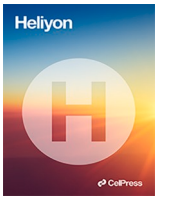
Heliyon 10 (2024) e37011

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Morphological and cytochemical characterization of the peripheral blood cells in *Paralichthys olivaceus*

Yingli Gao [a,b,c,*], Lu Qiang [a], Ni Wu [a], Hui Wang [a], Ying Hao [a]

[a] Lab of Pathology and Immunology of Aquatic Animals, Jiangsu Ocean University, Lianyungang, 222005, China
[b] Jiangsu Key Laboratory of Marine Biotechnology, Jiangsu Ocean University, Lianyungang, 222005, China
[c] Marine Resource Development Institute of Jiangsu (Lianyungang), Lianyungang, 222005, China

ARTICLE INFO

Keywords:
Paralichthys olivaceus
Peripheral blood cells
Microstructure
Ultrastructure
Cytochemical characteristics

ABSTRACT

The flounder (*Paralichthys olivaceus*) is a highly nutritious, cultured bony fish with a high economic value. The health of the fish is closely related to its blood cells, which are critical for oxygen transport, natural defense, and immunity. The microstructures, classification, counting and size of peripheral blood cells in *P. olivaceus* were observed and measured by Wright-Giemsa staining, and the cytochemical characteristics of peripheral blood cells were investigated by different cytochemical staining methods including periodic acid-Schiff (PAS), Sudan black B (SBB), acid phosphatase (ACP), alkaline phosphatase (ALP), peroxidase (POX), and α-naphthol acetate esterase (NAE). Besides, the ultrastructures of different cells were detected by the transmission electron microscope. The results showed that erythrocytes, thrombocytes, lymphocytes, monocytes, and neutrophils constituted the peripheral blood cells in *P. olivaceus*. More heterochromatins were found in erythrocytes, thrombocytes and neutrophils; however, more organelles with fewer heterochromatins were found in monocytes. Endoplasmic reticulums and phagocytic vesicles were abundant in neutrophils. Lymphocytes were the most abundant in leukocytes, followed by monocytes and neutrophils. The cytochemical staining results showed that all leukocytes were positive for SBB. Most of the lymphocytes were positive for PAS, and monocytes were positive for PAS, ACP, and POX. As for neutrophils, ACP and POX were positive. Both monocytes and neutrophils showed positive for SBB, ACP and POX, indicating that the two kinds of cells play a vital role in phagocytosis and bactericidal action. Only lymphocytes were positive for ALP, indicating that they were important in inflammation and immune response. Some characteristics similarities in peripheral blood cells were showed in *P. olivaceus* just as the other fishes.

1. Introduction

Flounder *Paralichthys olivaceus* belongs to the order Pleuronectiformes, family Bothidae, genus *Paralichthys*. It is an important mariculture species in China and also the most productive mariculture species in Chile, Korea, Japan and Germany [1]. Due to the intensive farming of flounder rising, diseases occur frequently, causing economic losses seriously.

The peripheral blood cells play an important role in the immune response of fish, which is an important part of the immune system to resist the pathogen infection [2], which is benefit to disease diagnosis [3]. Therefore, the study on the morphology, quantity and

* Corresponding author. Lab of Pathology and Immunology of Aquatic Animals, Jiangsu Ocean University, Lianyungang 222005, China.
E-mail address: yingli.gao@jou.edu.cn (Y. Gao).

https://doi.org/10.1016/j.heliyon.2024.e37011
Received 24 April 2024; Received in revised form 25 August 2024; Accepted 26 August 2024
Available online 27 August 2024

cytochemical characteristics of peripheral blood cells in fish is of significance for the detection of water environment, diseases diagnosis and production practice [4,5]. The experimental procedure and judging standard of blood cell detection have been established in human and veterinary medicine, however, there is no uniform index in aquatic animal medicine [6]. There are many kinds of fish, and the composition and structure of peripheral blood cells vary greatly among species [7].

Traditional Wright's staining can be used to study the microstructure and classification of fish blood cells [8]. The biological macro-molecules and the activity of enzymes in the cells usually are detected by the cytochemical staining, and these results help to improve our understanding of the blood cells and cell lineages, such as function and physiological state [9]. The periodic acid Schiff (PAS) reaction and Sudan black B (SBB) staining may display intracellular glycogen and lipid particles, respectively [10]. Acid phosphatase (ACP) and alkaline phosphatase (ALP) are significant lysosomal enzymes that serve as representative marker enzymes of cell phagocytosis, and peroxidase (POX) within leukocytes plays an important role in defence against bacterial infections [11]. α-naphthol acetate esterase (NAE) participate in intracellular processing and antigen transport [12].

A number of studies have been carried out on the classification, and morphology of peripheral blood cells in many fish species. The percentage of monocytes in *Synechogobius hasta* was greater than that in *Sebastes schlegelii*, however, the opposite results were obtained in the percentage of lymphocytes and thrombocytes [7]. No difference was found for the percentages of neutrophils and monocytes in different freshwater fishes (*Ctenopharyngodon idella, Megalobrama amblycephala* and *Pelteobagrus fulvidraco*) [13]. Lymphocytes were the dominant leukocytes, followed by segmented neutrophils in *Salvelinus fontinalis* [14]. The above results showed that the composition of blood cells in different fish had a little different and this would serve as an important model for examining comparative physiology of blood cells in different fish.

The cytochemical characteristics of the peripheral blood cells in fish have also been extensively investigated. PAS staining was positive in neutrophils, monocytes, lymphocytes and thrombocytes, but not in erythrocytes of *Schizothorax prenanti* [12], however POX-positive staining was observed in neutrophils and monocytes, but not in erythrocytes, lymphocytes and thrombocytes. Except for erythrocytes, the other blood cells stained positively for ACP. Only neutrophils and monocytes were positive for NAE. None of the cells studied were positive for ALP. According to our previous study [7], *S. hasta* and *S. schlegelii* shared common cytochemical-staining results, which showed that monocytes were strongly positive for PAS and positive for SBB and NAE; lymphocytes were negative for SBB, POX and NAE; neutrophils were positive for SBB and NAE; and thrombocytes were negative for SBB, ALP, POX and NAE. These studies indicated that the major groups and micromorphology of peripheral blood cells in different fish were generally similar. However, there were obvious species-specific differences in the leukocytes, the proportion of various leukocyte types, and the cytochemical characteristics of blood cells. These studies have important significance for enriching the blood databases of aquatic animal. In addition, the study of cytochemical properties of peripheral blood cells can explore the composition of various cellular enzymes in different cells, thus revealing and providing functional information on immune defence in peripheral blood cells of different fishes.

At present, only a few studies have been reported on the composition and the physiological and biochemical characteristics of peripheral blood cells in *P. olivaceus* [15,16]. However, these researches did not investigate the cytochemical features of the above cell types to confrm their identities by examining traditional morphology with cytochemical markings. Studies on blood leukocytes can reveal the characteristics of the immune systems of different fish species. Hematological investigations have relied on classical staining with the Wright, May, Grünwald and Giemsa used to identify leukocytes, but cell-based classifications of *P. olivaceus* cells are not always reliable using classical staining methods because the staining procedures vary, which can lead to errors in the identification of a cell type. Electron microscope detection can pinpoint the elaborate structure of cells, making the results more intuitive, while cytochemical analysis is more practical and can be used for rapid detection without special instruments. The combination of the two methods is more beneficial to fully grasp the characteristics of the blood cells in flounder and lay a good foundation for further study. Thus, cytochemical staining with ultramicroscopic examination of leukocytes in blood may be particularly useful for identifying cell lineages and may indicate cell function. Consequently, we investigated the morphology, microstructure, and cytochemical characteristics of the peripheral blood cells of *P. olivaceus* using the Wright-Giemsa staining, different cytochemical staining methods and the transmission electron microscope to provide basic data of physiology and immunology for monitoring the health of artificially-bred *P. olivaceus*.

2. Materials and methods

2.1. Animals and staining kits

Healthy adult specimens of *P. olivaceus* (35–40 cm, 0.9–1 kg) were obtained from a flounder aquaculture factory, Lian Yungang, Jiangsu Province, China. Then, the fish were transferred to one aquarium and acclimatized for 7 days with flowing seawater at 10–15 °C, 30 ‰ salinity, and the dissolved oxygen concentration at 7 mg/L. A total of thirty individuals were used for the experiment. Neither visible lesions nor gross abnormalities were observed in any of the fish during the experiment. All the staining kits, including WG (Wright-Giemsa), PAS (periodic acid Schiff), ACP (acid phosphatase), ALP (alkaline phosphatase), NAE (α-naphthol acetate esterase), SBB (Sudan black B) and POX (peroxidase), were obtained from a commercial company (Solarbio, Beijing, China).

2.2. Blood smear preparation

Fish were anaesthetized with tricaine methane sulfonate (MS-222, 10 mg/L), and fish were moderately anaesthetized, floating on the surface, and not sensitive to stimulation. The blood collection was done by caudal venepuncture with a syringe. The blood samples were immediately used to prepare blood smears and air-dried. For each staining method, ten blood smears per fish were prepared and

performed with the subsequent staining method.

2.3. Staining methods

According to the staining method and judging method established in our laboratory [7], the prepared blood smears were stained respectively. WG staining results were used for classification count and microscopic observation. PAS positive staining results showed red granules with blue nuclei. SBB positive staining results were black with purplish red nuclei. ACP positive reaction showed red granules with blue nuclei and AKP positive reaction was blue with red nuclei. POX positive reaction showed brown-black with blue nuclei. NAE positive reaction was black with green nuclei. The results of cytochemical staining were expressed in terms of the intensity of cytochemical reactions according to the evaluation method described by our previous studies [7].

2.4. Microscopic observation

All the staining smears were photographed by Nikon 90i electric microscope to identify the blood cells by the cell size, nucleus size, nucleus morphology, karyoplasmic ratio and cytosolic colouration. The cell smears stained with WG were randomly divided into 3 parts, and 24 view fields were taken to count the cells at 400 magnification each part. 50 cells of different cell types were randomly selected to take pictures for measuring the size of cells and nucleus at 1000 magnification.

2.5. Ultramicroscopic observation

The isolation of peripheral blood cells in flounder was conducted according to the established method [17]. The red blood cells and white blood cells were separated by the Percoll gradient solution. Subsequently, the cell suspensions of blood were laid over a discontinuous Percoll gradient and centrifuged (840 g, 30 min). The leukocytes at the Percoll interface were collected and washed 3 times with PBS containing 5 % (v/v) newborn calf serum and then centrifuged (640 g, 10 min) and then suspended in PBS for staining [17]. According to the method of *Cynoglossus semilaevis* [18], the isolated leukocytes were respectively fixed with phosphate buffer solution (0.1 M, pH 7.4) containing 1 % osmium tetroxide for cell pellets. The isolated erythrocytes were treated as the same way. After fixing, the cell pellets were dehydrated by acetone, then embedding and sectionging, stained by uranyl acetate and lead citrate. The ultrastructure of erythrocytes and leukocytes were observed by the electron transmission microscope (HITACHIHT7650, Japan).

2.6. Data analysis

The results were expressed in the form of mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics (Version 19).

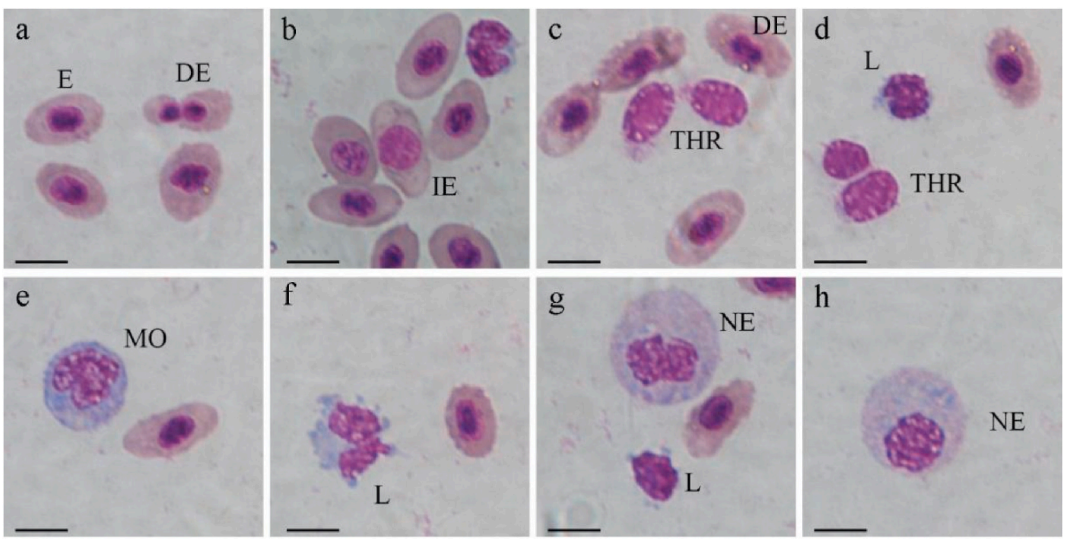


Fig. 1. The microscopic structures of the peripheral blood cells in *Paralichthys olivaceus* with the WG staining. a. the mature erythrocyte and the dividing erythrocyte; b. the immature erythrocyte; c. the dividing erythrocyte and thrombocyte; d. the thrombocyte and lymphocyte; e. monocyte; f. lymphocyte; g. neutrophils and lymphocyte; h. neutrophils; E, erythrocyte; DE, dividing erythrocyte; IE, immature erythrocyte; THR, thrombocyte; L, lymphocyte; MO, monocyte; NE, neutrophil. Scale bar = 5 μm.

3. Results

3.1. Morphology of different blood cells

According to the morphology and size of cells and nuclei, nucleo-cytoplasmic ratio, the presence or absence of particles, and tinctorial feature in the cytoplasm by WG staining, the peripheral blood cells of *P. olivaceus* were consisted of erythrocytes, leukocytes and thrombocytes. Leukocytes were further classified into lymphocytes, monocytes and neutrophils (Fig. 1).

The mature erythrocytes were oval with evenly red cytoplasm, and the oval or round nucleus was located in the centre of the cell with dense and purplish red chromatin (Fig. 1a). The immature erythrocytes were occasionally visible with loose and light purple chromatin (Fig. 1b). The dividing erythrocytes could be observed during the staining (Fig. 1a and c).

The cytoplasm of the thrombocytes was hardly stained, exhibiting less greyish blue cytoplasm, and the elliptic (Fig. 1c) or short rod-shaped (Fig. 1d) nucleus was observed during the staining.

The cytoplasm of the lymphocytes was stained blue, exhibiting basophilic, and the nucleus was divided into two shapes, oval (Fig. 1g) or two-leaves shape (Fig. 1d and 1f) with dense and purplish red chromatin.

The monocytes were round with dark blue cytoplasm, and the nucleus was laterally biased with purple chromatin. The karyoplasmic ratio was more than 0.5 (Fig. 1e).

The neutrophils were round with cytoplasm containing bluish-purple granules, and the nucleus was divided into two shapes, two-leaves shape (Fig. 1g) or oval (Fig. 1h) with purple chromatin. The karyoplasmic ratio was less than 0.5.

3.2. The ultrastructure of different blood cells

Under the transmission electron microscope, the surface of erythrocytes was smooth, and the nuclear membrane and nuclear pores were clearly visible (Fig. 2a). The heterochromatin in the nucleus was abundant with sheet-like distribution along the inner side of the nuclear membrane and with a band-like distribution in the centre of the nucleus. The cytoplasm was uniform and compact with high electron density. No organelles were found in the cytoplasm of erythrocytes.

The thrombocytes had a clear nuclear membrane and nuclear pore with abundant heterochromatin in the nucleus. The cytoplasm was even and dense with high electron density, and no organelles were observed (Fig. 2b).

There were protrusions on the surface of the lymphocyte with a clear nuclear membrane (Fig. 2c). Little heterochromatin was present in the nucleus, and it was distributed patchily in the central nucleus and in bands along the inner side of the nuclear membrane. Low electron density and loose cytoplasm were present. The endoplasmic reticulum and vesicles were seen in the lymphocyte.

The surface of the monocyte was rough, and the heterochromatin in the nucleus was few, distributed along the nuclear membrane

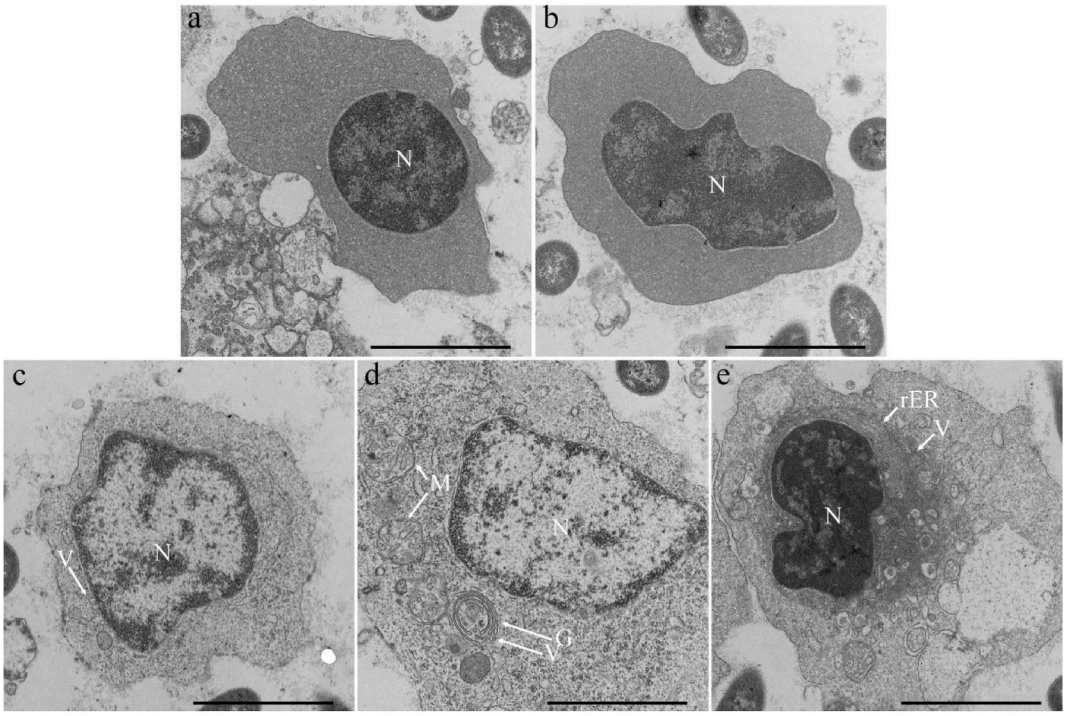


Fig. 2. The ultrastructures of the peripheral blood cells in *Paralichthys olivaceus.* a. erythrocyte; b. thrombocyte; c. lymphocyte; d. monocyte; e. neutrophil; N. nucleus; V. vesicle; M. mitochondrion; G. Golgi apparatus; rER. rough endoplasmic reticulum. Scale bar = 2 μm.

and scattered in the central nucleus (Fig. 2d). The cytoplasm was loose with low electron density. Mitochondria were abundant and cristae were clear in the Golgi apparatus, and a lot of vesicles were found around the trans-cystic cavity.

The neutrophils were characterized by finger-like protrusions with abundant heterochromatin in the nucleus (Fig. 2e). Golgi apparatus, ribosomes, and granules were observed in the cytoplasm with abundant rough endoplasmic reticulum and numerous vesicles.

3.3. Cell measurement and the relative account

The cell size of the blood cells in *P. olivaceus* were listed in Table 1. The size of peripheral blood cells and nuclei of flounder varied among different cells, with the largest neutrophil 10.01 ± 1.64 μm × 9.26 ± 2.02 μm. The percent of erythrocytes in total blood cells was 97.54 ± 0.38 %, and thrombocytes accounted for 1.12 ± 0.19 %. The percentage of lymphocytes in the total number of leukocytes was 49.13 ± 2.67 %, followed by monocytes 36.65 ± 2.56 % and neutrophils 14.22 ± 0.12 %.

3.4. Cytochemical properties of the blood cells

To further understand the cell populations, six different cytochemical stains were used to investigate the cytochemical characterizations, including a combination of enzymatic stains (ACP, ALP, POX, and NAE) and nonenzymatic stains (SBB and PAS). The cytochemical findings of the present study were shown in Fig. 3 and Table 2. All of the six cytochemical stainings of erythrocytes and thrombocytes were negative, and the results of cytochemical staining of different leukocytes differed.

The results of PAS staining revealed that monocytes had red glycogen granules in the cytoplasm (Fig. 3, a4), which was PAS-positive. Most lymphocytes were PAS-positive with irregular red glycogen granules distributed at the edges of cells, due to their large karyoplasmic ratio (Fig. 3, a3 L-o), while a few lymphocytes were PAS-negative with no red glycogen particles (Fig. 3, a3 L-n). All of the leukocytes were SBB-positive with obvious brown-black granules in the cytoplasm (Fig. 3, b3-b5). Monocytes and neutrophils showed ACP-positive with red granules in the cytoplasm (Fig. 3, c4-c5). Lymphocytes were ACP-negative with blue cytoplasm (Fig. 3, c3). After ALP staining, only the lymphocytes were positive with blue cytoplasm (Fig. 3, d3), while all other cells were negative with pale yellow. For the POX-staining results, monocytes and neutrophils were POX-positive with brownish black granules distributed in the cytoplasm (Fig. 3, e4-e5), while all other cells were negative (Fig. 3, e1-e3). All of the blood cells were negative for NAE with yellowish cytoplasm(Fig. 3, f1-f5).

A1. erythrocytes stained with PAS negatively; a2. thrombocytes stained with PAS negatively; a3. lymphocytes stained with PAS positively or negatively; a4. monocytes stained with PAS positively; a5. neutrophils stained with PAS negatively; b1. erythrocytes stained with SBB negatively; b2. thrombocytes stained with SBB negatively; b3. lymphocytes stained with SBB positively; b4. monocytes stained with SBB positively; b5. neutrophils stained with SBB positively; c1. erythrocytes stained with ACP negatively; c2. thrombocytes stained with ACP negatively; c3. lymphocytes stained with ACP negatively; c4. monocytes stained with ACP positively; c5. neutrophils stained with ACP positively; d1. erythrocytes stained with ALP negatively; d2. thrombocytes stained with ALP negatively; d3. lymphocytes stained with ALP positively; d4. monocytes stained with ALP negatively; d5. neutrophils stained with ALP negatively; e1. erythrocytes stained with POX negatively; e2. thrombocytes stained with POX negatively; e3. lymphocytes stained with POX negatively; e4. monocytes stained with POX positively; e5. neutrophils stained with POX positively; f1. erythrocytes stained with α-NAE negatively; f2. thrombocytes stained with α-NAE negatively; f3. lymphocytes stained with α-NAE negatively; f4. monocytes stained with α-NAE negatively; f5. neutrophils stained with α-NAE negatively; E, erythrocyte; THR, thrombocyte; L, lymphocyte; MO, monocyte; NE, neutrophil. L-o, lymphocytes were PAS-positive with irregular red glycogen granules; L-n, Lymphocytes were PAS-negative with no red glycogen particles. Scale bar = 5 μm.

4. Discussion

Erythrocytes, thrombocytes, lymphocytes, monocytes and neutrophils were consisted in the peripheral blood cells of *Paralichthys olivaceus*, and these results were consistent with *Ctenopharyngodon idella*, *Megalobrama amblycephala*, *Pelteobagrus fulvidraco*, *Synechogobius hasta*, *Sebastes schlegelii*, *Parupeneus forsskali* and *Thalassoma klunzingeri* [7,13,19].

The mature erythrocytes were the majority in the peripheral blood cells of *P. olivaceus*, and the immature erythrocytes and the dividing erythrocytes could be detected occasionally, similar results were observed in *Glyptosternum maculatum* [20], *Acipenser sinensis* [21], three freshwater fishes (*Ctenopharyngodon idella*, *Megalobrama amblycephala* and *Pelteobagrus fulvidraco*) [13], two carnivorous

Table 1
The cell size and nucleus size of peripheral blood cells in *Paralichthys olivaceus*.

Cell types	Cell size (μm)		Nucleus size (μm)	
	Length	Width	Length	Width
Erythrocytes	8.97 ± 1.07	5.33 ± 0.76	3.94 ± 0.42	2.76 ± 0.47
Lymphocytes	7.76 ± 1.26	6.75 ± 0.97	5.86 ± 1.14	4.16 ± 0.52
Monocytes	9.47 ± 1.66	8.78 ± 1.24	6.46 ± 0.81	4.21 ± 1.07
Neutrophils	10.01 ± 1.64	9.26 ± 2.02	5.66 ± 1.10	4.52 ± 0.66
Thrombocytes	7.60 ± 1.81	4.29 ± 0.56	5.58 ± 0.74	3.92 ± 0.39

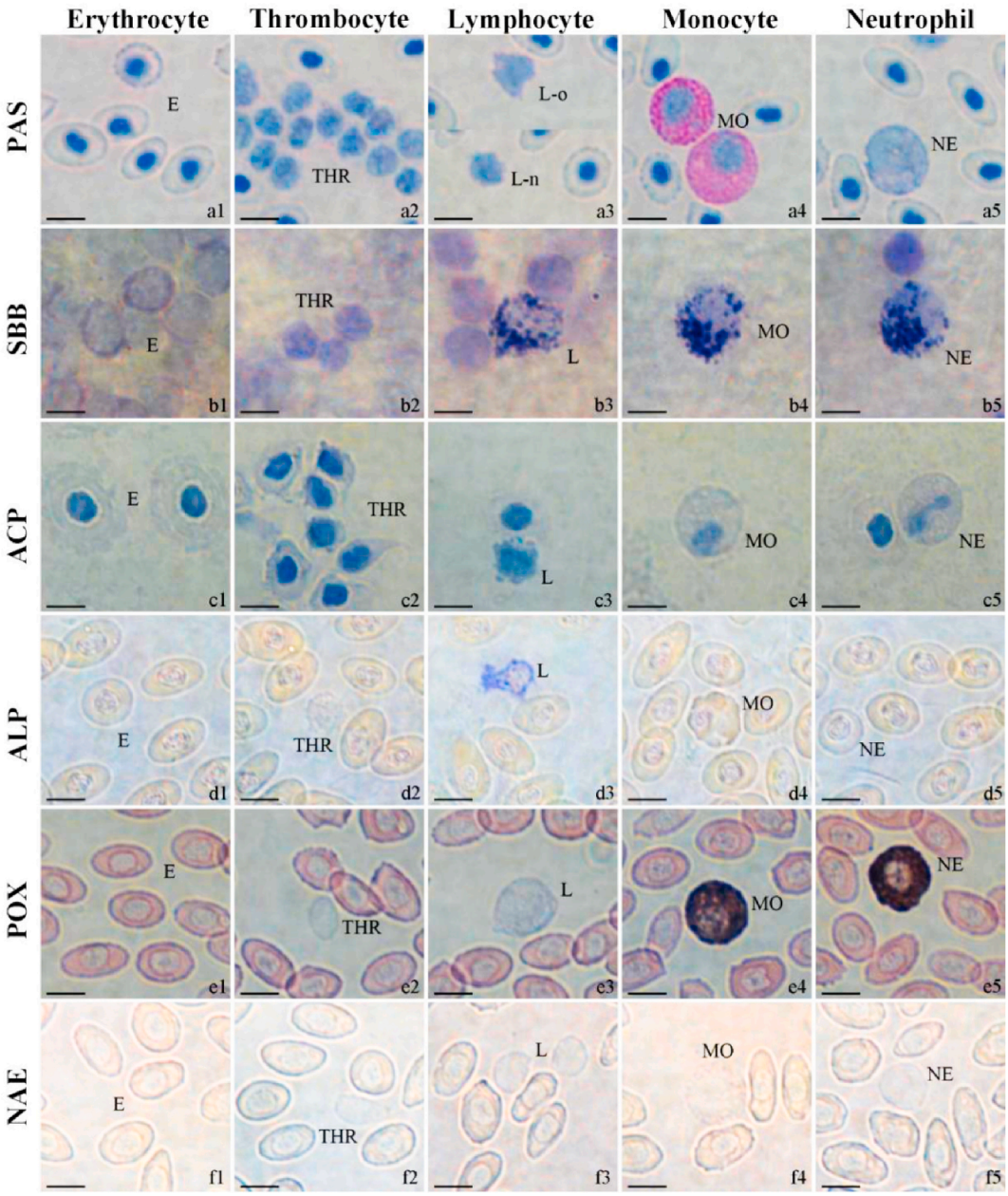


Fig. 3. The cytochemical staining of peripheral blood cells in *Paralichthys olivaceus*.

Table 2
The cytochemical characteristics of peripheral blood cells in *Paralichthys olivaceus*.

	PAS	SBB	ACP	ALP	POX	α-NAE
Erythrocytes	−	−	−	−	−	−
Lymphocytes	+ / −	+	−	+	−	−
Monocytes	+	+	+	−	+	−
Neutrophils	−	+	+	−	+	−
Thrombocytes	−	−	−	−	−	−

Note: " + " indicates positive staining, " − " indicates negative staining, " + / − " indicates both positive and negative reactions.

fishes (*Parupeneus forsskali* and *Thalassoma klunzingeri*) [19], indicating that the process of erythrocytes proliferation and maturation existed in the peripheral blood of different fishes. The metabolism of erythrocyte in fish was relatively vigorous with mitochondria [13], however, under transmission electron microscopy, erythrocytes of *P. olivaceus* had no organelles, which were similar to those of *Gymnocyprinis eckloni* [22], *Schizothorax Prenanti* [12], *Ctenopharyngodon idellus*, *Megalobrama amblycephala*, and *Pelteobagrus fulvidraco* [13]. Currently, it has been accepted that whether mitochondria can be observed in fish erythrocyte under transmission electron microscopy may be related to the species, age, or the development status of erythrocyte. Besides, the size of erythrocytes in *P. olivaceus* was smaller than that in the other two marine fish studies in our previous studies [7]. The size of erythrocytes is inversely proportional to the metabolic activity of the organism. Thus, erythrocytes in *P. olivaceus* were smaller, and had no organelles to adapt to lower oxygen consumption, as in previous studies [23–25].

Thrombocytes in fish had nuclei and played an important role in coagulation, antigen presentation and phagocytosis [2], which was similar to the mammalian platelets. The number of thrombocytes in fish fluctuated greatly between individuals and was significantly influenced by smear preparation methods [26]. In this study, a large number of oval thrombocytes could be congregated together occasionally, in addition, the number of thrombocytes was more than that in erythrocytes in one vision field, which may be related to their hemostatic function [27].

The lymphocytes in the peripheral blood of *P. olivaceus* were the most abundant leukocyte (49.13 %), the same as the previous results [28]. In the work of our previous study [7], the percent proportion of lymphocytes in leukocytes of *Synechogobius hasta* and *Sebastes schlegelii* reached 66.14 % and 72.17 %, respectively. It was proposed that variations in components of leukocytes in vertebrate species might be associated with species, age, season, and habitat conditions. Some studies have classified fish lymphocytes into big lymphocytes and small lymphocytes, for example, our previous studies have identified the two types of lymphocytes in two marine fishes based only on cell size rather than on functional characteristics [7]. In this study, the different sizes of lymphocytes were not observed in *P. olivaceus*, however, lymphocytes with two different nucleus shapes were observed, which might be related to the different functional states or the different developmental stages of lymphocytes. In addition, lymphocytes with few organelles and a large number of vacuoles were observed during the experiment, suggesting that lymphocytes had phagocytosis, which was similar to *Brycon orbignyanus* [29]and *Ictalurus punctatus* [30].

Monocytes played an important role in the immune system of fish ([31,32] and they had chemotaxis and phagocytosis, which made them highly sensitive to environmental changes [22]. There were vacuoles in monocytes of *P. olivaceus*, and some pseudopods extended into the surrounding cell space, which was consistent with other fish ([22,29] and indicated that monocytes were in the process of phagocytosis [22]. Studies on the peripheral blood of Chinese sturgeon (*Acipenser sinensis*) [21] and Chinook salmon (*Oncorhynchus tshawytscha*) [33] have shown that monocytes had the ability to extend pseudopodia and engulf materials. In addition, monocytes had dense nuclear electrons, abundant mitochondria, Golgi apparatus and vesicles, suggesting that monocytes were in the process of protein synthesis.

Fish neutrophils could resist the invasion of extracellular pathogens through degranulation and formation of extracellular antimicrobial peptides, and these cell particles and antimicrobial peptides were able to target microbes more specifically [34]. There were abundant phagocytic vacuoles and organelles in neutrophils, which indicated that they had active phagocytic function.

The lymphocytes of *P. olivaceus* showed both positive and negative PAS staining results, and PAS staining revealed the intracellular glycogen. Glycogen is a vital source of cellular energy storage and plays an important role in innate defense, especially phagocytosis [35]. It was suggested that the intracellular glycogen in *P. olivaceus* lymphocytes changed greatly in different functional states, therefore some *P. olivaceus* lymphocytes might be PAS-negative. The monocytes of flounder were PAS-positive and the neutrophils were PAS-negative. However, granulocytes generally were phagocytic and PAS-positive [36], which was inconsistent with the negative PAS in neutrophils of flounder, however, it showed the species specificity of flounder.

The SBB staining might display intracellular neutral lipid particles, which was an important structural material and energy source for cells [10]. The SBB positive results in lymphocytes of *P. olivaceus* suggested that lymphocytes could be supplied by neutral lipid. In addition, both monocytes and neutrophils were SBB positive. Combining the results of PAS staining in monocytes and neutrophils, we suggested that the phagocytic function of *P. olivaceus* neutrophils might be mediated by lipid rather than glycogen and that *P. olivaceus* monocytes could utilize both glycogen and lipid.

ACP was a lysosomal marker enzyme, with phagocytosis, sterilization and other functions [37]. ALP was an important conserved defence enzyme that fights pro-inflammatory responses [38]. POX, an important lysosomal enzyme involved in intracellular digestion, indicates the absence of eosinophils and neutrophils [39]. POX was a heme-containing enzyme that eliminates the toxic effects of hydrogen peroxide and various small molecules such as methanol, ethanol, nitrite, or formic acid. Both monocytes and neutrophils in *P. olivaceus* were ACP-positive and ALP-negative, indicating that both the two cells lacked ALP enzymes and were rich in ACP enzymes, so they had strong phagocytic and bactericidal abilities. Both monocytes and neutrophils contained large amounts of peroxidase, which fitted their phagocytic and bactericidal functions and was consistent with studies in *Ophiocephalus argus* Cantor [40].

In conclusion, the peripheral blood cells of *P. olivaceus* were composed of erythrocytes, thrombocytes, lymphocytes, monocytes, and neutrophils. No organelles were found in mature erythrocytes and thrombocytes, but abundant organelles were found in leukocytes. SBB, ACP and POX were positive for monocytes and neutrophils. Only lymphocytes were ALP positive. This study can enrich the hematological data of *P. olivaceus* and provide basic data for cell biological function and health evaluation in *P. olivaceus* aquaculture.

Funding

This work was supported by the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (22KJB240001), the Postdoctoral Research Foundation of Lianyungang (LYG20230012), the Scientific Research Foundation Program of Jiangsu Ocean

University (KQ16032) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Ethics declarations

This study was reviewed and approved by the Ethics Committee of Jiangsu Ocean University with the approval number 20220301 according to the regulations of <Regulations of Experimental Animal Ethics Review Committee of Jiangsu Ocean University ([2020] 37)>.

Data availability statement

All the data analysis results obtained during this study are included in the manuscript. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Yingli Gao: Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Lu Qiang:** Writing – original draft, Software, Investigation, Formal analysis. **Ni Wu:** Investigation, Formal analysis. **Hui Wang:** Investigation. **Ying Hao:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] FAO, The State of World Fisheries and Aquaculture 2024. Blue Transformation In Action (2024). Rome, https://www.fao.org/home/en/.
[2] F. Ferdous, T.R. Scott, A comparative examination of thrombocyte/platelet immunity, Immunol. Lett. 163 (2015) 32–39.
[3] W.E. Furtado, L. Cardoso, A.B. Figueredo, N.C. Marchiori, M.L. Martins, Histological and hematological alterations of silver catfish *Rhamdia quelen* highly parasitized by *Lernaea cyprinacea*, Dis. Aquat. Org. 135 (2) (2019) 157–168.
[4] M.A. Burgos-Aceves, L. Lionetti, C. Faggio, Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish, Sci. Total Environ. 670 (2019) 1170–1183.
[5] F. Fazio, C. Saoca, V. Ferrantelli, et al., Relationship between arsenic accumulation in tissues and hematological parameters in mullet caught in Faro Lake: a preliminary study, Environ. Sci. Pollut. Control Ser. 26 (9) (2019) 8821–8827.
[6] Junior G. Bandeira, A.C. Dos Santos, C.F. Souza, et al., *Citrobacter freundii* infection in silver catfish (*Rhamdia quelen*): hematological and histological alterations, Microb. Pathog. 125 (2018) 276–280.
[7] G.S. Han, H.J. Yao, L. Qiang, X.N. Chen, Y.L. Gao, Comparative study of peripheral blood cells in two marine fishes (*Synechogobius hasta* and *Sebastes schlegelii*): Morphological and cytochemical characterization, Tissue Cell 73 (2021) 101633.
[8] F.M. Zhang, R.R. Feng, W. Fang, Y.H. Shi, L.G. An, G.W. Yang, Cytochemical characterization of peripheral blood cell populations of two Cyprinidae, *Carassius auratus* and *Ctenopharyngodon idellus*, Anat. Histol. Embryol. 48 (1) (2019) 22–32.
[9] B. Massar, S. Dey, R. Barua, K. Dutta, Microscopy and microanalysis of hematological parameters in common carp, *cyprinus carpio*, inhabiting a polluted lake in North East India, Micros, Microscopy & Microanalysis 18 (5) (2012) 1077–1087.
[10] M. Chawla, V. Verma, M. Kapoor, S. Kapoor, A novel application of periodic acid-Schiff (PAS) staining and fluorescence imaging for analysing tapetum and microspore development, Histochem. Cell Biol. 147 (1) (2017) 103–110.
[11] E.L. Thomas, P.M. Bozeman, M.M. Jefferson, et al., Oxidation of bromide by the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase, J. Biol. Chem. 270 (7) (1995) 2906–2913.
[12] J. Fang, K. Chen, H.M. Cui, X. Peng, T. Li, Z.C. Zuo, Morphological and cytochemical studies of peripheral blood cells of *Schizothorax prenanti*, Anat. Histol. Embryol. 43 (5) (2014) 386–394.
[13] H.J. Chen, G.L. Yuan, J.G. Su, X.L. Liu, Hematological analysis of *Ctenopharyngodon idella, Megalobrama amblycephala* and *Pelteobagrus fulvidraco*: morphology, ultrastructure, cytochemistry and quantification of peripheral blood cells, Fish Shellfish Immunol. 90 (2019) 376–384.
[14] D. Suljević, M. Mitrašinović-Brulić, The first record of brook trout (*Salvelinus fontinalis*, Salmonidae) blood cell characteristics and hematological profile: the influence of fish sex on leukocyte count, Aquacult. Int. 28 (2020) 2505–2516.
[15] T. Matsuyama, C. Nakayasu, M. Sano, Immunocytochemical studies of the ontogeny of peripheral blood leucocyte subpopulations in Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immunol. 29 (2) (2010) 362–365.
[16] K. Zang, Y.J. Xu, X.Z. Liu, B. Shi, Y.Y. Wang, Comparative analysis of blood physiological and biochemical charateristics between pond and indoor-culture of Japanese flounder *Paralichthys olivaceus*, Progress in fishery science. 34 (5) (2013) 36–42.
[17] Y.L. Gao, X.Q. Tang, X.Z. Sheng, J. Xing, W.B. Zhan, Immune responses of flounder *Paralichthys olivaceus* vaccinated by immersion of formalin-inactivated *Edwardsiella tarda* following hyperosmotic treatment, Dis. Aquat. Org. 116 (2015) 111–120.
[18] Z.X. Sha, L.Q. Wang, L.M. Sun, Y.D. Chen, Y. Zheng, M. Xin, C. Li, S.L. Chen, Isolation and characterization of monocyte/macrophage from peripheral blood of half smooth tongue sole (*Cynoglossus semilaevis*), Fish Shellfish Immunol. 65 (2017) 256–266.
[19] A.E.H. Sayed, U.M. Mahmoud, O.I. Muhammad, Comparative study of two carnivorous fish (*Parupeneus forsskali* and *Thalassoma klunzingeri*) from the Red Sea: hemato- biochemical parameters and cellular characterization, Tissue Cell 63 (2020) 101316.
[20] H.J. Zhang, C.X. Xie, D.P. Li, H.P. Liu, X.F. Yang, Blood cells of a sisorid catfish *Glyptosternum maculatum* (siluriformes: sisoridae), in Tibetan plateau, Fish Physiol. Biochem. 37 (1) (2011) 169–176.
[21] Z.X. Gao, W.M. Wang, Y. Yang, K. Abbas, D.P. Li, G.W. Zou, J.S. Diana, Morphological studies of peripheral blood cells of the Chinese sturgeon, *Acipenser sinensis*, Fish Physiol. Biochem. 33 (3) (2007) 213–222.
[22] Z.X. Zheng, Y. Tang, J. Fang, X. Peng, J.D. Fan, H.M. Cui, L.Z. Yang, Ultrastructural and cytochemical properties of peripheral blood cells of piebald naked carp (*Gymnocypris eckloni*), Anat. Histol. Embryol. 46 (1) (2017) 17–24.
[23] S. Maciak, A. Kostelecka-Myrcha, Regularities of variation of the red blood indices characterizing the respiratory function of blood in selected fish, Zool. Pol. 56 (1–4) (2011) 35–48.
[24] S. Longeville, L.R. Stingaciu, Hemoglobin diffusion and the dynamics of oxygen capture by red blood cells, Sci. Rep. 7 (1) (2017) 10448.
[25] V. Parrino, T. Cappello, G. Costa, C. Cannavà, F. Sanfilippo, F. Fazio, S. Fasulo, Comparative study of haematology of two teleost fish (*Mugil cephalus* and *Carassius auratus*) from different environments and feeding habits, European Zool J 85 (1) (2018) 193–199.

[26] J.G. Zinkl, W.T. Cox, C.S. Kono, Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish, Comp Haematol Int 1 (4) (1991) 187–195.
[27] F. Peng, X.X. Chen, T. Meng, E. Li, Y.K. Zhou, S.Z. Zhang, Hematology and serum biochemistry parameters of captive Chinese alligators (*Alligator sinensis*) during the active and hibernating periods, Tissue Cell 51 (2018) 8–13.
[28] G.I. Pronina, A.G. Mannapov, Some aspects of fish breeding for immune resistance as shown through a study of common carp, AACL Bioflux 13 (5) (2020) 3004–3014.
[29] M. Tavares-Dias, F.R. Moraes, Morphological, cytochemical, and ultrastructural study of thrombocytes and leukocytes in neotropical fish, *Brycon orbignyanus* Valenciennes, 1850 (Characidae, Bryconinae), J. Submicr. Cytol. Pathol. 38 (2–3) (2006) 209–215.
[30] M. Tavares-Dias, M.F. Ruasde, Leukocyte and thrombocyte reference values for channel catfish (*Ictalurus punctatus* Raf), with an assessment of morphologic, cytochemical, and ultrastructural features, Vet. Clin. Pathol. 36 (1) (2007) 49–54.
[31] T.M. Clauss, A.D.M. Dove, J.E. Arnold, Hematologic disorders of fish, Vet Clin North Am Exot Anim Pract. 11 (3) (2008) 445–462.
[32] Y. Okabe, R. Medzhitov, Tissue biology perspective on macrophages, Nat. Immunol. 17 (1) (2016) 9–17.
[33] R. Lulijwa, A.C. Alfaro, F. Merien, M. Burdass, T. Young, J. Meyer, T.V. Nguyen, C. Trembath, Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit, Fish Shellfish Immunol. 88 (2019) 179–188.
[34] A.M. Rieger, D.R. Barreda, Antimicrobial mechanisms of fish leukocytes, Dev. Comp. Immunol. 35 (12) (2011) 1238–1245.
[35] I.K. Ueda, M.I. Egami, W. Sasso, E.R. Matushima, Cytochemical aspects of the peripheral blood cells of *Oreochromis (Tilapia) niloticus* (Linnaeus, 1758) (Cichlidae, Teleostei): Part II, Braz. J. Vet. Res. Anim. Sci. 38 (6) (2001) 273–277.
[36] A.T. de Oliveira, J.R.G. Lemos, M.Q.C. Santos, J. Pantoja-Lima, P.H.R. Aride, M.L.G. de Araújo, M. Tavares-Dias, J.L. Marcon, Morphological, cytochemical and ultrastructural aspects of blood cells in freshwater stingray species in the middle Rio Negro basin of Amazonian Brazil, Sci. Rep. 11 (1) (2021) 15685.
[37] W.F. Da Silva, M.I. Egami, A.A. Santos, M.M. Antoniazzi, M. Silva, R.C. Gutierre, M.J.R. Paiva, Cytochemical, immunocytochemical and ultrastructural observations on leukocytes and thrombocytes of fat snook (*Centropomus parallelus*), Fish Shellfish Immunol. 31 (4) (2011) 571–577.
[38] J.-P. Lallès, Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation, Rev. Aquacult. 12 (2) (2020) 555–581.
[39] A.T. Oliveira, W.R. Cruz, J. Pantoja-Lima, S.B. Araújo, M.L.G. Araújo, J.L. Marcon, M. Tavares-Dias, Morphological and cytochemical characterization of thrombocytes and leukocytes in hatchings of three species of Amazonian freshwater turtles, Vet. Arh. 81 (5) (2011) 657–670.
[40] X. Wang, Z.J. Wu, S.M. Wu, X.X. Chen, M. Hanif, S.Z. Zhang, Hematological and cytochemical characteristics of peripheral blood cells in the argus snakehead (*Ophiocephalus argus* Cantor), PeerJ 9 (2021) e11234.