EnvironmentAsia
The international journal by the Thai Society of Higher Education Institutes on Environment

EnvironmentAsia 12(2) (2019) 79-86
DOI 10.14456/ea.2019.29
ISSN 1906-1714; ONLINE ISSN: 2586-8861

# Morphological Characterization of Blood Cells in Five Important Estuarine Fish Species in Thailand During Juvenile Stages

Naruebet Singkhanan[1], Jes Kettratad[1], Sinlapachai Senarat[1*], Theerakamol Pengsakul[2], Chamnan Para[3], and Gen Kaneko[4]

[1]*Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand*
[2]*Faculty of Medical Technology, Prince of Songkla University, Songkhla, 90110, Thailand*
[3]*Department of Western Languages and Linguistics, Faculty of Humanities and Social Sciences, Mahasarakham University, Thailand*
[4]*School of Arts and Sciences, University of Houston-Victoria, 3007 N. Ben Wilson St. Victoria, Texas 77901, USA*

*Corresponding author: Senarat.S@hotmail.com
Received: October 14, 2018; Revised: December 5, 2018; Accepted: January 3, 2019

## Abstract

Hematological profiles have been extensively used as a biomarker of fish, but such information is still limited for juvenile fish in mangrove forests of Thailand. The current study aimed to investigate the morphological characteristics of blood cells in five ecologically important estuarine fish species from the Estuary Pranburi River (EPR), Thailand: *Ambassis kopsii*, *Chanos chanos*, *Chelon subviridis*, *Dermogenys pusilla*, and *Leiognathus decorus*. We determined erythrocyte size of these species, demonstrating that *C. subviridis* and *D. pusilla* have eclipse erythrocytes. Three kinds of leukocyte including lymphocyte, monocyte, and neutrophil were also identified in this study. Lymphocytes were found in all species whilst monocytes and neutrophils were identified only in *C. chanos*, *C. subviridi*, and *D. pusilla*. Our hematological analysis has added new information to the literature and will help future hematology-based health monitoring of fish in Thailand and other countries.

***Keywords:*** Blood cell morphology; Estuarine fishes; Estuary Pranburi River; Thailand

## 1. Introduction

Hematology provides a sensitive and informative biomarkers in fish, and has therefore been used to assess fish condition in both field and laboratory (Corredor-Santamaría *et al*., 2016; Seriani *et al*., 2011; Meraj *et al*., 2017). For example, Şahan *et al*. (2007) compared conditions of *Anguilla anguilla* caught from two regions in Ceyhan River, Turkey, and found that neutrophils and leukocytes are increased in fish from a highly polluted region. Similarly, the

number of leukocytes was significantly higher in *Prochilodus lineatus* from a heavily polluted region than those from a less polluted region (Cazenave *et al*., 2009). On the other hand, Lohner *et al.* (2001) reported that Lepomis sp. living near the Coal Power Plant had fewer leukocytes, lymphocytes, and neutrophils compared to fish from a reference site. These findings validate the use of hematology profiles as a biomarker, but also suggest that the effect of pollutions on hematology profiles is not straightforward – possibly depending on species and the degree of pollution. It is therefore important to acquire hematology data from various species and habitats in order to establish hematology as a more robust method.

The estuary of Pranburi River (EPR) is an economically important region in Thailand that directly supplies water for the local community, aquaculture, and industries. As in the case of other rivers flowing into the Gulf of Thailand, the discharge of sewage, especially heavy metals and petroleum hydrocarbons, has been a problem in EPR (Environmental Health Division, 1984; Cheevaporn and Menasveta, 2003). However, the level of heavy metals is still lower in EPR than other rivers in the Gulf of Thailand (Cheevaporn and Menasveta, 2003). Thus, there is an urgent need for hematology profiling of fish in EPR, which will provide the empirical basis for the long-term monitoring. In this study, we report morphological characterizations of blood cells and the morphometric analysis in five economically important fish species in EPR: *Ambassis kopsii*, *Chanos chanos, Chelon subviridis*, *Dermogenys pusilla*, and *Leiognathus decorus*. This information will help future hematology-based health monitoring of fish in EPR.

## 2. Materials and Methods

*2.1 Fish collections and study area*

Only healthy juvenile fish with no parasite infection were used in this study. Species include *A. kopsii* (n = 10, approximately 9.77 ± 0.61 cm in total length), *C. chanos* (n = 10, approximately 14.5 ± 0.98 cm of total length), *C. subviridis* (n = 10, approximately 23.28 ± 3.29 cm in total length), *D. pusilla* (n = 10, approximately 6.9 ± 3.73 cm in total length), and *L. decorus* (n = 10, approximately 6.67 ± 0.39 cm in total length). Fish were collected during October–December 2016 from five stations (ST) in the EPR, Thailand [ST1: N 12°24'16.5"/ E 099°59'20.2", ST2: N 12°24'16.5"/ E 099°59'20.2", ST3: N 12°24'06.3"/ E 099°58'58.0", ST4: N 12°24'18.5"/ E 099°58'36.0" and ST5: N 12°24'15.3"/ E 099°58'28.6"]. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723004).

*2.2 Analysis of blood cells*

All fish specimens were euthanized by a rapid cooling shock (Wilson *et al*., 2009). After that 0.4-0.5 milliliters of blood were collected into a heparinized tube by using a 21G × 1" needle injected to the ventral surface of the isthmus through pericardial cavity following the standard method in Watson *et al.* (1989). Blood smears on slides were fixed in methanol for 10 min, air-dried, and stained with Giemsa for 25 min. After washing with phosphate-buffered saline (PBS, pH 7.4) three times, all slides were mounted by Permount (mounting medium). Blood cells (n = 10 in each fish species) were characterized according to guidelines of the standard hematology (Theml *et al*., 2004) and photographed with a Leica DM1000 light microscope equipped with a 1000x oil-immersion lens. Schematic diagrams of blood cells were drawn by using Adobe Illustrator (version CS6).

*2.3 Morphometric analysis of blood cells*

To determine the size of erythrocytes, three representative blood smear slides from each individual were examined. Blood cell characteristics, such as erythrocyte size (length, width, nucleus length, and nucleus width) and white blood cell size, were determined using 50 randomly selected cells per slide (total 150 cells). Leica light microscope (60X and immersion oil) and the program LAS version 4.9 were used for this analysis.

*2.4 Statistical analyses*

All morphometric parameters of blood cells are presented as the mean ± standard deviations (SD). The normality of data distribution was tested by the Kolmogorov-Smirnov Test (K-S Test). The difference between species was tested by One-Way Analysis of Variance (ANOVA) followed by Duncan's post-hoc test at the significance level of $P < 0.05$ by SPSS Version 22.

## 3 Results

*3.1 Morphological characterization and morphometric analysis of erythrocytes*

Table 1 summarizes morphological characteristics of mature erythrocytes for the five species determined by the high-magnification light micrograph observation. In all species, erythrocyte was the most abundant cell type. Mature erythrocytes showed an oval or eclipse shape (Figures 1A-1D). The nuclei of erythrocytes were also in the oval to round shape and are shown in magenta surrounded by the pink cytoplasm. No reticulocyte was observed in this study.

The ellipse major axis of erythrocytes from the 5 species ranged from 6.62 to 11.29 μm (Table 1). Erythrocytes of the longest and shortest ellipse major axes were found in *C. subviridis* (11.29 ± 0.26 μm) and *L. decorus* (6.62 ± 0.62), respectively. The ellipse minor axis of erythrocytes ranged from 6.03 μm to 8.59 μm, and those with the shortest and longest ellipse minor axes were found in *D. pusilla* (6.03 ± 0.33 μm) and *L. decorus* (8.59 ± 0.62 μm), respectively. Most differences in erythrocyte sizes were statistically significant. Erythrocytes of *C. chanos* appeared to have an eclipse shape by morphological observation (Figure 1C), but the quantification of the erythrocyte size (EL/EW ratio) showed that erythrocytes of *C. subviridis* and *D. pusilla* are more eclipsed than those of other species (Table 1).

The lengths of nuclei ranged from 4.60 μm to 5.17 μm. The longest and shortest lengths were found in *A. kopsii* (5.17 ± 0.65 μm) and *D. pusilla* (4.60 ± 0.40 μm), respectively. Meanwhile, the widths of nuclei ranged from 4.28 μm to 3.38 μm. The longest and shortest widths of nuclei were found in *A. kopsii* (4.28 ± 0.64 μm) and *C. chanos* (3.38 ± 0.54 μm), respectively. Most differences in nucleus sizes were statistically significant. The nucleus of *C. chanos* had a higher (1.49) EL/EW ratio than the other fishes (Table 1).]

*3.2 Comparative characterization and morphometric analysis of leucocytes*

Three types of leucocyte were identified in the five species tested (Table 2; Figures 1E-1L). Neutrophil had a round shape and the band or multi-lobed (bilobed, or trilobed) nucleus (Figure 1E, 2A). Neutrophils were found

**Table 1.** Six morphometric characters (Erythrocyte length, erythrocyte width, nucleus length, and nucleus width, and their ratio) of the erythrocytes from 150 total cells of *Ambassis kopsii*, *Chanos chanos*, *Chelon subviridis*, *Dermogenys pusilla*, and *Leiognathus decorus*

| Species | Erythrocytes | | | | | |
|---|---|---|---|---|---|---|
| | EL μm (Mean±SD) | EW μm (Mean±SD) | EW/EL | NL μm (Mean±SD) | NW μm (Mean±SD) | NL/NW |
| *Ambassis kopsii* | 9.54 ± 0.89[a] | 7.10 ± 0.84[a] | 1.33 | 5.17 ± 0.65[a] | 4.28 ± 0.64[a,d] | 1.21 |
| *Chanos chanos* | 10.04 ± 0.73[b,d] | 7.01 ± 0.60[b] | 1.43 | 5.04 ± 0.60[b] | 3.38 ± 0.54[b,d,e] | 1.49 |
| *Chelon subviridis* | 11.29 ± 0.26[b,d] | 7.30 ± 0.26[b] | 1.55 | 4.85 ± 0.27[b] | 4.15 ± 0.24[b,c,e] | 1.17 |
| *Dermogenys pusilla* | 9.26 ± 0.01[c] | 6.03 ± 0.33[b] | 1.53 | 4.60 ± 0.40[b] | 3.51 ± 0.42[b,d] | 1.31 |
| *Leiognathus decorus* | 6.62 ± 0.62[b,d] | 8.59 ± 0.66[b] | 1.30 | 4.98 ± 0.59[b] | 4.13 ± 0.53[b,d,e] | 1.21 |

**Note**; EL: Erythrocyte Length, EW: Erythrocyte Width, NL: Nucleus Length, NW: Nucleus Width.
The letter a, b, c, d indicates a significant difference ($P<0.05$) when the data are compared with other groups

**Table 2.** Morphometric characteristics of white blood cells from five fish species

| Species | Leukocytes | | | Thrombocyte (Mean±SD) |
|---|---|---|---|---|
| | Lymphocyte (Mean±SD) | Monocyte (Mean±SD) | Neutrophil (Mean±SD) | |
| *Ambassis kopsii* | 8.59 ± 1.35 | - | - | 5.72 ± 0.48 |
| *Chanos chanos* | 10.54 ± 0.50 | 10.79 ± 0.25 | 17.21* | 5.02 ± 1.00 |
| *Chelon subviridis* | 10.73 ± 0.56 | 14.68 ± 2.26 | 11.75 ± 2.36 | 5.66 ± 1.23 |
| *Dermogenys pusilla* | 8.84 ± 0.60 | 10.10* | 8.55* | 4.63 ± 0.60 |
| *Leiognathus decorus* | 9.16 ± 1.07 | - | - | 7.44 ± 0.40 |

*Only a single cell was found in this study.

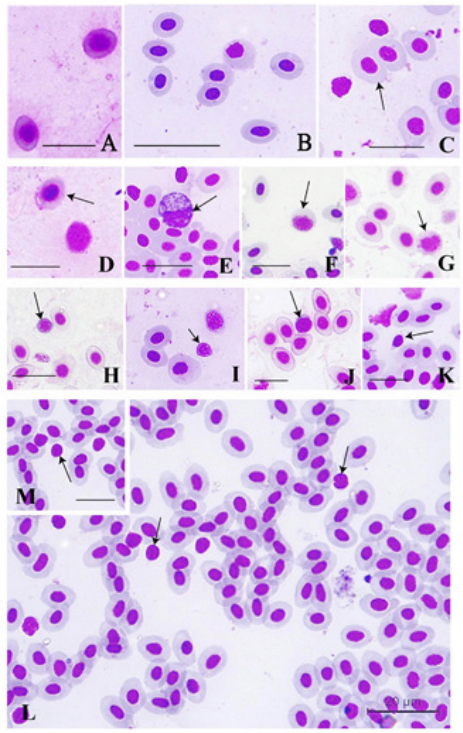


**Figure 1.** Representative light microscopic photographs of blood cells from the five selected species. (A) Erythrocytes of *Ambassis kopsii*. (B) Erythrocytes of *Chanos chanos*. (C) Erythrocytes of *Dermogenys pusilla*. (D) Erythrocytes of *Leiognathus decorus.* The arrow indicates a representative erythrocyte. (E) A representative picture of *Dermogenys pusilla* blood smear containing a neutrophil (arrow) and erythrocytes. (F) A representative picture of blood smear of *Chelon subviridis* containing a monocyte (arrow) and erythrocytes. (G) A representative picture from *C. chanos* blood smear containing a monocyte (arrow) and erythrocytes. (H) A representative picture from *C. chanos* blood smear containing a lymphocyte (arrow) and erythrocytes. (I) A representative picture of blood smear from *Chanos chano* a lymphocyte (arrow) and erythrocytes. (J) A representative picture of blood smear from *Chelon subviridis* containing a lymphocyte (arrow) and erythrocytes. (K) A representative picture of blood smear from *Chelon subviridis* containing a thrombocyte (arrow) and erythrocytes. (M-L) A representative picture of blood smear from *C. chanos* containing thrombocytes (arrow) and erythrocytes. Scale bar: A, C-K, M = 10 μm; B = 20 μm.

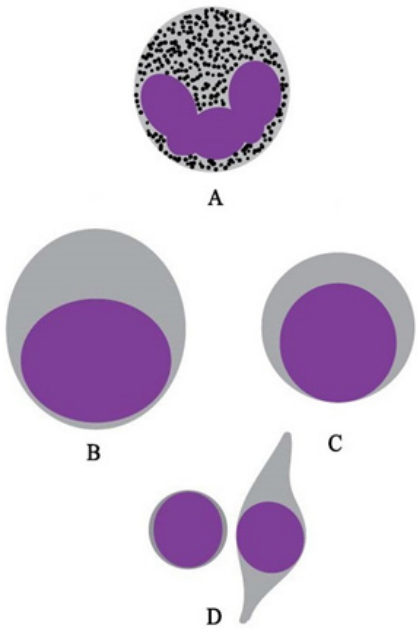


**Figure 2.** Schematic diagrams of morphological characteristics of four different blood cell types: neutrophil (A), monocyte (B), lymphocyte (C), and thrombocyte (D). Purple, gray, and black colors represent the nucleus, cytoplasm, and granules, respectively.

in three fishes, *C. chanos*, *C. subviridis*, and *D. pusilla*. Small granules were also detected in the cytoplasm (Figure 1E).

Monocytes were characterized by a large nucleus in an eccentric position, which was usually ovoid and filled with slight basophilic nucleoplasm (e.g., Figures 1F-1G, 2B). These cells were found only in *C. chanos*: *C. subviridis*, and *D. pusilla*. The largest monocyte was observed in *C. subviridis* (14.68 ± 2.26 μm). Lymphocytes were characterized by the presence of the small amount of cytoplasm with a large centrally located nucleus (Figure 2C). Blood smear from all fish species contained lymphocytes (e.g., Figures 1G-1J). The size of lymphocytes varied considerably among five fish species, and the largest size was observed in *Chelon subviridis* (10.73 ± 0.56 μm).

Thrombocytes were also found in this study. These cells had various shapes such as round, ovoid and spindle-shapes (Figures 1K-1L, 2D), and contained a purple-stained nucleus surrounded by the light purple cytoplasm (Figures 1K).

## 4. Discussion

The goal of the current study is to obtain the basic knowledge about hematological profiles of five important estuarine fishes in Thailand: *A. kopsii, C. chanos, C. subviridis, D. pusilla*, and *L. decorus* during their juvenile stages. To our knowledge, no hematological data are available for these species in the literature. In this study, we observed nucleated erythrocytes with normal characteristics, which are comparable to previous reports on other fish species under the healthy condition (Fang *et al*., 2014; Yilmaz *et al*., 2015). Furthermore, we did not observe any reticulocytes in our fish blood samples, suggesting that our specimens were not exposed to extreme stresses such as anoxia or pollutions (Lecklin and Nikinmaa, 1998). Hence, our hematological profiles will be suitable as a future reference to assess the estuarine condition from biological perspectives.

The morphometric parameters of erythrocytes are in general similar to those

from previous studies on other fish species (*Piaractus mesopotamicus, Brycon orbignyanus, Oreochromis niloticus and Rhamdia quelen*) (Dal'Bó *et al*., 2015). A potential significance of determining these parameters is that erythrocyte sizes could be related to the species-specific metabolism (Smith, 1925) such as aerobic swimming ability (Lay and Baldwin, 1999) and physiological adaptation (Campbell and Murru, 1990). Several investigators have proposed that fish adapt to the environment by changing the erythrocyte size and haemoglobin properties (Herbing and Cashon, 2004; Koldkjaer and Berenbrink, 2007; Lay and Baldwin, 1999). More specifically, Holland (1970) and Jones (1979) reported that the erythrocyte volume is positively correlated to oxygen transition and oxygen exchange rate. It is therefore possible that the *C. subviridis,* which has the biggest erythrocytes as demonstrated in this study, requires a higher amount of oxygen than other fish species do, and might be more vulnerable to pollution. This hypothesis needs to be further tested under laboratory studies.

The present study found two types of agranulocytes (lymphocyte and monocyte) and one type of granulocyte (neutrophil) by the blood smear examination. In general, lymphocyte is the most abundant leukocyte in fish. Murtha *et al*. (2003) reported that lymphocytes occupy 71-92 % in zebrafish leukocytes. Indeed, we observed lymphocytes from all species tested. On the other hand, monocytes and neutrophils were found from three species in this study, but only one cell was found in two out of the three fish species (*C. chanos* and *D. pusilla*). These cells have been frequently observed in fish blood analyses (Serpunin and Korobeinikova, 1997), but the absence of monocytes has also been reported in several species (Saunders, 1966). The presence or absence of monocytes, as well as neutrophils and thrombocytes, may depend on sample size, sampling periods, sex, habitats, and natural history of each fish. Although the function of these cells has not been investigated in fish tested in this study, their role in the immune response to environmental pollutants is generally accepted (Clauss *et al*., 2008; Xu *et al*., 2018; Serpunin and Korobeinikova, 1997).

## 5. Conclusion

Blood cells of five important estuarine fishes in Thailand were morphologically characterized for the first time in this study. Normal erythrocyte morphology and the absence of reticulocytes suggest that our fish specimens were under a healthy condition. Basic knowledge obtained by this study will help us to use hematological profiles in environmental monitoring in future studies.

## References

Cazenave J, Bacchetta C, Parma MJ, Scarabotti PA, Wunderlin DA. Multiple biomarkers responses in *Prochilodus lineatus* allowed assessing changes in the water quality of Salado River basin (Santa Fe, Argentina). Environmental Pollution 2009; 157(11): 3025– 3033.

Campbell T, Murru F. An introduction to fish hematology. Compendium on Continuing Education for the Practicing Veterinarian 1990; 12(4): 525– 532.

Clauss TM, Dove AD, Arnold JE. Hematologic disorders of fish. Veterinary clinics of North America: Exotic animal practice 2008; 11(3): 445– 462.

Cheevaporn V, Menasveta P. Water pollution and habitats degradation in the Gulf of Thailand, Marine Pollution 2003; 47: 43–51.

Corredor-Santamaría W, Gómez MS, Velasco-Santamaría YM. Using genotoxic and haematological biomarkers as an evidence of environmental contamination in the Ocoa River native fish, Villavicencio—Meta, Colombia. Springerplus 2016; 5: 351.

Dal'Bó GA, Sampaio FG, Losekann ME, Queiroz J FD, Luiz AJB, Wolf VHG, Carra ML. Hematological and morphometric blood value of four cultured species of economically important tropical foodfish. Neotropical Ichthyology 2015; 13(2); 439– 446.

Environmental Health Division. Survey of the water quality in the estuary. Proceeding of the 3rd Seminar on the Quality of Living Resources in Thai Waters, National Research Council of Thailand, Bangkok 1984. p. 62–78.

Fang J, Chen K, Cui HM, Peng X, Tang L, Zuo ZC. Morphological and cytochemical studies of peripheral blood cells of Schizothorax prenanti. Anatomia, Histologia, Embryologia 2014; 43: 386–394.

Herbing H, Cashon R. Hemoglobin sickling in Boreal fishes: Adaptation to the cold? Cardio-Respiratory Responses of Fish to Hypoxia, Hypercarbia and Temperature 2004; 49-53.

Holland RA. Factors determining the velocity of gas uptake by intracellular hemoglobin. In: Blood oxygenation. Springer US; 1970. p. 1-23.

Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. Nature cell biology 2008; 10(3): 314-321.

Koldkjaer P, Berenbrink M. In vivo red blood cell sickling and mechanism of recovery in whiting, *Merlangus merlangus*. Journal of Experimental Biology 2007; 210: 3451–3460.

Jones DA. The important of surface area/volume ratio to the rate of oxygen uptake by red cells. The Journal of general physiology 1979; 74(5): 643–646.

Lecklin T, Nikinmaa M. Erythropoiesis in Arctic charr is not stimulated by anaemia. Journal of Fish Biology 1998; 53: 1169–1177.

Lay PA, Baldwin J. What determines the size of teleost erythrocytes? Correlations with oxygen transport and nuclear volume. Fish Physiology and Biochemistry 1999; 20: 31–35.

Lohner T W, Reash RJ, Willet VE, Rose LA. Assessment of tolerant Sunfish populations (*Lepomis sp*.) inhabiting selenium-laden coal ash effluents: 1. Hematological and population level assessment. Ecotoxicology and Environmental Safety 2001; 50(3): 203-216.

Meraj M, Nizam MW, Maqbool S, Ali MN, Ganai BA, Bhat FA. Alteration in hematology of *Cyprinus carpio* under the stress of pollution of water bodies of Kashmir valley. International Journal of Fisheries and Aquatic Studies 2017; 5: 176–179.

Murtha JM, Weici W, Keller ET. Hematologic and serum biochemical values for the zebrafish (Danio rerio). Comparative Medicine 2003; 53: 37–4.

Şahan A, Altun T, Çevik F, Cengizler İ, Nevsat E, Genç E. Comparative study of some haematological parameters in European eel (*Anguilla anguilla* L., 1758) caught from different regions of Ceyhan river (Adana, Turkey). Journal of Fisheries and Aquatic Science 2007; 24: 167–171.

Saunders DC. Differential blood cell counts of 121 species of marine fishes of Puerto Rico. Transactions of the American Microscopy Society 1966; 85: 427-449.

Serpunin G G, Korobeinikova EG. Response of the blood system of carp (*Cyprinus carpio* L.) to the effects of heavy metals, in proceedings of the 1st Congress of Ichthyologists of Russia (NPO BIOS, Astrakhan 1997. p. 237–238.

Seriani R, Abessa DMS, Kirschbaum AA, Pereira CDS, Romano P, Ranzani-Paiva M J T. Relationship between water toxicity and hematological changes in *Oreochromis niloticus*. Brazilian Journal of Aquatic Science and Technology 2011; 15: 47-53.

Smith HM. Cell size and metabolic activity in Amphibia. The Biological Bulletin 1925; 48(5): 347–378.

Theml H, Haferlach T, Diem H. Color Atlas of Hematology: Practical Microscopic and Clinical Diagnosis. Thieme. 2004.

Watson CF, Baer KN, Benson WH. Dorsal gill incision: a simple method for obtaining blood samples in small fish. Environmental Toxicology and Chemistry 1989; 8(5): 457-461.

Wilson JM, Bunte RM, Carty AJ. Evaluation of rapid cooling and tricaine

methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). Journal of the American Association for Laboratory Animal Science 2009; 48(6): 785-789.

Xu, H., Zhang X, Li H, Li C, Huo XJ, Hou LP, Gong Z.. 2018. Immune response induced by major environmental pollutants through altering neutrophils in zebrafish larvae. Aquatic Toxicology 2018; 201: 99-108."

Yilmaz M, Guven O, Mutaf BF. Comparative morphometry of erythrocyts of different fish species. Journal of Cell and Molecular Biology 2015; 1(1): 002.