with TAM and CK-MB with CKT ($r < 0.20$; $p < 0.05$) (Figure S11), as well as the same negative association profile of LIP activity with CK-MB and CKT ($r > -0.20$; $p < 0.05$) (Figure S11). Interestingly, the positive association of AST activity with LDH, CKT and CK-MB is common in coho and Atlantic salmon, whereas in rainbow trout the positive association was observed with ALP, LIP and TAM activity ($r < 0.20$; $p < 0.05$) (Figure S11). Similarly, ALT activity in coho and Atlantic salmon showed a positive association with AST, LDH and LIP, but it was positively associated with the activity of LDH, ALP, CKT, AST and TAM in rainbow trout ($r < 0.20$; $p < 0.05$) (Figure S11). A positive association between CK-MB activity with CKT, LDH and ALP was observed only in Atlantic salmon ($r < 0.20$; $p < 0.05$) (Figure S11). ALT demonstrated a significant positive association with AST in smolt and postsmolt, but not in presmolt or adults (Figure S12). Similarly, LIP presented a significant positive association with TAM in smolt and adult, but not in presmolt and postsmolt (Figure S12), while CK-MB revealed a significant positive association with other important enzymes of skeletal and cardiac muscle tissue such as ALP, AST and CKT in smolt and postsmolt (Figure S12). The 33.7% of the total variability of the plasma enzymes profile was captured by two-dimensional analysis both salmonid species and age ranges, mainly driven by TAM, CK-MB and CKT (positive) and AST and ALP (negative) (Figure 4). Clusters were observed between ALP, AST and LDH, and between TAM, CK-MB, CKT, LIP and ALT (Figure 4). The enzyme profile demonstrated significant variability between species ($R_{\text{ANOSIM}} = 0.2109$, $p = 0.0001$), mainly attributed to Atlantic salmon, since the distribution of observations was similar between rainbow trout and coho salmon (Figure 4). Regarding age range, the enzyme profile showed low variability ($R_{\text{ANOSIM}} = 0.07708$, $p = 0.0001$), explained by a uniform distribution between presmolt and smolt, although distanced from postsmolt and adults (Figure 4). Taken together, our results demonstrate that the activity of most plasma enzymes depends on the salmonid species, age range and/or interaction between them, and they are often biologically related to each other according to the functionality of fish systems, organs and tissues.

Table 5. Reference intervals (RIs) for plasma enzymes biomarkers in presmolt and smolt (freshwater) and postsmolt and adult (seawater) of Atlantic salmon, coho salmon, and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | | | |
|----------------------|--------------|-----------------|-----------------|-----------|--------|--------|---------------|---------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|--------------|----------------------|---------------|--------------|---------------|
| Alkaline phosphatase | ALP | U/L | Coho salmon | Smolt | 43.00 | 48.74 | 25.86 | 1.85 | b | 18.93–115.23 | 15.0–122.0 | Kruskal-Wallis, Dunn | | | | | | |
| | | | | Postsmolt | 67.00 | 65.80 | 22.58 | 2.18 | a | 20.56–110.74 | 15.04–116.44 | | | | | | | |
| | | | | Adult | 39.00 | 46.68 | 26.40 | 1.65 | c | 13.00–106.2 | 11.0–255.49 | | | | | | | |
| | | | Atlantic salmon | Presmolt | 177.00 | 179.77 | 35.12 | 2.73 | a | 107.55–247.14 | 99.80–255.49 | Kruskal-Wallis, Dunn | | | | | | |
| | | | | Smolt | 176.00 | 176.56 | 38.76 | 2.18 | a | 99.25–252.11 | 93.88–258.23 | | | | | | | |
| | | | | Postsmolt | 156.00 | 153.59 | 41.11 | 2.10 | b | 70.50–232.67 | 64.90–238.67 | | | | | | | |
| | | | Rainbow trout | Adult | 171.00 | 170.62 | 34.77 | 2.10 | a | 101.98–239.18 | 96.24–244.93 | Kruskal-Wallis, Dunn | | | | | | |
| | | | | Presmolt | 164.00 | 161.00 | 27.71 | 5.54 | a | 101.37–218.62 | 84.47–234.88 | | | | | | | |
| | | | | Smolt | 83.00 | 90.14 | 41.69 | 2.74 | c | 8.42–171.85 | 1.57–178.71 | | | | | | | |
| | | | | | | | Postsmolt | 99.00 | 104.74 | 46.80 | 3.09 | b | 7.95–194.24 | 0.57–204.26 | Kruskal-Wallis, Dunn | | | |
| | | | | | | | Adult | 65.50 | 74.77 | 36.68 | 2.53 | d | 22.28–157.73 | 20.0–162.0 | | | | |
| | | | | | | | Smolt | 15.90 | 15.86 | 7.41 | 0.59 | b | 1.35–30.38 | 0.05–31.68 | | | ANOVA, Tukey | POWER BOX COX |
| Alanine transaminase | ALT | U/L | Coho salmon | Postsmolt | 10.30 | 10.91 | 3.61 | 0.36 | a | 3.13–17.73 | 1.98–18.91 | ANOVA, Tukey | POWER BOX COX | | | | | |
| | | | | Adult | 14.80 | 15.38 | 5.39 | 0.35 | c | 4.31–25.73 | 3.54–26.78 | | | | | | | |
| | | | | Presmolt | 19.95 | 19.70 | 7.81 | 0.64 | c | 4.62–35.63 | 2.70–37.38 | | | | | | | |
| | | | Atlantic salmon | Smolt | 15.20 | 16.88 | 7.41 | 0.41 | b | 1.04–30.98 | 0.09–32.38 | ANOVA, Tukey | POWER BOX COX | | | | | |
| | | | | Postsmolt | 14.10 | 14.47 | 6.75 | 0.36 | a | 1.25–27.69 | 0.44–28.50 | | | | | | | |
| | | | | Adult | 12.65 | 12.73 | 4.68 | 0.30 | a | 3.28–21.74 | 2.45–22.68 | | | | | | | |
| | | | Rainbow trout | Presmolt | 6.60 | 7.44 | 1.70 | 0.37 | c | 3.00–11.08 | 1.74–12.27 | ANOVA, Tukey | POWER BOX COX | | | | | |
| | | | | Smolt | 14.50 | 15.87 | 7.83 | 0.48 | a | 5.40–33.90 | 5.37–34.67 | | | | | | | |
| | | | | Postsmolt | 10.90 | 11.61 | 4.84 | 0.37 | b | 1.47–20.85 | 0.39–22.15 | | | | | | | |
| | | | | | | | Adult | 8.20 | 10.33 | 4.95 | 0.38 | d | 5.50–25.79 | 5.14–29.30 | ANOVA, Tukey | POWER BOX COX | | |
| | | | | | | | Smolt | 245.50 | 269.36 | 134.75 | 9.11 | b | 52.0–582.85 | 38.10–601.50 | | | ANOVA, Tukey | POWER BOX COX |
| | | | | | | | Postsmolt | 271.30 | 293.45 | 92.50 | 8.70 | a | 86.95–465.02 | 52.98–500.03 | | | | |
| Atlantic salmon | Adult | 259.30 | 291.12 | 125.28 | 6.91 | c | 45.57–536.67 | 26.50–555.74 | ANOVA, Tukey | POWER BOX COX | | | | | | | | |
| | Presmolt | 346.00 | 353.18 | 92.48 | 7.96 | c | 160.02–528.15 | 136.88–555.85 | | | | | | | | | | |
| | Smolt | 352.10 | 367.40 | 106.57 | 6.20 | b | 134.57–558.92 | 115.90–584.67 | | | | | | | | | | |
| | | | | Postsmolt | 267.10 | 272.41 | 107.76 | 4.99 | a | 49.99–474.44 | 34.97–490.75 | ANOVA, Tukey | POWER BOX COX | | | | | |

Table 5. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-----------------------|--------------|-----------------|-----------------|-----------|---------|-------------|-----------|-----------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Total amylase | TAM | U/L | Rainbow trout | Adult | 278.10 | 286.64 | 112.45 | 6.16 | a | 52.49–496.83 | 35.13–518.01 | ANOVA, Tukey | POWER BOX COX |
| | | | | Presmolt | 346.65 | 362.46 | 112.69 | 20.57 | c | 110.81–584.47 | 51.37–653.85 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 349.20 | 348.92 | 124.85 | 7.80 | a | 99.19–591.83 | 73.07–615.53 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 276.45 | 290.74 | 110.98 | 7.25 | b | 54.44–496.13 | 31.02–519.56 | ANOVA, Tukey | POWER BOX COX |
| | | | Coho salmon | Adult | 307.00 | 316.66 | 129.73 | 9.00 | d | 50.78–565.15 | 24.77–594.71 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 990.50 | 972.68 | 502.26 | 44.39 | c | 216.13–2366.88 | 163.50–2662.95 | ANOVA, Tukey | BOX COX |
| | | | | Postsmolt | 1539.00 | 1593.84 | 417.81 | 39.30 | a | 711.29–2388.73 | 599.84–3517.48 | ANOVA, Tukey | BOX COX |
| | | | Atlantic salmon | Adult | 1281.50 | 1267.71 | 544.66 | 34.18 | b | 143.61–2294.0 | 53.19–2404.72 | ANOVA, Tukey | BOX COX |
| | | | | Presmolt | 609.00 | 656.76 | 303.19 | 22.85 | a | 62.53–1250.99 | 3.15–1310.37 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 880.00 | 924.00 | 303.60 | 16.61 | b | 306.71–1515.83 | 264.88–1562.42 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 967.90 | 987.36 | 381.63 | 17.91 | b | 208.88–1712.55 | 140.99–1777.90 | Kruskal-Wallis, Dunn | |
| | | | Rainbow trout | Adult | 1177.00 | 1180.59 | 341.57 | 21.22 | c | 491.25–1832.17 | 422.24–1898.45 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 445.00 | 493.08 | 220.81 | 43.30 | c | 160.0–1050.0 | 160.0–1050.0 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 876.00 | 887.59 | 445.30 | 27.78 | b | 155.60–1583.10 | 135.45–2414.80 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 934.00 | 991.61 | 399.42 | 25.89 | a | 160.73–1754.25 | 79.31–1836.25 | Kruskal-Wallis, Dunn | |
| | | | Lipase | LIP | U/L | Coho salmon | Adult | 1120.00 | 1069.92 | 352.98 | 26.02 | a | 357.87–1768.88 |
| Smolt | 5.40 | 5.53 | | | | | 0.83 | 0.07 | b | 4.04–6.89 | 3.88–7.06 | ANOVA, Tukey | BOX COX |
| Postsmolt | 5.70 | 5.69 | | | | | 0.40 | 0.04 | ab | 4.91–6.50 | 4.79–6.64 | ANOVA, Tukey | BOX COX |
| Atlantic salmon | Adult | 5.90 | | | | 5.85 | 0.88 | 0.05 | a | 4.19–7.59 | 4.05–7.72 | ANOVA, Tukey | BOX COX |
| | Presmolt | 6.20 | | | | 6.02 | 0.86 | 0.06 | a | 4.35–7.83 | 4.05–8.12 | Kruskal-Wallis, Dunn | |
| | Smolt | 5.90 | | | | 5.63 | 1.21 | 0.06 | b | 3.39–8.26 | 3.14–8.48 | Kruskal-Wallis, Dunn | |
| | Postsmolt | 5.20 | | | | 5.33 | 1.70 | 0.08 | c | 2.01–8.46 | 1.81–8.68 | Kruskal-Wallis, Dunn | |
| Rainbow trout | Adult | 5.60 | | | | 5.42 | 1.44 | 0.08 | c | 2.83–8.12 | 2.59–8.34 | Kruskal-Wallis, Dunn | |
| | Presmolt | 6.60 | | | | 6.50 | 0.57 | 0.13 | a | 5.23–7.70 | 4.79–8.14 | Kruskal-Wallis, Dunn | |
| | Smolt | 5.30 | | | | 5.33 | 0.74 | 0.04 | c | 3.83–6.76 | 3.70–6.91 | Kruskal-Wallis, Dunn | |
| Coho salmon | Postsmolt | 5.60 | 5.62 | 0.81 | 0.05 | b | 3.96–7.15 | 3.80–7.30 | Kruskal-Wallis, Dunn | | | | |
| | Adult | 5.60 | 5.47 | 1.34 | 0.09 | b | 2.88–8.21 | 2.59–8.44 | Kruskal-Wallis, Dunn | | | | |
| Creatine kinase total | CKT | U/L | Coho salmon | Smolt | 5212.50 | 5980.72 | 4527.46 | 377.29 | b | 23.0–16,269.25 | 16.0–16,967.75 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 7926.00 | 8324.64 | 6839.67 | 687.41 | a | 22.0–23,723.00 | 22.0–26,210.0 | ANOVA, Tukey | POWER BOX COX |

Table 5. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-----------------------------------|--------------|-----------------|-----------------|-----------|---------|----------------|-------------------|----------------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Cardiac creatine kinase isoenzyme | CK-MB | U/L | Atlantic salmon | Adult | 5181.00 | 7207.93 | 5716.01 | 340.99 | c | 457.0–22,267.10 | 293.0–23,667.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Presmolt | 7548.00 | 8670.21 | 4816.54 | 455.12 | c | 1398.98–20,848.55 | 1014.0–21,725.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 7029.50 | 8638.65 | 4422.61 | 351.84 | b | 2827.35–18,632.50 | 2522.90–21,403.50 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 4637.00 | 4787.97 | 1878.53 | 114.11 | a | 883.94–8317.86 | 552.21–8674.11 | ANOVA, Tukey | POWER BOX COX |
| | | | Rainbow trout | Adult | 5670.50 | 6144.78 | 3025.80 | 198.65 | a | 1921.18–14,481.05 | 1347.0–16,082.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Presmolt | 2705.00 | 3414.33 | 2052.56 | 374.74 | c | 1380.0–9180.0 | 1380.0–9180.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 5294.00 | 6660.63 | 5120.90 | 368.61 | b | 265.75–20,177.30 | 24.0–20,617.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 5250.00 | 7235.51 | 6037.84 | 390.56 | ab | 751.95–23,438.70 | 421.0–23,963.0 | ANOVA, Tukey | POWER BOX COX |
| | | | Coho salmon | Adult | 5965.00 | 7875.02 | 5099.61 | 386.60 | a | 1553.48–21,792.40 | 1273.0–22,850.0 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 3419.50 | 3628.47 | 1410.47 | 190.19 | b | 626.15–6386.21 | 45.34–6982.88 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 9707.00 | 10,268.18 | 5107.02 | 484.74 | a | 1706.40–22,365.60 | 1248.0–23,554.0 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 3811.00 | 4445.89 | 2760.02 | 235.80 | b | 1244.57–13,392.15 | 1059.78–19,625.45 | Kruskal-Wallis, Dunn | |
| Atlantic salmon | Presmolt | 11,277.00 | 11,012.54 | 6265.61 | 934.02 | c | 169.67–22,306.20 | 17.31–22,362.0 | Kruskal-Wallis, Dunn | | | | |
| | Smolt | 16,970.00 | 16,911.02 | 4926.72 | 703.82 | b | 6946.41–26,975.05 | 4820.19–29,099.54 | Kruskal-Wallis, Dunn | | | | |
| | Postsmolt | 3951.50 | 4361.31 | 2036.19 | 176.56 | a | 370.44–8352.18 | 126.15–8596.47 | Kruskal-Wallis, Dunn | | | | |
| | Adult | 3812.90 | 4352.02 | 2502.36 | 347.02 | a | 428.48–12,130.70 | 174.0–12,743.0 | Kruskal-Wallis, Dunn | | | | |
| Rainbow trout | Presmolt | 2285.00 | 2713.33 | 1799.22 | 328.49 | b | 790.0–7500.0 | 790.0–7500.0 | Kruskal-Wallis, Dunn | | | | |
| | Smolt | 3939.00 | 3890.86 | 2372.30 | 199.78 | a | 597.90–9265.55 | 351.80–12,833.83 | Kruskal-Wallis, Dunn | | | | |
| | Postsmolt | 3306.85 | 3443.51 | 1808.86 | 137.92 | a | 578.61–7795.09 | 347.0–8608.0 | Kruskal-Wallis, Dunn | | | | |
| | Adult | 4206.60 | 4258.64 | 2108.70 | 274.53 | a | 651.70–8941.30 | 189.0–8948.60 | Kruskal-Wallis, Dunn | | | | |
| Coho salmon | Smolt | 775.00 | 781.61 | 284.63 | 25.46 | a | 194.81–1324.83 | 87.63–1424.18 | Kruskal-Wallis, Dunn | | | | |
| | Postsmolt | 619.50 | 686.95 | 301.19 | 28.21 | b | 330.0–1572.38 | 292.0–1626.0 | Kruskal-Wallis, Dunn | | | | |
| | Adult | 848.00 | 823.44 | 309.42 | 19.23 | c | 181.70–1405.37 | 113.53–1465.71 | Kruskal-Wallis, Dunn | | | | |
| Lactate dehydrogenase | LDH | U/L | Atlantic salmon | Presmolt | 980.00 | 1007.72 | 248.13 | 25.46 | b | 492.44–1488.65 | 407.86–1571.51 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 1018.00 | 1122.48 | 446.28 | 39.76 | b | 431.75–2342.0 | 296.25–2496.50 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 873.00 | 869.34 | 364.41 | 17.03 | a | 131.86–1564.84 | 60.42–1629.83 | Kruskal-Wallis, Dunn | |
| | | | Rainbow trout | Adult | 952.00 | 954.57 | 309.14 | 17.97 | c | 343.34–1559.91 | 258.42–1639.88 | Kruskal-Wallis, Dunn | |
| | | | | Presmolt | 1130.00 | 1215.77 | 539.96 | 105.89 | c | 400.0–2140.0 | 400.0–2140.0 | Kruskal-Wallis, Dunn | |
| | | | | Smolt | 733.00 | 835.04 | 392.65 | 27.03 | a | 65.46–1604.61 | 0.87–1669.21 | Kruskal-Wallis, Dunn | |
| Postsmolt | 615.00 | 696.93 | 342.99 | 23.45 | a | 233.13–1615.0 | 174.0–1788.38 | Kruskal-Wallis, Dunn | | | | | |
| Adult | 870.00 | 882.25 | 308.17 | 23.03 | d | 219.87–1440.16 | 155.0–1529.68 | Kruskal-Wallis, Dunn | | | | | |

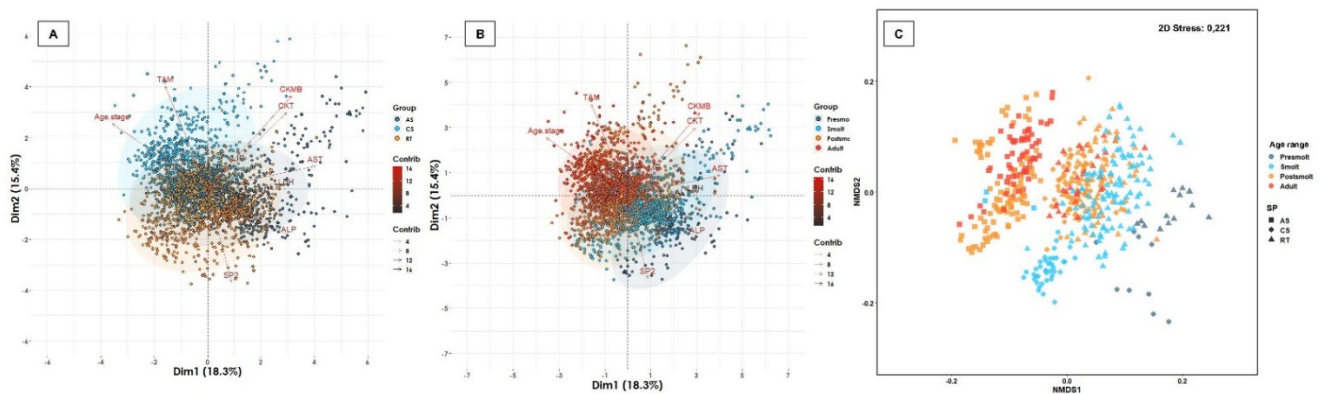


Figure 4. Multivariate analysis of plasma enzymes biomarkers. Salmonid species and range contribute significantly to the total variation of the plasma enzymes profile. (A) Spatial sorting of the plasma enzymes according to salmonid species and (B) age ranges. The two-dimensional analysis captures 33.7% of the total variance of the plasma substrate biomarkers profile. CKT, CK-MB and AST contribute significantly to the total variance of the plasma enzymes in dimension 1, while the ALP and TAM are the biomarkers that contributes the most to dimension 2. A cluster was observed between ALP, AST and LDH, and TAM, CK-MB, CKT, LIP and ALT. (C) The multivariate analysis of interdependence of the plasma enzymes profile shows significant differences between salmonid species ($R_{ANOSIM} = 0.2109$; $p = 0.0001$), suggesting a uniform distribution between Atlantic salmon and rainbow trout, but a different distribution in coho salmon. Concurrently, no significant differences were observed between age ranges ($R_{ANOSIM} = 0.0771$; $p = 0.083$), suggesting a similar distribution of plasma enzymes between freshwater- and seawater-reared fish.

3.5. Plasma Electrolytes and Minerals, Cortisol and Blood Gases

All plasma minerals analyzed demonstrated a normal distribution (e.g., Ca, P, Mg and Fe) (Table 6, Figure S13), whereas plasma electrolytes in coho and Atlantic salmon (e.g., Na, K and Cl) and COR did not have a normal distribution (Table 6, Figure S13). Biomarkers of blood gasometry and COR did not demonstrate a normal distribution (Table 6, Figure S13). While the median plasma cortisol concentration was similar in the smolts of the three salmonid species (34.69, 39.28 and 39.17 ng/mL in coho salmon, Atlantic salmon and rainbow trout, respectively), the median cortisol was significantly different in postsmolts among salmonid species (77.65, 55.60 and 43.26 ng/mL in coho salmon, Atlantic salmon and rainbow trout, respectively). In adult fish, median plasma cortisol was similar in coho salmon and rainbow trout (30.90 and 30.33 ng/mL, respectively), but significantly higher in Atlantic salmon (59.10 ng/mL) (Table 6). The highest plasma Na and Cl concentrations were observed in postsmolt and adult Atlantic salmon (Figures S14 and S15). A positive association between Na, Cl, Mg, Fe, K and Ca ($r < 0.20$; $p < 0.05$) and a negative association between Cl and K was observed ($r > -0.20$; $p < 0.05$) (Figure S14). Interestingly, a significant positive association between COR with Na and with Cl was noted only in Atlantic salmon ($r < 0.20$; $p < 0.05$) and essentially between freshwater and saltwater age ranges (Figure S15). Biomarkers of blood gasometry did not show a normal distribution (Table 6, Figure S16). Likewise, the highest $p\text{CO}_2$, HCO_3^- and Mg concentrations were recorded in presmolt and smolt regardless salmonid species (Figure S17). A positive association between HCO_3^- and $p\text{CO}_2$ was detected in all salmonid species ($r < 0.20$; $p < 0.05$) and a negative association between pH and $p\text{CO}_2$ especially in coho salmon ($r > -0.20$; $p < 0.05$) (Figure S17). Likewise, pH demonstrated a positive significant association with HCO_3^- , but a negative significant association with $p\text{CO}_2$ in presmolt and smolt (Figure S18). Table 6. Reference intervals (RIs) for plasma electrolytes, minerals, cortisol, and blood gases parameters in presmolt and smolt, postsmolt and adult Atlantic salmon, coho salmon and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

Table 6. Reference intervals (RIs) for plasma minerals and blood gasometry biomarkers and cortisol in presmolt and smolt (freshwater) and postsmolt and adult (seawater) of Atlantic salmon, coho salmon, and rainbow trout reared in Chile. The respective confidence intervals (CIs) for the respective RIs are included. Letters indicate significant differences between age ranges ($p < 0.05$).

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-----------|--------------|-----------------|-----------------|-------------|-----------|--------|---------------|---------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|
| Sodium | Na | mmol/L | Coho salmon | Smolt | 159.00 | 158.17 | 6.17 | 0.41 | c | 146.47–171.04 | 145.12–172.25 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 171.05 | 170.99 | 4.16 | 0.39 | a | 162.67–179.22 | 160.93–181.0 | | |
| | | | | Adult | 166.00 | 164.66 | 9.26 | 0.54 | b | 147.67–184.12 | 145.97–185.55 | | |
| | | | Atlantic salmon | Presmolt | 160.00 | 159.63 | 6.57 | 0.48 | a | 147.05–173.05 | 144.74–175.17 | | |
| | | | | Smolt | 170.00 | 167.33 | 8.83 | 0.48 | b | 150.02–186.37 | 148.58–187.42 | | |
| | | | | Postsmolt | 173.00 | 173.32 | 10.36 | 0.47 | c | 153.43–194.23 | 152.04–195.49 | | |
| | | | Rainbow trout | Adult | 180.00 | 176.93 | 10.62 | 0.57 | d | 158.55–202.07 | 156.05–203.90 | | |
| | | | | Presmolt | 179.90 | 179.40 | 3.30 | 0.62 | a | 173.09–187.06 | 170.64–188.46 | | |
| | | | | Smolt | 157.00 | 156.01 | 10.45 | 0.63 | c | 135.89–177.28 | 133.89–179.17 | | |
| | | | | Postsmolt | 156.00 | 156.12 | 13.10 | 0.85 | cb | 130.37–182.11 | 127.89–184.51 | | |
| | | | | Adult | 168.00 | 164.61 | 12.61 | 0.87 | b | 142.03–193.58 | 138.60–197.61 | | |
| | | | | Smolt | 3.40 | 4.08 | 2.69 | 0.18 | b | 1.17–10.49 | 1.0–12.30 | | |
| Potassium | K | mmol/L | Coho salmon | Postsmolt | 0.99 | 1.17 | 0.72 | 0.07 | c | 0.53–3.85 | 0.45–5.21 | Kruskal-Wallis, Dunn | |
| | | | | Adult | 4.40 | 5.02 | 2.86 | 0.17 | a | 1.20–13.28 | 1.10–14.26 | | |
| | | | | Presmolt | 3.70 | 3.90 | 1.95 | 0.14 | b | 0.83–8.76 | 0.45–9.29 | | |
| | | | Atlantic salmon | Smolt | 3.60 | 3.69 | 1.48 | 0.08 | b | 0.59–6.45 | 0.29–6.75 | | |
| | | | | Postsmolt | 3.00 | 3.37 | 1.89 | 0.09 | a | 0.87–8.32 | 0.80–9.60 | | |
| | | | | Adult | 4.30 | 4.50 | 2.25 | 0.12 | c | 1.07–9.73 | 0.90–10.60 | | |
| | | | Rainbow trout | Presmolt | 2.80 | 2.78 | 1.23 | 0.23 | bc | 0.76–6.02 | 0.76–6.02 | | |
| | | | | Smolt | 2.30 | 2.81 | 1.95 | 0.12 | c | 0.70–8.57 | 0.70–8.88 | | |
| | | | | Postsmolt | 3.90 | 4.29 | 2.06 | 0.13 | a | 1.20–9.50 | 1.10–10.28 | | |
| | | | | Adult | 3.70 | 3.62 | 1.58 | 0.11 | b | 0.80–8.17 | 0.70–8.80 | | |
| | | | | Smolt | 130.85 | 130.38 | 11.12 | 0.73 | c | 108.90–152.87 | 106.78–154.61 | | |
| | | | | Coho salmon | Postsmolt | 141.25 | 141.92 | 4.38 | 0.41 | a | 132.66–150.27 | | |
| Adult | 139.25 | 134.26 | 13.13 | | 0.75 | b | 109.23–164.61 | 106.51–166.66 | | | | | |
| Presmolt | 119.00 | 120.09 | 6.91 | | 0.50 | a | 105.85–133.50 | 104.30–135.09 | | | | | |
| Chloride | Cl | mmol/L | Atlantic salmon | Smolt | 125.70 | 127.38 | 12.08 | 0.67 | a | 101.58–149.61 | 99.85–152.02 | Kruskal-Wallis, Dunn | |
| | | | | Postsmolt | 134.05 | 138.07 | 12.43 | 0.57 | c | 111.44–162.83 | 110.17–164.46 | | |

Table 6. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing | | | |
|---------------|--------------|-----------------|-----------------|-------------|--------|-------------|-----------|------------|--------------------------|------------------------|---------------------------|----------------------|-----------------|--------------|--------------|---------------|
| Calcium | Ca | mmol/L | Rainbow trout | Adult | 143.10 | 143.17 | 12.57 | 0.67 | d | 118.62–168.17 | 116.8–169.81 | Kruskal-Wallis, Dunn | | | | |
| | | | | Presmolt | 141.80 | 142.16 | 2.04 | 0.39 | a | 138.16–146.16 | 137.12–147.21 | ANOVA, Tukey | BOX COX | | | |
| | | | | Smolt | 120.60 | 122.58 | 12.47 | 0.76 | c | 97.30–147.14 | 94.96–149.08 | ANOVA, Tukey | BOX COX | | | |
| | | | | Postsmolt | 119.00 | 120.26 | 9.23 | 0.60 | c | 101.03–137.83 | 99.28–139.80 | ANOVA, Tukey | BOX COX | | | |
| | | | | Adult | 131.25 | 129.03 | 15.07 | 1.04 | b | 99.68–159.91 | 96.72–162.55 | ANOVA, Tukey | BOX COX | | | |
| | | | Coho salmon | Smolt | 8.75 | 8.91 | 1.58 | 0.21 | a | 5.66–12.08 | 5.14–12.64 | ANOVA, Tukey | Normal | | | |
| | | | | Postsmolt | 12.25 | 12.12 | 1.56 | 0.15 | c | 9.31–15.52 | 8.81–16.02 | ANOVA, Tukey | Normal | | | |
| | | | | Adult | 13.56 | 13.25 | 2.96 | 0.51 | b | 8.64–18.98 | 7.34–20.22 | ANOVA, Tukey | Normal | | | |
| | | | Atlantic salmon | Presmolt | 14.45 | 14.78 | 1.66 | 0.26 | a | 11.34–17.53 | 10.57–18.45 | ANOVA, Tukey | POWER BOX COX | | | |
| | | | | Smolt | 11.60 | 11.68 | 1.44 | 0.19 | b | 8.61–14.41 | 8.06–15.04 | ANOVA, Tukey | POWER BOX COX | | | |
| | | | | Postsmolt | 11.96 | 12.13 | 2.14 | 0.29 | b | 8.22–16.33 | 7.60–17.08 | ANOVA, Tukey | POWER BOX COX | | | |
| | | | | Adult | 12.09 | 12.04 | 1.40 | 0.26 | b | 9.09–14.87 | 8.16–15.69 | ANOVA, Tukey | POWER BOX COX | | | |
| Rainbow trout | Presmolt | 16.99 | | 15.78 | 2.83 | 0.57 | a | 9.73–22.72 | 7.89–24.36 | ANOVA, Tukey | BOX COX | | | | | |
| | Smolt | 13.32 | | 13.33 | 1.96 | 0.21 | b | 9.33–17.15 | 8.83–17.80 | ANOVA, Tukey | BOX COX | | | | | |
| Magnesium | Mg | mmol/L | | Coho salmon | Smolt | 4.60 | 4.56 | 1.12 | 0.15 | a | 2.26–6.81 | 1.89–7.22 | ANOVA, Tukey | BOX COX | | |
| | | | Postsmolt | | 1.31 | 1.33 | 0.32 | 0.03 | b | 0.68–1.97 | 0.60–2.05 | ANOVA, Tukey | BOX COX | | | |
| | | | Adult | | 1.06 | 1.15 | 0.26 | 0.04 | c | 0.51–1.63 | 0.39–1.80 | ANOVA, Tukey | BOX COX | | | |
| | | | Atlantic salmon | Presmolt | 3.46 | 3.56 | 0.61 | 0.11 | b | 2.17–4.71 | 1.73–5.14 | ANOVA, Tukey | BOX COX | | | |
| | | | | Smolt | 6.25 | 5.96 | 1.12 | 0.15 | a | 3.70–8.39 | 3.27–8.77 | ANOVA, Tukey | BOX COX | | | |
| | | | | Postsmolt | 5.36 | 5.75 | 1.45 | 0.22 | a | 2.58–8.75 | 1.59–9.43 | ANOVA, Tukey | BOX COX | | | |
| | | | | Adult | 2.94 | 3.24 | 0.81 | 0.15 | b | 1.40–5.02 | 0.99–5.44 | ANOVA, Tukey | BOX COX | | | |
| | | | Rainbow trout | Presmolt | 3.70 | 3.81 | 0.85 | 0.16 | a | 2.0–5.62 | 1.66–5.98 | ANOVA, Tukey | BOX COX | | | |
| | | | | Smolt | 3.31 | 3.33 | 0.44 | 0.05 | b | 2.43–4.21 | 2.26–4.36 | ANOVA, Tukey | BOX COX | | | |
| | | | | Postsmolt | 1.57 | 1.57 | 0.23 | 0.04 | c | 1.10–2.05 | 0.97–2.17 | ANOVA, Tukey | BOX COX | | | |
| | | | Iron | Fe | μmol/L | Coho salmon | Smolt | 72.50 | 108.86 | 73.56 | 10.62 | a | 26.95–244.50 | 26.50–244.40 | ANOVA, Tukey | POWER BOX COX |
| | | | | | | | Postsmolt | 58.30 | 64.27 | 25.30 | 2.41 | c | 25.12–131.99 | 17.30–170.0 | ANOVA, Tukey | POWER BOX COX |

Table 6. Cont.

| Parameter | Abbreviation | Unit of Measure | Species | Age Stage | Median | Mean | S. D | S. E | Difference between Means | 95% Reference Interval | Confidence Interval (95%) | Test | Data Processing |
|-------------------------------|-------------------------------|-----------------|-----------------|-----------|--------|--------|-------------|-------------|--------------------------|------------------------|---------------------------|--------------|-----------------|
| Phosphorus | P | mmol/L | Atlantic salmon | Adult | 103.00 | 106.99 | 30.76 | 5.20 | b | 45.58–169.08 | 31.28–186.60 | ANOVA, Tukey | POWER BOX COX |
| | | | | Presmolt | 125.15 | 122.42 | 26.37 | 4.81 | c | 87.09–159.23 | 76.95–171.46 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 114.50 | 119.62 | 31.60 | 4.08 | b | 51.04–180.10 | 37.43–193.84 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 75.60 | 70.81 | 19.87 | 2.61 | a | 34.31–111.09 | 27.83–116.32 | ANOVA, Tukey | POWER BOX COX |
| | | | Rainbow trout | Presmolt | 6.93 | 8.19 | 4.26 | 0.78 | b | 1.97–17.09 | 1.97–17.09 | ANOVA, Tukey | Normal |
| | | | | Smolt | 13.45 | 13.43 | 4.06 | 0.44 | a | 5.37–21.59 | 4.02–22.97 | ANOVA, Tukey | Normal |
| | | | | Postsmolt | 10.67 | 11.34 | 3.07 | 0.57 | a | 4.58–17.72 | 2.85–19.49 | ANOVA, Tukey | Normal |
| | | | Coho salmon | Smolt | 29.00 | 29.38 | 3.65 | 0.48 | a | 21.90–36.76 | 20.77–38.09 | ANOVA, Tukey | sqrt(x) |
| | | | | Postsmolt | 16.39 | 16.71 | 2.35 | 0.22 | c | 11.90–21.34 | 11.32–21.96 | ANOVA, Tukey | sqrt(x) |
| | | | | Adult | 20.86 | 20.11 | 3.42 | 0.59 | b | 13.05–27.53 | 11.29–28.99 | ANOVA, Tukey | sqrt(x) |
| | | | Atlantic salmon | Presmolt | 21.58 | 21.37 | 2.85 | 0.37 | c | 15.86–27.40 | 14.65–28.32 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 28.25 | 26.67 | 7.45 | 1.01 | b | 11.54–42.48 | 8.83–45.66 | ANOVA, Tukey | POWER BOX COX |
| | | | | Postsmolt | 30.39 | 30.79 | 3.14 | 0.41 | a | 24.01–36.78 | 22.92–38.16 | ANOVA, Tukey | POWER BOX COX |
| | | | | Adult | 11.72 | 11.69 | 1.81 | 0.33 | d | 7.87–15.36 | 6.65–16.34 | ANOVA, Tukey | POWER BOX COX |
| | | | Rainbow trout | Presmolt | 23.15 | 23.86 | 3.68 | 0.67 | a | 15.85–31.51 | 13.91–33.41 | ANOVA, Tukey | POWER BOX COX |
| | | | | Smolt | 21.28 | 21.85 | 4.35 | 0.46 | b | 12.69–30.22 | 11.22–31.78 | ANOVA, Tukey | POWER BOX COX |
| Postsmolt | 24.68 | 25.07 | | 3.17 | 0.58 | a | 18.29–31.59 | 16.75–33.38 | ANOVA, Tukey | POWER BOX COX | | | |
| Bicarbonate ion concentration | HCO ₃ ⁻ | mmol/L | Coho salmon | Postsmolt | 7.43 | 7.43 | 1.12 | 0.14 | NA | 5.15–9.65 | 4.78–10.05 | NA | |
| | | | Atlantic salmon | Presmolt | 10.71 | 10.86 | 1.37 | 0.18 | a | 7.86–13.44 | 7.21–14.12 | ANOVA, Tukey | log(x + 1) |
| | | | | Postsmolt | 8.41 | 8.53 | 0.90 | 0.13 | b | 6.60–10.28 | 6.31–10.69 | ANOVA, Tukey | log(x + 1) |
| | | | Rainbow trout | Presmolt | 10.92 | 11.24 | 1.39 | 0.26 | a | 8.17–14.15 | 7.57–14.89 | ANOVA, Tukey | Normal |
| | | | | Smolt | 7.72 | 7.61 | 1.35 | 0.26 | b | 4.86–10.55 | 4.01–11.35 | ANOVA, Tukey | Normal |

The 36.4% of the total variability of the plasma electrolytes and minerals profile was captured by two-dimensional analysis both in salmonid species and age ranges (Figure 5), mostly driven by a cluster between Na and Cl, and Mg and P. On the other hand, the 70.8% of the total variability of the blood gasometry profile was captured by two-dimensional analysis considering salmonid species and age ranges (Figure 6). The plasma electrolytes and minerals profile demonstrated significant differences between salmonid species ($R_{ANOSIM} = 0.4227$; $p = 0.0001$), attributed particularly by rainbow trout because the electrolytes and minerals profile in Atlantic salmon and coho salmon demonstrated a high degree of similarity. Furthermore, significant differences between age ranges were found ($R_{ANOSIM} = 0.1997$; $p = 0.0001$), demonstrating an appreciably different distribution between freshwater and seawater-farmed fish (Figure 5). Blood gasometry biomarkers were significantly different between salmonid species ($R_{ANOSIM} = 0.2039$; $p = 0.0001$) and age ranges ($R_{ANOSIM} = 0.5876$; $p = 0.0001$), suggesting a considerably different distribution of the gases profile between freshwater and seawater-farmed fish (Figure 6). In general, the Na and Cl concentration increases during the smolt and post-smolt stages associated with the physiological preparation of fish for transfer to seawater commanded by cortisol and gas concentration decreases as fish are cultured in seawater. The distributions of all blood biomarkers and the correlograms based on salmonid species and age ranges are proved in Supplementary Figures S1–S18.

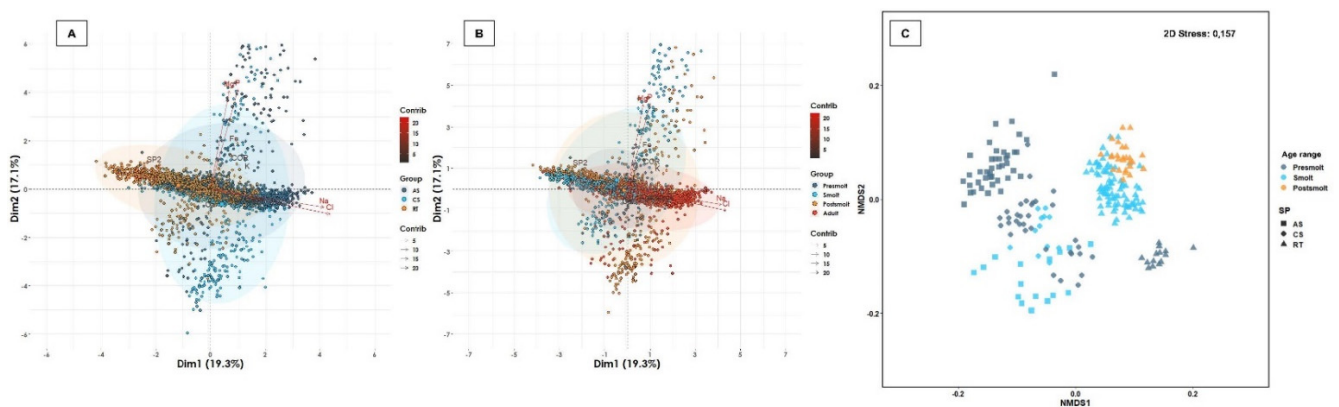


Figure 5. Multivariate analysis of plasma electrolytes and minerals biomarkers. Salmonid species and range contribute significantly to the total variation of the plasma electrolytes and minerals profile. (A) Spatial sorting of the plasma electrolytes and minerals according to salmonid species and (B) age ranges. The two-dimensional analysis captures 36.4% of the total variance of the plasma substrate biomarkers profile. Na and Cl contribute significantly to the total variance of the plasma enzymes in dimension 1, while the Na, Cl, P and Mg are the biomarkers that contribute the most to dimension 2. A cluster between Na and Cl, and Mg and P was detected. (C) The multivariate analysis of interdependence of the plasma electrolytes and minerals profile demonstrates significant differences between salmonid species ($R_{ANOSIM} = 0.4227$; $p = 0.0001$), suggesting a different distribution for each salmonid species. In addition, significant differences between age ranges were found ($R_{ANOSIM} = 0.1997$; $p = 0.0001$), suggesting a very different distribution of plasma electrolyte and mineral profile between freshwater and seawater-farmed fish.

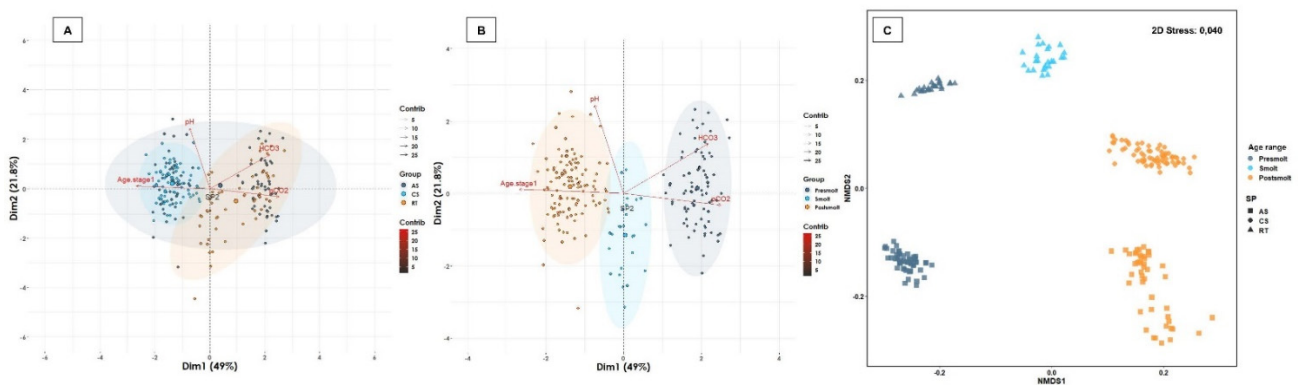


Figure 6. Multivariate analysis of blood gases. Salmonid species and range contribute significantly to the total variation of the blood gases profile. (A) Spatial sorting of the plasma electrolytes and minerals according to salmonid species and (B) age ranges. The two-dimensional analysis captures 70.8% of the total variance of the blood gases profile. HCO_3 and pCO_2 contribute significantly to the total variance of the blood gases in dimension 1, while the pH is the biomarker that contributes the most to dimension 2. A cluster between HCO_3 and pCO_2 , and pH was detected. (C) The multivariate analysis of interdependence of the blood gases profile demonstrates significant differences between salmonid species ($R_{\text{ANOSIM}} = 0.2039$; $p = 0.0001$), suggesting a uniform distribution between Atlantic salmon and rainbow trout, but a different distribution in coho salmon. In addition, significant differences between age ranges were found ($R_{\text{ANOSIM}} = 0.5876$; $p = 0.0001$), proposing a different distribution of blood gasometry profile between freshwater and seawater-farmed fish.

4. Discussion

The mission of veterinary clinical pathology is to support the diagnostic process by using tests to measure different blood biomarkers to support decision making about farmed fish health and welfare. Consequently, here we report for the first time and with an integrated approach the RIs for 44 different blood biomarkers only from healthy individuals of the three salmonid species and age ranges farmed in Chile. Forty-one of the 44 blood biomarkers analyzed in this study changed significantly with age range (and consequently with salinity), salmonid species and/or their interaction. Taken together, our results confirm that fish growth over the production cycle, and especially the change from freshwater (smolt) to seawater environment (post-smolt and adult), is especially critical in the differences found in the different blood biomarker profiles. In particular, the smolt stage, where one of the major physiological, morphological and behavioral changes in anadromous salmonid species occur, is the turning point for several of the biomarkers.

Blood biochemistry is based on the quantification of different elements such as enzymes, substrates, minerals, electrolytes, hormones, among others. The methods used in mammals have been adapted for aquatic animals, but the interpretation of the results may be different since they are directly or indirectly influenced by intrinsic (e.g., species, age range, sex, nutritional and reproductive status, etc.) and extrinsic factors (e.g., stress, environmental conditions, population density, catching and sampling methods, etc.). This variability supports the need to estimate RIs with respect to the normality of the indicators in fish under productive conditions, according, at least, to the species and age range. An RI corresponds to a range within which the values of a biological variable are found in the majority of individuals (95%) of a clinically healthy population [48]. Population-based RI is one of the most widely used laboratory tools in the clinical decision-making process. Technically, each laboratory must generate its own RIs according to its specific pre-analytical, analytical and post-analytical procedures, but high costs are a critical limitation to its practical implementation. Although the analytical phase of clinical biochemistry is usually well controlled in laboratories, it is known that pre-analytical technical variables can influence analyte concentrations or activities.

Although there are some reports that can be compared, at least in part, with our study in different species of salmonids [19–21,25,29–31,42,46,50–53], our study is the first one that has incorporated the greatest biological variability for the estimation of RIs. The lack of a standardized protocol for blood collection and processing prior to analysis has historically limited the practical application of blood biochemistry in clinical diagnosis and disease monitoring in aquaculture, as it has generally resulted in the generation of RIs that are too wide between the maximum and minimum range, even those estimated from healthy fish. There are some studies that have investigated a range of pre-analytical treatments such as tube type, time to obtain serum or plasma, storage time and temperature, and freeze–thaw cycles, sampling methods, among others, to examine their effect on analyte concentrations and activities [6,30,46]. However, the standardization of protocols has not been an easy task, because salmonid species have also demonstrated high individual variation in blood biomarkers, and each laboratory calculates RIs based on the fish samples that come into the laboratory precisely for analysis; therefore, they are often not completely healthy. To this analysis we must add logistical and operational variables related to the fact that salmon farms are often geographically distant from established clinical laboratories and not all have the same practical conditions, starting with trained personnel, for the execution of pre-analytical protocols under strictly equal conditions.

Although previous studies have demonstrated that many preanalytical technical treatments lead to statistically significant differences in Atlantic salmon, often not large enough to be clinically significant [46], clinical practice demonstrates the opposite when RIs are calculated considering a representative biological variability. For example, our results for adult Atlantic salmon demonstrate differences from the RIs described by [46] for almost all enzymes, substrates and minerals. Thus, while our RIs were broader for LIP, PTO, ALB, GLO, Na, Cl, K, P, Mg and Ca, they were narrower for CRE, ALP, ALT, AST, CKT and LDH. That is, our RIs for substrates and minerals were consistently broader, while for enzymes they were consistently narrower. The only biomarkers that presented similar results were TAM, TCH and GLU. The variation in the results of some enzyme assays between laboratories could be related to the use of different methodologies, different manufacturers of diagnostic kits, among others, so each diagnostic laboratory should generate the RIs based on their own methods and equipment.

Blood biomarkers profiling in salmonids has been used most frequently in the characterization of infectious diseases, defining and quantifying systemic functional profiles such as liver, renal, cardiac, pancreatic and other functions [54–69]. Furthermore, the number and percentages of the different types of leukocytes present have been demonstrated to change in response to infection and stress in salmonids [29,30,70–80].

In fish, COR is the predominant glucocorticoid released as part of the primary stress response, and is critical in mediating metabolic, physiological and behavioral adaptive adjustments [81], which can impact health and welfare, and threaten aquaculture sustainability [82]. Recently, stress-induced cortisol production has been demonstrated to be associated with an altered gut microbiome in Atlantic salmon, specifically with a marked decrease in lactic acid bacteria *Carnobacterium* sp. and an increase in the abundance of *Clostridia* and *Gammaproteobacteria* [83]. Plasma cortisol concentration in farmed fish is a validated and recognized biomarker for monitoring fish welfare and health, but often exhibits high biological variability when an adequate and standardized sampling and sample collection protocol is not used. The plasma COR concentration in our study was significantly higher in seawater than in freshwater regardless of species, although the highest concentration was observed in Atlantic salmon. Overall, these values are slightly higher than those described in salmonids in experimental studies [84,85], included during the smoltification process [86], but are similar to those described in field conditions [30]. Under field conditions in Chile, higher plasma cortisol concentrations have been described in Atlantic salmon infested by parasitic copepod *Caligus rogercresseyi* [87].

The osmoregulatory process is essential for salmonid fish survival specially after seatransfer [88]. The concentration of Na and Cl increased with age range from freshwater

to seawater in Atlantic salmon, but the concentration of Mg decreased with age range from freshwater to seawater. These results are consistent with previous reports in Atlantic salmon, which are associated with the salinity challenge experienced by the fish post-entry to the sea [35]. Blood gasometry biomarkers ($p\text{CO}_2$, HCO_3 and pH) were found significantly higher in freshwater regardless of the salmonid species. Atlantic salmon farmed on RAS and exposed to CO_2 for 12 weeks demonstrated significantly increased blood pH, K, HCO_3 and PCO_2 and decreased plasma Na and Cl concentrations, suggesting that CO_2 concentrations below the 15 mg/L threshold continue to impact Atlantic salmon [89].

Our results demonstrated that MON and NEU counts and Hct were found to be significantly higher in seawater regardless of the salmonid species; the LYM counts, MCHC, MVC and Hgb were significantly higher in freshwater, also regardless of the species. In rainbow trout, RIs for red blood cell indices, substrates, serum enzymes and electrolytes for freshwater farmed fish have been established [29]. PCA revealed that certain serum components were effectively differentiated between fish life stages (92.7% of the variance) than hematological principal components (80% of the variance). On contrary, our results demonstrated that while leukogram and erythrogram biomarkers captured between 62.4 and 85.1% of the variance, respectively, the blood biochemistry biomarkers captured between 46.3% to 77.1% of the variance. PTO, ALB, GLO and BTI in Atlantic salmon, DBI in rainbow trout, and NH_3 in coho salmon were higher in seawater, but TCH and TGR were higher in Atlantic salmon and rainbow trout during the freshwater stage. This is interesting, given that TCH is crucial for the biosynthesis of steroid hormones such as cortisol [90], and has also been demonstrated to increase both the enthalpy and entropy of activation for Na^+ K^+ enzyme activity during the Atlantic salmon smoltification process [91]. The interaction between TCH and COR is especially important in the context of Atlantic salmon seawater adaptation [92].

Regarding the plasma enzymes, ALP was found to be significantly lower in coho salmon regardless of the age range. ALT, AST and CK-MB were found significantly higher in freshwater regardless of the salmonid species, but TAM and LIP were observed at higher levels in seawater. There are several studies reporting that body size influences blood biomarkers in different salmonid species [21,26,29,30], and there are even findings showing that in rainbow trout farmed in Italy the values of RBC, Hct, TCH and TPO were significantly lower than in Turkish trout, although no significant differences were found for WBC and Hgb [93]. In fact, it has recently been reported that between 1 and 13% of 80 Atlantic salmon farms tested during a seawater grow-out between 2017 and 2019 in Scotland demonstrated suspected anemia or clinical anemia, suggesting an eventual association with blood loss from the gills [94].

5. Conclusions

RIs for salmonid species- and age-range-specific blood biomarkers are essential to help improve clinical, husbandry and nutritional management for farmed fish health and welfare. Our results indicate that variability in most blood biomarkers depends on salmonid species, age range and/or interaction between them, but they are often biologically related to each other. The establishment of the RIs in this study is valuable, not only because little work has been conducted previously in farmed salmonid species, but also because no previous work has presented normal RIs for 44 blood biomarkers considering more than 3000 individuals of different age ranges and from three different species with high commercial value that came from 78 different farms. Our study provides the Chilean salmon industry with standardized protocols for routinely sampling their fish populations, since a standardized system for sampling methods and sample processing is essential for implementing comprehensive fish health monitoring. To our knowledge, this is the reference interval study for blood biomarkers that has been performed with the highest number of fish and farms of different salmonid species and age ranges using the same pre-analytical protocol and described comprehensively in the same report. The practical application of these blood biomarkers under a preventive medicine vision contributes

concretely to the technical support of strategic decision making that optimizes the health and husbandry management of salmon farmers, veterinarians and health managers.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biology11071066/s1>, Figure S1. Histograms represent the count of erythrogram biomarker observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for erythrogram biomarkers. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S2. Correlogram between the different erythrogram biomarkers according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between Hct, Hgb, RBC, MCV, MHCH in Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S3. Correlogram between the different erythrogram biomarkers according to age ranges. Pearson correlation coefficient (r) and p -value (p) between Hct, Hgb, RBC, MCV, MHCH in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S4. Histograms represent the count of leukogram biomarker observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for leukogram biomarkers. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S5. Correlogram between the different leukogram biomarkers according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between WBC, LYM, NEU, MON, and TCC in Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S6. Correlogram between the different leukogram biomarkers according to age ranges. Pearson correlation coefficient (r) and p -value (p) between WBC, LYM, NEU, MON, and TCC in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S7. Histograms represent the count of plasma substrates biomarkers observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for plasma substrates biomarkers. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S8. Correlogram between the different plasma substrates biomarkers according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between TPO, ALB, GLO, TBI, DBI, CRE, GLU, LAC, NH₃, URE, AUC, TCH, TRG, HDL, LDL, and COR in Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S9. Correlogram between the different plasma substrates biomarkers according to age ranges. Pearson correlation coefficient (r) and p -value (p) between TPO, ALB, GLO, TBI, DBI, CRE, GLU, LAC, NH₃, URE, AUC, TCH, TRG, HDL, LDL, and COR in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S10. Histograms represent the count of plasma enzymes biomarkers observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for plasma enzymes biomarkers. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S11. Correlogram between the different plasma enzymes biomarkers according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between ALP, ALT, AST, TAM, LIP, CKT, CKMB, and LDH in Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S12. Correlogram between the different plasma enzymes biomarkers according to age ranges. Pearson correlation coefficient (r) and p -value (p) between ALP, ALT, AST, TAM, LIP, CKT, CKMB, and LDH in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S13. Histograms represent the count of plasma electrolytes and minerals biomarkers observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for plasma electrolytes and minerals biomarkers. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S14. Correlogram between the different plasma electrolytes and minerals biomarkers according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between Na, K, Cl, Ca, Mg, Fe, and P in Atlantic salmon (AS), coho salmon (CS) and rainbow

trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S15. Correlogram between the different plasma electrolytes and minerals biomarkers according to age ranges. Pearson correlation coefficient (r) and p -value (p) between Na, K, Cl, Ca, Mg, Fe, and P in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S16. Histograms represent the count of blood gases observations and density plots show the distribution of data by estimating kernel density among salmonid species and age ranges. Box plots show the overall IRs (black lines) for blood gases. Dashed black lines show CIs for both the maximum and minimum range of RIs. AS: Atlantic salmon; CS: Coho salmon; RT: Rainbow trout. Figure S17. Correlogram between the different blood gases according to salmonid species. Pearson correlation coefficient (r) and p -value (p) between HCO_3 , pCO_2 , and pH in Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie. Figure S18. Correlogram between the different blood gases according to age ranges. Pearson correlation coefficient (r) and p -value (p) between HCO_3 , pCO_2 , and pH in presmolt, smolt, postsmolt and adult from Atlantic salmon (AS), coho salmon (CS) and rainbow trout (RT) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). SP: Specie.

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Institutional Review Board Statement: The ethical review and approval of this study was waived because the Executive Council of the Aquaculture Management Program (PGSA), financed by the Economic Investment Fund (FIE), determined that the study was conducted on salmonid species intensively farmed on a commercial scale (2015-V014). Therefore, the fish were all healthy and being farmed under the husbandry, health and animal welfare protocols of each of the salmon producing companies in Chile, which have the certifications of the Aquaculture Stewardship Council (ASC) and Best Aquaculture Practices (BAP), among others; and all of them also belong to the Association of Salmon Producers of Chile (SalmonChile A.G.) or the Salmon Council Association, which also define good farming practices for their associates. The research team of the present study was limited to collecting the fish and taking the samples in each farm but did not intervene in the productive management of the animals, since the objective of the study was precisely the evaluation of blood biomarkers in field conditions and in healthy fish.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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