A Characterization And Enumeration Of Blood Cells In The Cutthroat Trout (Salmo clarki)

Arleen Sorensen

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A CHARACTERIZATION AND ENUMERATION OF BLOOD CELLS IN THE CUTTHROAT TROUT \textit{(Salmo clarki)}

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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March 24, 1981
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Dr. John Christenson, Advisor

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March 24, 1981
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ACKNOWLEDGEMENTS

A thesis is a work which can be greatly enhanced by the contributions of other people. Many of these people donate their time and/or equipment as an act of kindness or out of an interest in science. Therefore, we would like to take this opportunity to thank some of the people whose aid was so important for the success of our project. We would like to thank Dr. George Holton and the Montana Department of Fish, Wildlife and Parks, especially Mr. Emmett Colley, for all of their cooperation, fish, and facilities. We wish to thank the Montana State Library and Dr. Bernard Winter for help in gathering information, and Eric Irwin for the use of his camera. We also thank Mike Holland for the use of his IBM Mag Card typewriter. Finally, we thank Dr. Christenson and Father Harrington for their guidance and encouragement.
ABSTRACT

Morphological characteristics of blood cells in the cutthroat trout (*Salmo clarki*) were explored. Such factors as mass, length, and sex were checked for relative occurrence of blood cells. Results indicate there is no correlation between mass and the number of erythrocytes or length and the number of erythrocytes. There may be a correlation between sex and the number of erythrocytes. In addition, the number of cells present of each type was determined. Lymphocytes were found to be more numerous than any other non-red blood cell. The lymphocytes are of two types—large and small. Other non-red blood cells observed include neutrophils, monocytes, and blast cells. Cutthroat also possess immature erythrocytes known as polychromatocytes. Several techniques used in fish hematology are discussed.
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INTRODUCTION

The westslope cutthroat trout (*Salmo clarki*) is native to the western part of Montana. These fish are closely related to the rainbow trout (*Salmo gairdneri*) and natural hybrids occur between the two types of fish. The pureblood cutthroat trout is being eliminated from Montana waters by the occurrence of these hybrids. We hope our small contribution to research may prove beneficial to Montana's State Fish (the cutthroat trout), fishery science, and biology.

Our study began in the area of fish immunology. We soon found the blood would have to be studied and described before immunology could be considered. Thus, we began our study of blood cells in the cutthroat trout. We intended to use fluorescent microscopy to determine whether or not T-like and B-like cells are present. We were unable to do this. We continued the study of blood cell types using Wright's and May-Grunwald Giemsa stains.

Overall, our work became a study of blood cell numbers, types, sizes, and correlations in the cutthroat trout. We have established a base from which future studies of the cutthroat trout can be made, provided information for the comparison with other Teleostei, and reviewed recent literature on this subject. Finally, the realization that all research is important is expressed in the following quote:

*Your average scientists and perhaps an even larger majority of professional technologists, whether engineers, physicians, or agricultural experts, feel uneasy when asked to discuss the purpose of what they are doing . . . . It has been easier to begin by*
discussing negative purposes, or costs. Positive purposes, which once seemed so obvious as to scarcely merit discussion, turn out to be very difficult to characterize precisely or measure quantitatively.

Robert S. Morison
Science Digest (June, 1980)
LITERATURE REVIEW

Research involving the blood of Teleostei is only recently being undertaken on a large scale. Such species as the rainbow trout, plaice, and carp have been studied\(^8\). Advancements in immunology have aided this research by providing functionally and morphologically based techniques. The sudden explosion of information thus generated has resulted in some confusion. We would like to review the information that is related to cutthroat trout hematology.

BASIC CONSIDERATIONS

Most studies use tricaine methanesulfonate (MS222) at a 1:10,000 dilution in water as an anesthetic. Some deleterious effects have been described after its use\(^{21}\). The use of a buffer is thought to diminish these problems. Quinaldine\(^{31}\) and benzocaine have also been used but the benzocaine has effects very similar to MS222\(^{21}\).

Cardiac puncture, venous puncture, and severance of the caudal peduncle\(^{17}\) are used as methods for bleeding fish. Fish less than 15 cm long usually require the caudal peduncle method. The use of plastic syringes\(^1\) and the removal of the needle before expelling the blood are necessary precautions against unwanted clotting.

Traditional anticoagulants such as mineral oil\(^{17}\), heparin, EDTA, citrates, and oxalates\(^{11}\) have been used. Heparin was considered best\(^{17}\) but has since been shown to alter staining affinity\(^8\). Recent investigations have used
the potassium salts of EDTA(1).

Hendricks'(17), Dacie's, Shaw's, and Rees-Ecker fluids are used as dilution fluids in fish hematology. Blaxhall and Daisley found modified Dacie's fluid best because of storage and staining properties. They also found standardized bulb-type diluting pipettes inappropriate. They preferred a 1:50 dilution(1). The erythrocytes(RBCs) must be counted with a hemocytometer because they are nucleated and indistinguishable to an automated counter. Abnormal amounts and/or types of RBCs may indicate pathological ailments(17).

CELL TYPES

The literature concerning teleost leukocytes is very confusing and contradictory. Some investigators claim fish have all the groups of leukocytes found in mammals(11). Others feel the leukocytes are of two types(2). Consequently, certain leukocytes are called monocytes by Ellis and macrophages by other investigators(18 cited in 8). The presence of thrombocytes is doubted by some investigators; others describe large numbers of them(8).

Neutrophils

Neutrophils have been observed in fish blood. They are described by morphology, staining reactions, and functions. However, they may not resemble their mammalian counterparts. Perhaps they should be called heterophils or Type I leukocytes instead(8). Romanowsky dyes stain their cytoplasm pale pink(1) to gray(8). The cytoplasm appears granular without distinct granules(1). The neutrophils in rainbow trout exist in three
age-associated forms. A cleft pale nucleus and finely granular cytoplasm characterize the juvenile form. The band form has a U-shaped nucleus with dense chromatin. The segmented form has a lobulated nucleus(21). Researchers describe round to oval nuclei in many non-salmonoid neutrophils. Two to five lobes occur in the salmonoid neutrophil nuclei(8).

Other considerations include benzidine-peroxidase testing and the presence of phagocytosis and chemotaxis. Neutrophils are considered benzidine-peroxidase positive by Ellis and Blaxhall and Daisley(1) but not by Kelényi(20). Phagocytosis of carbon particles is absent from plaice(8) and rainbow trout(21) neutrophils. Goldfish neutrophils are highly phagocytic to thorotrast(34 cited in 8). Neutrophil chemotaxis has not been adequately demonstrated(8).

Eosinophils

Klontz(21) denies the existence of eosinophils in rainbow trout, but Ellis does not(8). The cells are identified by large pink cytoplasmic granules when stained with Romanowsky dyes(8). Electron microscopy is being used to determine the structure to these granules(20). Ezzat claims the cells are 13.7 to 14.3 μm in diameter with nuclei smaller than those of neutrophils(11). Eosinophils are also characterized by benzidine-peroxidase testing. They are benzidine-peroxidase positive(20). In addition, goldfish and guppy eosinophils demonstrate phagocytosis(8).

Basophils

Research on fish basophils is very limited. They are
absent from the trout(31,1) and plaice(8) but occur in salmon(31). These cells are characterized using human basophil properties including purple-blue granules from Romanowsky staining(8).

**Thrombocytes**

Fish have thrombocytes rather than platelets. They function in the clotting mechanism and are referred to as "spindle cells"(8) or "flask shaped" cells(1). Blood smears prepared without anticoagulents and stained with Romanowsky dyes show four forms of thrombocytes: spiked, spindle, oval, and lone nucleus. The lone nucleus form appears similar to small lymphocytes; therefore, differential counts of these two cell types may be unreliable(8). Live thrombocytes are distinguishable when using phase contrast microscopy. They are also periodic acid-Schiff(PAS) and benzidine-peroxidase negative(31). Ferguson found plaice blood thrombocytes phagocytic to carbon particles(12 cited in 8).

**Monocytes**

Some authors claim monocytes are present(11). Others feel that these cells are actually macrophages(18 cited in 8). Klontz observed their phagocytic nature during the acute phase of the immune response. He feels this indicates the cell is a macrophage rather than a monocyte(21). They are produced by stem cells in the head kidney. Plaice monocytes phagocytize carbon particles(8).

The cells are characterized as being 17.2 μm in diameter with a eccentric oval or kidney-shaped nucleus(11). They
resemble mammalian monocytes histochemically and morphologically(31), and they have basophilic cytoplasm. Monocytes were not found in brown trout(1).

Blast Cells

Blaxhall and Daisley group all the primitive hemopoietic cells as blast cells. They are too similar to adequately differentiate morphologically, and they are too few in number to separate histochemically. They consist of cells with diameter near 15.1 μm, a light basophilic blue cytoplasm without granules, and a round to oval nucleus having a stippled appearance(1). Weinreb and Weinreb grouped the hemocytoblast, lymphoid hemoblast, and lymphoblast as blast cells(34 cited in 1).

Lymphocytes

Literature regarding fish lymphocytes is based on different techniques of enumerating and distinguishing the population and/or subpopulations of lymphocytes. Some papers are based on morphological evidence, others on functional criteria. The question of T-cell and B-cell analogues is of interest.

Fish lymphocytes are separated into two size categories. They are referred to as small lymphocytes and large lymphocytes. Small lymphocytes range from 4.5 μm in the plaice (8) to 10 μm in the rainbow trout(21). They are spherical with very dark round nuclei. Each has only a small lip of basophilic cytoplasm. Small lymphocytes are thought to have variable phagocytic activities(21). Some contain reddish purple granules when stained with Romanowsky dyes(8).

Large lymphocytes have a diameter from 8.3 μm in the
gervais(11) to 15 μm in the rainbow trout(21). They are spherical to ovoid with eccentric round or irregular nuclei. Some cells have a small indentation in the nucleus. This indentation faces the cytoplasm. The cytoplasm is nongranular and stains dark blue(1) to pale blue with Romanowsky dyes(11).

Work has been done to establish the T-like and B-like cell subpopulations using fluorescent antibody tagging, mitogenic responses, and ecotaxis. In mammals, the B-cells have more surface immunoglobulin than the T-cells. Quantitative cytofluorometry has produced some evidence for heterogeneity of fish lymphocytes(7). The strength of fluorescence was greatest for spleen, then head kidney, and finally thymus. This indicates the T-cells may come from the thymus. However, lymphocytes in fish have immunoglobulin on their surface in relatively large amounts. This has been shown in the plaice(8) and in the carp(7). "Capping," as described for murine B-cells, takes place in the fish lymphocytes, but it is not temperature dependent(8).

Mitogens are substances capable of causing non-specific proliferation of cells. In mammals, phytohaemagglutinin(PHA) and Concanavilin A(Con A) are predominantly specific to T-cells. Lipopolysaccharides(LPS) stimulate B-cells(19 cited in 8). Recent work has been carried out by Etlinger on the rainbow trout(9). He found thymocytes were stimulated by Con A but not by the purified protein derivative of tuberculin(PPD) or LPS. In addition, head kidney lymphocytes were stimulated by LPS but not PPD or Con A. This suggests heterogeneity of T-like and B-like cell lines. The thymus and
anterior kidney represent hemopoietic regions analogous to those in mammals(9).

Another useful technique is Ecotaxis(29). This procedure involves the release of tagged cells into an organism. The cells are followed to cell specific locations. Mammalian T-cells and B-cells occupy clearly defined compartments of the spleen, lymph nodes, and Peyer's patches. This kind of experiment has been performed by Parrott and DeSousa and Ellis on plaice(8). The results were very similar to those seen in rats by Goldschneider and McGregor(15 cited in 8). Labeled cells did not appear in the thymus but they did in spleen and head kidney. The cells segregated into two populations, one that incorporated the label into their RNA and another that did not(8).
MATERIALS AND METHODS

ANIMALS

We obtained westslope cutthroat trout (*Salmo clarki*) from the Anaconda Fish Hatchery and maintained them at the Giant Springs Hatchery in Great Falls, Montana. The fish were kept in a 4.6 m by 46 cm tank with a 47 l per min supply of 12°C spring water. The fish were fed once a day and the tank was cleaned ½ hr after feeding. The fish stayed in this environment for 62 days before we began actual experiments. When used, the fish had a mean length of 17.6 cm (s.d.=2.4) and a mean mass of 57.4 g (s.d.=24.9).

We also used seven trout from the Murray Springs Trout Hatchery at Eureka. These fish were taken from the parental stock of our smaller fish. They were an average size of 28 cm long and 247 g.

A male Californian rabbit and a male Australian White rabbit were maintained on Purina rabbit chow in the Carroll College animal facilities. They were used to produce antibodies. The rabbits were approximately 4 mo old when immunization procedures were begun.

PHOTOGRAPHY

Microphotographs of blood, kidney, thymus, and spleen cells were taken with a Cannon AE-1 camera and Kodachrome 64 film. The microscope was a Zeiss Standard RA. Fluorescent pictures were taken using the HBO 200 W/4 Super-pressure mercury lamp as an illuminator.
ANESTHESIA

Fish were anesthetized with MS222. The fish were placed in a 10 gal aquarium containing 10 l of a 1:10,000 dilution of the MS222. Complete anesthesia, determined by lack of response to tactile stimulation, occurred after 3 to 4 min.

COLLECTION OF EXPERIMENTAL TISSUES

Blood

Blood was collected from anesthetized fish by cardiac puncture after a medial incision was made to reveal the heart. A 20-gauge, 1-in needle was inserted into the ventricle and as much blood as possible was withdrawn into a 5-cc plastic syringe. The blood was stored for 3 days in 5 mg EDTA Vacutainers at 4°C.

Thymus

The thymus is a paired ovoid organ attached to the medial side of each operculum(22). It was teased from under its membranous covering and placed in phosphate buffered saline (PBS, see appendix) for transfer and 3 days of storage at 4°C.

Anterior Kidney

Kidney tissue anterior to the bifurcation was removed as anterior kidney tissue. The viscera were removed, the parietal peritoneum on the lateral sides of the kidney was teased and removed, and the anterior kidney was excised. The kidney tissue was transferred and stored for 3 days in PBS at 4°C.

Spleen

The spleen is found along the greater curvature of the
stomach. It was removed by cutting the gastro-splenic omentum near the hilum. The spleen was transferred and stored for 3 days at 4°C in PBS.

**PREPARATION OF SLIDES**

Slides were made from blood, kidney, thymus, and spleen. Blood smears were made and allowed to air dry. They were stained with Wright's and May-Grunwald Giemsa stains (see appendix). Kidney, thymus, and spleen slides were made using an imprinting technique. A piece of tissue was cut to expose an inner section. This section was then pressed onto the slide to transfer some of the cells to the glass. The smears were allowed to air dry before staining.

Wright's stain was poured over and allowed to stand on an air-dried smear for 1 min. An equal amount of water was added and allowed to stand 3 min. The slide was then flooded with water, drained, and allowed to dry.

A second set of blood slides and the kidney, thymus, and spleen slides were stained with May-Grunwald Giemsa stain. The air-dried smears were fixed in absolute methanol for 5 min. The slides were then placed in Jenner's stain for 4 min and transferred directly to Giemsa stain for 15 min. Stained slides were then rinsed with water and allowed to dry.

**COUNTING OF BLOOD CELLS**

We used an Improved Neubauer hemocytometer (1/400 sq. mm; 1/10 mm deep) to count a 1:50 dilution of RBCs in Dacie's fluid. We counted the cells in the four outer areas and the middle area. Each area consisted of 16 small squares, each with a volume of 1/4000 mm³. Eighty of these squares were counted
and our two dilutions (1/10 and 1/50) gave a total factor of 1/25,000. Therefore, all RBC counts were multiplied by 25,000 to obtain cells/mm
3. The cells of stained slides were counted in random areas until 300 cells had been counted on each slide. All cells were classified and their percents of occurrence were calculated.

FLUORESCENT ANTIBODY TECHNIQUE

Purification of Antigen

Two antigens were used. The first was the immunoglobulin fraction from cutthroat trout blood. This fraction was separated by salting out and subsequent dialysis. Blood was pooled from seven large (approximately 247 g) cutthroat trout and kept in EDTA. Plasma was obtained by centrifugation of the pooled blood at 1469 RCF for 15 min. The plasma was diluted with an equal volume of PBS and fibrinogen was removed by bringing the plasma to 20% saturation with ammonium sulfate solution drop by drop (see appendix). The solution was allowed to stand for at least 30 min before centrifuging at 13,218 RCF for 15 min. Ammonium sulfate solution was again added to bring the saturation to 50%. The solution stood for at least 30 min and was centrifuged at 13,218 RCF for 15 min. The supernatent was decanted and the precipitate was dissolved in PBS (same volume as original volume of plasma). This precipitation step was repeated three times and the final precipitate was dissolved in a small amount of PBS. The remaining ammonium sulfate was removed by dialysis through cellulose against PBS. The PBS was changed approximately every 5 hr until no white precipitate was visible when 0.5 M BaCl was added to the used PBS. All of
the procedures were carried out in a cold room and all solutions were cooled to 4°C.

Secondly, we prepared a thymus cell suspension. Thymus tissue was collected from the same seven fish and homogenized in a minimal amount of Hank's buffered saline. The procedure was performed using an ice bath.

**Preparation of Immune Serum**

Both antigens were mixed into Freund's complete adjuvant in equal volumes by using a plastic syringe without a needle. The antigen and adjuvant were forcefully expelled from the syringe until the antigen was completely emulsified. Emulsification was determined by placing a drop of the mixture into cold water. If the drop kept its shape, the mixture was considered emulsified. The antigens were mixed with Freund's incomplete adjuvant in the same manner.

**Injection of Rabbits**

The rabbits' necks were shaved on the dorsal side and 0.2 ml injections were made subcutaneously with a 20-gauge needle and 1-cc syringe. Shots of 0.1 ml were also given into the hind foot pads. The Californian rabbit received 1.2 ml in the neck and 0.2 ml in the foot pads. The injection material had a concentration of 0.4 mg/ml; thus, the rabbit received a total of 0.64 mg of protein. The Australian White received 0.5 ml in the neck and no shots in the foot pads; thus receiving a total of 0.22 g of the thymus cell material.

Each rabbit also received a booster shot 10 days before they were bled. The Californian rabbit received 0.8 ml immunoglobulin suspension 40 days after its first injection. The
Australian White rabbit received 0.5 ml of the thymus suspension 30 days after its first injection. The boosters were given subcutaneously in the neck.

**Bleeding of Rabbits**

Fifty ml of blood were taken from each rabbit by cardiac puncture and kept in the refrigerator (4°C). Cardiac puncture required the use of 18-gauge needles and 50-cc plastic syringes. The blood was allowed to clot in the refrigerator for 24 hr. The extruded serum was removed using sterile pasteur pipettes and placed in 2-ml aliquots which were stored at -10°C.

**Preparation of Absorption Tissue**

We mixed 1.5 g of fish liver tissue with 1.5 ml PBS, added 12 ml acetone and centrifuged at 1469 RCF for 10 min. We recovered the precipitate, added 6 ml PBS and let this sit for 8 hr in the cold. The precipitate was recollected by centrifugation and resuspended in 1.5 ml saline. This was repeated until no hemoglobin pigment (red color) was discernable in the supernatent. The final precipitate was collected using a Büchner funnel and dried ½ hr in a 37°C incubator.

Absorption powder (0.23 g) was added to the serum of each rabbit. After standing 1 hr at room temperature, the material was centrifuged at 23,499 RCF for 25 min. The supernatent was collected and purified as absorbed antibody.

**Purification of Primary Antibody**

The frozen serum was allowed to thaw to room temperature and then absorbed using the above method. Next, we purified out the gammaglobulin(IgG) fraction by the following method:
sodium sulfate (1.8 g) was added to 10 ml serum and centrifuged at 13,218 RCF for 15 min. The precipitate was saved and dissolved in 4 ml PBS and 0.48 g sodium sulfate was added to this and centrifuged again. The precipitate was dissolved in 2 ml PBS and 0.26 g sodium sulfate was added. The solution was centrifuged once more. The precipitate was dissolved in a small amount of PBS and dialyzed against PBS for 48 hr. The solution of PBS was changed every 8 hr.

**Fluorescent Staining**

Nonfluorescent slides were cleaned in ethanol and rinsed 20 times in distilled water. The following procedures were done in reduced light. A drop of blood was placed on the slide, allowed to air dry, and fixed for 1 min with 95% ethanol. A drop of primary antibody was added and the slide was placed into a petri dish containing a Bacto-FA buffer-moistened paper (pH=7.2). The dish was placed in a 35°C incubator for 30 min. The slide was then rinsed in two changes of buffer and a drop of FITC labeled antibody (conjugate) was added. The slide was again incubated at 35°C for 30 min and rinsed in two changes of buffer. The cover slip was mounted with Bacto mounting fluid.

**Determination of Titer**

We used a precipitation test to determine the titer of our solutions. The conjugate was diluted 10 times with PBS and 0.25 ml was added to each of the nine tubes. IgG fraction (0.25 ml/tube) was added, using doubling dilutions starting with a 1:2 dilution in tube 1. The IgG fraction from the Australian White rabbit was unabsorbed while that of the
Californian rabbit was absorbed. We titered blood from both rabbits against conjugate a second time but diluted the conjugate only by two. This time, both fractions were absorbed. In the above cases, a 1:2 dilution of IgG served as a control.

The titer between fish immunoglobulin and the Californian rabbit was also determined. Fish immunoglobulin was diluted by combining 0.5 ml with 2 ml of PBS. This was placed over unabsorbed IgG from the rabbit (which had been diluted by two), again using doubling dilutions for the nine tubes. The control consisted of the diluted fish immunoglobulin.

Biuret

Protein concentrations were determined using the Biuret procedure. A standard curve was prepared with serum albumin. We then determined protein concentrations of various solutions; 0.1 ml of solution + 0.1 ml 10% sodium deoxycholate + 1.3 ml 0.9% NaCl + 1.5 ml of biuret reagent was allowed to stand for 30 minutes. The solution was then analyzed using a Beckman Model B spectrophotometer. Absorbances were measured at 540 nm. The spectrophotometer was zeroed with a blank consisting of 0.1 ml 10% sodium deoxycholate + 1.4 ml 0.9% NaCl + 1.5 ml biuret reagent. Resulting absorbances were compared to our standard curve and multiplied by 10 to give the total mg/ml protein.
RESULTS

Differential Blood Cell Counts and Cell Sizes

We counted blood cells from 18 fish. Differential counts from the stained, permanent slides are given in Table 1. Hypothesis testing was utilized to detect any difference in staining methods. Results showed that only for small lymphocytes could the hypothesis that the mean count with Wright's stain is equal to the mean count with May-Grunwald Giemsa stain be rejected at a confidence level of 90%. We also checked the hypothesis that the number of large and small lymphocytes are equal for each staining method. We could only reject the hypothesis in the case of Wright's staining.

RBC counts made with Dacie's fluid had a mean count of 1,930,000 RBCs/mm$^3$ with a standard deviation of 1,170,000/mm$^3$. We graphed RBC count versus mass and RBC count versus length. These results are in Fig. 1 and 2. Thirteen of the 18 fish were male. These fish had a mean RBC count of 2,055,000/mm$^3$. The remaining five fish were female with a mean RBC count of 1,615,000/mm$^3$. Hypothesis testing showed that with a 90% confidence level, we could not reject the hypothesis that the number of RBCs in females equals the number in males.

Size determinations were made using selected slides from both staining procedures. The results can be found in Table 2. Thrombocytes were not measured.

Titer

The titer of all the needed antigen-antibody complexes except the Australian White rabbit versus thymus was performed
Table 1. Differential cell counts of cutthroat trout blood.
Results are given for the counts obtained from the
two staining procedures. The mean counts are given
as cells per 300 total cells. The % white cells
includes small and large lymphocytes, neutrophils, and
monocytes.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mean Count</th>
<th>Standard Deviation</th>
<th>% of Total Cells</th>
<th>% of White Cells</th>
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<tr>
<td>Wright's Stain</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Large Lymphocyte</td>
<td>12.8</td>
<td>11.3</td>
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<td>77.4</td>
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<tr>
<td>Small Lymphocyte</td>
<td>2.8</td>
<td>3.2</td>
<td>0.9</td>
<td>16.9</td>
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<td>Thrombocyte</td>
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<td>1.5</td>
<td>0.4</td>
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<td>0.7</td>
<td>0.1</td>
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</tr>
</tbody>
</table>
with a precipitation test. The titer of rabbit antifish globulin and fish immunoglobulin was 320. The titer of the Californian rabbit against conjugate was 16. The titer of the Australian White rabbit against conjugate was 32.

Biuret

Biuret determinations showed the Californian rabbit absorbed IgG concentration to be 4.4 mg/ml. Its unabsorbed concentration was 19.6 mg/ml. The Australian White rabbit had an absorbed concentration of IgG of 1.67 mg/ml and unabsorbed concentration of 2.1 mg/ml. Goat antirabbit-labeled conjugate had a concentration of 2.1 mg/ml, and the fish immunoglobulin concentration was 3.5 mg/ml.

Table 2. Cell sizes in cutthroat trout blood. Sizes were determined from slides stained with Wright's and May-Grunwald Giemsa stains.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th># cells counted</th>
<th>Size (μm)</th>
<th>Standard Deviation(μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Lymphocyte</td>
<td>32</td>
<td>9.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Small Lymphocyte</td>
<td>31</td>
<td>5.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>17</td>
<td>9.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Blast Cells</td>
<td>31</td>
<td>13.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Monocyte</td>
<td>17</td>
<td>7.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Polychromatocyte</td>
<td>31</td>
<td>8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>RBC</td>
<td>30</td>
<td>9.4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Fig. 1 Number of RBCs versus length in cutthroat trout blood. The line is the calculated line of best fit. $y = 17.5 + 0.0029x$. It has a correlation coefficient of 0.05.
Fig. 2 Number of RBCs versus mass in cutthroat trout blood. The line is the calculated line of best fit. $y = 62.25 - 0.033x$. It has a correlation coefficient of 0.06.
DISCUSSION

Much of the pertinent research done on fish involves the rainbow trout. We decided to examine the cutthroat trout because it is native to Montana and is closely related to the rainbow trout. We hoped to obtain data which could shed light on the cellular components of teleost blood.

MS222 is the most widely used anesthetic for fish. It can affect staining affinities and may alter the number of lymphocytes present. We feel the above problems are related to repeated use. Our fish were sacrificed as soon as the blood sample was drawn; therefore, this should not have been a factor in this study. Anesthetic improved reliability by reducing trauma to the fish.

The fish used were too small for venous puncture. Severing of the caudal peduncle and subsequent collection of blood would have introduced unnecessary dilution errors from the peritoneal fluid and slime. Consequently, we chose cardiac puncture and made a medial incision because the ability to see the heart is an advantage. Fish blood is very fast clotting; thus, we used plastic syringes and worked as quickly as possible. We attempted to prevent any small clots from entering the tubes since small clots facilitate the clotting of the rest of the blood.

We selected Dacie's diluting fluid because of its staining ability. Dacie's fluid contains Brilliant Cresyl Blue, a nuclear stain. The darkened nuclei helped us to see the RBCs and aided counting. One five area count was performed
for each fish. This should have been sufficient to eliminate any bias. More counts per fish, however, would be preferred.

The value of $1.93 \times 10^6$ RBCs/mm$^3$ is within the ranges shown by other researchers. For example, Ellis states the RBC counts in Teleostei ranged from $1.05 \times 10^6$ to $3.0 \times 10^6$ cells/mm$^3$ (8). In addition, the size data obtained for RBCs seems acceptable.

We used Wright's stain, a standard of human hematology, and May-Grunwald Giemsa, a preferred stain in fish hematology, to compare the two stains. RBC counts were plotted against mass and length to look for any correlations. We also tested for differences between cell counts in the male and female fish. A statistically valuable correlation coefficient for mass versus RBC or for length versus RBC was not demonstrable.

A difference may exist in the number of RBCs in males and females even though the data when analyzed statistically did not show this. With the exception of one female, the female RBC counts were statistically lower than the RBC counts for males. The small sample size (only five female) makes the data very difficult to interpret.

Neutrophils were definitely present in the cutthroat trout. Their nuclei were multilobed (2-5) and very easily seen. The neutrophils had a mean size of 9.8 μm in comparison to 9.1 μm in brown trout as recorded by Blaxhall and Daisley (1). Some differences in cytoplasmic color were seen but we were unable to discern whether the cells were neutrophils, eosinophils, or basophils; and elected to consider them all as neutrophils. Neutrophils are said to make up 20.0 to 66.1% of the white
cells(11). In our counts, they make up only 2.6 to 2.9%; therefore, counting all polymorphonucleated cells as neutrophils seems appropriate.

Thrombocytes were not very common in the cutthroat trout. They were about 14 times less common than lymphocytes. We were hoping to better enumerate them by use of fluorescence but were unsuccessful. Ellis has used fluorescence to count thrombocytes in plaice and found them to be only one-fourth as common as lymphocytes. We feel this is not the case in cutthroat trout. If we consider the possibility that we counted an appreciable amount of thrombocytes as lymphocytes, then we would have an unusually low number of lymphocytes present. Since lymphocyte counts are more firmly established, we feel they are more likely correct. Consequently, we think thrombocytes may be less prevalent in cutthroat trout than in plaice.

We feel monocytes are present. We applied criteria based on other reports(8) and found monocytes made up 2.2 to 3.0% of the leukocytes. This value is low in comparison to other members of the genus Salmo. We found it to be in close agreement with Anguilla japonica in which 2.5% of the leukocytes were monocytes(24 cited in 11). We were surprised to determine their average size to be 7.7 μm, a value considerably smaller than the 12.2 μm described for Tilapia zilli(11). Possibly these cells are age related forms of neutrophils rather than monocytes. Blaxhall and Daisley feel this is the case in brown trout(Salmo trutta)(1). If they are in fact younger neutrophils, this could account for their smaller size(7.7 μm compared to 9.8 μm for neutrophils.) This could also help
explain our low neutrophil percentages.

The fact that we were unable to distinguish specific types of blast cells led us to adopt Blaxhall and Daisley's viewpoint. Thus we grouped them all into a single category referred to as blast cells. We found the cutthroat trout blast cells to be slightly smaller than those in the brown trout (13.8 µm compared to 15.1 µm for brown trout)(1). They have a pale pink cytoplasm and very light pale blue mottled nuclei.

Our data, along with those of other investigators, show lymphocytes to be the most common of the non-red blood cells present in trout blood. They were of two morphological types, large and small. Size measurements on these subpopulations are very closely related to those in the brown trout(1). We felt the large lymphocytes are more common than the small lymphocytes. However, Blaxhall and Daisley found the small population to be most prevalent. We feel the young age of our cutthroat trout may account for our large lymphocytes being more prevalent. One theory that supports this suggests the large lymphocytes are precursors to the small lymphocytes. We feel small lymphocytes represent the competent form of lymphocytes and that they should by rarer in younger fish which have not had as varied an immunological experience.

The number of lymphocytes is appreciably greater in fish than in humans. For example, we counted 8.5x10^4 to 10.0x10^4 cells/mm^3 compared to 2.0x10^3 cells/mm^3 in man. We feel this reflects an increased need for lymphocytes in fish because of either their cold-blooded condition and/or deficiencies
in their immunological system. Further work should center on these topics.

We were not successful in our attempts to fluorescently tag subpopulations of lymphocytes. We do not think that it was because of properties of the fish cells but rather a collective affect caused by procedural difficulties. These might include absorption of our active fraction and too low of an antibody titer. Consequently, we are unable to provide new data to support or deny the existence of T-like and B-like populations in fish lymphocytes. We feel other evidence cited in the literature review and rational arguments based on phylogeny suggest separate populations probably do exist. We did find that RBCs appear to have immunoglobulin on their surface. This observation should be skeptically accepted since it may have been non-specific staining or autofluorescence.

We also made imprint slides of the head kidney, spleen, and thymus. We did not find these particularly useful. They were not conducive to cell differentiation because we could not make positive cell identifications. Also, some types of cells do not transfer well and are therefore misrepresented on imprints.

In conclusion, the study of the cutthroat trout has provided several bases for continued study. We developed a picture of the cellular components of blood which is very basic. The question of subpopulations of lymphocytes remains to be solved. Histochemical properties of the blood cells should be explored and studies of the spleen, thymus, and head kidney should be done with sections. Further study is also
needed in the humoral aspects of teleost immunology. We hope these studies may be aided by the cellular information we described.
APPENDIX

PREPARATION OF VARIOUS FLUIDS

PHOSPHATE BUFFERED SALINE (14)

8 g NaCl
0.2 g KCl
1.15 g anhydrous Na₂HPO₄
0.2 g KH₂PO₄

These are dissolved in 1 l distilled water and adjusted to a pH of 7.2-7.4. A 10-times concentrated stock solution without NaCl was prepared. It was diluted and NaCl added just before use.

MAY-GRUNWALD GIEMSA STAIN (26)

Jenner solution (stock)
1 g Jenner stain, dry powder
400 ml alcohol, methyl

Giemsa solution (stock)
1 g Giemsa powder
66 ml Glycerin
66 ml alcohol, methyl

Jenner solution (working)
25 ml Jenner stock
25 ml distilled water

Giemsa solution (working)
50 drops Giemsa stock
50 ml distilled water

make fresh, do not reuse

MODIFIED DACIE'S FLUID (1)

10 cm³ 40% formaldehyde
31.3 g Trisodium Citrate
1 g Brilliant Cresyl Blue
1 l distilled water

Filter before use
AMMONIUM SULFATE SOLUTION

400 g ammonium sulfate (without iron)
500 ml water (distilled)

Heat to 70-80°C and stir for 20 min and allow to cool to room temperature. Crystals form at the bottom and the supernatent is saturated with a pH of 5.0. The pH should be adjusted to 7.0 with 28% ammonia solution. Ammonium sulfate should be prepared in advance to allow time for saturation. Filter before use.
REFERENCES


