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Hematologic and Serum Biochemical Values for Zebrafish (*Danio rerio*)

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The zebrafish (*Danio rerio*) has proven an excellent model for study of vertebrate development and genetics. Mutagenesis studies have produced many blood mutants with defects ranging from hematopoiesis to coagulation. The overwhelming majority of zebrafish studies have focused on development and mutational effects in embryos, whereas effects in mature zebrafish have gone largely unexplored. We believe that zebrafish will prove a valuable model for study of aging and age-related diseases, and we have sought to characterize some of the basic features of mature zebrafish. Accordingly, blood was collected from adult zebrafish and was analyzed to determine reference hematologic and biochemical parameters. White blood cell differential counts indicated predominantly lymphocytes, with mean proportion of 82.95%. Total red blood cell counts averaged 3.02×10^6 cells/μl. Except for increases in alanine transaminase (ALT), amylase, and phosphorus values, serum biochemical analytes were within the range of reported values for mammals and other species of fish. Accurate analysis of the many zebrafish mutants generated requires determination of normal characteristics of zebrafish. We believe results such as these will help define normal adult zebrafish, which have a tremendous potential for use in the study of human disease and aging.

The zebrafish (*Danio rerio*) has proven an excellent model for study of vertebrate development and genetics (1, 2). In recent years, several studies have documented the effects of mutagenesis, such as hematopoietic defects in the offspring of mutagenized fish (3, 4) and the development of cancers in the mutagenized fish themselves (5), on mature zebrafish. One unique aspect of zebrafish among currently used vertebrate animal models is that the species is readily amenable to large-scale mutagenesis studies. Allowing the offspring of mutagenized zebrafish to reach maturity could lead to identification of phenotypes that model various human diseases and, subsequently, discovery of the genes responsible for these diseases. For example, a mutant zebrafish line, *sauternes* (*sau*), has been reported to model congenital sideroblastic anemia (CSA) in humans and represents the first animal model of this disease (6). To fully exploit the potential of these unique animal models, we have sought to characterize some of the basic features of mature zebrafish by examining hematologic and serum biochemical parameters in wild-type, healthy, adult zebrafish.

Materials and Methods

Animals. Mature zebrafish (approx. one year old) were obtained from Scientific Hatcheries (Huntington Beach, Calif.). All fish were healthy and free of signs of disease. Our zebrafish supplier screens their facility for *Capillaria* and *Mycobacterium* spp., *Flavobacterium columnare*, external skin and gill parasites (*Gyrodactylus*, *Oodinium*, *Ichthyophthirius*, and *Trichodina* spp.), *Aeromonas* spp., and microsporidia, and it has been free of these disease-causing organisms. All fish were maintained and treated humanely, and experimental protocols were approved by our Institutional Animal Care and Use Committee.

Husbandry. All fish were allowed to acclimate to their new environment for at least one week after arrival. Fish were housed in 10-gallon aquaria containing conditioned tap water at 28°C in groups of approximately 25 animals/tank, resulting in a stocking density of 2.5 fish/gal. The water was conditioned by mixing tap water (adjusted to 28°C) with AmQuel (Novalek, Inc., Hayward, Calif), an instant water detoxifier that removes ammonia, chlorine, and chloramines, at a ratio of 1 tsp of AmQuel/10 gal of tap water. This water was immediately added to the tanks during cleaning, which involved removing 25 to 33% of the tank water and replacing it with the conditioned water once a week. Each tank had an external three-way filtration system (Whisper Power Filter, Tetra, Blacksburg, Va.), which included mechanical (floss screen), chemical (carbon), and biological (aerobic bacteria) filtration components. Water flow rate was approximately 125 gal/h. Water quality was assessed weekly by measuring pH, ammonia, nitrite, and nitrate values, using Aquarium Pharmaceuticals Inc. (Chalfont, Pa.) water test kits. Water pH was maintained between 6.8 and 7.4, and ammonia, nitrite, and nitrates concentrations at 0, 0, and 0 to 20 ppm, respectively. The light:dark cycle was maintained at 14:10 h. Fish were fed a 50:50 mixture of commercial flake food and freeze-dried brine shrimp twice daily, and fresh brine shrimp once daily.

Blood collection. Each fish was quickly euthanized by immersion in MS-222 (3 g in 1000 ml ice water) (Sigma Chemical Co., St. Louis, Mo.). Blood was immediately collected from the dorsal aorta as described (7). Briefly, a transverse incision was made just caudal to the dorsal fin as follows. The skin was punctured initially by inserting the sharp ends of a slightly opened pair of dissecting scissors. Closure of the scissors resulted in a 0.3- to 0.5-cm incision. Blood welling up from this incision was

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rapidly collected by use of a micropipette tip. Blood yields from individual fish ranged from one to 10 µl.

Smears were immediately prepared from fresh whole blood. For total erythrocyte counts, the micropipette tip was coated with EDTA prior to blood collection, and whole blood from groups of five fish was pooled in an EDTA-coated microtube. For serum acquisition, whole blood from groups of 10 zebrafish was pooled in a 0.5-µl microcentrifuge tube, allowed to clot, and spun for 10 min at 2,500 rpm; then serum was pipetted off the top. We used these parameters because we determined that the amount of time required to collect blood from groups of fish larger than 10, and centrifuge speeds > 2,500 rpm, resulted in marked hemolysis of the sample. Sera from approximately 50 zebrafish were then pooled to assay biochemical analytes in each sample.

Hematologic analysis. Blood smears were treated with Diff-Quik stain (Dade Behring Inc., Newark, Del.), and leukocyte differential counts were performed on smears from 50 individual zebrafish. For total erythrocyte counts, anticoagulated whole blood from three groups of five fish each was diluted, using a Unopette microcollection system (Becton Dickinson and Company, Franklin Lakes, N.J.), and was counted by use of a hemacytometer. Due to their similar size and appearance on the hemacytometer, we were unable to determine total platelet and leukocyte counts using the Unopette system. Staining with Rees-Ecker diluent, which reportedly stains leukocytes and platelets differentially (8), failed to distinguish between these cell types.

Serum biochemical analysis. Full serum biochemical panels were obtained from the pooled serum of five groups of approximately 50 zebrafish, using the VetScan Diagnostic Profile II (Abaxis, Inc., Union City, Calif.).

Results

Leukocyte differential counts. Counts from the smears of zebrafish (n = 50) indicated predominantly lymphocytes, with smaller numbers of monocytes, neutrophils, eosinophils, and basophils (Table 1). Erythrocytes measured approximately 7 × 10 µm, and were easily recognized by their eosinophilic cytoplasm and ovoid nuclei oriented parallel to the long axis of the cell. Lymphocytes were round to ovoid in shape, with a large nucleus and thin rim of cytoplasm, and were approximately 5 to 8 µm in diameter (Fig. 1). Monocytes were recognized by their large size (approx. 12 to 18 µm in diameter), abundant, foamy, basophilic cytoplasm, and irregularly shaped nuclei (Fig. 2). There is considerable controversy regarding the correct terminology for describing granulocytic leukocytes in fish, and they reportedly may have either neutrophils or heterophils, or both (8). Heterophils contain distinct granules that are usually rod-shaped and eosinophilic and frequently obscure nuclear detail when viewed under light microscopy (19). The principal granulocytic cells that we observed lacked these distinct granules, had pale blue color and grainy appearance to their cytoplasm, and most closely resembled neutrophils, with segmented and band-type cells being present (Fig. 3). The indented nuclei and more abundant, grainy-appearing cytoplasm distinguished them from the lymphocytes. Neutrophils measured approximately 9 to 10 µm in diameter. Thrombocytes were similar in appearance to small lymphocytes (Fig. 4), though slightly smaller (3 to 4 µm in diameter). The denser chromatin pattern and frequent aggregation of the thrombocytes allowed them to be distinguished from the lymphocytes by use of the Diff-Quik stain.

Table 1. White blood cell differential counts for zebrafish

	Mean ± SD (%)	Range (%)
Lymphocytes	82.95 ± 5.47	71 - 92
Monocytes	9.68 ± 2.44	5 - 15
Neutrophils	7.10 ± 4.75	2 - 18
Eosinophils	0.15 ± 0.53	0 - 2
Basophils	0.13 ± 0.40	0 - 2

Total erythrocyte counts. Total red blood cell counts from three groups of zebrafish averaged 3.02 (range, 2.89 to 3.25) × 10^6 cells/µl. We were unable to obtain total white blood cell and platelet counts due to the similar size and appearance of the cells on the hemacytometer.

Serum biochemical analytes. Results of serum biochemical analysis are listed in Table 2. We were able to measure the samples readily, using an automated serum chemistry analysis

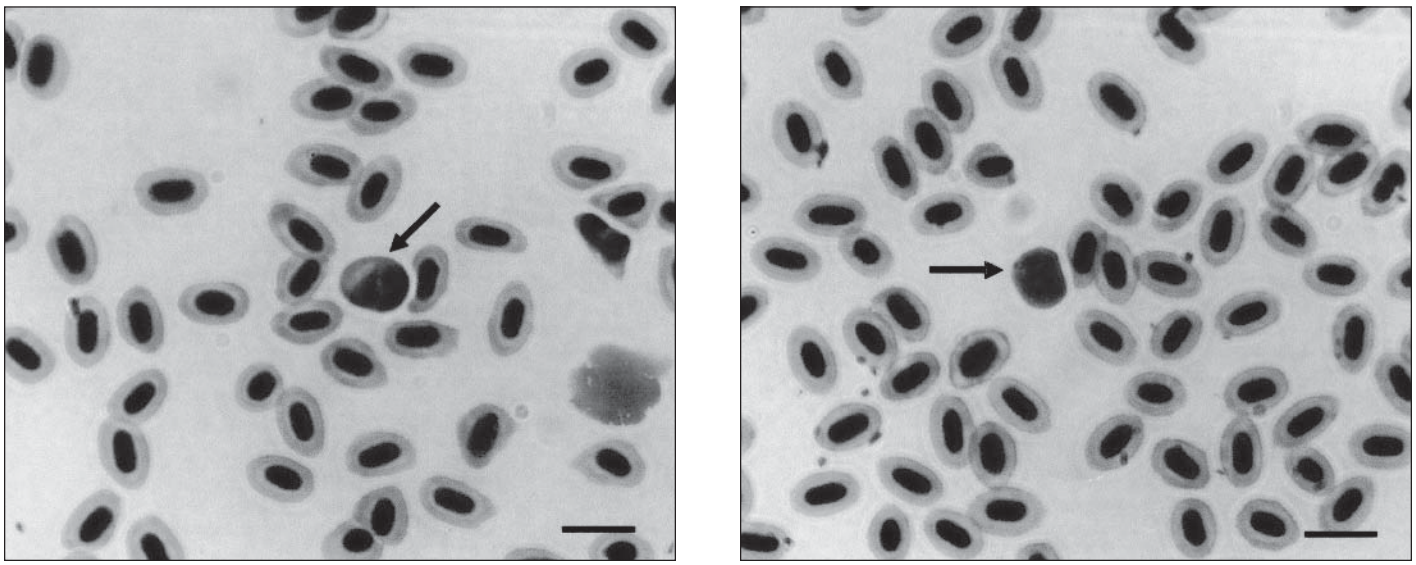

Figure 1. Photomicrograph of zebrafish lymphocytes (arrows). The other cells are erythrocytes, which are easily recognized with their eosinophilic cytoplasm and ovoid nuclei oriented parallel to the long axis of the cell. Diff-Quik stain; bar = 10 µm (1,000× magnification).

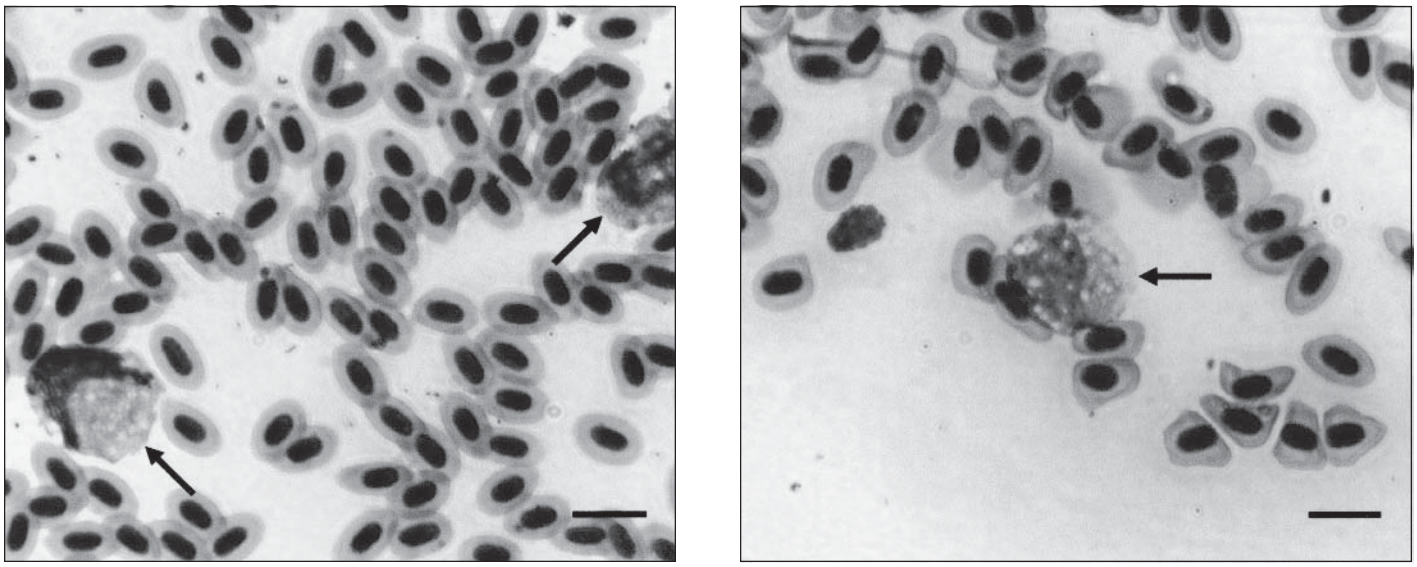

Figure 2. Photomicrograph of zebrafish monocytes (arrows), which are recognized by their large size, abundant, foamy, basophilic cytoplasm, and irregularly-shaped nuclei. Diff-Quik stain; bar = 10 µm (1,000× magnification).

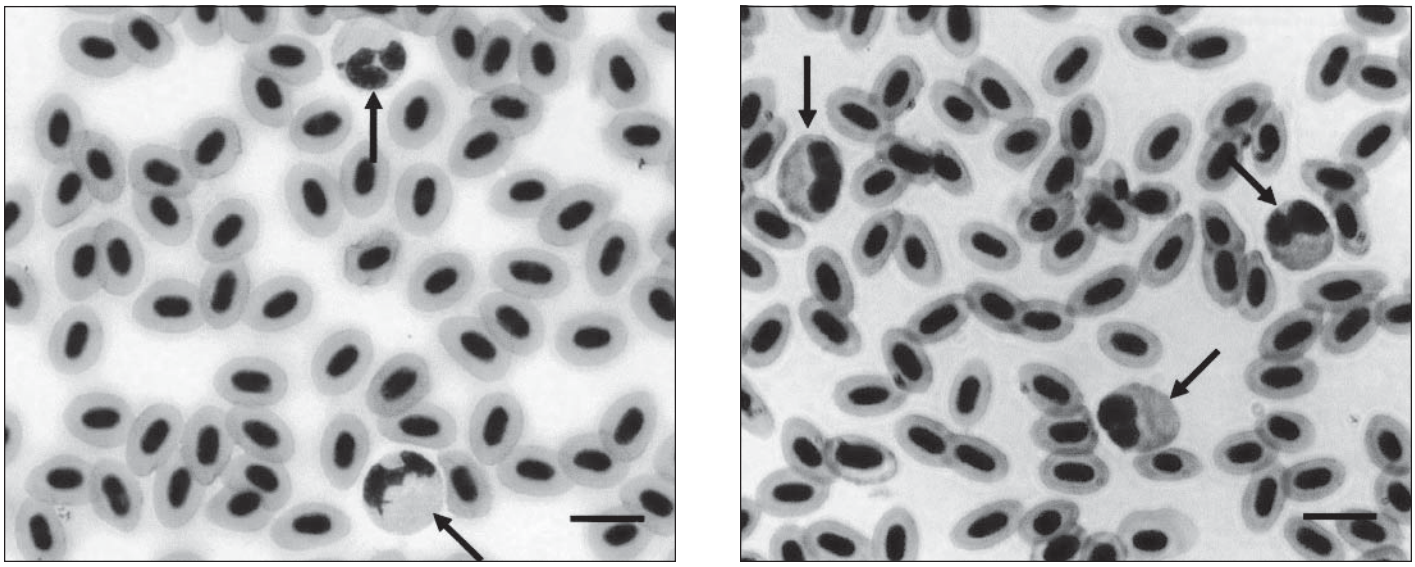

Figure 3. Photomicrograph of zebrafish neutrophils (arrows) appearing as segmented and band-type granulocytes. The indented nuclei and more abundant, grainy-appearing cytoplasm distinguished them from lymphocytes. Diff-Quik stain; bar = 10 µm (1,000× magnification).

system. There was moderate hemolysis in the samples; however, we were able to obtain values for all biochemical analytes tested.

Discussion

In the study reported here, we determined hematologic and clinical chemical parameters in adult zebrafish. We believe these values will be useful in future studies that examine various disease models in zebrafish, normal aging of zebrafish, and in screens of mutagenic zebrafish lines.

In recent years, there has been a tremendous increase in the number of zebrafish with genetic mutations. There is great potential for the use of these mutants as models of human disease. Several anemias of mutant zebrafish with phenotypes that resemble human disorders have been described. These mutants have altered erythrocyte indices, compared with those of wild-type zebrafish. For example, the zebrafish mutant *sauternes* (*sau*) has a microcytic, hypochromic anemia due to a mutation in the gene coding for the enzyme δ-aminolevulinate synthase (ALAS2) (6). Mutations in ALAS2 are known to cause CSA in humans, and the *sau* mutant zebrafish represents the first animal model of this disease. Positional cloning also has revealed the gene responsible for the hypochromic anemia of the zebrafish mutant *weissherbst* (*weh*) (9). The function of this gene, ferroportin1, may be perturbed in mammalian disorders of iron deficiency or overload.

In addition to hematologic mutants such as these, zebrafish also have been documented as potential models for the study of other human diseases, such as hepatoerythropoietic porphyria (HEP) (10), Huntington's disease (11), Alzheimer's disease (12), multiple endocrine neoplasia type 1 (MEN1) (13, 14), and congenital coarctation of the aorta (15), to name a few. The field of transgenics has also begun to emerge in the zebrafish community (16, 17).

Although hematologic data have been reported for several

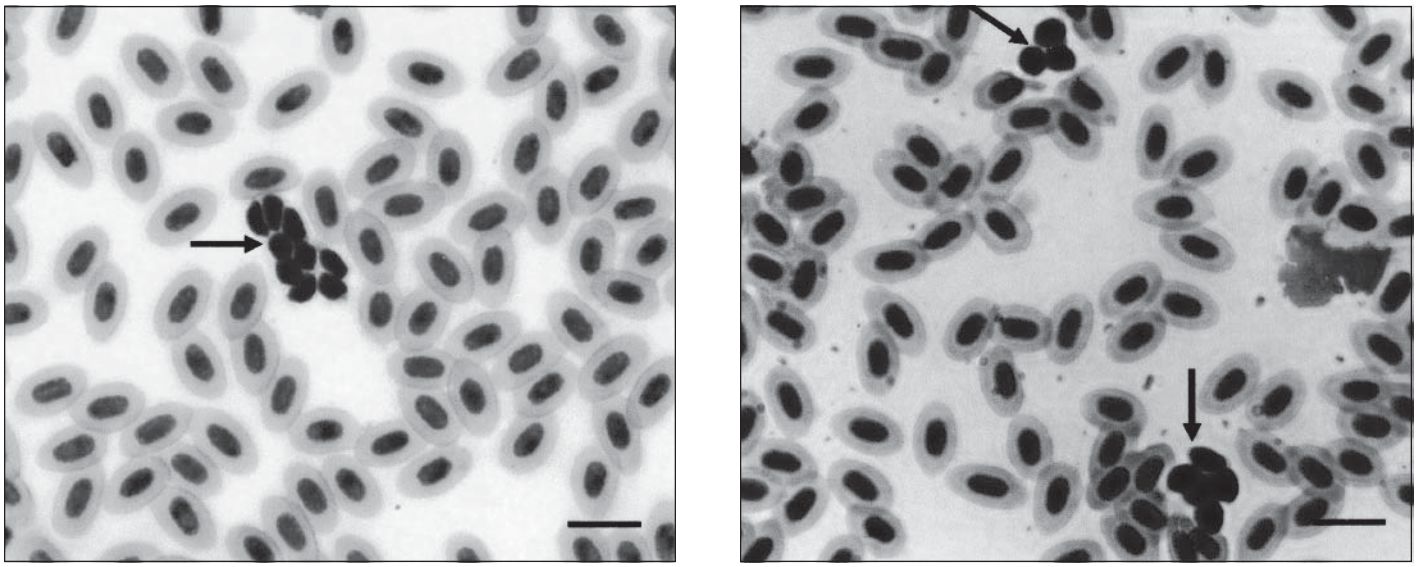

Figure 4. Photomicrograph of zebrafish thrombocytes (arrows). These cells are similar in appearance to small lymphocytes; however, the denser chromatin pattern and frequent aggregation of the thrombocytes allowed them to be distinguished. Diff-Quik stain; bar = 10 µm (1,000× magnification).

Table 2. Serum biochemical analytes for zebrafish

	Mean ± SD	Range
Albumin	3.0 ± 0.2 g/dl	2.7 - 3.3 g/dl
ALP	2.0 ± 4.5 U/L[a]	0.0 - 10.0 U/L[a]
ALT	367.0 ± 25.3 U/L	343.0 - 410.0 U/L
Amylase	2331.4 ± 520.6 U/L	1898.0 - 3195.0 U/L
Total bilirubin	0.38 ± 0.1 mg/dl	0.2 - 0.6 mg/dl
BUN	3.2 ± 0.4 mg/dl	3.0 - 4.0 mg/dl
Calcium	14.7 ± 2.3 mg/dl	12.3 - 18.6 mg/dl
Phosphorus	22.3 ± 1.5 mg/dl	20.3 - 24.3 mg/dl
Creatinine	0.7 ± 0.2 mg/dl	0.5 - 0.9 mg/dl
Glucose	82.2 ± 12.0 g/dl	62.0 - 91.0 g/dl
Potassium	6.8 ± 1.0 mEq/L	5.2 - 7.7 mEq/L
Total protein	5.2 ± 0.5 g/dl	4.4 - 5.8 g/dl
Globulins	2.1 ± 0.6 g/dl	1.3 - 2.8 g/dl

[a]ALP values were inconsistent and ranged from negative results to 10 U/L. ALP = Alkaline phosphatase; ALT = alanine transaminase; BUN = blood urea nitrogen.

species of fish, the results vary considerably within and between species. The values reported here for the zebrafish are within the ranges reported for mammalian species and fish (8, 18-20). Serum biochemical studies of healthy fish are scarce, and results vary widely. With the exception of increases in ALT, amylase, and phosphorus values, results of serum biochemical analysis were within the normal ranges reported for mammalian species and fish (18-20). Phosphorus values were only slightly higher than the upper range reported in some publications (20). Alanine transaminase and amylase activities were most likely falsely increased due to some hemolysis in the samples (21).

One potential drawback to this technique is the need to pool together samples from many fish. As microanalysis techniques improve, this problem will be minimized. However, pooling minimizes the variability due to individual biologic variation, which allows better representation of the population as a whole.

Accurate analysis of the many zebrafish mutants generated requires determination of the normal characteristics of zebrafish. We have described some of the basic hematologic and serum biochemical characteristics of mature zebrafish. We believe results such as these will help define normal adult zebrafish, which have a tremendous potential for use in the study of human disease and aging.

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