Hematological and cytochemical characteristics of peripheral blood cells in argus snakehead (*Ophiocephalus argus* Cantor)

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Background. Argus snakehead (*Ophiocephalus argus* Cantor) is a freshwater cultured bony fish with high nutritional and economic value. Blood cells play a critical role in oxygen transport, defensive and immunization, which are closely related to the health of fish. The purpose of this study was to investigate the morphometry, microstructure and cytochemical characteristics of peripheral blood cells in *O. argus*. The results could provide the necessary basic references for the health monitoring in the large-scale cultivation of *O. argus*.

Methods. The number of blood cells in *O. argus* was counted on a blood count board, and the size of which was measured by a micrometer under light microscope. The morphology and classification of blood cells were studied by wright's staining, and the cytochemical characteristics was investigated by seven chemical staining including peroxidase(POX), Sudan black B(SBB), periodic acid-Schiff(PAS), acid phosphatase(ACP), alkaline phosphatase(ALP), chloroacetic acid AS-D naphthol esterase(AS-D) and α-naphthol acetate esterase(α-NAE) staining.

Results. The peripheral blood cells in *O. argus* could be divided into erythrocytes, leukocytes and thrombocytes, the number of which was 2.9597 million /mm^3, 88,400 /mm^3 and 43,600 /mm^3 respectively in females; 3.0105 million /mm^3, 105,500 /mm^3 and 34,000 /mm^3 respectively in males. Leukocytes consisted of neutrophils, monocytes, large lymphocytes and small lymphocytes, and eosinophils and basophils were not found. Monocytes were the most numerous leukocytes, followed by neutrophils and small lymphocytes, large lymphocytes were the fewest. Cytochemical staining showed that erythrocytes were only positive for PAS staining. Neutrophils were strongly positive for POX and SBB, and positive for all the other cytochemical staining. Monocytes showed strongly positive for ACP, positive for PAS and α-NAE, and weakly positive for AS-D staining. Large lymphocytes exhibited positive for PAS, and weakly positive for ALP, AS-D and α-NAE staining. Small lymphocytes were positive for PAS, and weakly positive for ACP, AS-D and α-NAE staining. Thrombocytes exhibited positive for PAS and AS-D, while negative for the remaining cytochemical staining. The morphology of peripheral blood cells in *O. argus* was generally similar to that of other fish species, while the cytochemical staining patterns have obvious species specificity.

Hematological and cytochemical characteristics of peripheral blood cells in argus snakehead (*Ophiocephalus argus* Cantor)

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Abstract

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/mm^3 respectively in females; 3.0105 million /mm^3, 105,500 /mm^3 and 34,000 /mm^3 respectively in males. Leukocytes consisted of neutrophils, monocytes, large lymphocytes and small lymphocytes, and eosinophils and basophils were not found. Monocytes were the most numerous leukocytes, followed by neutrophils and small lymphocytes, large lymphocytes were the fewest. Cytochemical staining showed that erythrocytes were only positive for PAS staining. Neutrophils were strongly positive for POX and SBB, and positive for all the other cytochemical staining. Monocytes showed strongly positive for ACP, positive for PAS and α-NAE, and weakly positive for AS-D staining. Large lymphocytes exhibited positive for PAS, and weakly positive for ALP, AS-D and α-NAE staining. Small lymphocytes were positive for PAS, and weakly positive for ACP, AS-D and α-NAE staining. Thrombocytes exhibited positive for PAS and AS-D, while negative for the remaining cytochemical staining. The morphology of peripheral blood cells in *O. argus* was generally similar to that of other fish species, while the cytochemical staining patterns have obvious species specificity.

Key words: Cell metrology, Cytochemistry, Microstructure, *Ophiocephalus argus*, Peripheral blood cells

Introduction

As in other vertebrates, blood is an extremely important tissue in fish, consisting of plasma and blood cells (Zhang et al., 2011). Fish blood cells can be divided into erythrocytes, leukocytes

and thrombocytes, which play a vital role in gas transportation, immune defense and coagulation respectively in the body (Chen et al., 2019; Palmer et al., 2015). Blood cells are very sensitive to changes in internal physiological conditions and stimulus by external environmental factors (Palmer et al., 2015). Variations in blood cell counts, morphology and various intracellular functional components can be used as direct markers to judge the health status of fish (Fang et al., 2014; Ishikawa, Ranzani-Paiva & Lombardi, 2008).

Traditional wright's staining can be used to study the microstructure and classification of fish blood cells (Zhang et al., 2019). Cytochemical staining is widely used to detect the content of biological macromolecules and the activity of enzymes, so as to understand the function and physiological state of blood cells and further understand the cell lineages(Shigdar, Harford & Ward, 2009). At present, there have been many reports on the classification, microstructure and cytochemical characteristics of peripheral blood cells in fish, especially in commercially cultured fish. Tripathi, Latimer & Burnley (2004) determined the hematological reference intervals for koi (*Cyprinus carpio*), including blood cell morphology, cytochemistry, and ultrastructure. Tavares-dias & Moraes (2006) described the morphology, cytochemistry and ultrastructure of thrombocytes and leukocytes in neotropical fish (*Brycon orbignyanus*). Tavares-dias (2006) studied the morphology and cytochemistry of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts: big head carp (*Aristichthys nobilis*), oscar (*Astrootus ocellatus*), traíra (*Hoplias malabarus*) and lambari (*Astyanax bimaculatus*). Fang et al. (2014) observed the morphology and cytochemistry of peripheral blood cell in *Schizothorax prenanti* by light and

electron microscopy. Bianchi et al. (2014) described the cell morphology and cytochemical characteristics of a native South America catfish (*Sorubim lima*). Zheng et al. (2016) investigated the ultrastructure and cytochemical properties of peripheral blood cells of piebald naked carp (*Gymnocypris eckloni*) by transmission electron microscopy. Zhang et al. (2019) compared the microstructure and cytochemical characteristics of peripheral blood cells in crucian carp (*Carassius auratus*) and grass carp (*Ctenpharyngodon idellus*). These studies indicated that the major groupes and micromorphology of peripheral blood cells in different fish were generally similar, but there were obvious species-specific differences in the subgroup of leukocytes, the proportion of various leukocyte types and the cytochemical characteristics of blood cells.

Argus snakehead (*Ophiocephalus argus* Cantor) belongs to the family Channidae, perciformes, which is widely distributed in the Yangtze River basin and the lower Yellow River in China, as well as various river systems in Korea, Japan and Russia (Courtenay & Williams, 2004).*O. argus* is a very popular, high-value and highly nutritious, economically farmed freshwater fish (Xiao et al., 2017). At present, the basic biology of *O. argus* has been extensively studied, including individual growth and reproductive behavior (Landis, Lapointe & Angermeier, 2011), population genetics (Xiao, Xia & Bao, 2015), SNP markers (Xiao et al., 2017). However, the hematology and cytochemistry of peripheral blood cells in *O. argus* have been rarely reported. In this study, the number, microstructure and cytochemical characteristics of peripheral blood cells in *O. argus* were investigated by the cell counts, wright's staining and cytochemical methods, which could enrich the basic data of fish hematology, provide reference for health

97 monitoring in artificial breeding of *O. argus*, and provide basic information for the further
98 studies on physiology and immunology of this species.

99

100 Materials & Methods

101 Animals and blood smears preparation

102 Twenty healthy adult *O. argus* (ten males and ten females) were selected, with an average
103 body length of 30.56-42.78 cm and weight of 960.20-1850.32 g. All the fish were obtained from
104 wuhu local aquaculture farm from May to September 2019. Blood samples were collected by
105 caudal vein puncture, and about 2 ml of blood was taken from each fish using a sterile 5 ml
106 syringe and 22 G needle. The K_2-EDTA was used as an anticoagulant to avoid blood coagulation
107 and blood smears should be prepared immediately after blood collection. This work was
108 approved by the ethics committee of Anhui Normal University (permit no. 20190312). All the
109 handling and sampling were carried out in accordance with standard vertebrate procedures and
110 veterinary practices, and in accordance with national and provincial guidelines.

111 Wright's staining

112 The prepared blood smears were air-dried naturally at room temperature and treated with
113 wright's reagent according to the guidelines set by Hefei Tianda Diagnostic Reagent Co., LTD.
114 (Hefei, China). In short, the blood smears were placed in a wet box, stained with wright's A
115 solution for 1 min at room temperature and treated with wright's B buffer for 10 min. Then

rinsed with distilled water for several times and finally air-dried again at room temperature. Stained blood smears were examined under the light microscope.

Cytochemical staining

Cytochemical staining was carried out according to the methods described by Chen et al. (2019) with minor improvements, which are briefly described as follows.

Peroxidase (POX) staining

Blood smears were stained with 0.5 ml benzidine solution (2 ml 0.1% tetramethylbenzidine ethanol solution mixed with 20 μl nitroso ferricone saturated solution) for 1 min at room temperature and added 0.7 ml 1% H_2O_2, which was blown evenly and oxidized for 6 min. After rinsing in distilled water for 3 times, the smears were stained with wright's reagent for 15-20 min.

Sudan black B (SBB)staining

Blood smears were fixed with formaldehyde vapor for 10 min, and then stained with SBB staining solution (300 mg SBB dissolved in 100 ml 70% ethanol) for 60 min at 37℃. Then rinsed in 70% ethanol solution and distilled water for 3 to 4 times, the smears were counterstained with wright's reagent for 20-30 min.

Periodic acid-Schiff (PAS) staining

Blood smears were fixed with 95% ethanol solution for 10 min and washed with distilled water, and then oxidated with 10 mg/ml periodate solution for 15-20 min. After rinsing with

distilled water, they were placed in Schiff's solution for 60 min at room temperature. After rinsing in sulfurous acid solution and distilled water, the smears were counterstained with 20 mg/ml methyl green solution for 15 min.

Acid phosphatases (ACP) staining

Blood smears were fixed with formaldehyde vapor for 10 min, washed with distilled water, and then stained with the incubation solution (74 ml distilled water, 12 ml pH4.7 acetic acid buffer, 2 ml 50 mg/ml lead nitrate, 4 ml 32 mg/ml β -sodium glycerophosphate) for 4-5 hours at 37°C. After rinsing in distilled water for 3 times, they were immersed in 2-10% ammonium sulfide solution for 30 min. Finally, the smears were counterstained with 10 mg/ml methyl green solution for 2 min.

Alkaline phosphatases (ALP) staining

Blood smears were fixed with 10% methanol-formaldehyde solution (10 ml formaldehyde mixed with 90 ml methanol) for 30s, rinsed in distilled water, and then stained with the incubation solution (20 mg leben-naphthol sodium phosphate dissolved in 20 ml 0.05 mol/L propylene glycol buffer, and mixed with 20 mg diazo solid blue, then filtered) at room temperature for 45 min. After rinsing in distilled water for 3 times, the blood smears were counterstained with 1 mg/ml hematoxylin for 10 min.

Chloroacetic acid AS-D naphthol esterase (AS-D) staining

Blood smears were fixed with 10% formaldehyde methanol solution (10 ml formaldehyde mixed with 90 ml methanol) for 30s , rinsed in distilled water, and then stained with staining solution (10 mg chloroacetic acid AS-D naphthol dissolved in 0.5 ml acetone, 10 mg diazo fast blue dissolved in 5 ml distilled water, 5 ml Veronal acetate buffer) for 30-45 min at 37℃. After rinsing in distilled water for 3 times, the smears were counterstained with 1mg/ml hematoxylin for 8 min.

α-naphthol acetate esterase (α-NAE) staining

Blood smears were fixed in 10% formaldehyde saline for 5 min, washed with distilled water, and then stained with the incubation solution (50 ml 0.067 mol/L phosphate buffer, 1 ml liquid: 4 mg/ml α-naphthol acetate dissolved in 50% acetone, 100 mg diazo fast blue) for 1h at 37°C. After rinsing in distilled water for 3 times, the smears were counterstained with 10 mg/ml methyl green solution for 5-15 min.

Evaluation of cytochemical staining results

According to the evaluation method described by Bianchi et al. (2014), the results of cytochemical staining were expressed in terms of the intensity of cytochemical reactions: negative reaction (-), weak positive reaction (+), positive reaction (+ +) and strong positive reaction (+ + +).

Blood cell counts and measurements

The total number of blood cells was calculated through Neubauer counter board under an Olympus BX61 microscope. The number of erythrocytes, leukocytes and thrombocytes were calculated according to the proportions of these cells counted on the wright's blood smears. The percentages of different leukocyte types were calculated after counting 3000 randomly selected leukocytes from male and female individuals, and the cell sizes(the length and width of various cells and nuclei) were obtained by measuring 20 randomly selected cells for each cell type from male and female individuals with an ocular micrometer scale.

Statistical analysis

The experimental data were represented by Mean ± SD. The significant differences in morphometric values among different cell types or between sexes were compared by one-way ANOVA analysis in SPSS 21.0 software, A P-value less than 0.05 was significant difference, and P-value less than 0.01 was extremely significant difference.

Results

Classification and counting of peripheral blood cells

The blood smears stained by wright's staining were observed under light microscope with oil-immersion at 1000× magnification. 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, the peripheral blood cells of *O. argus* could be divided into erythrocytes, leukocytes

and thrombocytes, and the leukocytes could be subdivided into neutrophils, monocytes, large lymphocytes and small lymphocytes.

The number of erythrocytes, leukocytes and thrombocytes in *O. argus* were calculated and shown in Table 1. There was no significant difference in the number of erythrocytes and leukocytes between sexes ($P > 0.05$), while the number of thrombocytes in females was significantly higher than that in males ($P < 0.05$). The percentages of different leukocytes were also shown in Table 1. Monocytes were the most abundant leukocytes in *O. argus*, followed by neutrophils and small lymphocytes, and the large lymphocytes were the least numerous(one-way ANOVA: $F_{4,11}=496.966.\ P < 0.01$). No statistically significant differences in the percentage of all types of leukocytes were found between females and males ($P > 0.05$).

The microstructure of peripheral blood cells

Erythrocytes

Mature erythrocytes (Fig. 1A) were oval in shape, smooth, and contained an ovoid or rod-shaped purple nucleus in the center of the cell, with light brown or yellowish cytoplasm. The size of the mature erythrocytes and their nuclei were shown in Table 2. The cell length and nuclear length and width of mature erythrocytes in females were significantly larger than those in males ($P < 0.01$). A small number of immature erythrocytes (Fig. 1A) were also observed on the blood smears stained by wright's staining, which were round and smaller than mature erythrocytes, with round or elliptic, dark purplish-stained nuclei.

208 **Neutrophils**

209 Neutrophils (Fig. 1B, C) were spherical or round in shape, with purplish stained nuclei. The
210 nuclei had a variety of shapes, including bilobate, trilobed, kidney-shaped or non-bilobed, and
211 bilobed nuclei were most frequently observed. The cytoplasm was rich and stained light blue,
212 containing numerous fine mauve and reddish particles.

213 **Monocytes**

214 Monocytes (Fig. 1D) were the largest leukocytes in *O. argus* (Table 2). Most of them were
215 round and oval, and a few were irregular. The nuclei were oval, pear-shaped or horseshoe-shaped,
216 and generally stained purple. The most obvious morphological feature of monocytes was that the
217 cytoplasm contained a large number of vacuoles of different sizes with pseudopodia
218 protuberances at the cell edges.

219 **Large lymphocytes**

220 Lymphocytes could be divided into large lymphocytes and small lymphocytes. Large
221 lymphocytes (Fig. 1E) were generally round or irregularly round, with large, oval nuclei on one
222 side of the cells, and the purplish nucleus occupied almost the entire cytoplasm. Some large
223 lymphocytes had smooth surfaces and some had small fingerlike protuberances on the surface of
224 the cells.

225 **Small lymphocytes**

Small lymphocytes (Fig. 1F) were oval in shape with an eccentric, purple, rounded, gapped nucleus that occupied almost the entire cell, and contained a thin rim of pale blue cytoplasm. Small lymphocytes were characterized by numerous microvilli protuberances from the cytoplasmic margins.

Thrombocytes

Thrombocytes showed different forms in the smears, including round, oval, long ovoid and spindle. Spindle-shaped thrombocytes (Fig. 1G) were usually isolated and their nuclei were consistent with the shape of the cells, most of which are centered and purplish, and the cytoplasm was approximately colorless. Round thrombocytes (Fig. 1H) usually appeared in clusters with multiple cells, and they had round, dark purple-stained nuclei, and less cytoplasm was flocculent around the nucleus.

The cytochemical staining characteristics of peripheral blood cells

POX staining

Neutrophils (Fig. 2A2) showed strongly positive with blue-black coarse and rod-shaped granules in the cytoplasm. Erythrocytes (Fig. 2A1), monocytes (Fig. 2A3), large lymphocytes (Fig. 2A4), small lymphocytes (Fig. 2A5) and thrombocytes (Fig. 2A6) were all negative, and their cytoplasm was light blue without granules.

SBB staining

Neutrophils (Fig. 2B2) exhibited strongly positive, the cytoplasm was covered with a large number of diffusely distributed dark black granules. Erythrocytes(Fig. 2B1), monocytes (Fig. 2B3), large lymphocytes (Fig. 2B4), small lymphocytes (Fig. 2B5) and thrombocytes (Fig. 2B6) were all negative with pale purple cytoplasm and no granules.

PAS staining

Erythrocytes, four types of leukocytes and thrombocytes were all positive for PAS staining, and their cytoplasm was purple or dark purple with diffusely granular matter (Fig. 2C1-C6).

ACP staining

Monocytes (Fig. 2D3) showed strongly positive, the cytoplasm contained a large number of brown-black granules or tablets. Neutrophils (Fig. 2D2) and small lymphocytes (Fig. 2D5) were weakly positive, with small amounts of brown granules in the cytoplasm. Erythrocytes (Fig. 2D1), large lymphocytes (Fig. 2D4) and thrombocytes (Fig. 2D6) were all negative with pale purple cytoplasm and no granules.

ALP staining

Both neutrophils (Fig. 2E2) and large lymphocytes (Fig. 2E4) exhibited weakly positive, with many fine uniformly distributed purple granules in the cytoplasm. Erythrocytes (Fig. 2E1), monocytes (Fig. 2E3), small lymphocytes (Fig. 2E5), and thrombocytes (Fig. 2E6) were all negative, and their cytoplasm was pale yellow without stained granules.

AS-D staining

Both neutrophils (Fig. 2F2) and thrombocytes (Fig. 2F6) showed positive, with many uniformly distributed red granules in the cytoplasm. Monocytes (Fig. 2F4), large lymphocytes (Fig. 2F5), and small lymphocytes (Fig. 2F6) showed weakly positive, and their cytoplasm was pale red with fine granules. Erythrocytes (Fig. 2f1) were negative with pale pink cytoplasm.

α-NAE staining

Both neutrophils (Fig. 2G2) and monocytes (Fig. 2G3) were positive, and their cytoplasm was filled with gray-black diffused or granular deposits. Large lymphocytes (Fig. 2G4) and small lymphocytes (Fig. 2G5) were weakly positive, with dark brown or purple granules in the cytoplasm. Erythrocytes (Fig. 2G1) and thrombocytes (Fig. 2G6) were negative, their cytoplasm was purplish without granules.

The cytochemical staining patterns of peripheral blood cells

The cytochemical staining patterns of peripheral blood cells in *O.argus* were summarized in Table 3. Erythrocytes were positive for PAS, and negative for POX, SBB, ACP, ALP, AS-D and α-NAE staining. Neutrophils exhibited strongly positive for POX and SBB, positive for PAS, AS-D and α-NAE, and weakly positive for ACP and ALP staining. Monocytes showed strongly positive for ACP, positive for PAS and α-NAE, and weakly positive for AS-D, while negative for POX, SBB and ALP staining. Large lymphocytes exhibited positive for PAS, and weakly positive for ALP, AS-D and α-NAE, while negative for POX, SBB and ACP staining. Small

lymphocytes were positive for PAS, and weakly positive for ACP, AS-D and α-NAE, while negative for POX, SBB and ALP staining. Thrombocytes showed positive for PAS and AS-D, while negative for all the other cytochemical staining.

Discussion

The number and morphology of erythrocytes in O. argus

Transporting oxygen and carbon dioxide through intracellular hemoglobin is the primary function of erythrocytes (Minasyan, 2014). Erythrocytes are the predominant blood cell type in the vast majority of fish (Satheeshknmar et al., 2011; Satheeshknmar et al., 2012), while the erythrocyte counts were significantly different among different fish. In this study, the number of erythrocytes in *O. argus* was 2.78-3.30 $\times 10^6/mm^3$, which was comparable to that of spotted rose snapper (*Lutjanus guttatus*) (0.75-3.71 $\times 10^6/mm^3$) (Rio-Zaragoza et al., 2011) and cichlid fish (*Cichlasoma dimerus*) (1.68-4.27 $\times 10^6/mm^3$) (Vázquez & Guerrero, 2007), and lower than that of Persian sturgeon (*Acipenser persicus*) (4.80-7.90$\times 10^6/mm^3$) (Milad et al., 2016) and shovelnose catfish (*Sorubim cuspicaudus*) (3.50-14.0 $\times 10^6/mm^3$)) (Negrete et al., 2010), and higher than that of Siamese fighting fish (*Betta splendens*) (1.70-2.21 $\times 10^6/mm^3$) (Motlagh et al., 2012), Asian sun catfish (*Horabagrus brachysoma*) (1.66-2.43 $\times 10^6/mm^3$) (Prasad & Charles, 2010) and piebald naked carp (1.48-1.78 $\times 10^6/mm^3$) (Tang et al., 2015). The number of erythrocytes in fish is related to various factors, such as diet habits, fish body length, age, sex,

water temperature, salinity and living environment (Jawad, Al-Mukhtar & Ahmed, 2004; Kori-Siakpere, Ake & Idoge, 2005; Martins et al., 2011). The above fish with higher erythrocyte counts were mostly carnivorous, with wide range of motion and high activity, while the fish with less erythrocytes were mostly omnivorous, with low activity. The results were consistent with previous reports that the fish with high activity needed to consume more oxygen and had a correspondingly higher number of erythrocytes (Engel & Davis, 1964; Rambhaskar & Rao, 1987). This study showed that there was no significant difference in the number of erythrocytes between males and females, which was consistent with most of fish, such as Persian sturgeon (Milad et al., 2016), Siamese fighting fish (Motlagh et al., 2012) and cichlid fish (Vázquez & Guerrero, 2007).

The morphological characteristics of mature erythrocytes of *O. argus* were similar to those of other fish (Ahmed & Sheikh., 2020; Chen et al., 2019; Negrete et al., 2010; Vázquez & Guerrero, 2007), which were usually oval in shape with an oval or long oval nucleus. This study showed that the size of erythrocytes in *O. argus* (14.33 ± 1.16 × 10.43 ± 1.05 μm) was smaller than Chinese sturgeon (*Acipenser sinensis*) (17.98 ± 0.96 × 12.65 ± 0.87 μm) (Gao et al., 2007) and sisorid catfish (*Glyptosternum maculatum*) (19.39 ± 2.48 × 15.15 ± 1.91 μm) (Zhang et al., 2011), and larger than siamese fighting fish (12.68 ± 1.13 × 9.52 ± 0.75 μm) (Molagh et al., 2012) and cichlid fish (9.4-10 × 6.2-7.3 μm) (Vázquez & Guerrero, 2007), and similar to the piebald naked carp (14.88 ± 0.76 × 10.02 ± 0.42 μm) (Tang et al., 2015). The erythrocyte size

reflects the capacity of transporting oxygen, small erythrocytes have a strong ability to transport oxygen (Fang et al., 2014).

In this study, a small number of immature erythrocytes were also found in the peripheral blood of *O. argus*, which was consistent with reports in other fish. However, the morphology of immature erythrocytes in *O. argus* were round with a smaller and mostly round nucleus, which was somewhat different from that of other fish, such as *S. prenanti* (Fang et al., 2014), spotted rose snapper (Rio-Zaragoza et al., 2011), piebald naked carp (Tang et al., 2015) and crucian carp and grass carp (Zhang et al., 2019), whose immature erythrocytes were mostly ovoid or oval in shape, with a elliptic and larger nucleus.

The proportion and morphology of leukocytes in O. argus

Four types of leukocytes: neutrophils, monocytes, large lymphocytes and small lymphocytes were found in the peripheral blood of *O. argus*. There were three types of granulocytes in vertebrates: neutrophils, eosinophils and basophils (Fang et al., 2014). Almost all of the bony fish contain neutrophils (or heterophils), but eosinophils and/or basophils exist only in certain species (Zhou et al., 2006). Few of fish have both eosinophils and basophils, such as Tilapia (*Oreochromis niloticus*) (Ueda et al., 2001), most of the fish have only eosinophils, without basophils (Gao et al., 2007; Milad et al., 2016; Motlagh et al., 2012; Rio-Zaragoza et al., 2011;Vázquez & Guerrero, 2007; Zheng et al., 2016), few of fish have only basophils,without eosinophils (Shigdar, Harford & Ward, 2009; Zhang et al., 2019), and some fishes have neither eosinophils nor basophils (Chen et al., 2019; Fang et al., 2014; Tavares-dias & Moraes, 2006;

339 Silva et al., 2011; Zhang et al., 2011). In this study, eosinophils and basophils were also not
340 found in the peripheral blood of *O. argus*.

341 The percentages of leukocytes were different among different fish species. Lymphocytes
342 were the most abundant leukocytes in most of the fish, such as sovel-nosed catfish (Bianchi et al.,
343 2014), turbot (*Psetta maxima*) (Burrows, Fletcher & Manning, 2001), Siamese fighting fish
344 (Motlagh et al., 2012), shovelnose catfish (Negrete et al., 2010), spotted rose snapper (Rio-
345 Zaragoza et al., 2011), piebald naked carp (Tang et al., 2015), cichlid fish (Vázquez & Guerrero,
346 2007) and sisorid catfish (Zhang et al., 2011). Neutrophils were the most common in some fish,
347 such as Persian sturgeon (Milad et al., 2016), *S. prenanti* (Fang et al., 2014). In this study,
348 monocytes were the most abundant leukocytes in *O. argus*, which was consistent with the
349 reported Chinese sturgeon (Gao et al., 2007).

350 Fish monocytes are mostly round and oval, and a few are irregular in shape, which have
351 phagocytic function and extremely sensitive to the environmental variations(Zheng et al., 2016).
352 Mononcytes were the largest leukocytes in *O. argus*, which were significantly larger than those
353 of *S. prenanti* (Fang et al., 2014), spotted rose snapper (Rio-Zaragoza et al., 2011), piebald naked
354 carp (Tang et al., 2015) and neotropical fish (Tavares-Dias & Moraes, 2006). The most obvious
355 morphological characteristics of monocytes in *O. argus* was that their cytoplasm contained
356 vacuoles of different sizes and pseudopodia protuberances on the cell edge, which was consistent
357 with the monocytes reported in other fish (Tavares-Dias, 2006; Tripathi, Latimer & Burnley,
358 2004; Zheng et al., 2016) and may be related to their phagocytic function (Zheng et al., 2016).

359 Lymphocytes belong to agranulocytes and play an important role in both innate and
360 acquired immunity (Shigdar, Harford & Ward, 2009). Compared with the lymphocytes in some
361 other fish (Tavares-dias, 2006; Zheng et al., 2016), the lymphocytes in *O. argus* were varible in
362 size, which can be divided into large lymphocytes and small lymphocytes. Most of large
363 lymphocytes had small fingerlike protuberances on the cell surface. Many microvilli
364 protuberances can also be found in the cytoplasmic edges of small lymphocytes. These features
365 have also been reported in other fishes (Burrows, Fletcher & Manning, 2001; Rio-Zaragoza et al.,
366 2011; Vázquez & Guerrero, 2007;Silva et al., 2011), and the protuberances on the surface of
367 lymphocytes may be related to immune function of antigen binding receptor molecules
368 (Scapigliati, 2013).

369 Neutrophils in *O. argus* were usually round or spherical with a bilobate nucleus, their
370 cytoplasm contained numerous fine mauve and reddish granules, these morphological
371 characteristics were somewhat different from the reports in some other fish. For instance, the
372 neutrophils of *S. prenanti* (Fang et al., 2014) were round or irregular-shaped, their nuclei were
373 usually kidney-shaped or trilobed, and their cytoplasm contained a large number of light blue or
374 pink granules, neutrophils of shovelnose catfish (Negrete et al., 2010) had an eccentric and round
375 nucleus with light blue granules in the cytoplasm, neutrophils of neotropical fish (Tavares-Dias
376 & Moraes., 2006) were round with an oval-shaped eccentric nucleus, their cytoplasm contained
377 many purple granules of different sizes, the heterophils of sisorid catfish (Zhang et al., 2011)

were round and regular in shape with kidney-shaped or round nuclei, the cytoplasm contained pale blue granules.

The morphology and number of thrombocytes in O. argus

Thrombocytes of *O. argus* were observed in different forms, including round, oval, oblong and spindle-shaped, which was consistent with the reports in other fish (Fang et al., 2014; Gao et al., 2007; Micha et al., 2019; Rio-zaragoza et al., 2011; Zhang et al., 2011; Zheng et al., 2016).The spindle-shaped thrombocytes often existed alone with some vacuoles in the cytoplasm, which were related to cell phagocytosis (Nagasawa, Somamoto & Nakao, 2015; Stosik et al., 2002). The round thrombocytes usually appeared in clusters with 2-8 cells, which may be related to their hemostatic function (Chen et al., 2019; Peng et al., 2018).

The number of thrombocytes in *O. argus* was 0.31-0.47 $\times 10^5/mm^3$, lower than that of Siamese fighting fish (0.96-0.98 $\times 10^5/mm^3$) (Motlagh et al., 2012), shovelnose catfish (0.8-19 $\times 10^5/mm^3$) (Negrete et al., 2010) and spotted rose snapper (0.35-1.13 $\times 10^5/mm^3$) (Rio-Zaragoza et al., 2011), higher than that of Chinese sturgeon (0.13 $\times 10^5/mm^3$) (Gao et al., 2007) and piebald naked carp (0.11-0.2 $\times 10^5/mm^3$) (Tang et al., 2015), and similar to grass carp (0.41 $\times 10^5/mm^3$), blunt snout bream (*Megalobrama amblycephala*) (0.31 $\times 10^5/mm^3$), yellow catfish (*Pelteobagrus fulvidraco*) (0.30 $\times 10^5/mm^3$) (Chen et al., 2019) and cichlid fish (0.17-0.48 $\times 10^5/mm^3$) (Vázquez & Guerrero, 2007). The differences in the number of thrombocytes in different species of fish may be related to biotic and abiotic factors and their adaptability to the environment (Pavlidis et al., 2007; Prasad & Charles, 2010).

The cytochemical staining patterns of blood cells in O. argus

In this study, the cytochemical characteristics of peripheral blood cells of *O. argus* were studied by seven staining methods of POX, SBB, PAS, ACP, ALP, AS-D and α-NAE for the first time. POX is an enzyme specific to neutrophils in mammals and participates in the defense mechanism of bacterial infection (Tavares-Dias, 2006). SBB and PAS staining were used to detect intracellular glycogen and lipids, which could provide energy for phagocytosis (Ueda et al., 2001). ACP and ALP are lysosomal enzymes involved in phagocytosis and degradation (Shigdar, Harford & Ward, 2009; Silva et al, 2011). AS-D is a specific esterase,which is associated with cellular defense and phagocytic material processing (Casaletti-Rosa & Lunardi, 1997; Tavares-Dias et al., 2007). As a non-specific esterase, α-NAE plays an important role in phagocytosis and antigen presentation (Fang et al., 2014).

Erythrocytes of *O. argus* in this study were only positive for PAS, which was different from that described in other fish, such as *S. prenanti* (Fang et al., 2014), Tilapia (Ueda et al., 2001), crucian carp and grass carp (Zhang et al., 2019) and piebald naked carp (Zheng et al., 2016), whoes erythrocytes showed negative for PAS. The erythrocytes in *O. argus* were negative for POX, SBB, ACP, ALP, AS-D and α-NAE staining, which was consistent with above fish. PAS positivity and SBB negativity indicated that glycogen is the main energy source of erythrocytes in *O. argus*. Vacuoles have been observed in the cytoplasm of some fish erythrocytes and thought to be related to phagocytosis (Neumann et al., 2001; Zheng et al., 2016). In this study, no

417 vacuoles were found in the cytoplasm of erythrocytes. The absence of POX, ACP, ALP, AS-D
418 and α-NAE suggested that erythrocytes in *O. argus* may not have phagocytic activity.

419 Neutrophils of *O. argus* exhibited strongly positive for POX and SBB, positive for PAS,
420 AS-D and α-NAE , and weak positive for ACP and ALP staining, which was generally similar to
421 the staining results of Murray cod (*Maccullochella peelii peelii*) (Shigdar, Harford & Ward,
422 2009), but different from reports in some other fish. For instance, neutrophils of fat snook
423 (Centropomus parallelus) (Silva et al., 2011) were positive for PAS, SBB, ACP and NAE, while
424 negative for ALP staining, American paddlefish (*Polyodon spathula*) (Petrie-Hanson &
425 Peterman, 2005) and piebald naked carp (*Gymnocypris eckloni*) (Zheng et al., 2016) were
426 positive for ACP, while negative for SBB staining, and Asian sun catfish (*Horabagrus*
427 *brachysoma*) (Prasad & Charles., 2010) were negative for ALP, NAE and ASD staining.
428 Neutrophils in human and mammals are mainly involved in the phogocytosis and degradation of
429 invading microorganisms (Azevedo and Lunardi, 2003; Rieger and Barreda, 2011; Wang et al.,
430 2019). The strongly positive reactions for POX and SBB and the positive reactions for PAS,
431 ACP, AS-D and α-NAE indicated that the neutrophils of *O. argus* were similar to those of
432 mammals, which had strong phagocytic and bactericidal abilities.

433 Monocytes of *O. argus* showed strongly positive for ACP, positive for PAS and α-NAE,
434 and weakly positive for AS-D, while negative for SBB, POX and ALP staining, which was
435 basically consistent with those of other fish, such as *S. prenanti* (Fang et al., 2014), American
436 paddlefish (Petrie-hanson & Peterman, 2005), Murray cod (Shigdar, Harford & Ward, 2009),

Tilapia(Ueda et al., 2001), fat snook (Silva et al., 2011) and piebald naked carp (Zheng et al., 2016), indicating that fish monocytes have phagocytosis and antigen presenting functions, glycogen is the main energy source of phagocytosis. Lack of POX and ALP suggested that fish monocytes had weaker bactericidal abilities.

Lymphocytes of *O. argus* were positive for PAS, which was different from those of sovel-nosed catfish (Bianchi et al., 2014), four freshwater teleosts（Tavares-Dias, 2006）, channel catfish (*Ictalurus punctactus*) (Tavares-Dias & Moraes, 2007) and fat snook (Silva et al., 2011), whose lymphocyte were negative for PAS, while consistent with *S. prenanti* (Fang et al., 2014), Tilapia (Ueda et al., 2001) and piebald naked carp (Zheng et al., 2016), suggesting that there were certain glycogen in the lymphocytes of *O. argus*. Large and small lymphocytes of *O. argus* were negative for POX and SBB, indicating that they may not have phagocytic and bactericidal ability. Large and small lymphocytes of *O. argus* showed weakly positive for AS-D and α-NAE, the large lymphocytes were weakly positive for ALP, and the small lymphocytes were weakly positive for ACP, indicating that they may have a weak antigen presentation function.

Thrombocytes were positive for PAS and AS-D, while negative for all other cytochemical staining, and the same results were also found in Murray cod (Shigdar, Harford & Ward, 2009), Tilapia (Ueda et al., 2001) and piebald naked carp (Zheng et al., 2016).The thrombocytes of lower vertebrates are functionally similar to platelets of mammals (Micha et al., 2019), playing an important role in the process of hemostasis and coagulation (Chen et al., 2018; Peng et al., 2018). Studies have considered that thrombocytes in some fish have phagocytosis (Michał et al.,

457 2019; Shigdar et al., 2007; Shigdar, Harford & Ward, 2009; Silva et al, 2011; Zhang et al., 2019).
458 In this study, thrombocytes of *O. argus* showed positive for PAS and AS-D staining, indicating
459 that they may have some phagocytic and material processing functions.

460 In summary, a comprehensive study on the morphological metrology, microstructure and
461 cytochemical characteristics of peripheral blood cells in *O. argus* has been conducted in this
462 study for the first time. The results showed that the number of erythrocytes and leukocytes in *O.*
463 *argus* was consistent with that of carnivorous fish. The morphology and microstructure of
464 peripheral blood cells in *O. argus* was basically similar to those of other fish, while the
465 cytochemical staining patterns have obvious species specificity. For example, all the blood cell
466 types of *O. argus* exhibited positive for PAS, neutrophils were strongly positive or positive for
467 all the seven kinds of cytochemical staining, monocytes, large lymphocytes and small
468 lymphocytes were negative for POX and SBB, thrombocytes were positive for AS-D. Our results
469 could enrich the understanding of the morphology and function of peripheral blood cells of fish,
470 and provide basic data for the health assessment in aquaculture of *O. argus*.

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Table 1(on next page)

The peripheral blood cell counts and percentage of leukocytes in argus snakehead.

*indicates the significant differences in blood cell counts between males and females ($P < 0.05$), different letters (a, b, c, d) in the same column indicate the significant differences among different leukoytes ($P < 0.05$).

Table 1: The peripheral blood cell counts and percentage of leukocytes in argus snakehead.

	Females	Males
Erythrocyte count（$\times 10^6/mm^3$）	2.96 ± 0.16	3.01 ± 0.24
Leukocyte count（$\times 10^5/mm^3$）	0.88 ± 0.05	1.06 ± 0.09
Neutrophil（%）	17.9 ± 0.75^c	17.07 ± 1.01^b
Monnocyte（%）	50.83 ± 2.05^d	45.93 ± 2.45^c
Large lymphocyte（%）	5.03 ± 0.58^a	6.37 ± 0.45^a
Small lymphocyte（%）	12.67 ± 1.20^b	15.1 ± 1.12^b
Thrombocyte count（$\times 10^5/mm^3$）	0.44 ± 0.02*	0.34 ± 0.02*

Notes: *indicates the significant differences in blood cell counts between males and females ($P < 0.05$), different letters (a, b, c, d) in the same column indicate the significant differences among different leukoytes ($P < 0.05$).

Table 2(on next page)

The size of the peripheral blood cells in argus snakehead (Mean ± SD, μm, N = 100).

** indicates that extremely significant differences between males and females ($P < 0.01$), different letters (a, b, c, d) in the same column indicate significant differences among different cells ($P < 0.05$).

Table 2: The size of the peripheral blood cells in argus snakehead (Mean ± SD, μm, N = 100).

Cell types	Females		Males	
	Cell length	Cell width	Cell length	Cell width
Erythrocytes	14.33 ± 1.16 **	10.43 ± 1.05	13.11 ± 0.88	10.47 ± 0.98
(nuclei)	(8.71 ± 0.88**)	(4.60 ± 0.70**)	(7.21 ± 0.90)	(3.55 ± 0.79)
Neutrophils	16.64 ± 2.16^{c}	14.83 ±2.45^{c}	15.21 ± 2.21^{c}	13.55 ± 2.16^{c}
Monocytes	16.60 ± 1.77^{c}	14.61 ± 1.43^{c}	15.51 ± 1.34^{c}	14.29 ±1.10^{c}
Large lymphocytes	11.38 ± 2.67^{b}	9.92 ± 2.50^{b}	11.94 ± 2.17^{b}	10.31 ± 2.09^{b}
Small lymphocytes	6.75 ± 1.36^{a}	6.00 ± 1.30^{a}	6.77 ± 1.13^{a}	6.17 ±1.06^{a}
Thrombocytes	19.18 ± 3.19^{d}	6.99 ± 0.74^{a}	14.70 ± 2.50^{c}	6.87 ± 1.03^{a}

Notes: ** indicates that extremely significant differences between males and females ($P < 0.01$), different letters (a, b, c, d) in the same column indicate significant differences among different cells ($P < 0.05$).

Table 3(on next page)

Cytochemical staining patterns of peripheral blood cells in argus snakehead.

"+ + +" strongly positive; "+ +" positive; "+" weakly positive; "-" negative.

1 **Table 3 Cytochemical staining patterns of peripheral blood cells in argus snakehead.**

Cell types	POX	SBB	PAS	ACP	ALP	AS-D	α-NAE
Erythrocytes	–	–	+ +	–	–	–	–
Neutrophils	+ + +	+ + +	+ +	+	+	+ +	+ +
Monocytes	–	–	+ +	+ + +	–	+	+ +
Large lymphocytes	–	–	+ +	–	+	+	+
Small lymphocytes	–	–	+ +	+	–	+	+
Thrombocytes	–	–	+ +	–	–	+ +	–

2 Notes: "+ + +" strongly positive; "+ +" positive; "+" weakly positive; "-" negative.

3

Figure 1

Microstructure of peripheral blood cells in argus snakehead (Wright's staining).

(A) mature erythrocyte (arrow) : oval with a long oval nucleus; immature erythrocyte (arrowhead) : round, with a round nucleus. (B) neutrophil: globular with a bilobate nucleus, pale blue cytoplasm contained many fine mauve and reddish particles. (C) neutrophil: round with an eccentric and oval-shaped nucleus, cytoplasm contained a large number of diffuse purple particles. (D) monocyte: oval with a horseshoe-shaped nucleus, cytoplasm contained many small vacuoles. (E) large lymphocyte: round or irregularly round, with many protuberances on the surface. (F) small lymphocyte: elliptic, with minimal cytoplasm, and some microvilli protuberances at the margin. (G) thrombocyte: spindle-shaped with an oval and mostly centered nucleus. (H) thrombocyte: round with flocculent cytoplasm, often appeared in clusters with multiple cells. Scale bars = 10 μm.

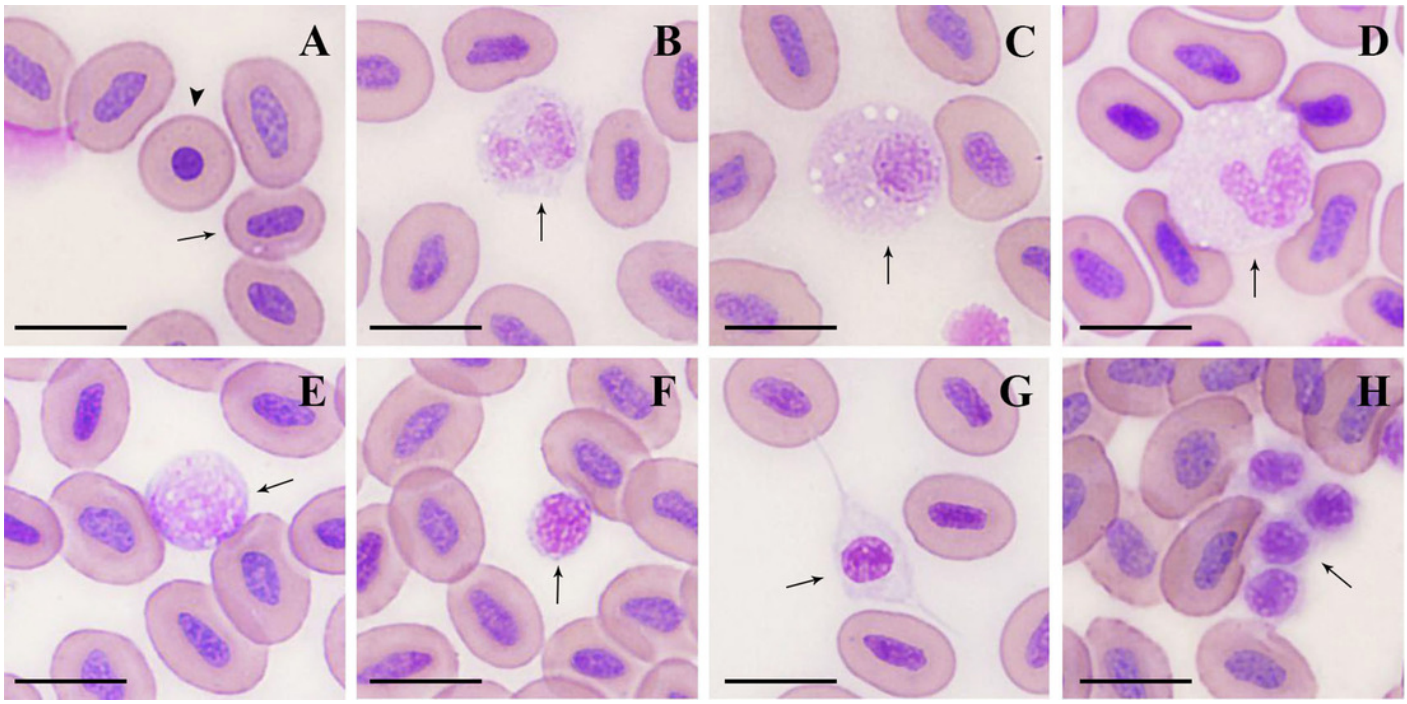
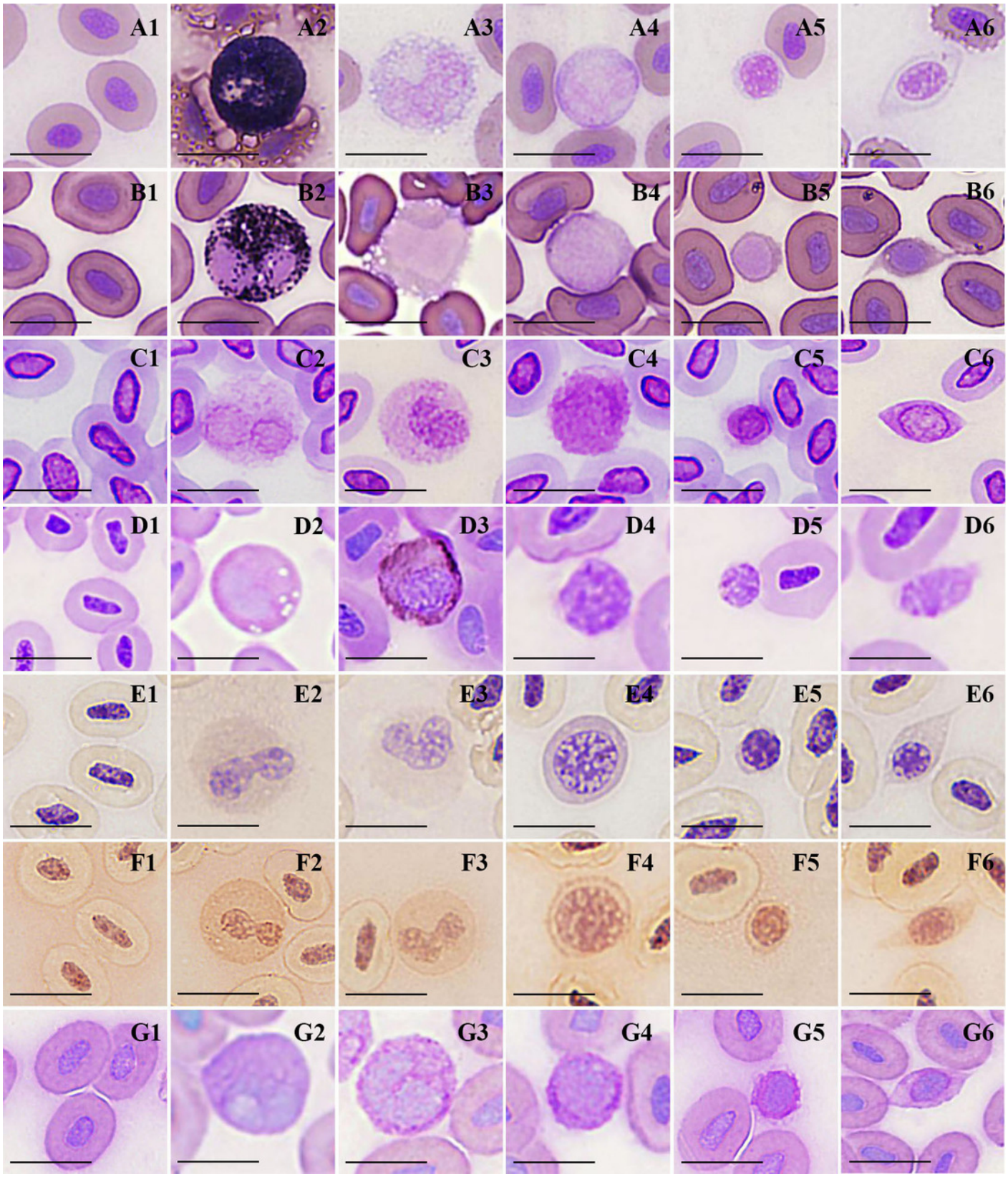


Figure 2

Cytochemical staining of peripheral blood cells in argus snakehead.

Erythrocytes were positive for PAS (C1) and negative for POX, SBB, ACP, ALP, AS-D and α-NAE staining (A1, B1, D1, E1, F1, G1); neutrophils were strongly positive for POX and SBB (A2, B2), positive for PAS, AS-D and α-NAE (C2, F2, G2) and weakly positive for ACP and ALP staining(D2, E2); monocytes were strongly positive for ACP (D3), positive for PAS and α-NAE (C3, G3) and weakly positive for AS-D (F3), while negative for POX, SBB and ALP staining (A3, B3, E3); large lymphocytes were positive for PAS (C4) and weakly positive for ALP, AS-D and α-NAE (E4, F4, G4), while negative for POX, SBB and ACP staining (A4, B4, D4); small lymphocyte were positive for PAS (C5) and weakly positive for ACP, AS-D and α-NAE (D5, F5, G5), while negative for POX, SBB and ALP staining (A5, B5, E5); thrombocytes were positive for PAS and AS-D (C6, F6) and negative for POX, SBB, ACP, ALP and α-NAE staining (A6, B6, D6, E6, G6).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B1
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B3
B4
B5
B6
C1
C2
C3
C4
C5
C6
D1
D2
D3
D4
D5
D6
E1
E2
E3
E4
E5
E6
F1
F2
F3
F4
F5
F6
G1
G2
G3
G4
G5
G6