# MANUAL
# OF CLINICAL PATHOLOGY IN SALMONIDS

SECOND EDITION

EDITED BY
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2020

Project financed by

PROGRAMA PARA LA GESTIÓN SANITARIA EN LA ACUICULTURA

Executed by

pathovet® LABORATORIO

Universidad Austral de Chile
Campus Patagonia

Supported by

SalmonChile

SERNAPESCA
Ministerio de Economía, Fomento y Turismo
Gobierno de Chile

Design, printing and translation sponsored by*

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# Contents

# Foreword

*M.A. Rozas-Serri*

Veterinary clinical pathology is a specialization of veterinary medicine that is dedicated to diagnostic support using laboratory tests on the blood and other body fluids of animals, including fish, providing necessary tools for decision-making in the diagnostic plan, treatment, control and prognosis of the population of animals being farmed.

Blood is a tissue made up of a liquid matrix called plasma, in which cells in suspension and proteins, glucose, ions, minerals, hormones and carbon dioxide in solution are found. Blood is distributed in all the tissues and organs of the organism through the vascular system; therefore, the presence or absence of specific components of the tissues in the blood allows to determine potential alterations in them. The total volume of blood in animals varies from 5% to 10% of body weight.

Erythrocytes are the predominant blood cells in the vast majority of fish species. One of the most important functions of erythrocytes is to take oxygen to the tissues and carbon dioxide to the gills for being eliminated. Leukocytes are the fundamental components of the immune response of fish, so they are used as indicators of health status. Thrombocytes or platelets are involved in the blood coagulation process. Unlike mammals, fish erythrocytes and thrombocytes are nucleated cells.

Hematology is the study of blood and its components, a health specialization that is becoming increasingly important in aquaculture due to its high value as a physiopathological indicator for monitoring the health status of the population of farmed fish. Hematologic parameters are evaluated by a laboratory test called hemogram, which includes blood cell count and morphology, and hemoglobin concentration.

In salmonids, the most frequent hematological disorders are associated with infectious diseases, metabolic diseases and stress. The alterations in the quantity of leukocytes and their differential count (lymphocytes, neutrophils, eosinophils, monocytes), represent important clinical indicators since they guide potential infectious causes, acute and/or chronic stress, among others.

Fish neutrophils exhibit myeloperoxidase activity in cytoplasmic granules, making them more similar to mammals' neutrophils than to bird and reptile heterophiles. Additionally, basophils have been identified only in 4 of 121 species of cartilaginous and teleost fish and 14 of 20 species of freshwater fish. In salmonids, basophils have not been described, but we also include them in this work for possible applications in other fish species.

On the other hand, 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 the analysis of fish; however, the interpretation of the results may be different since they are directly or indirectly influenced by different intrinsic factors (species, production stage, sex, nutritional and reproductive status) and extrinsic ones (environmental conditions, population density, catching and sampling method, among others).

This variability supports the need to estimate reference intervals (RI) with respect to the normality of the indicators in fish under productive conditions, according, at least, to the species and productive stage of the animals. A RI corresponds to a range within which the values of a biological variable are found in most individuals (95%) of a clinically healthy population.

Population-based RI is one of the most widely used laboratory tools in the clinical decision-making process. Technically, each laboratory should generate its own RI according to the specific technique and working conditions; however, the high costs are a critical limitation to implement it. Biochemical profiles in salmonids have been used more frequently in the characterization of infectious diseases, defining and quantifying profiles of systemic functionality such as liver, kidney, cardiac and pancreatic function, among others.

However, the spectrum of application of these indicators has been broadened towards the evaluation of the health status of farmed fish, with the aim of predictively quantifying the response of animals and, in this way, evaluating the efficacy of functional diets, vaccines and fish that are resistant to diseases. In this way, the application of these indicators with the vision of preventive medicine, specifically contributes to technically supporting strategic decision-making that optimizes the health and productive management of the industry.

An interesting contribution of this work is also the diffusion of RI for some parameters associated with acid-base status and blood gases in salmonids. These indicators must be measured immediately after obtaining the blood sample, which determines the application of portable devices or gasometers (Point-of-Care) capable of making fast measurements under field conditions. On the other hand, endocrinology in salmonid fish has had a significant impact on the understanding of the function and evolution of several neurochemical messengers and systems. In this way, the measurement of the concentration of different hormones in the blood is a quick tool to evaluate key physiopathological processes such as smoltification, stress and reproduction.

In this way, clinical laboratory tests are a fundamental tool in aquatic animal medicine, since they support both the diagnosis and the prognosis of farmed fish and their treatment. The objective of this Manual is to contribute to the knowledge, identification and interpretation of the most widely used clinical laboratory tests in salmon farming and to support the clinical diagnosis of our colleagues who daily develop their profession in aquatic animal medicine.

Finally, in addition to the theoretical and practical content, this Manual describes the RI of indicators of hematology, clinical biochemistry, blood gasometry and hormones for farmed salmonids, contributing to the development and transfer of an important public asset for the industry in general and for veterinarians in particular.

# CHAPTER 1

# Blood Sampling

# Blood Sampling

*V.F.D. Jaramillo, R.S. Ildefonso & M.A. Rozas-Serri*

Obtaining blood samples in fish can be done by lethal (euthanasia) or non-lethal (light sedation) sampling, depending on the size of the animals and the objective of the study. The use of anesthetic drugs by immersion in water is the most widely used method to induce euthanasia and mild sedation in salmonids, with the aim of minimizing the level of stress in fish during the procedure carried out to obtain the blood sample and preventing eventual alterations in the laboratory results.

Among the most widely used anesthetics in fish, we can mention benzocaine, metomidate, etomidate, phenoxyethanol, quinaldine, isoeugenol, tricaine methanesulfonate (MS-222) and eugenol. However, the most widely used anesthetic in fish is benzocaine at 20% at a dose of 15 to 20 mL (30 to 40 mg/L) for every 100 L of water. Fish should be kept in the anesthetic solution for 2 to 5 minutes depending on whether you are trying to induce deep sedation or euthanasia.

It is recommended to induce sedation in no more than two fish simultaneously and avoid excessive handling of the fish to minimize stress. To obtain a blood sample by lethal sampling, the complete absence of opercular movements in the fish must be first confirmed.

# Taking the blood sample

## Materials:

- Nitrile or latex gloves.
- Syringes and needles according to the size of the fish.
- Anesthetics.
- Tubes to arrange blood according to the objective of the study.
- Expanded polystyrene boxes or cooler to transport samples.
- Gelpack or ice to maintain the cold chain during the transport of samples to the laboratory.
- Disposable datalogger for temperature control during transport.
- Chemical disinfectant.

## Method:

- Blood collection must be aseptic, smooth and careful because it coagulates quickly and is highly susceptible to hemolysis.
- The fish must be taken from their respective farming units by trained personnel, avoiding excessive handling and placing them in containers with fresh water.
- Depending on the objective of the sampling, the fish should be selected in a random or directed way.
- The procedure for obtaining and analyzing blood samples involves two phases. The preanalytical phase that includes all activities from the selection and collection of fish at the farming site to the arrival of the blood samples at the analytical laboratory. The analytical phase considers the detection and quantification of the blood indicators and parameters of interest.
- Before obtaining the samples, the type of tube with or without anticoagulant should be selected according to the objective of the analysis (Table 1), their expiration date, the size of the fish and the size of the needles and syringes ( Table 2).

**Table 1.**
Blood collection tubes most used in clinical practice in aquamedicine.

| Lid Color | Additive and mechanism of action | Sample | Use | Tube |
|---|---|---|---|---|
| Red | Without anticoagulant | Serum | Biochemistry, Endocrinology | |
| Green | Heparin: thrombin inhibition | Whole blood, Plasma | Biochemistry, Hematology | |
| Purple | EDTA (ethylenediaminetetraacetic acid): forms calcium chelates | Whole Blood | Hematology | |
| Light Blue | Sodium citrate: forms calcium chelates | Plasma | Coagulation | |
| Grey | Sodium fluoride: forms calcium chelates | Plasma | Lactate Glucose | |

**Table 2.**
Considerations prior to obtaining the blood sample according to the size of the fish and after the selection of the analyzes to be carried out.

| Stage | Needles | Tube size |
|---|---|---|
| Alevins < 20 g | Capillary 27.5 G x 1/2" | 0.5 to 1 ml |
| Alevins > 20 g | 23 G x 1" 25 G x 5/8" | 1 ml |
| Smolts | 21 G x 1 1/2" 23 G x 1" | 1 to 2 ml |
| Adults | 21G x 11/2" | 2 to 4 ml |

Blood samples can be extracted by caudal vein puncture, intracardiac puncture, dorsal aortic puncture, and peduncle cut, depending on the objective of the analysis and the size of the animals.

## Caudal vein puncture:

- Place the fish on a lateral decubitus position on a non-slip surface.
- Induce light anesthesia only to reduce its movement and maintain the heart rate, in order to obtain enough quantity of blood.
- Insert the needle perpendicularly (Figure 1) or laterally (Figure 2), approximately 1 to 2 cm posterior to the pelvic fins.
- Insert the needle until the point of contact with the backbone.
- Gently withdraw the needle, remove it from the syringe and dispose of it in a container for sharp objects.
- Slowly empty the blood sample from the syringe using the inner wall of the collection tube.
- Complete the tube up to the mark indicated by the manufacturer, since the blood volume must agree with the amount of anticoagulant. Excess blood causes coagulation and a small amount causes erythrocyte crenation and sample dilution.
- If the tube has anticoagulant, shake it gently by inversion between 10 to 15 times until the correct homogenization is achieved.
- In the case of non-lethal sampling, immediately return the fish to a container with fresh water and monitor the recovery process.
- Place the tubes in an expanded polystyrene box with gelpack or ice and datalogger to transport the samples to the laboratory.

**Figure 1.** *Caudal vein puncture through a perpendicular approach in Atlantic salmon presmolts.*

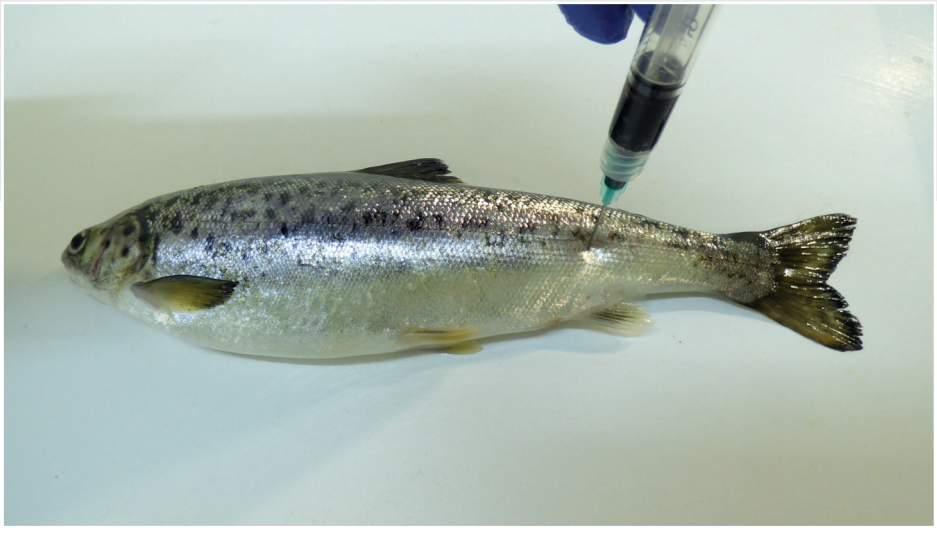


**Figure 2.** *Caudal vein puncture through a lateral approach in Atlantic salmon presmolts.*

## Cardiac puncture:

- Place the fish on a lateral decubitus position on a non-slip surface.
- Induce light anesthesia only to reduce its movement.
- Puncture the bulbus arteriosus or directly the heart, anatomically located between the gills and the thymus (Figure 3).
- Consider cardiac contractions at the time of sampling to avoid the needle from leaving the heart.
- Gently withdraw the needle, remove it from the syringe and dispose of it in a container for sharp objects.
- Slowly empty the blood sample from the syringe using the inner wall of the collection tube.
- Complete the tube up to the mark indicated by the manufacturer.
- If the tube has anticoagulant, shake it gently by inversion between 10 to 15 times until the correct homogenization is achieved.
- Place the tubes in an expanded polystyrene box with gelpack or ice and datalogger to transport the samples to the laboratory.
- If the procedure is performed according to protocol and the sampling is not lethal, the individuals can be immediately returned to the container with fresh water. However, handling can be lethal if irreversible damage to cardiac tissue is produced.

**Figure 3.** *Cardiac puncture by ventral approach in Atlantic salmon presmolts.*

## Dorsal aortic puncture:

- Place the fish on a dorsal decubitus position or supine on a non-slip surface.
- This method can be lethal or non-lethal, but the mortality risk of the handled fish is higher than the previously described methods.
- Induce light anesthesia only to reduce its movement or to induce euthanasia.
- Insert the needle at a 45° angle with the bevel pointing upwards, along the dorsal midline of the palate, just after the junction of the second branchial arch (Figure 4).
- Gently withdraw the needle, remove it from the syringe and dispose of it in a container for sharp objects.
- Slowly empty the blood sample from the syringe using the inner wall of the collection tube.
- Complete the tube up to the mark indicated by the manufacturer.
- If the tube has anticoagulant, shake it gently by inversion between 10 to 15 times until the correct homogenization is achieved.
- Place the tubes in an expanded polystyrene box with gelpack or ice and datalogger to transport the samples to the laboratory.

**Figure 4.** *Dorsal aortic puncture by mouth approach in Atlantic salmon presmolts.*

## Peduncle cut:

- This technique is used in small fish because it is not possible to obtain a blood sample by puncture. This procedure is always lethal. The amount of blood that can be obtained is small and comes in the form of a leak from the tail.
- Place the fish on a lateral decubitus position on a non-slip surface.
- Cut the peduncle area in the shape of a bevel and collect blood in heparinized capillaries by capillarity.
- Seal the capillary and refrigerate or arrange the tubes in an expanded polystyrene box with gelpack or ice and datalogger to transport the samples to the laboratory.

## BOX 1.

**The main considerations during the preanalytical phase or when obtaining, arranging and transporting the blood samples are: (1) Homogenization of the sample by gently shaking the tube 10 to 15 times; (2) Transport temperature by arranging the tubes with the samples in suitable containers to maintain a cold chain between 4 to 8°C and protection from direct sunlight; (3) Transport time to the laboratory according to the type of analysis to be performed: (i) Hematology from 12-24 h maximum; (ii) Non-centrifuged blood from 12-24 h maximum; (iii) Plasma/serum ideally refrigerated 24 h; (v) Frozen plasma/serum, it can be > 24 h.**

# CHAPTER 2

# Hematology in Salmonids

# Hematology in Salmonids

*R.Y. Walker-Vergara, V.C. Arnés, A. Müller, R.S. Ildefonso & M.A. Rozas-Serri*

A complete hemogram is a blood test used to assess general health status and detect a wide variety of diseases in fish, including anemias and infections. An abnormal increase or decrease in cell counts could indicate the presence of a disease (Table 1). In salmonids, the hemogram is routinely carried out manually because erythrocytes and thrombocytes are cells with nuclei, such as leukocytes.

## ERYTHROGRAM

The erythrogram or red series includes erythrocyte count and their morphological evaluation, hematocrit or Agglomerated Globular Volume (AGV), hemoglobin concentration (Hb) and Wintrobe's erythrocyte indeces – Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC) (Table 1).

### Erythrocyte count

Fish erythrocytes are oval to ellipsoidal with a centrally positioned oval to ellipsoidal basophilic nucleus and pale eosinophilic cytoplasm. Anisocytosis and mild polychromasia are normal findings in fish blood, that is, erythrocytes with rounder nuclei, less condensed chromatin, and more basophilic cytoplasm.

**Table 1.**
Hematological parameters of a complete hemogram.

| Erythrogram | Measurement unit | Function |
|---|---|---|
| Erythrocyte count (RBC) | x $10^6$ µL | Oxygen transport |
| Immature Erythrocytes or Reticulocytes | x $10^6$ µL | Immature red blood cells highly demanded in circulation in anemia cases |
| Hemoglobin (Hb) | g/L | Erythrocyte protein that transports oxygen |
| Mean Corpuscular Volume (MCV) | fL | Mean erythrocyte individual volume (size) |
| Mean Corpuscular Hemoglobin Concentration (MCHC) | g/L | Amount of HG that erythrocytes contain in a given volume |
| Hematocrit (AGV) | % | Erythrocyte ratio compared to blood plasma |

| Leukogram | Measurement unit | | Function |
|---|---|---|---|
| Leukocyte count (WBC) | cel/µL | | Cells executing the immune response |
| **Differential count** | **Absolute** | **Relative** | |
| Basophils | cel/µL | % | Inflammatory cells predominant in allergic reactions |
| Eosinophils | cel/µL | % | Inflammatory cells predominant in antiparasitic responses and allergic reactions |
| Neutrophils | cel/µL | % | Cell that is homologous to neutrophils in mammals and its function is predominantly phagocytic |
| Baciliforms | cel/µL | % | Immature heterophile that appears in periods of high demand |
| Lymphocytes | cel/µL | % | Enforcers of the humoral and cell-mediated immune response |
| Monocytes | cel/µL | % | They leave the circulation to become macrophages in the tissues, whose role is predominantly phagocytosis and the presentation of antigens to lymphocytes |
| **Thrombocytes** | cel/µL | | They participate in blood coagulation |
| Total Plasma Proteins (TPP) | g/L | | It considers the albumin that prevents liquid from escaping out of blood vessels, and globulins are an important part of the immune system |

Erythropoiesis is the process of formation of erythrocytes in hematopoietic tissue and can be affected by events of anemias, water temperature, seasonality, production stage, among other factors; directly influencing the quantity of circulating erythrocytes and reticulocytes or immature erythrocytes. Thus, immature erythrocyte count could be useful as an indicator of the efficacy of the regenerative response of hematopoietic tissue after a period of high systemic oxygen demand.

The total erythrocyte count in fish is determined using the manual method of the hemocytometer or count with Neubauer chamber, which is a quantitative study of the figurative elements of blood per volume unit (N° cells/µL). The main stain for the specific count of erythrocytes and leukocytes in fish blood is Natt-Herrick.

## Materials:

- Prepare a 1:200 dilution by adding 20 µL of blood in 3,980 mL of Natt-Herrick (Table 2).
- Neubauer count chamber or hemocytometer.

## Método:

- Arrange the stain in the Neubauer chamber.
- Let the cells settle for 5 minutes.
- Count cells using a light microscope with 400X magnification.
- Use the central square of the camera that is divided into 25 squares, of which 5 are counted (4 of the corners and the central one). Each of these is divided into 16 small squares (Figure 1).
- The number obtained is multiplied by 10,000 to calculate the total erythrocyte count per mL of blood. This factor is obtained from the formula: **EC = E x 5 x 10 x D**, where **EC**: total erythrocyte count per mm³ of blood; **E**: number of erythrocytes counted in the 5 squares; **D**: dilution factor.

**Table 2.**
Preparation of the Natt-Herrick solution.

| Reactive | Quantity |
|---|---|
| Sodium chloride [NaCl] | 3.88 g |
| Sodium sulfate [$NaSO_4$] | 2.50 g |
| Sodium phosphate [$Na_2HPO_4$] | 1.74 g |
| Potassium phosphate [$KH_2PO_4$] | 0.25 g |
| Formalin [37%] | 7.50 mL |
| Gentian violet | 0.10 g |

* Adjust pH to 7.3, add colorant and complete up to 1000 mL with distilled water. Filter through filter paper # 10.

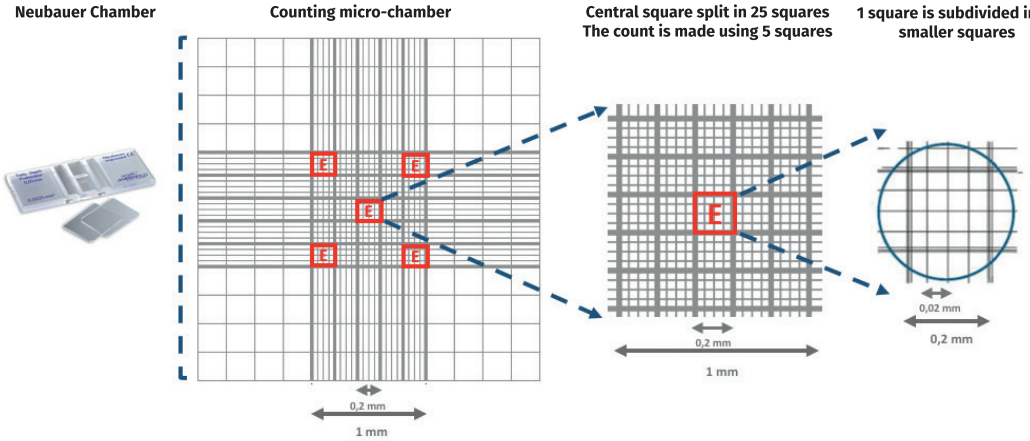



**Figure 1.** *General description of the method of erythrocyte count in Neubauer chamber (E: areas where the erythrocyte count is performed).*

## Hematocrit or AGV

The standard manual technique for determining AGV in fish is the use of micro-hematocrit capillary tubes and centrifugation.

## Materials:

- 75 x 1.5 mm micro-hematocrit tubes, capillary tube sealer, micro-hematocrit centrifuge and hematocrit reader.

## Method:

- Fill the micro-hematocrit capillary tubes up to 70 to 90% of its capacity holding it horizontally.
- Close one end of the tube by pressing it into the sealer once or twice.
- Clean excess blood on the outside of the capillary with absorbent paper.
- Arrange the tubes in the micro-hematocrit centrifuge and program it at 12,000 G for 5 minutes.
- Confirm the presence of the three layers in the tube from top to bottom: plasma, buffy coat layer and, finally, erythrocytes packed at the bottom (Figure 2). The buffy coat layer is made up of nucleated cells and thrombocytes.
- Overlap the micro-hematocrit tube on the reader, first aligning the erythrocyte-clay interface of the tube on line 0 of the reader and the top of the plasma column on line 100 thereof.
- Read the position of the upper part of the erythrocyte column on the reader and obtain the value of AGV expressed as a percentage (%) (Figure 2).

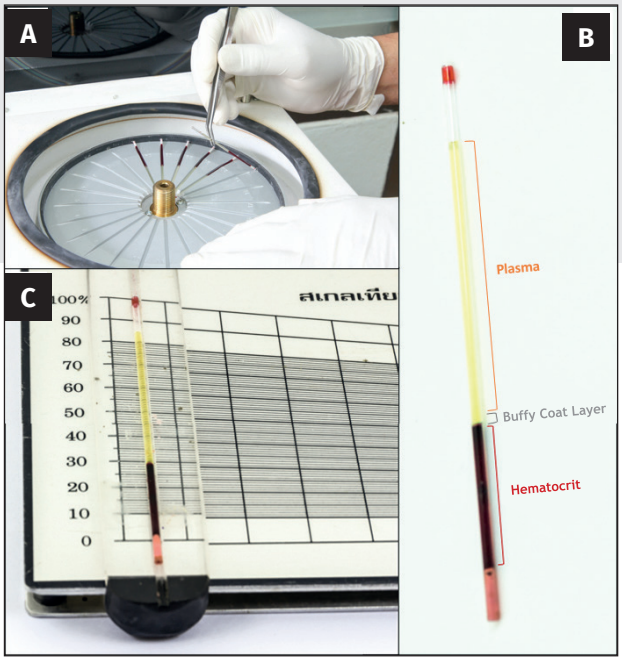



**Figure 2.** *Hematocrit determination. (A) Centrifugation of the micro-hematocrit capillaries. (B) Arrangement of the different cell layers and plasma in micro-hematocrit capillary: Plasma, Buffy Coat Layer and Hematocrit. (C) Superposition of the micro-hematocrit tube on the hematocrit reader.*

## Hemoglobin

Hemoglobin is a hemoprotein in the blood that transports oxygen from the gills to the tissues, and carbon dioxide from the tissues to the gills. In addition, it participates in the regulation of blood pH. The concentration of hemoglobin in blood can be quantified and expressed in grams per liter (g/L) by the cyanometahemoglobin method.

## Materials:

- Prepare Drabkin's reagent according to the indications in Table 3, which induces a quick lysis of the erythrocytes, releasing hemoglobin in the liquid phase.
- Hemoglobinometer.

## Method:

- Dilute 20 µL of blood with anticoagulant in 5 mL of Drabkin's reagent
- Wait 3-5 minutes for hemolysis to occur and centrifuge at 1500-2000 G for 10 minutes for sedimentation of free nuclei.
- Measure Hg concentration using a hemoglobinometer, which estimates the absorbance at 540 nm by spectrophotometry. Light absorption is proportional to hemoglobin concentration.

**Table 3.**
Preparation of Drabkin's reagent.

| Substance | Quantity |
|---|---|
| Sodium bicarbonate [$NaHCO_3$] | 1.00 g |
| Potassium cyanide [KCN] | 0.05 g |
| Potassium ferrocyanide [$Na_4Fe(CN)_6$] | 1.74 g |
| Distilled water | 1000 mL |

## Wintrobe's erythrocyte indices

MCV is proportional to the average size of erythrocytes expressed as femtoliter (fL) and is calculated from AGV and erythrocyte count: **MCV (fL) = AVG (%) x10/N° of erythrocytes in millions/µL.** Example: If AGV is 45% and N° of erythrocytes is 1.2 million; then MCV = 45 x 10/1.2 = 375 fL.

The MCHC provides an index of the amount of Hg relative to AGV expressed in grams per liter (g/L): **MCHC (g/L) = Hg (g/L) x 100/AGV (%).** Example: If Hg is 100 g/L and VGA is 45%, then MCHC = 100 x 100/45 = 222 g/L.

## Interpretation of erythrogram results

Individual variations in AGV and/or Hg are associated with changes in water temperature, dissolved oxygen concentration, farming density, photoperiod, and/or sexual maturity. Fish with a hematocrit > 60% can be considered in a dehydrated status, particularly when accompanied by an increase in the plasma level of total protein. On the other hand, fish that are anemic usually show a significantly low hematocrit (<20%).

In production animals, the results are also interpreted in groups between fish from different farming units (tanks or cages), between fish with different treatments or handling, between fish in different physiological status, among others. For example, individuals of Atlantic salmon, *Salmo salar,* have a higher hematocrit during the pre-spawning stage compared to the spawning stage.

The handling of the animals to obtain blood samples can induce alterations in the results of the erythrogram, e.g., an increase of the hematocrit by up to 25%. The handling of salmonids for more than 20 seconds determines the release of catecholamines that cause hemoconcentration and increase of the size of erythrocytes.

## BOX 1.

**The results of the erythrogram are interpreted individually with respect to the deviations of RIs for the species and the determined productive stage, since the hematological parameters in fish vary according to the physiological, productive, sanitary and/or environmental condition to which they are subjected. Thus, the interpretation of the results must consider RIs that allow to discriminate between physiological and pathological variations.**

Anemia is a decrease in the erythrocyte mass and the concentration of Hg in the erythrocytes below the minimum RI limit. The final result is reduced oxygen transport to the tissues. Anemias can be classified according to etiological or morphological criteria, but the diagnostic approach must contemplate both criteria in a complementary way (Figure 3).

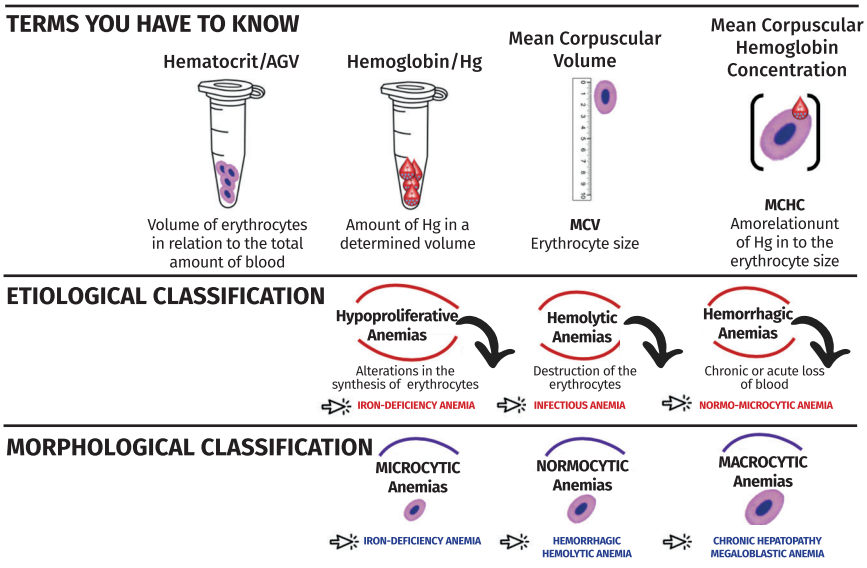



**Figure 3.** *General concepts and classification of anemias.*

From the etiological point of view, anemias can be classified as disorders associated with the inability to produce erythrocytes with an adequate shape and quantity (e.g. depression of hematopoietic tissue) and disorders resulting from increased destruction (hemolysis) or loss of erythrocytes (hemorrhage).

On the other hand, the alteration of MCV and MCHC allow to classify anemia from the morphological point of view according to the size of the erythrocyte and its hemoglobin concentration, respectively (Figure 3).

- **Hypochromic microcytic anemia.** It is characterized by smaller erythrocytes (low MCV) and with a concentration of Hg lower than normal (MCHC). This group includes iron-deficiency anemia and thalassemia, and the anemias that accompany chronic infections.

- **Normochromic macrocytic anemia.** It is characterized by larger than normal erythrocytes (high MCV) and with a normal Hg concentration (normal MCHC). It includes megaloblastic anemia, either secondary to folic acid or vitamin $B_{12}$ deficiency. Liver disease and some cases of aplastic anemia. In general, they are non-regenerative anemias.

- **Normochromic normocytic anemia.** It is characterized by erythrocytes of normal size (normal MCV) and normal Hg concentration (normal MCHC). A characteristic cause is anemia that is secondary to acute hemorrhage or to intrinsic or extrinsic hemolysis.

Anemias are also classified according to the reticulocyte or polychromasia response in regenerative and non-regenerative anemias. The reticulocyte count reflects the activity status of the erythropoietic tissue. In regenerative anemias, a high reticulocyte response is observed, which indicates an increase in erythropoietic regeneration, as occurs in hemolytic and hemorrhagic anemias. Non-regenerative anemias are those that occur with a low reticulocyte response or low or no polychromasia, which is why they are associated with low activity tissue. In general, most chronic anemias are non-regenerative anemias.

## Anemia due to nutritional deficit

Iron and vitamins C, E, K, $B_{12}$, $B_8$, $B_5$ are necessary elements for erythropoiesis, so the deficit is associated with hypochromic microcytic anemia. Several studies indicate that the deficiency of folic acid or vitamin C does not show an effect on the erythrogram, but they agree that the nutritional deficits associated with chronic infections or intoxications generate non-regenerative anemia. In this way, morphological changes of the nucleus of erythrocytes and the release of erythrocytes without nucleus to the peripheral blood can be related to the deficiency of folic acid or vitamin E.

## Anemia associated to toxic agents

Intoxication with nitrites induces hemolytic anemia characterized by a decrease in AGV and Hg, which oxidizes and transforms into methemoglobin. Acute intoxication results in moderate to severe hemolytic anemia characterized by decreased AGV, Hg, erythrocytes, and MVC, and increased MCHC. Hemolysis and changes in MCV and MCHC may not be observed in chronic intoxications.

Intoxications with heavy metals reduce the half-life of erythrocytes by increasing hemolysis, in addition to influencing erythropoiesis. In this way, copper and zinc induce non-regenerative hemolytic anemia, characterized by a decrease in AGV, Hg, erythrocyte count and MCHC, while MVC can increase or remain normal.

## Anemia induced by infectious diseases

Salmonids infected with IHNv show hemolytic anemia and fish infected with ISAv show hemorrhagic anemia, whereas fish infected with IPNv show non-regenerative anemia due to liver damage. Fish with Vibriosis show hemolytic anemia associated with toxins that induce apoptosis of erythrocytes and an increase in circulating immature erythrocytes.

Fish chronically infected with *Piscirickettsia salmonis* show regenerative hypochromic microcytic hemorrhagic anemia and AGV between 10 and 34%. Furthermore, acute infection due to *P. salmonis* could induce intravascular hemolytic anemia. External parasites such as sea lice induce hemorrhagic anemia and microsporid protozoa such as *Nucleospora salmonis* and *Mycrosporidium spp.* induce hemorrhagic and hemolytic anemia.

## Leukogram

The analyzes incorporated in the leukogram include total and differential count of leukocytes identified by blood smear. Leukocytes in salmonids include: eosinophils, neutrophils, bacilliforms, lymphocytes, and monocytes.

## Total leukocyte count

Because the fish have nucleated erythrocytes and thrombocytes, it is not possible to carry out a leukogram in automated equipment such as in mammals; therefore, the main method for counting leukocytes in fish is manual using the hemocytometer or Neubauer chamber and Natt-Herrick stain.

The total leukocyte count (LC) is obtained by counting all the leukocytes in the nine large squares of Neubauer chamber and, the addition is multiplied by 222, a factor obtained from the following formula: LC (µL) = Total N° leukocytes × 222. L**C = L/9 x 10 x D**, where; **LC**: total leukocyte count per $mm^3$ of blood; **L**: total N° of leukocytes in the 9 squares; **D**: dilution factor.

The advantage of this method is that the total count of erythrocytes and thrombocytes can also be performed in the same procedure. One disadvantage is that differentiation of thrombocytes from small lymphocytes is often difficult, so a single count in a chamber is recommended for leukocytes plus thrombocytes. Then, in the differential count of leukocytes in smears, the correction of thrombocytes and leukocytes is made.

## Differential leukocyte count

The differential leukocyte count can be relative or absolute. The relative count refers to the percentage (%) of a certain type of leukocyte with respect to the total white cells. The absolute count is calculated by multiplying the relative percentage obtained by the number of leukocytes counted in one mm3 of blood divided by 100 (N° leukocytes/µL).

To perform a differential leukocyte count, a blood smear and Giemsa stain must be performed on a slide (Figure 4):

- Take a clean slide between the thumb and middle finger.
- Clean with the brush.
- On one end, place a small drop of blood previously mixed with the anticoagulant or directly from the sample taken.

- Extend it with another slide with perfectly smooth edges, move the slide in front of the drop until it makes contact with it and slide it gently forward trying to obtain a monolayer. The angle of the slide determines the thickness of the film, so it is suggested to use 30°.
- Dry in the heat of the burner, in the air or with a hair dryer to minimize the presentation of blood smear artifacts.
- Cover dry smears with absolute methyl alcohol for 3 to 5 min. Pour the excess and then air dry.
- Prepare fresh and diluted Giemsa stain: 1 mL of phosphate buffer pH 6.9 per 2 drops of Giemsa solution.
- For routine study, leave the slide in the stain for 15 min.
- Eliminate the excess, wash in mild running water and let it dry at temperature.
- Under the microscope, count 100 leukocytes under immersion objective (1000X) and obtain a percentage of each type of cell: eosinophils, neutrophils, lymphocytes and monocytes. Additionally, the blood smear is used to evaluate the morphology of leukocytes, erythrocytes, and thrombocytes, but also to search for parasites or bacteria.

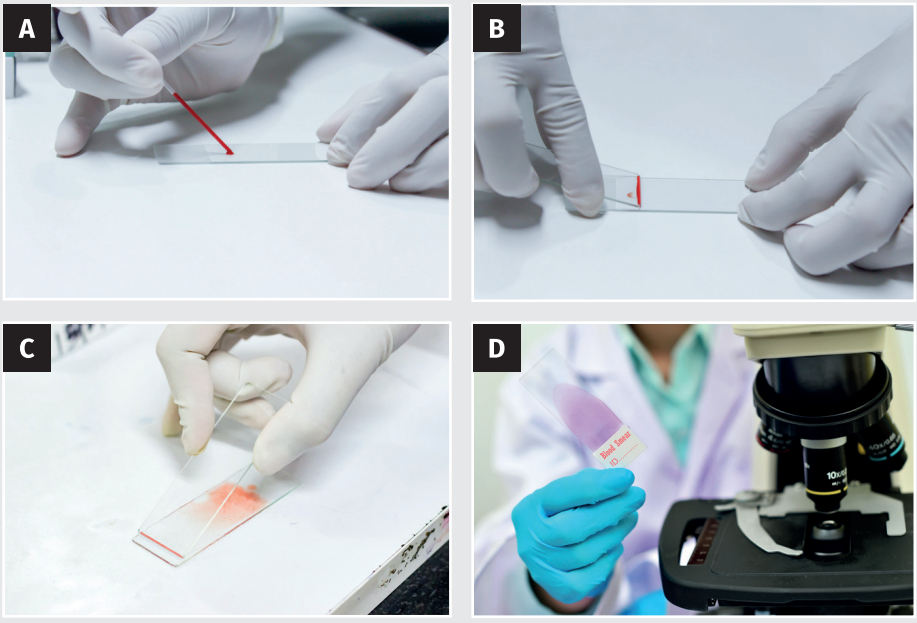



**Figure 4.** *Blood smear. (A) On one end, place a small drop of blood previously mixed with the anticoagulant. (B) Use another slide with perfectly smooth edges and move it in front of the drop until it makes contact with it. (C) Move it gently forward trying to obtain a monolayer. (D) Carry out Giemsa staining of the smears, observe and count 100 leukocytes under a microscope (1000X) and obtain the differential count (%).*

Physiological factors such as reproductive status, growth, smoltification, and environmental stress factors such as farming density, water quality, and productive handling, can generate a moderate increase in the leukocyte count, a finding called leukocytosis (Figure 6). Juvenile fish have higher leukocyte counts and lymphocytes compared to adult fish. The opposite effect occurs in fish under stress, since a decrease in the leukocyte count or leukopenia with lymphopenia and a relative increase in granulocytes are observed.

Leukocytes are divided into granulocytes (neutrophils, eosinophils, and basophils) and agranulocytes (lymphocytes and monocytes), which are distinguished by their morphological and functional characteristics (Figure 5).

Los leucocitos se dividen en granulocitos (neutrófilos, eosinófilos y basófilos) y agranulocitos (linfocitos y monocitos), los cuales se distinguen por sus características morfológicas y funcionales (Figura 5).

## Granulocytes

**Neutrophils:** Fish neutrophils have very similar properties to mammalian neutrophils and, unlike heterophiles, can be distinguished by the presence of myeloperoxidase in the cytoplasmic granules. They measure between 6.5 to 8.2 µm in diameter and have a multilobed, eccentric, purple-color nucleus (Figure 5). The cytoplasm contains granules and may present vacuoles. Neutrophils correspond to about 20% of the circulating leukocyte population and have a high capacity for phagocytosis, chemotaxis and bactericidal functions (Figure 6).

An increase in neutrophil count, neutrophilia, is usually seen in infectious diseases (Figure 7). The presence of active neutrophils is observed in severe systemic diseases in response to inflammation, e.g. in septicemia, fungal infections and severe tissue necrosis.

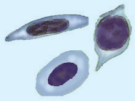
| | TYPE OF CELL | ILLUSTRATION | DESCRIPTION (GIEMSA) | FUNCTION |
|---|---|---|---|---|
| Granulocytes | ***LEUKOCYTES*** | | | |
| | **Neutrophils** |  | Multilobed nucleus; cytoplasmic granules; 6.5 to 8.5 µm in diameter | Phagocytic and chemotactic capacity and bactericidal functions |
| | **Eosinophils** |  | Bilobed nucleus; eosinophilic cytoplasmic granules; 11.4 to 12.6 µm | Response against parasites and allergies |
| | **Basophils** |  | Bilobed nucleus; large cytoplasmic and blue-purple granules; 11.4 to 12.6 µm | Release of histamine and other inflammatory mediators |
| Agranulocytes | **Lymphocytes** |  | Spherical or indented nucleus; pale blue cytoplasm; 4.5 to 8.0 µm | Cytotoxic immune response (T cells) or antibodies (B cells) |
| | **Monocytes** |  | U-shaped or kidney-shaped nucleus; blue-gray cytoplasm; 12 to 15 µm | Phagocytosis because they differ to macrophages in tissues |
| | ***THROMBOCYTES*** |  | Variable shape, round, elongated or spindle-shaped; colorless to faint blue cytoplasm; condensed nucleus and may contain eosinophilic granules; 7.8 to 8.6 µm | Involved in blood coagulation, phagocytosis, and other immune functions |

**Figure 5.** *General description and function of leukocytes and thrombocytes in salmonids.*

**Eosinophils:** They measure from 11 to 19 µm in diameter, they have a bilobed, eccentric, violet-color nucleus that occupies approximately 30% of the cell. The cytoplasm has numerous orange-to-red granules (Figure 5). They occur in a proportion less than 5% in the circulation (Figure 6).

Eosinophils in fish are usually found in low concentrations, some studies indicate that these cells participate in the inflammatory response along with neutrophils and macrophages. The increase in the eosinophil count, eosinophilia, in fish suggests an inflammatory response associated with parasitic infections or antigenic stimulation (Figure 7).

**Basophils:** These cells have been identified only in 4 of 121 species of cartilaginous and teleost fish and in 14 of 20 species of freshwater fish. In salmonids, basophils have not been described, but we include them in this section for possible applications in other fish species. They have a bilobed nucleus and large blue-purple cytoplasmic granules. They measure between 11.4 to 12.6 µm in diameter and, like eosinophils, basophils play an important role in antiparasitic defense and inflammation that amplifies when they pour the contents of their cytoplasmic granules into the extracellular medium (Figure 5).

## Agranulocytes

**Lymphocytes:** They measure from 6.5 to 8.0 µm in diameter, are spherical and have an eccentric, violet-color nucleus that occupies about 70% of the cell. They have a scarce slightly basophilic cytoplasm with no granules (Figure 5). They are the predominant leukocyte in the circulating blood of fish, corresponding to 70 to 80% (Figure 6). Functionally, they divide into B and T lymphocytes, being responsible for the humoral adaptive and cell-mediated immune response, respectively.

Lymphocytosis or increase in the count of circulating lymphocytes is seen in immunogenic stimulation processes, while lymphopenia is seen in immunosuppressive conditions such as stress (Figure 7). The highest lymphocyte and total leukocyte counts are found in fish farmed in high densities, poor water quality and/or with high bacterial load. Leukocytosis accompanied by neutrophilia and lymphocytosis has been observed in the initial stage of the experimental infection with *P. salmonis*.

**Monocytes:** Its nucleus occupies about 50% of the cell, it is irregular, kidney-shaped, lax chromatin and violet in color. The cytoplasm is blue-gray in color and may contain vacuoles (Figure 5). Monocytes are large cells that correspond to 1 to 5% of leukocytes in salmonids (Figure 6).

Monocytes are actively phagocytic cells that specialize in macrophages in tissues. Monocytosis or increase in the count of circulating monocytes occurs in neutrophil-like conditions, and may be present in both acute and chronic inflammatory responses and associated with an infectious agent, foreign body, and/or post-vaccination reactions (Figure 7). In infectious diseases, monocytes activate and prominent cytoplasmic vacuoles, basophilia, pleomorphic nucleus, and cytoplasmic granules are observed.

## THROMBOCYTE COUNT

Fish thrombocytes are smaller than erythrocytes, they can be round, elongated or spindle-shaped depending on the stage of maturity or the degree of reactivity (Figure 5). Thus, immature thrombocytes are round and mature thrombocytes tend to be oval, whereas grouped spindle-shaped thrombocytes appear to be reactive forms (Figure 6). The nucleus of the thrombocytes is condensed and can contain a variable quantity of eosinophilic cytoplasmic granules.

Thrombocytes are responsible for blood coagulation and control of liquid loss in a superficial injury. Clot formation occurs with fibrinopeptides that form after cleavage of the fibrinogen that is controlled by thrombin. However, these fibrinopeptides chemically differ in fish compared to mammals.

Thrombocyte aggregation converts arachidonic acid into prostaglandins with little or no thromboxane formation, unlike in mammals, where it is a powerful inducer of platelet aggregation. Clot formation in teleosts usually occurs about 5 min after an injury has occurred and is primarily dependent on the extrinsic path of coagulation.

The excess of glucocorticoids tends to decrease the concentration of thrombocytes and increase the coagulation time; therefore, it is an important factor to consider in the handling of sampling (Figure 7). Stress factors such as a prolonged photoperiod and high water temperature induce thrombocytopenia. In salmonids, prolonged coagulation times also occur due to vitamin K deficiency.

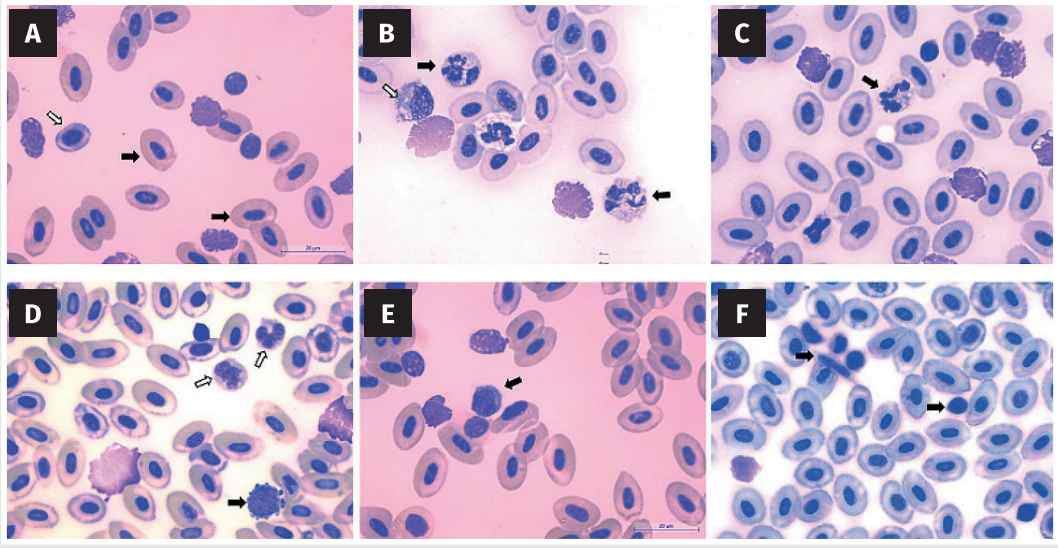



**Figure 6.** *Main blood cells in salmonids. (A) Nucleated erythrocytes (black arrows) and immature erythrocytes or reticulocytes (white arrow) (Bar 20 µm); (B) Neutrophils (black arrows) and monocyte (white arrow) (Bar 20 µm); (C) Neutrophils (black arrow) (Bar 20 µm); (D) Neutrophils (white arrows) and reactive lymphocyte (black arrow) (Bar 20 µm); (E) Lymphocyte (Bar 20 µm); (F) Round and spindle-shaped thrombocytes (black arrows) (Bar 20µm).*

## TOTAL PLASMA PROTEINS

Plasma proteins are measured using the plasma column in the micro-hematocrit tube. The tube is broken above the buffy coat layer and the part containing the plasma is used to load the refractometer. The instrument is maintained so that an ambient light source can pass through the plasma-wet prism and the light refraction is read on a scale by means of an eyepiece. The result of the total plasma protein obtained has to be multiplied by 10 in order to express the result in g/L.

### BOX 2.

**Phagocytosis plays a fundamental role in nonspecific immunity in fish, activity carried out mainly by professional phagocytes such as neutrophils, macrophages and, to a lesser extent, B lymphocytes. Fish immunology has advanced rapidly in recent years; however, the shortage of validated monoclonal antibodies persists to characterize leukocyte subpopulations. Thus, there is still limited knowledge regarding specific markers of immune cells and their activation and differentiation processes. In this sense, the leukogram continues to be a valid and inexpensive tool to evaluate leukocyte cells.**

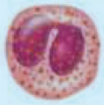
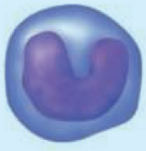
| | TYPE OF CELL | ILLUSTRATION | REFERENCE INTERVAL *(Salmo Salar)* | INCREASE | DECREASE |
|---|---|---|---|---|---|
| | ***LEUKOCYTES*** | | 4.904 a 11.394 / µL | **Leukocytosis** Bacterial and viral infections, Acute stress | **Leukopenia** Severe septicemic infections, Chronic stress |
| Granulocytes | **Neutrophils** |  | | **Neutrophilia** Bacterial and viral infections, Inflammation and tissue damage, metabolic processes | **Neutropenia** Parasitic infections, vitamin deficiencies, hematopoietic tissue alterations |
| Granulocytes | **Eosinophils** |  | 3 to 17% | **Eosinophilia** Allergic response, parasitic infections, vasculitis, nephropathies | **Eosinopenia** Bacterial and viral infections, aplastic anemia, stress |
| Granulocytes | **Basophils** |  | | **Basophilia** Infections, allergic inflammation | **Basopenia** Stress, severe allergy, immunosuppression |
| Agranulocytes | **Limphocytes** |  | 75 to 89% | **Lymphocytosis** Bacterial and viral infections, Chronic inflammation, vasculitis, acute stress | **Lymphopenia** Chronic viral infections, hematopoietic tissue alterations, chronic stress |
| Agranulocytes | **Monocytes** |  | 0.1 to 3% | **Monocytosis** Infections and chronic inflammation, autoimmune response | **Monocytopenia** Vitamin B deficiency, hematopoietic tissue alterations |

**Figure 7.** *Interpretation of the increase or decrease in the population of circulating leukocytes in salmonids.*

# CHAPTER 3

# Clinical Biochemistry in Salmonids

# Clinical Biochemistry in Salmonids

*R.Y. Walker-Vergara, V.C. Arnés, A. Müller & M.A. Rozas-Serri*

Clinical biochemistry consists in the quantification of the concentration of various chemical substances transported by the blood at a given moment, which inform the veterinarian regarding the diagnosis, the evolution of the disease and the usefulness of a treatment. The parameters provide guidance on the functionality of different fish organs and systems, e.g. liver, kidney, heart, etc. (Table 1).

During the preanalytical phase of obtaining the blood sample, it must be considered that the handling of the fish for more than 30 seconds can induce changes in the biochemical parameters. *In vitro* changes after blood collection can be minimized by rapidly separating plasma from erythrocytes after obtaining the blood.

There are different methods for clinical biochemistry in fish, either based on dry chemistry or wet chemistry, so they require equipment and trained personnel. Additionally, portable equipment for field use is available, which require low sample volumes and minimal training. In the case of wet chemistry, it must be ensured that the technique has the desired sensitivity and linear detection limit, as well as knowing the interferents that could alter the results (hemolysis, jaundice).

**Table 1.**
Main parameters incorporated in the evaluation of the blood biochemistry of salmonids

| Group | Analyte | Abreviation | Unit of measurement |
|---|---|---|---|
| **Electrolytes** | Sodium | Na | mmol/L |
| | Chlorine | Cl | mmol/L |
| | Potassium | K | mmol/L |
| **Minerale** | Phosphorus | P | mmol/L |
| | Iron | Fe | mmol/L |
| | Magnessium | Mg | mmol/L |
| | Calcium | Ca | mmol/L |
| **Enzymes** | Alkaline Phosphatase | ALP | U/L |
| | Total amylase | AMT | U/L |
| | Pancreatic amylase | AMP | U/L |
| | Lipase | LIP | U/L |
| | Lactate dehydrogenase | LDH | U/L |
| | Creatine kinase | CK | U/L |
| | Aspartate aminotransferase | AST | U/L |
| | Alanine aminotransferase | ALT | U/L |
| **Substrates** | Cholesterol | COL | mmol/L |
| | High density lipoprotein | HDL | mmol/L |
| | Low density lipoprotein | LDL | mmol/L |
| | Triglycerides | TRG | mmol/L |
| | Total protein | PTO | g/L |
| | Albumin | ALB | g/L |
| | Globulins | GLO | g/L |
| | Glucose | GLU | mmol/L |
| | Lactate | LAC | mmol/L |
| | Creatinine | CRE | µmol/L |
| | Urea | URE | mmol/L |
| | Uric acid | AUR | µmol/L |
| | Ammonium | NH4 | µmol/L |

There are several techniques to measure clinical biochemistry parameters, but absorbance spectrophotometry is one of the most widely used techniques and the one we will describe in this work. Absorbance spectrophotometry is a semi-automated or automated analytical technique in which the concentration of substances is determined by directing a beam of light through a solution containing the substance of interest and then, the amount of light they absorb is measured.

Absorbance spectrophotometry can be performed using the endpoint assay or the kinetic assay. Endpoint assays are applied when measuring the concentration of a pre-existing substance in serum or plasma. In that test, reagent is added to a quantity of serum or plasma and the resulting product is measured by spectrophotometry. Then, using a calibration curve and/or a calibration constant, the concentration of the substance is calculated. An example of an endpoint reaction is that one used for determination of serum albumin.

Kinetic assays are used to indirectly measure the enzyme activity or the speed with which an enzyme converts a substrate into a product, which requires multiple measurements of the concentration of the product over time. So, the faster the product is obtained, the greater the activity of the enzyme is. An example of a kinetic reaction is that one used for the determination of the activity of transaminase enzymes (ALT, AST).

## Interpretation of the results of blood biochemistry

### Liver and metabolic functionality

The liver tissue of teleost fish has high activity of transaminases such as AST and ALT. Therefore, the plasma activity of these enzymes can be elevated with severe hepatocellular disease. ALP concentration is usually increased in cases of bile duct damage and obstruction or cholestasis. However, the level of these enzymes is not as high in chronic diseases. Additionally, liver functionality should be evaluated with the measurement of metabolites produced in the liver and that have an implication in the metabolism of carbohydrates, lipids and proteins.

Total plasma proteins are mainly represented by albumins and globulins. Hypoproteinemia or decrease of albumins and globulins in fish is indicative of malnutrition, low hepatic synthesis and loss or degradation in infections. The first sign of hypoproteinemia is reduced growth, which results in an alteration of hepatic and hematopoietic function and a decrease in calcium concentration since it is transported in association with albumin. The increase in total protein concentrations can occur due to dehydration status, with an increase mainly in albumin or due to an increase in globulins, in cases of antigenic stimulation with the production of antibodies, such as in some inflammatory/infectious processes, including vaccination.

Bile pigments in most fish include bilirubin and biliverdin; however, the percentages of these pigments vary between species. Serum/plasma is usually light yellow in color due to the presence of bilirubin; however, liver disease in fish may not lead to an increase in plasma bilirubin concentrations as these will also be affected by pre-liver conditions, like hemolysis.

The plasma glucose concentration in fish is variable. The source of plasma glucose comes from the hepatic metabolism of glycogen; therefore, depletion of hepatic glycogen reserves can lead to hypoglycemia. Plasma glucose concentration also varies with age, nutritional and reproductive status, and stress. The duration and magnitude of postprandial hyperglycemia in fish depend on the intake of carbohydrates in the diet, the effect of starvation on plasma glucose concentration depends on the species and time, since many fish species have normal concentrations of blood glucose (up to 150 days).

The variation in glucose concentration also changes with the reproductive status and sex of the animals and may be lower in males than in females during the spawning period. Cortisol-induced hyperglycemia in stress events is common in salmonids and the duration depends on the intensity of the stress. The plasma hyperglycemia associated with glycogenolysis in the liver and muscle is probably due to the increase in catecholamines.

It is not clear if the changes in plasma concentration of cholesterol are significant with respect to its concentration in hepatic diseases. Most fish have higher blood cholesterol concentrations than mammals, and approximately 60% of cholesterol is carried by high-density lipoproteins (HDL). Males undergoing active spermatogenesis have higher cholesterol values compared to inactive males. Females have lower blood cholesterol concentrations compared to males.

Triglycerides are lipids synthesized in the liver and constitute one of the main forms of energy storage in fish. Triglycerides are a useful index of nutritional status. Decreased plasma TRGs are associated with prolonged fasting or acute and chronic hepatic failure, although it is not necessarily observed with values

below the reference limits. Plasma TRG concentrations rise temporarily after administration of a high fat diet, as lipids are absorbed by the intestine and transported to the liver for further processing.

## Renal functionality

In fish, the kidney contributes only partially to the excretion of nitrogenous wastes; therefore, the interpretation of plasma concentrations of urea, uric acid and creatinine could be insufficient to evaluate renal functionality. In general, creatinine is formed from creatine and is secreted through the kidneys, so an increase in plasma creatinine concentration has been associated with kidney disease, although urea concentrations often remain normal.

However, the gills are the main organ for urea excretion in most fish. Therefore, the increase in plasma urea concentration may be more indicative of an alteration in branchial functionality rather than renal in teleosts. Among the pathological processes that can alter kidney function, the following can be mentioned: tubular necrosis, infectious salmon anemia (ISA), bacterial kidney disease (BKD), piscirickettsiosis (SRS), acute heavy metal intoxication, among others.

## Gill functionality

Gills are essential in fish since they ensure osmoregulation, respiration and excretion functions of some nitrogenous compounds, representing a wide surface of exchange with the environment. The normal plasma concentrations of sodium and chloride of freshwater teleosts are lower those of marine teleosts. These concentrations are affected by changes in environmental salinity, gill function, and stress.

Within minutes of fish handling, catecholamines and cortisol are released into the bloodstream along with the release of lactic acid from the muscles. The stress-induced release of catecholamines causes an increase in blood pressure, which leads to increased electrolytic permeability of the gills, causing a rapid decrease in sodium and chloride in freshwater fish and an increase in ions in marine teleosts. Hyponatremia and hypochloremia in freshwater fish may be associated with gill and/or kidney disease or with acidic environments.

In this way, plasma electrolytes are an important indicator in smoltification since they are a fundamental part of homeostasis. During the smoltification process, a gradual increase in sodium and chlorine is observed with a peak prior to transfer to seawater, which is maintained after arriving at the sea until acclimatization occurs.

Salmonids are among the fish groups with the least tolerance to ammonia. Ammonia in water exists mainly as non-ionized ammonia ($NH_3$) and ammonium ion ($NH_4$+). Total ammoniacal nitrogen (TAN) is the main excretion product of protein metabolism and is eliminated through the gills. In production systems where open water flow is used, TAN does not represent a significant problem; however, in water recirculation systems and fish transport, it is a critical variable.

$NH_3$ toxicity increases along with pH, temperature, exercise, and stress, being small fish more sensitive. Hyperammonemia can occur in freshwater fish with damage to branchial epithelium, which can go hand in hand with an increase in the concentration of urea and uric acid, as a way to compensate.

## Musculoskeletal and mineral functionality

The skeletal structure forms the basic framework for the growth of a healthy animal and minerals are an important group of essential nutrients for skeletal development. The fish skeleton is made up of an organic bone matrix and inorganic minerals, consisting mainly of collagen and hydroxyapatite (HAP), respectively. In fish, bones represent an important and highly conserved reservoir of calcium (Ca), phosphorus (P), zinc

(Zn) and manganese (Mn). The concentration of these minerals in the vertebrae is considered the most relevant indicator of the mineral status and quantifies dietary needs.

P is a structural component of bones, teeth, and scales, in addition to playing a role in various metabolic processes. The decrease in phosphorus concentrations, hypophosphatemia, is mainly associated with the decrease in dietary intake and produces a decrease in growth rate, reduction in dietary efficiency and bone deformities.

In addition to reducing bone mineral content, hypophosphatemia has also been shown to increase alkaline phosphatase (ALP), probably as a compensatory mechanism in mineral deficiency. Other causes of hypophosphatemia are intestinal malabsorption or an increase in calcium contribution in the diet. An increase in the requirements of P in the diet is not correlated with the increase in P in the blood due to the homeostatic regulation exerted by vitamin D.

Ca is the most abundant mineral in fish bones and comprises about 20-25% of dry bone mass. The importance of calcium as a structural mineral in bone is related to HAP, $Ca_{10}(PO_4)6\ (OH)_2$, the primary materials of the bone matrix, with a Ca:P ratio of 1.6 to 2 in salmonid bones.

The absorption of calcium from the environment will depend on the species, availability of Ca in the water and supplementation in the diet. In teleosts, approximately 99% of the Ca fraction of the whole body is incorporated into bones and scales and, in addition to its structural functions, it plays an important role in muscle contraction, hemostasis and coagulation, transmission of the nerve impulse, cellular integrity , acid-base balance and enzymatic activation.

An important point to consider is the Ca:P ratio, which varies with the stage of life. In Atlantic salmon, a Ca:P mass ratio of 0.2-0.4 has been observed before the first feeding, 0.86-1.04 during the growth stage, decreasing to 0.6 during and after smoltification to then, stay between 0.7-0.8 in the adult stage. In the case of freshwater salmonids, 30-40% of the total plasma calcium is bound to the proteins; therefore, changes in plasma proteins will affect the total plasma calcium concentration.

Mg deficiency in rainbow trout determines injuries to the backbone. The increase in P content in the diet probably caused by the formation of the insoluble Ca-P-Mg complex in the intestine, inhibits Mg absorption and could induce hypomagnesemia.

Musculoskeletal tissue diseases are mainly of the inflammatory type, associated with viral infections (PD or pancreatic disease, HSMI or heart and skeletal muscle inflammation), bacterial infections (BKD, SRS, Atypical Furunculosis, Streptococcosis, Tenacibaculosis, Flavobacteriosis), and fungal infections (Phaeohyphomicosis).

AST and CK activities increase after muscle injury or intense muscle activity associated with hypoxia of muscle tissue. LDH activity may also be increased in acute inflammatory processes, due to its involvement in anaerobic energy metabolism. Therefore, lactate concentration could also increase under conditions of intense muscular activity and oxygen restriction.

It is important to consider that the plasma concentration of enzymes could vary according to the stage of development. AST levels are high during spawning and subsequently decrease, along with the activity of other transaminases, glycolytic enzymes, and muscle proteins. Periods of starvation and inactivity generate low LDH values, which is also positively correlated with water temperature and pH.

# CHAPTER 4

# Blood Gasometry in Salmonids

# Blood Gasometry in Salmonids

*R.Y. Walker-Vergara & M.A. Rozas-Serri*

The parameters associated with the acid-base status and blood gases need to be measured immediately after the sample is collected, which generates a technical inconvenience to transfer the samples to the laboratory. However, portable devices or gasometers *(Point-of-Care; POC)* have been developed, they are capable of accurately assessing a variety of relevant blood parameters in mammals, including blood gases and the acid-base status, allowing measurements to be made in field conditions and in a short time.

The limitation of this type of measurement is the technical and operational validation of the equipment to be used in fish. Factors that can generate measurement biases are ambient temperature, nucleated erythrocytes, blood gas concentration, and acid-base status that vary with $PaCO_2$, especially in teleosts.

RIs for blood gas variables defined and described for salmonids in this manual were defined using the IRMA Trupoint kit (ITC, Edison, NJ, USA) (Figure 1). Other equipment widely used in field conditions are i-STAT (Abbott Point-of-Care, Princeton, NJ, USA) and EPOC (Siemens Healthcare Diagnostic, Malvern, PA, USA) (Figure 1).

## Methodology for blood gasometry

Gasometers are made up of a pH electrode, a $CO_2$ electrode and an $O_2$ electrode, by means of which the equipment can measure the concentration of the analytes in whole blood using potentiometric, amperometric and conductometric techniques (Table 1). Additionally, the equipment can mathematically calculate other parameters such as: bicarbonate ($HCO\text{-}_3$), total carbon dioxide ($TCO_2$) and excess base.

**Table 1.**
Measurement techniques in blood gas and pH analyzers

| Sensor | Measurement technique |
|---|---|
| pH, $pCO_2$ | Potentiometric measurement based on the electrode principles of MacInnes and Dole (for pH) and of Severinghaus and Bradley (for $pCO_2$) |
| $pO_2$ | Amperometric measurement based on the principles of the Clark electrode |
| Hematocrit | Conductometric measurement |

## Materials

- Blood must be collected using syringes that include lithium heparin (heparinized) of 1, 2 or 3 mL depending on the volume of blood to be analyzed (Figure 1).
- Portable gasometer.
- Before using the equipment, the electrodes must be calibrated with known concentrations of standard buffers and calibration solutions.
- The form of calibration varies between different equipment, so it must be carried out according to the manufacturer's recommendations.

## Method

- Collect fresh arterial or venous whole blood using a heparinized syringe.
- Extract the residual air in the syringe immediately after collecting the blood. If you want to separate part of the sample for other analyses, do not expose the sample to air.
- Homogenize the sample by gently shaking the syringe to homogenize the blood with the anticoagulant.
- Close the end of the syringe to prevent the entrance of air, which can lead to analytical errors and store it in ice (approximate temperature of 4ºC) until analysis.
- The maximum time elapsed between taking the sample and its subsequent analysis should not exceed 2 h.
- Inject a minimum volume of 125 to 200 µL of whole blood into the equipment sample chamber.
- When the blood comes into contact with the electrodes in the chamber, there is an output of electricity that corresponds to a pH value or a partial pressure. Blood gas analyzers automatically monitor the electrode response and after stabilization, they print out the measured values.
- Portable equipment works in a temperature range between 12 to 30°C, so ambient temperature in field conditions during winter could be a problem for gas measurements, specifically $pO_2$.
- Portable equipment can correct results according to the individual's body temperature at the time of obtaining the sample. This is important because the equipment is designed for being used in homeothermic individuals; therefore, the working temperature is 37ºC. In fish, the temperature of the water should be considered before analyzing the sample so that it measures corrected blood gases at that temperature.

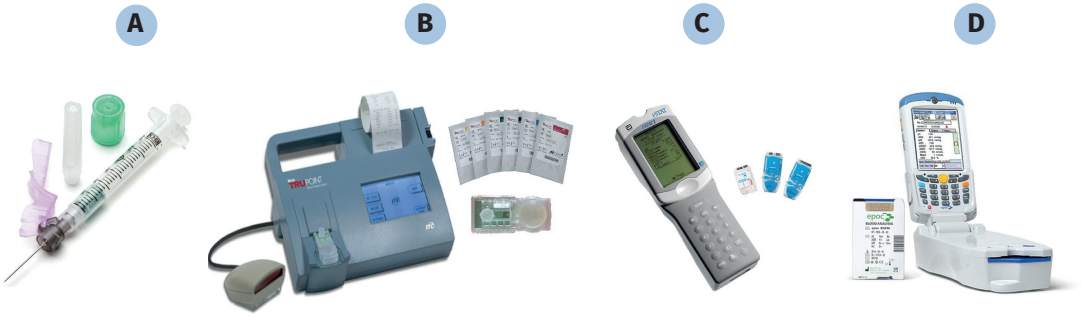



**Figure 1.** *Basic equipment to perform blood gas measurements in field conditions.*

## Interpretation of blood gasometry results

Blood gasometry results must always be interpreted integrally with the clinical, environmental and productive history of each farming site. The measurement of blood gases directly provides results of partial pressure of $CO_2$ ($PaCO_2$), partial pressure of $O_2$ ($PaO_2$) and pH, with which bicarbonate ($NaHCO_3$) and total $CO_2$ ($TCO_2$) can be calculated.

## $CO_2$ partial pressure

$PaCO_2$ is a measure of ventilation efficiency, an indicator of the effectiveness of elimination or excretion of carbon dioxide or the intensity factor of $CO_2$ dissolved in plasma. Additionally, it is an indicator of the amount of carbonic acid present in the plasma. So, $PaCO_2$ is a reflection of the respiratory component of acid-base balance.

$PaCO_2$ is abnormally high when there is an excess of carbonic acid in the plasma, that is, respiratory acidosis. On the other hand, when $PaCO_2$ is abnormally low, it is because there is a deficit of carbonic acid in the plasma, that is, respiratory alkalosis.

## $O_2$ partial pressure

$PaO_2$ is the blood oxygenation index, an indicator of the intensity of the presence of molecular oxygen in solution in plasma. Thus, $PaO_2$ is the expression of the efficiency of branchial ventilation/perfusion and diffusion to achieve the normal transfer of oxygen from inside the lamella to the branchial capillary blood.

## Potential for hydrogen (pH)

It is a measure to determine the degree of alkalinity or acidity of the plasma. pH is expressed through the Henderson-Hasselbalch equation that defines pH in terms of the relationship between salt and acid, that is, the pH of the extracellular liquid depends on the relationship between the amount of bicarbonate and the amount carbonic acid; $pH = pK + \log HCO^-_3 / H_2CO_3$.

Then, pH is the most important indicator of the severity and magnitude of an acid-base alteration, more than $PaCO_2$ or bicarbonate ($HCO^-_3$). pH varies according to the $HCO^-_3$ / $H2CO_3$ ratio, but the following definition is established: (1) high pH always means alkalosis; and, (2) low pH always means acidosis. However, pH does not make it possible to differentiate whether it is a respiratory or metabolic acidosis, or a respiratory or metabolic alkalosis.

## Bicarbonate

$HCO^-_3$ is the metabolic component of acid-base balance, the chemical compound that contains almost all the organism's $CO_2$. $PaCO_2$ is the intensity factor that determines the amount of carbonic acid, but $HCO^-_3$ is the $CO_2$ amount factor. While $PaCO_2$ refers to molecular $CO_2$ dissolved in plasma, $HCO^-_3$ refers to $CO_2$ in combination.

$HCO^-_3$ is the most important base of the organism's buffer system, which maintains pH at normal values or the acid-base balance. Although there are various buffer systems in the organism, pH is controlled only by the $HCO^-_3$ / $H2CO_3$ ratio. Taken together, these indicators allow for the evaluation of the acid-base balance and define the type of alteration in simple or mixed disorders.

Acid-base abnormalities have a metabolic or respiratory etiology. The $CO_2$ content of the serum alone is not the best indicator of the acid-base balance, but rather the amount of $HCO\text{-}_3$. CO2 and $H2CO_3$ in solution contribute only with a few millimoles. In this way, frank respiratory acidosis or alkalosis can be observed in the acute phase without an alteration in the $CO_2$ content of the plasma being observed. For this reason, pH and $PaCO_2$ must be measured in a fresh blood sample.

In practice, complex respiratory and/or metabolic situations can occur, and the values of blood gases can mean phenomena of compensation for the primary alteration or they can represent coexisting primary alterations. The following can be observed:

- **Respiratory acidosis:** elevated $PaCO_2$, normal or slightly increased $HCO\text{-}_3$ and acid pH.
- **Metabolic acidosis:** decreased $HCO\text{-}_3$, decreased $PaCO_2$ and acid pH.
- **Respiratory alkalosis:** markedly decreased $HCO\text{-}_3$, decreased $PaCO_2$ and alkaline pH.
- **Metabolic alkalosis:** markedly high $HCO\text{-}_3$, high $PaCO_2$ and alkaline pH.

## Total carbon dioxide ($TCO_2$)

The value measures the total carbon dioxide dissolved in the blood and is determined by combining $HCO\text{-}_3$ and $PaCO_2$, multiplied by a factor (usually 0.03) that estimates the amount of pure $CO_2$ that is dissolved in its natural form.

Finally, with these indicators it is also possible to calculate the base excess. In physiology, **base excess** refers to the amount of base required to return an individual's blood pH to normal. Usually the value is reported in units of (mEq/L) and fluctuates between -2 and +2.

# CHAPTER 5

# Endocrinology in Salmonids

# Endocrinology in Salmonids

*R.Y. Walker-Vergara & M.A. Rozas-Serri*

Endocrinology in fish has had a significant impact on the understanding of the function and evolution of several neurochemical messengers and systems. In this way, measuring the concentration of different hormones in the blood is a quick and useful tool in key processes such as smoltification, stress and reproduction, among others.

## Methodology to quantify serum hormones

Hormone measurement can be done in serum or plasma and, depending on the hormone, a blood sample in tubes without anticoagulant or tubes with lithium heparin is required. The minimum plasma/serum volume is 200 µL, which should ideally be separated from whole blood within 12 hours. The sample can be kept at -20°C until further analysis.

There are several methods for measuring the concentration of serum hormones, such as chemical, spectrophotometric, chromatographic, and immunoassay methods. Immunoassays are based on an antigen-antibody reaction and, at the same time, there are several techniques:

- **Radioimmunoanalysis (RIA):** It is based on an antigen-antibody reaction with labeled radioactive isotopes. The measured radioactivity concentration indicates the concentration of the analyte. Although it can detect and quantify very low concentrations of hormones, it is currently only used for specific measurements.
- **Enzyme-linked Immunosorption Assay (ELISA):** It is a method that allows the concentration of an antigen or an antibody to be determined in a similar way to RIA, but the labeling is done with an enzyme.
- **Chemiluminescence:** It is based on the principle of light emission through an enzyme-substrate reaction. Chemiluminescence is based on the light emission caused by the products of an immunoreaction, e.g. acridine ester, peroxide-acid, sodium hydroxide, alkaline phosphatase.
- **High Performance Liquid Chromatography (HPLC):** It is a technique used to separate the components of a mixture based on different types of chemical interactions between the analyzed substances and the chromatographic column. It is the method chosen for quantification of catecholamines and steroidal hormones, but it has a high cost.

## Hormonal profiles in salmonids

Notwithstanding the fact that there are still questions regarding the use of hormonal profiles in fish, there is consensus on the importance of the neuroendocrine system in the regulation of different physiological processes, especially osmoregulation, stress and reproduction.

## Osmorregulation

Euryhaline fish such as salmonids have the ability to adapt to both freshwater (hypo-osmotic media) and seawater (hyper-osmotic media), depending on the biological or productive phase in which they are found. The metamorphosis by which freshwater fish adapt their physiology to adapt and survive in seawater is called smoltification (Figure 1).

The osmoregulatory system of teleost fish is controlled by the endocrine system through a wide variety of pituitary hormones (prolactin, growth hormone, thyroid, among others) and extrapituitary hormones (arginine, vasotocin, urotensins, stanocalcin, among others). In this way, the adaptation of the fish to changes in the salinity of the water produces the activation of various factors that are involved in the processes of secretion and/or absorption of ions and water, as well as their control by various hormones, through its specific receptors, triggering the necessary cellular pathways to integrate its physiological action.

The osmoregulatory hormones involved in this adaptive process are divided into: (1) Short and fast-acting hormones that regulate the activity of pre-existing transporter proteins such as atrial natriuretic peptide (ANP), angiotensin II, among others; (2) Slow and long-acting hormones such as prolactin and cortisol for adaptation in freshwater and growth hormone, thyroid hormones and cortisol for adaptation in seawater.

The application of these neuroendocrine markers as indicators of the progress of smoltification have been widely described and can complement the measurement of plasma electrolytes, the activity of the $Na^+K^+$ ATPase pump and/or the expression of genes associated with osmoregulation.

## Prolactin

Prolactin is a hormone that promotes adaptation to hypo-osmotic or freshwater environments. During the freshwater phase, prolactin stimulates the secretion of mucus to reduce the loss of ions and water through the skin, which is why its concentration begins to decrease during the smoltification process and subsequent transfer to the sea, since ion uptake should be promoted.

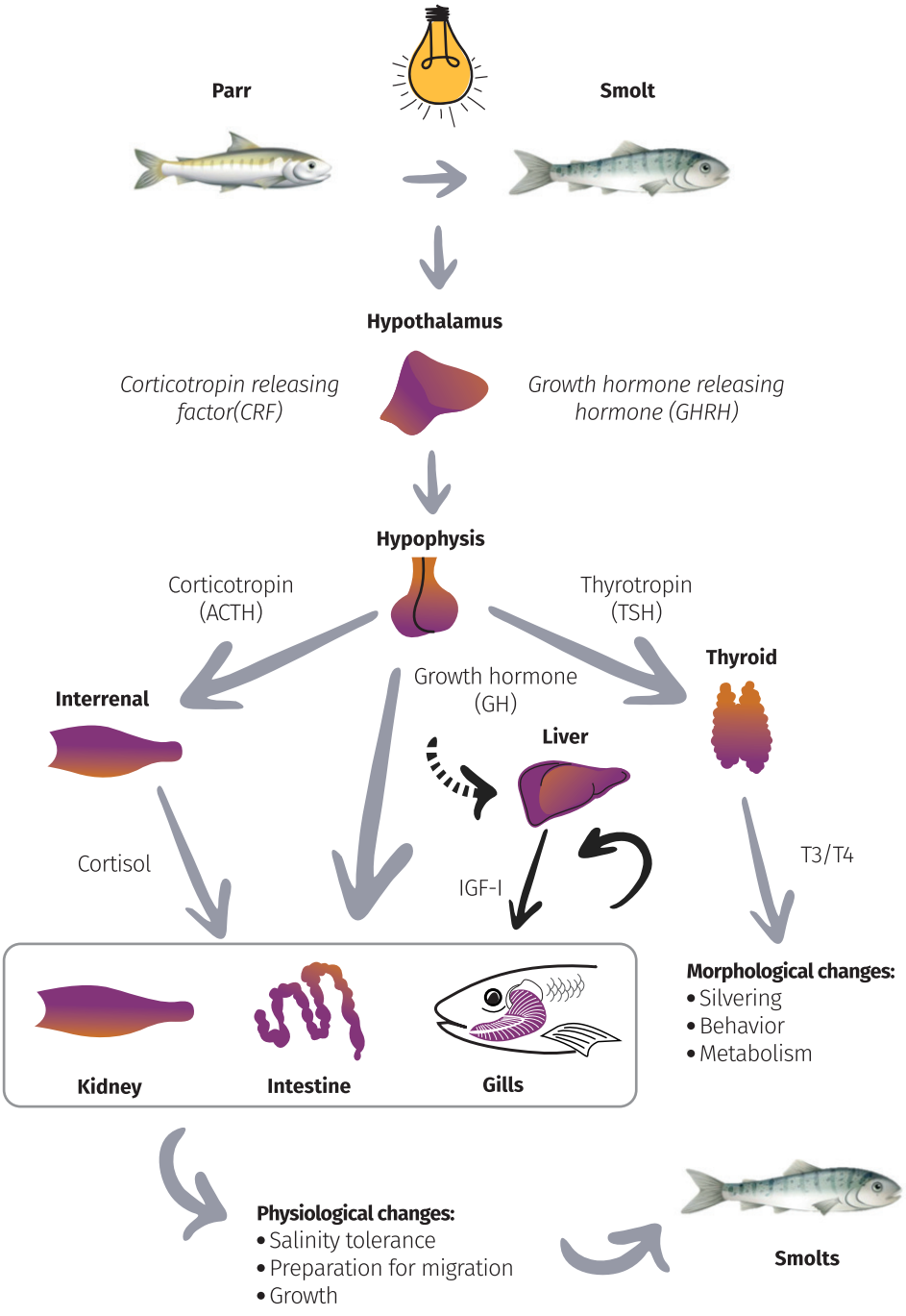



**Figure 1.** *Scheme of neuroendocrine control in the development of smolt.*

Additionally, it modulates branchial, intestinal and renal permeability to water, probably by regulating tight junctions, changes in the composition of membranes and aquaporins. It decreases the $Na^+K^+$ ATPase activity in the gills and reduces the number and/or size of chloride cells in the gill epithelium. At the renal level, prolactin stimulates the reabsorption of $Na^+$ in the renal tubules by increasing $Na^+K^+$ ATPase activity, favoring adaptation in freshwater.

## Growth hormone and somatomedins

Growth hormone (GH) plays a fundamental role in the growth and metabolism of vertebrates, influencing the metabolism of lipids, carbohydrates and proteins. Growth-promoting effects refer to weight growth (muscle) and length growth (skeletal). GH increases during smoltification, before and after transfer to the sea, probably associated with the increased metabolic demand for migration and the requirement for greater tolerance of fish to the saline environment.

GH can act directly on tissues and organs related to osmoregulation or indirectly through so-called somatomedins or insulin-like growth factors (IGF-I and IGF-II), which mediate the actions of GH. At the same time, the synergistic action of GH has been demonstrated with cortisol and thyroid hormones.

## Adrenocorticotropic hormone

Adrenocorticotropic hormone (ACTH) is part of the so-called hypothalamic-pituitary-interrenal axis. Corticotropin-releasing factor (CRF) stimulates the secretion of ACTH in the anterior pituitary, which acts on the interrenal tissue and induces cortisol release.

Cortisol is a hormone that promotes adaptation to saline environments, stimulating the growth and differentiation of chloride cells and increasing Na+K+ ATPase activity in the gills. When GH concentration is high and prolactin concentration is low, cortisol promotes the secretion of salts from the fish into the water.

### BOX 1.

**Summarizing, in freshwater, the concentration of prolactin is high until the start of smoltification, moment at which the concentration decreases along with the increase in the concentration of T4, GH and cortisol. The neuroendocrine physiological mechanisms are pulsatile; therefore, a single measurement in time does not always succeed in providing consistent information.**

## Thyroid hormones

The main function of the thyroid hormones - Triiodothyronine ($T_3$) and Thyroxine ($T_4$) - is to induce morphological and metabolic changes in fish that are preparing to adapt to the sea; therefore, serum concentration of $T_3$ and, especially $T_4$, increases during the smoltification process.

The increase in $T_4$ is basically associated with the requirement to increase the $Na^+K^+$ ATPase activity and the number of chloride cells. Additionally, thyroid hormones show a significant positive interaction with the endocrine axes of cortisol and GH/IGF to increase the ability to adapt to saline environments in salmonids and non-salmonids.

## Stress response

In general, aquaculture has gradually incorporated the concept of animal welfare. The consensus is that an animal has animal welfare if it develops in a suitable aquatic environment, if it is healthy, well-nourished, without physical and/or thermal discomfort, if it can express innate forms of behavior and if it does not suffer unpleasant sensations of pain or fear. In this way, animal welfare is understood as the low or inexistent level of stress under conditions of production, transport or slaughter.

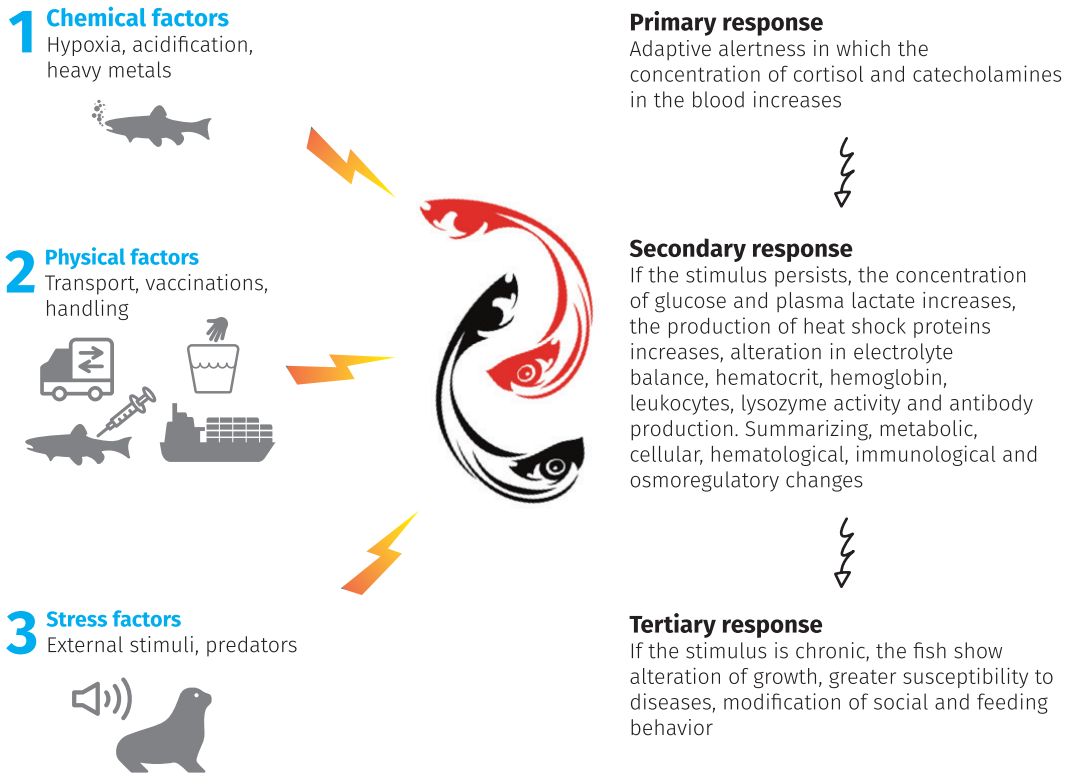



**Figure 2.** *Stressful events and stress response in fish.*

The main stress events in fish are related to overcrowding, transfer, vaccination and the change from fresh to seawater, and harvest, among others. Chronic stress is the main negative effect of intensive fish farming, since it affects the immune system. Therefore, animal welfare directly favors the productivity and profitability of aquaculture by means of obtaining a better-quality product and, consequently, a better price and greater consumer acceptance.

The stress response in fish can be classified as primary, secondary or tertiary (Figure 2). When a fish is subjected to acute stress (biometrics, vaccinations, transport, etc.), there is an immediate increase in catecholamines and cortisol, the peak of which varies depending on the time of exposure and the intensity of the stress.

When the stressful agent persists, it is classified as chronic stress and is mainly observed in the secondary (glucose and lactate) and tertiary response to stress (Figure 2). In this case, the measurement of cortisol has less diagnostic power than in acute stress; however, recent studies indicate that the measurement of cortisol in scales could be an important tool for the evaluation of chronic stress.

Cortisol is the hormone that is used to quantify stress levels in productive conditions, increasing in situations such as capture, high density, handling, intense exercise, incorporation of toxins, changes in salinity, pH, temperature, among others. An increase in cortisol levels is associated with a response to an acute stressful stimulus and together with secondary response indicators (glucose, lactate), it allows for the evaluation of the intensity of stress.

Cortisol increases gluconeogenesis in the liver, promotes the activity of the enzyme glycogen synthetase to synthesize glycogen rapidly, increases proteolytic activity in white muscle, increases plasma amino acid levels, interferes with neural centers of satiety, decreases the absorption of nutrients in the intestine, decreases the number of cellular receptors for GH, decreases the number of estrogen receptors in the liver, also affecting the synthesis of vitelogenin and decreases the immune response.

Additionally, when fish perceive a stressful agent, neuronal signals activate the hypothalamus to synthesize ACTH, which initiates the downward activation of sympathetic fibers that stimulate chromaffin cells in the kidney to release catecholamines adrenaline and noradrenalin into the bloodstream. Catecholamines prepare the fish to respond to the stressful agent by increasing gluconeogenesis and glycogenolysis, lipid degradation, among others.

## BOX 2.

**The primary response is adaptive and directed by the release of catecholamines and cortisol. If the stressful factor persists, the secondary response characterized by metabolic, hematological, cellular, osmoregulatory and immunological changes is activated. Finally, the tertiary response is characterized by alterations in the behavior patterns and productive performance of the fish.**

## Reproduction

Fish have a high sensitivity to environmental factors, which have an important control over reproduction. In fish, as in all vertebrates, reproduction is regulated by the brain through the gonadotropin-releasing hormone (GnRH) that is produced specifically in the hypothalamus (Figure 3). This decapeptide stimulates the production of gonadotrophins (GtH) by the hypophysis or pituitary gland. In some fish dopamine is a negative inhibitor of the production and release of GtH by the pituitary gland.

The pituitary gland controls reproduction through the follicle-stimulating hormone (FSH), which regulates vitellogenesis in females and spermatogenesis in males, and luteinizing hormone (LH), which is responsible for controlling the final maturation of the oocyte in females (Figure 3). Farming conditions affect females more significantly than males, since males seem to adapt better to farming conditions and are able to mature and produce viable gametes more easily.

During vitellogenesis in females, FSH or LH stimulate the production of testosterone in the theca cells and its aromatization to 17ß-estradiol (E2) in the granulosa cells of the ovarian follicle (Figure 3). The increase in plasma E2 stimulates the production of vitelogenin (VGT) in the liver, which is recognized by membrane receptors and incorporated by the oocyte through micropinocytosis, a process that is regulated by FSH. At the end of vitellogenesis, plasma levels of LH increase, producing an increase in plasma levels of the maturation-inducing steroid (MIS), which acts on the oocyte membrane inducing its final maturation (Figure 3).

In males, gonadotrophins regulate spermatogenesis through the production of androgens by Leydig cells in the testicle, specifically 11-ketotestosterone (11-KT). There is a significant increase in plasma 11-KT levels during spermatogenesis and a decrease before or during the spermiation period. The action of 11-KT finally stimulates Sertoli cells that are responsible for activating spermatogenesis.

The increase in plasma LH levels at the beginning of spawning induces an increase in steroidogenesis, in addition to the production of MIS. Then, LH and MIS induce increased semen production through stimulation of seminal plasma production. Finally, MIS stimulates the movement capacity of sperm by increasing the pH of the seminal plasma.

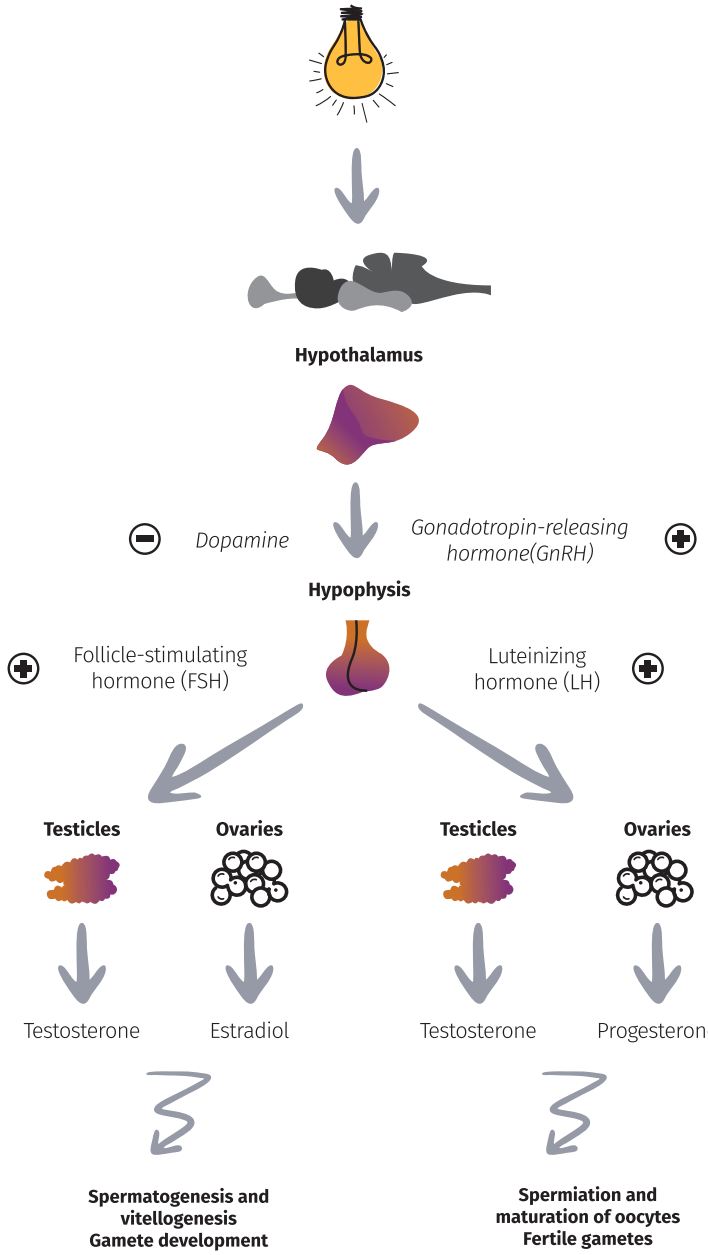



**Figure 3.** *Schematic representation of the reproductive axis in fish, main components and phases, environmental and endocrine control.*

# References


1. Baeverfjord G, Antony Prabhu P, Fjelldal PG, Albrektsen S, Hatlen B, Denstadli V, Ytteborg E, Takle H, Lock EJ, Berntssen MH, Lundebye AK, Åsgård T, Waagbø R. 2019. Mineral nutrition and bone health in salmonids. Rev Aquacult 11: 740-765.
2. Barton A. 2002. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. Integr and Comp Biol, 42(3): 517–525.
3. Brauner C, Randall D. 1998. The linkage between oxygen and carbón dioxide transport. In SF Perry II, BL Tufts, eds, Fish Physiology, Vol. XVII: Fish Respiration. Academic Press, San diego, pp 283-320.
4. Burtis CA. 2007. Tietz Fundamentals of Clinical Chemistry. 6ª ed. Saunders, USA, Pp 1104.
5. Cadwell S, Rummer, J Brauner J. 2006. Blood samplig techniques and storage duration: efects on the presence and magnitude of the red blood cell B-adrenergic response in rainbow trout *(Oncorhynchus mykiss)*. Com Biochem Physiol a Mol Inegr Physiol, 162: 94-100.
6. Campbell, Ellis.2007. Avian, Exotic Animal Hematology Citology. Third Edition. Blackwell, Pp93.
7. Campbell T. 2017. Exotic animal hematology and cytology. 4ª ed. Wiley-Blackwell, USA, Pp 424.
8. DiMaggio M, Ohs CL, Petty BD. 2010. Evaluation of a Point-of-Care Blood Analyzer for Use in Determination of Select Hematological Indices in the Seminole Killifish. N Am J Aquacult, 72:3, 261-268.
9. Duman M, Saticioglu IB, Suzer B., Altun S. 2019. Practices for Drawing Blood Samples from Teleost Fish. North Am J Aquaculture, 81: 119-125.
10. Fraser D, Weary DM, Pajor EA, Milligan BN. 1997. A scientific conception of animal welfare that reflects ethical concerns. Animal Welfare, 6: 187-205.
11. Fregeneda-Grandes J, Hernandez-Navarro S, Fernandez-Coppe I, Correa-Guimaraes A, Ruíz- Potosme N, Navas- Gracia L, Aller-Gancedo M, Martín-Gil F, Martín-Gil J. 2013. Seasonal and sex related variations in serum steroid hormone leves in wild and farmed Brown trout Salmo trutta L. in the north-west of Spain. J Water Health, 11: 720-728.
12. Gallagher A, Frick L, Bushnell P, Brill R, Mandelman J. 2010. Blood gas, oxygen saturation, pH, and lactate values in elasmobranch blood measured with a commercially available portable clinical analyzer and standard laboratory instruments. J Aquat Anim Health, 22: 229-234
13. Harrenstien LA, Tornquist SJ, Miller-Morgan TJ, Fodness BG, Clifford KE. 2005. Evaluation of a point-of-care blood analyzer and determination of reference ranges for blood parameters in rockfish. J Am Vet Med Assoc, 226: 255-65.
14. Harter T, Shartau R, Brauner C, Farrell A. 2014. Validation of the i-STAT system for the analysis of blood parameters in fish. C Physiology, 2: 1-12.
15. Haugland GT, Jordal AEO, Wergeland HI. 2012. Characterization of Small, Mononuclear Blood Cells from Salmon Having High Phagocytic Capacity and Ability to Differentiate into Dendritic like Cells. PLOS ONE 7(11): e49260.
16. Herrera E. 2004. Perfil metabólico de Salmón Atlántico Salmo salar y Trucha Arcoiris *Oncorhynchus mykiss* de tres pisciculturas en fase de agua dulce en el sur de Chile. Memoria de título, Escuela de Medicina Veterinaria, Universidad Austral de Chile, Valdivia, Chile.
17. Hossain MD, Yoshimatsu T. 2014. Dietary calcium requirement in fishes. Aquacult Nutr, 20: 1-11.
18. Kaneko JJ, Harvey JW, Bruss ML. 2008. Clinical Biochemistry of Domestic Animals. 6ª ed. Academic Press, USA, Pp 928.
19. Mancera J, Mc Cormick SD. 2007. Role of Prolactin, Growth Hormone, Insulin-like Growth Factor I and Cortisol in Teleost Osmoregulation In: Fish Osmoregulation. Ed. B.G. Kapoor, Science Publishers. Pp 497-515.

20. McCornick SD, O´Dea M, Moeckel A, Thrandur B. 2003. Endocrine and physiological changes in Atlantic salmon smolts following hatchery release. Aquaculture, 222:D 45-57.
21. McCormick S. 2013. Smolt Physiology and Endocrinology. In: Fish Physiology, Eurhaline fishes, 1st ed, Academic Press, Elsevier Inc. Pp199-251.
22. Neiffer DL, Stamper MA. 2009. Fish Sedation, Anesthesia, Analgesia, and Euthanasia: Considerations, Methods, and Types of Drugs, ILAR journal, Oxford University Press.24. Olabuena SE. 2000. Fish Immune System. Gayana (Concepc.), vol.64, n.2, pp.205-215.
23. Noro M, Wittwer F. 2012. Hematología de Salmonídeos. 1ª Ed. Imprenta America, Chile. Pp 45.
24. Olabuena SE. 2000. Fish Immune System. Gayana (Concepc.), vol.64, n.2, pp.205-215.
25. Preston AC, Taylor JF, Fjelldal PG, et al. 2017. Effects of temperature on feed intake and plasma chemistry after exhaustive exercise in triploid brown trout (Salmo trutta L). Fish Physiol Biochem, 43: 337–350.
26. Rozas-Serri MA, Ildefonso R, Peña A, Enríquez R, Barrientos S, Maldonado L. 2017. Comparative Pathogenesis of Piscirickettsiosis in Atlantic salmon (*Salmo salar* L.) post-smolt experimentally challenged with LF-89-like and EM-90-like *Piscirickettsia salmonis* isolates. *J Fish Dis* 40, 1451-1472.
27. Rozas-Serri M, Lobos C, Correa R, Ildefonso R, Vásquez J, Muñoz A, Maldonado L, Jaramillo V, Coñuecar D, Oyarzún C, Walker R, Navarrete C, Gayosa J, Mancilla P, Peña A, Senn C and Schwerter F (2020) Atlantic Salmon Pre-smolt Survivors of Renibacterium salmoninarum Infection Show Inhibited Cell-Mediated Adaptive Immune Response and a Higher Risk of Death During the Late Stage of Infection at Lower Water Temperatures. Front. Immunol. 11:1378. doi: 10.3389/fimmu.
28. Tavares-Dias M. 2006. A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. J Fish Biol, 68: 1822 - 1833.
29. Thrall MA, Baker DC, Campbell TW, DeNicolla D, Fettman MJ, Lassen ED, Rebar A, Weiser G. 2012. Veterinary Hematology and Clinical Chemistry. Lippincott Williams & Wilkins, USA, Pp 776.
30. Valdevenito I. 2005. Terapias hormonales utilizadas en el control artificial de la madurez sexual en peces de cultivo: una revisión. Arch Med Vet, 40: 115-123.
31. Vargas-Chacoff L, Muñoz J, Hawes C, Oyarzún R, Pontigo J, Saravia J, González MP, Morera FJ, Labbé BS, Bertrán C, Mardones O, Pino., Wadsworth S. 2016. Atlantic salmon *(Salmo salar)* and Coho salmon *(Oncorhynchus kisutch)* display differential metabolic changes in response to infestation by the ectoparasite *Caligus rogercresseyi*. Aquaculture, 464: 469-479.
32. Vielma J, Lall S. 1998. Control of phosphorus homeostasis of Atlantic salmon *(Salmo salar)* in fresh water. Fish Physiol Biochem, 19: 83–93.
33. Weiss DJ, Wardrop KJ. 2010. Schalm´s Veterinary Hematology. 6ª ed. Wiley-Blackwell, USA, Pp 1232.
34. Witeska M. 2015. Anemia in teleost fishes. Anemia in teleost fishes. Bull Eur Assoc Fish Pathol, 35(4): 148-160.
35. Wittwer F. 2012. Manual de patología clínica veterinaria, 2nd Ed. Imprenta América, Valdivia, Chile.
36. World Organization of Animal Health. 2008. Introduction to the recommendations for animal welfare, Article 7.1.1. Pages 235-236 in Terrestrial Animal Health Code 2008. World Organization for Animal Health (OIE), Paris, Francia.
37. Zinkl, J. G., Cox, W. T., & Kono, C. S. 1991. Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish. Comp Haematol Int. 1(4): 187–195.

## Appendix 1

### Blood samples for the different analyzes in salmonids

| Anlysis | Type of sample | | Tube collects | Observations | |
|---|---|---|---|---|---|
| | 1st option | Other | | Inteferences | Other |
| **BIOCHEMISTRY** | | | | | |
| TOTAL PROTEINS | Serum | Plasma | W.A, Hep, EDTA | Hemólisis | |
| ALBUMIN | Serum | Plasma | W.A, Hep, EDTA | Hemólisis | |
| TOTAL CHOLESTEROL | Serum | Plasma | W.A, Hep, EDTA | | |
| HDL | Serum | Plasma | W.A, Hep | | |
| LDL | Serum | Plasma | W.A, Hep | | |
| TRIGLYCERIDES | Serum | Plasma | W.A, Hep, EDTA | | |
| BILIRRUBIN (Total or indirect) | Serum | Plasma | W.A, Hep, EDTA | Hemolysis Avoid direct light | The use of EDTA plasma can cause slightly decreased values |
| GLUCOSE | Plasma | - | NaF, Hep | | Separate < 1 hr (Hep sample) |
| LACTATE | Plasma | - | NaF, Hep | Hemolysis | Separate < 1 hr (Hep sample). Transport in water/ ice or in a cooler. |
| CREATININ | Serum | Plasma | W.A, Hep, EDTA | Hemolysis | |
| UREA | Serum | Plasma | W.A, Hep, EDTA, NaF | Hemolysis | |
| URI ACID | | | | | |
| AMMONIUM | Plasma | | EDTA | Hemolysis | Separate < 20 min. Transport in water/ ice or in a cooler. |
| ALT/GPT | Serum | Plasma | W.A, Hep, EDTA | Hemolysis | |
| AST/GOT | Serum | Plasma | W.A, Hep, EDTA | Hemolysis | |
| GGT | Serum | Plasma | W.A, Hep, EDTA | Hemolysis | |
| ALK. PHOSPHATASE (ALP) | Serum | Plasma | W.A, Hep | Hemolysis | |
| α-AMYLASE | Serum | Plasma | W.A, Hep | Hemolysis | |
| LIPASE | Serum | Plasma | W.A, Hep | Hemolysis | |
| CK TOTAL | Serum | Plasma | W.A, Hep | Hemolysis | |
| LDH | Serum | Plasma | W.A, Hep | Hemolysis | |
| Ca | Serum | Plasma | W.A, Hep | Hemolysis, Lipemia | |
| P | Serum | Plasma | W.A, Hep | Hemolysis, Lipemia | |
| Fe | Serum | Plasma | W.A, Hep | Hemolysis, Lipemia | |
| Mg | Serum | Plasma | W.A, Hep | Hemolysis, Lipemia | |
| **HORMONES** | | | | | |
| PROGESTERONE | Serum | Plasma | W.A, Hep | | |
| CORTISOL | Serum | Plasma | W.A, Hep | | |
| T3 | Serum | Plasma | W.A, Hep | | |
| T4 | Serum | Plasma | W.A, Hep | | |
| TESTOSTERONE | Serum | Plasma | W.A, Hep | | |
| **ELECTROLYTES** | | | | | |
| Na, Cl, K | Plasma | Suero | W.A, Hep | Hemolysis, Lipemia | Only with lithium heparin |
| **GASES** | | | | | |
| ph, Blood gases | Blood | | Heparinzed syringe | | The syringe must not contain air bubbles. If the sample is not processed within 5 minutes of collection: - Extract all the air from the syringe. - Block or close the extreme of the device used to collect the sample. - Store the sample in ice. |
| **HEMATOLÓGICO** | | | | | |
| Hemogram | Hep-blood | Blood-EDTA | Hep, EDTA | Avoid clot formation | Mix the sample with the anticoagulant by inversion (minimum 8 times) |
| Erytrogram | | | | | |
| Leukogram | | | | | |
| Thrombocytes | | | | | |

Hep-blood: heparinized blood
W.A: without anticoagulant; Hep: Lithium heparin; EDTA: ethylenediaminetetracetic acid; NaF: sodium fluoride.

## Appendix 2
## Hematology

| Parameter | Unit | Rainbow Trout | | | Atlantic Salmon | | | Coho Salmon | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pre smolt-smolt | Post smolt | Broodstock | Pre smolt-smolt | Post smolt-Adult | Broodstock | Smolt | Post smolt - Adult | Broodstock |
| Erythrocytes | $10^6$ cél/µL | 0,6 - 1,3 | 0,8 - 1,3 | 0,6 - 1,3 | 0,6 - 1,3 | 0,6 - 1,1 | 0,5 - 1,1 | 0,7 - 1,3 | 0,6 - 1,2 | 0,7 - 1,7 |
| AGV | % | 37 -60 | 39 - 74 | 40 - 72 | 30 - 58 | 33 - 59 | 36 - 62 | 38 - 67 | 35 - 68 | 42 - 70 |
| HB | g/L | 44,7 - 95,0 | 63,1 - 139,1 | 42,7 - 99,0 | 41,3 - 108,6 | 53,6 - 111,0 | 52,1 - 135,9 | 48, 0 -113,8 | 50,8 - 100,0 | 78,6 - 138,8 |
| MCV | fL | 376,5 - 798,7 | 347,3 - 735,7 | 320,0 - 743,6 | 250,9 - 747,9 | 392,5 - 773,6 | 363,6 - 732,0 | 239,2 - 766,4 | 399,1 - 775,6 | 288,6 - 723, 1 |
| MCHC | g/L | 100,0 -198,9 | 110,3 - 259,9 | 102,9 - 235,0 | 111,9 - 210,8 | 112,5 - 201,9 | 107,2 - 281,0 | 81,7 - 212,5 | 91,0 - 209,6 | 141,4 - 293,6 |
| Leukocytes | cel/µL | 6544 - 24149 | 5486 - 10315 | 5210 - 24023 | 4653 - 21290 | 4547 - 21414 | 6695 - 23693 | 5189 - 24278 | 4689 - 23572 | 3070 - 20923 |
| Monocytes | % | 0 - 3 | 0 - 2 | 0-3 | 0-4 | 0-4 | 0 - 4 | 0 - 4 | 0 - 4 | 0 -2 |
| | cel/µL | 0 - 680 | 0 - 206 | 0 - 660 | 0 - 720 | 0 - 431 | 0 - 417 | 0 - 741 | 0 - 694 | 0 - 389 |
| Lymphocytes | % | 68 - 86 | 69 - 89 | 68 - 86 | 54 - 88 | 60 - 82 | 68 - 88 | 69 - 89 | 66 - 88 | 72 - 92 |
| | cel/µL | 5320 - 17500 | 3785 - 9180 | 4340 - 18200 | 3869 - 17193 | 2781 - 15499 | 5068 - 18896 | 3020 - 19732 | 2566 - 19027 | 2579 - 16749 |
| Eosinophils | % | 0 - 1 | 0 | 0 | 0 | 0 - 1 | 0-1 | 0 | 0 - 1 | 0 |
| | cel/µL | 0 - 194 | - | - | - | 0 - 119 | 0-11 | - | 0 - 170 | - |
| Heterophils | % | 15 - 30 | 4 - 19 | 16 - 33 | 12 - 33 | 18 - 38 | 12 - 31 | 11 - 31 | 12 - 32 | 8 - 28 |
| | cel/µL | 722 - 5928 | 219 - 1960 | 868 - 6080 | 749 - 4530 | 807 - 6051 | 1213 - 5468 | 914 - 5730 | 532 - 6194 | 391 - 5159 |
| Thrombocytes | cel/µL | 2120 - 11853 | 2257 - 5725 | 3415 - 12985 | 2183 - 12966 | 1144 - 15700 | 5295 - 14220 | 3512 - 18735 | 5399 - 24615 | 4600 - 5686 |

## Appendix 3
## Biochemistry

| Parameter | Unit | Rainbow Trout | | | Atlantic Salmon | | | Coho Salmon | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pre smolt-smolt | Post smolt | Broodstock | Pre smolt-smolt | Post smolt-Adulto | Broodstock | Smolt | Post smolt - Adulto | Broodstock |
| Total Proteins | g/L | 22,0 - 24,3 | 30,2 -60,5 | 20,4 - 61,7 | 28,0 - 65,0 | 29,0 - 59,4 | 41,9 - 72,0 | 27,0 - 56,8 | 29,3 - 57,7 | 33,9 - 74,8 |
| Albumin | g/L | 9,2 - 21,7 | 12,3 - 24,5 | 7,6 - 27,2 | 11,4 - 25,0 | 13,2 - 23,3 | 15,8 - 28,2 | 11,2 - 22,0 | 11,6 - 22,0 | 10,7 - 29,0 |
| Globulins | g/L | 14,2 - 35.4 | 15,6 - 38,4 | 12,1 -45,4 | 9,4 - 48,4 | 12,8 - 39,0 | 26,0 - 45,2 | 12,3 - 37,8 | 13,6 - 38,8 | 18,2 - 45,7 |
| Cholesterol | mmol/L | 3,1 - 15,9 | 2,1 - 12,9 | 2,4 - 21,3 | 11,4 - 25,0 | 3,0 - 13,9 | 6,7 - 19,0 | 3,4 - 14,1 | 2,8 - 14,0 | 2,5 - 18,7 |
| HDL | mmol/L | 0,3 - 7,1 | 0,6 - 4,4 | 0,1 - 7,6 | 1,1 - 8,9 | 0,9 - 8,7 | 0,3 - 13,3 | 0,5 - 6,7 | 0,2 - 8,0 | 0,1 - 0,6 |
| LDL | mmol/L | 0,4 - 12,5 | 0,6 - 7,2 | 0,1 - 12,2 | 1,0 -6,4 | 0,5 - 2,9 | 0,9 - 8,7 | 0,2 - 4,3 | 0,6 - 5,4 | 0,1 -7,0 |
| Triglycerides | mmol/L | 1,6 - 14,8 | 1,5 - 9,3 | 2,4 - 19,8 | 1,7 - 8,8 | 1,0- 6,6 | 1,5 - 7,3 | 1,0 - 6,5 | 0,5 - 5,9 | 0,9 - 4,7 |
| Lactate | mmol/L | 2,3 - 15,0 | 1,9 - 9,9 | 1,4 - 15,5 | 3,1 - 11,3 | 1,9 - 10,9 | 1,7 - 6,4 | 1,3 - 13,2 | 2,8 - 10,1 | 2,0 - 10,9 |
| Glucose | mmol/L | 1,0 - 9,4 | 1,2 - 12,2 | < 19,6 | 3,5 - 11,5 | 1,4 - 8,1 | 1,2 - 12,5 | 1,8 - 12,1 | < 12,6 | < 13,8 |
| TBIL | µmol/l | <4,4 | <4,1 | <5,1 | <3,8 | < 5,3 | * | 2,5 - 4,7 | < 5,2 | <4,1 |
| DBIL | µmol/l | <3,4 | <2,2 | <3,2 | <2,4 | <3,5 | * | 1,6 - 3,7 | < 3,3 | <3,1 |
| ALT | U/L | 5,4 - 36,1 | 5,3 - 32,8 | <24 | 6,4 - 37,6 | 5,5 - 37,5 | <17 | 5,2 - 44,5 | < 33,2 | <30 |
| AST | U/L | 107 - 612 | 117 - 589 | <877 | 210 - 626 | 106 - 546 | <665 | 52 - 588 | 120 - 590 | <775 |
| ALP | U/L | 21,5 - 226 | 23 - 221 | <84 | 106 - 277 | 79,0 - 282 | <283 | 18,9 - 115 | 13 - 184 | <72 |
| LDH | U/L | 367 - 2708 | 264 - 2007 | <1884 | 436 - 3827 | 240 - 3419 | <3171 | 250 - 2350 | 268 - 1738 | <766 |
| CK | U/L | < 19212 | < 23518 | <17221 | < 25524 | < 16915 | <26352 | < 16256 | < 22944 | <8300 |
| Lipase | U/L | 3,9 - 16,0 | 3,2 - 7,7 | 5,4 - 7,2 | 2,8 - 7,4 | 2,1 - 9,1 | 5,0 - 7,8 | 4,2 - 22,5 | 4,1 - 7,4 | 6,9 - 11,0 |
| Amilase | U/L | 160 - 1652 | 419 - 1916 | <1799 | 380 - 1492 | 387 - 2026 | <1668 | 20 -2366 | 261 - 2727 | <2090 |
| Creatinin | µmol/l | < 52,9 | < 55,7 | <58,9 | < 41,6 | < 45,8 | <52,8 | <54,4 | < 51,0 | * |
| Urea | mmol/L | 0,8 - 1,5 | 1,0 - 1,8 | 0,5 - 1,4 | 0,7 - 1,8 | 0,6 - 2,7 | 0,9 - 2,6 | 1,3 - 2,3 | 0,5 - 2,1 | 0,5 - 0,8 |
| Uric Acid | µmol/l | <83,7 | <36,0 | <48,3 | <43,3 | <23,3 | <40,5 | <26,9 | <44 | <77 |
| NH3 | mmol/L | <2,2 | <2,2 | < 2,1 | < 2,4 | <2,4 | < 1,4 | < 3,7 | < 3,0 | <1,3 |
| Calcium | mmol/L | 2,30 - 4,93 | 3,17 - 3,88 | 1,65 - 7, 38 | 2,38 - 4,58 | 2,00 - 4,03 | 1,85 - 3,73 | 1,38 - 3,05 | 1,90 - 4,35 | 3,00 - 9, 00 |
| iCa | mmol/L | 0,7- 1,6 | 1,2-1,9 | 0,4-1,7 | 0,2-0,6 | 0,6-1,6 | 0,6-1,2 | 0,4-1,4 | 0,2-1,4 | 0,5-1,4 |
| Magnesium | mmol/L | 1,03 - 2,01 | 1,06 - 2,01 | 0,66 - 2,75 | 1,19 - 3,21 | 0,74 - 3,53 | 1,11 - 2,88 | 0,8 - 3,2 | 0,70 - 2,01 | 0,82 - 2,01 |
| Iron | µmol/l | 2,3 - 22,4 | 5,9 - 18,6 | 5,91 - 26,42 | 11,60 - 34,67 | 4,51 - 19,49 | 2,74 - 31,67 | 4,85 - 50,81 | 4,94 - 29,82 | 2,47 - 20,69 |
| Phosphorus | mmol/L | 4,83 - 10,27 | 6,12 - 10,37 | 1,61 - 9,79 | 4,35 - 12,20 | 2,83 - 12,24 | 3,51 - 12,20 | 7,21 - 11,98 | 4,12 - 7,82 | 3,09 - 6,73 |
| Sodium | mmol/L | 134 - 182 | 131 - 180 | 134,2 - 182,3 | 148 - 186 | 148 - 190 | 126,5 - 164,0 | 142 - 169 | 137 - 169 | 153,9 - 165,7 |
| Potassium | mmol/L | 0,7 - 8,5 | 1,0 - 9,5 | 0,5 - 11,2 | 1,1 - 7,4 | 0,9 - 9,7 | 0,6 - 9,2 | 1,2 - 13,3 | 1,1 - 16,4 | 1,1 - 7,3 |
| Chlorine | mmol/L | 102 - 147 | 101 - 152 | 95,5 - 129,3 | 111 - 159 | 118 - 163 | 95,5 - 129,3 | 108 - 149 | 105 - 151 | 119,6 - 130,3 |
| Osm NaCl | mmol/L | 139 - 284 | 212 - 300 | 218 - 295 | 238 -306 | 246 - 331 | 203 - 266 | 231 - 286 | 222 - 299 | 105 - 269 |

*: Results obtained below the detection limit of the technique.

## Appendix 4
### Gases

| | | Rainbow Trout | | Atlantic Salmon | | | Coho Salmon | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Unit | Pre smolt-smolt | Broodstock | Pre smolt-smolt | Post smolt-Adult | Broodstock | Post smolt | Broodstock |
| pH | | 7,03 - 7,45 | 7,21 - 7,54 | 7,13 - 7,46 | 7,02 - 7,56 | 7,24 - 7,64 | 7,28 - 7,54 | 7,17 - 7,47 |
| $pCO_2$ | mmHg | 14,0 - 24,8 | 12,0 - 29,8 | 11,6 - 29,5 | 11,7 - 20,6 | 11,2 - 21,9 | 7,8 - 17,1 | 16,0 - 25,8 |
| $HCO-_3$ | mmol/L | 4,9 - 14,2 | 8,4 - 21,4 | 8,7 - 14,4 | 6,7 - 10,5 | 8,3 - 13,6 | 5,3 - 9,8 | 12,6 - 16,7 |
| $TCO_2$ | mmol/L | 5,3 - 14,9 | 9,1 - 23,2 | 9,5 - 15,1 | 7,9 - 11,6 | 8,8 - 14,3 | 5,6 - 10,3 | 14,0 - 18,3 |

## Appendix 5
### Hormones

| | | Rainbow Trout | | | Atlantic Salmon | | | Coho Salmon | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Unit | Pre smolt-smolt | Post smolt Adult | Broodstock | Pre smolt-smolt | Post smolt-Adult | Broodstock | Pre Smolt-Smolt | Post smolt - Adult | Broodstock |
| Cortisol | ng/mL | 8,1 - 98,2 | 7,0 - 116,6 | 8,4 - 411,4 | 2,2 - 102,4 | 0,4 - 212,6 | 9,2 - 241,5 | 37,9 - 162,3 | 0,8 - 232,6 | 18,0 -379,7 |
| T3 | nmol/L | 0,7 - 28,5 | * | 1,8 - 47.8 | 11,5 - 22,2 | 24,2 -62,2 | 3,3 - 88,8 | 3,7 - 17,3 | 1,9 - 22,0 | 4,9 - 76,5 |
| T4 total | nmol/L | 5,2 - 11,2 | * | 5,8 - 72,5 | 7,0 - 85,4 | 5,1 -24,5 | 3,6 - 38,7 | * | - | * |
| Testosterona | ng/mL | 0,01 - 0,3 | * | 0,04 - 8,4 | * | * | * | <2,0 | <1,8 | * |
| Progesterona[a] | ng/mL | - | - | 0,04 - 0,4 | - | - | <0,3 | - | - | 0,1 - 1,8 |

T3: triiodothyronine; T4: total thyroxine
a: Hormone measured only in broodstock
*: 80% or more of the values below the detection limit of the test. It is not possible to determine the reference interval.

## Appendix 6
### Conversion factors to the International System of Units (S.I.U.)

| Parameter | Conventional Unit | Factor | S.I. Unit |
|---|---|---|---|
| Uric Acid | mg/dL | 60 | µmol/L |
| Albumin | g/dL | 10 | g/L |
| Ammonium | ug/dL | 0,587 | µmol/L |
| Direct Bilirrubin | mg/dL | 17,1 | µmol/L |
| Total Bilirrubin | mg/dL | 17,1 | µmol/L |
| Calcium | mg/dL | 0,25 | mmol/L |
| Chloride | mEq/L | 1 | mmol/L |
| Cholesterol | mg/dL | 0,026 | mmol/L |
| Cholesterol HDL | mg/dL | 0,026 | mmol/L |
| Cholesterol LDL | mg/dL | 0,026 | mmol/L |
| Cortisol | ug/dL | 27,6 | nmol/L |
| Creatinin | mg/dL | 88,4 | µmol/L |
| Iron | µg/dL | 0,179 | µmol/L |
| Inorganic phosphate | mg/dL | 0,323 | mmol/L |
| Glucose | mg/dL | 0,056 | mmol/L |
| Hemoglobin | g/dL | 10 | g/L |
| Lactate | mg/dL | 0,111 | mmol/L |
| Magnesium | mg/dL | 0,411 | mmol/L |
| Potassium | mEq/L | 1 | mmol/L |
| Progesterone | ng/mL | 3,19 | nmol/L |
| Total Proteins | g/dL | 10 | g/L |
| Sodium | mEq/L | 1 | mmol/L |
| Testosterone | ng/mL | 3,46 | nmol/L |
| Thyroxine: T4 | ug/dL | 12,87 | nmol/L |
| Triiodothyronine: T3 | ng/dL | 0,015 | nmol/L |
| Triglycerides | mg/dL | 0,0113 | mmol/L |
| Urea | mg/dL | 0,167 | mmol/L |

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ISBN: 978-956-401-932-1

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The second edition of the Manual of Clinical Pathology in Salmonids has been sponsored by Elanco.