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, SBB and ACP, and positive for all the other cytochemical staining. Monocytes showed positive for PAS and α-NAE, and weakly positive for ACP and 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 AS-D and α-NAE staining. Thrombocytes exhibited positive for PAS, and weakly positive for ACP 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 SBB and ACP, and positive for all the other cytochemical staining. Monocytes showed positive for PAS and α-NAE, and weakly positive for ACP and 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 AS-D and α-NAE staining. Thrombocytes exhibited positive for PAS, and weakly positive for ACP 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., 2019b; 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 (Massar et al., 2012; 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

77 light and electron microscopy. Bianchi et al. (2014) described the cell morphology and
78 cytochemical characteristics of a native South America catfish (*Sorubim lima*). Zheng et al.
79 (2016) investigated the ultrastructure and cytochemical properties of peripheral blood cells of
80 piebald naked carp (*Gymnocypris eckloni*) by transmission electron microscopy. Zhang et al.
81 (2019) compared the microstructure and cytochemical characteristics of peripheral blood cells in
82 crucian carp (*Carassius auratus*) and grass carp (*Ctenpharyngodon idellus*). These studies
83 indicated that the major groups and micromorphology of peripheral blood cells in different fish
84 were generally similar, but there were obvious species-specific differences in the subgroup of
85 leukocytes, the proportion of various leukocyte types and the cytochemical characteristics of
86 blood cells.

87 Argus snakehead (*Ophiocephalus argus* Cantor) belongs to the family Channidae,
88 perciformes, which is widely distributed in the Yangtze River basin and the lower Yellow River
89 in China, as well as various river systems in Korea, Japan and Russia (Courtenay & Williams,
90 2004). *O. argus* is a very popular, high-value and highly nutritious, economically farmed
91 freshwater fish (Xiao et al., 2017). In Asia, especially in China, the annual production of *O.*
92 *argus* is about 510 000 tons (worth ~1.6 billion US dollars) (Sagada et al., 2017). With the
93 continuous expansion of cultivation scale, the incidence of diseases in *O. argus* increased
94 significantly (Xu et al., 2017). Fish hematology is one diagnostic tool that can provide useful
95 information in guiding treatment options (Grant, 2015). However, upto date, the hematology and
96 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 under the light microscope, which could enrich the basic data of fish hematology, provide reference for health monitoring in artificial breeding of *O. argus*, and provide basic information for the further studies on physiology and immunology of this species.

Materials & Methods

Animals and blood smears preparation

Thirty healthy adult *O. argus* (15 males and 15 females) were selected, with an average body length of 30.56-42.78 cm and weight of 960.20-1850.32 g. All the fish were obtained from wuhu local aquaculture farm from May to September 2019. Blood samples were collected by caudal vein puncture, and about 2 ml of blood was taken from each fish using a sterile 5 ml syringe and 22 G needle. The K_2-EDTA was used as an anticoagulant to avoid blood coagulation and blood smears were prepared immediately after blood collection. This work was approved by the ethics committee of Anhui Normal University (permit no. 20190312). All the handling and sampling were carried out in accordance with standard vertebrate procedures and veterinary practices, and in accordance with national and provincial guidelines.

Wright's staining

115 The prepared blood smears were air-dried naturally at room temperature and treated with
116 wright's reagent according to the guidelines set by Hefei Tianda Diagnostic Reagent Co., LTD.
117 (Hefei, China). In short, the blood smears were placed in a wet box, stained with wright's A
118 solution for 1 min at room temperature and treated with wright's B buffer for 10 min. Then
119 rinsed with distilled water for several times and finally air-dried again at room temperature.
120 Stained blood smears were examined under the light microscope (BM2000, Jiangnan Yongxin
121 Co., Ltd. Nanjing, China).

122 **Cytochemical staining**

123 Cytochemical staining was carried out according to the methods described by Xu (2003)
124 with minor improvements. The prepared blood smears were fixed by formaldehyde vapor for
125 Sudan black B (SBB) and acid phosphatase (ACP) staining, 10% methanol-formaldehyde
126 solution for alkaline phosphatase (ALP) and chloroacetic acid AS-D naphthol esterase (AS-D)
127 staining, and 95% ethanol solution for periodate-schiff's (PAS) and α-naphthol esterase (α-NAE)
128 staining. The specific staining procedures are briefly described as follows.

129 POX staining: 2 ml 0.1% tetramethylbenzidine ethanol solution (0.1 g tetramethylbenzidine
130 (Sangon, 54827-17-7) dissolved in 100 ml 88% ethanol solution) was mixed with 20 μl sodium
131 nitroferricyanide (Sangon, 13755-38-9) saturated solution and dropped on the smears. Then 0.7
132 ml dilute H_2O_2 solution (50 μl 1% H_2O_2 solution mixed with 10 ml distilled water) was added
133 after standing for 1 min , which was blown evenly and oxidized for 6 min.

SBB staining: the dried blood smears were placed in Sudan Black B (Sangon, 4197-25-5) staining solution for 60 min at 37℃, then rinsed in 70% ethanol solution and distilled water for 1-2 min.

PAS staining: blood smears were oxidized with 10 mg/ml periodate for 18-20 min, and rinsed in distilled water for 2 min. Then they were placed in Schiff's solution for 60-90 min at 37℃. After rinsing in sulfuric acid solution (0.6 g sodium bisulfite (Sangon, 7681-57-4) dissolved in 5 ml 1mol/l hydrochloric acid and 100 ml distilled water) for 3-4 times, the smears were washed with distilled water for 2-3 min.

ACP staining: blood smears were stained with the reaction solution (0.1 g lead nitrate (Xilong, 10099-74-8) and 0.128 g β-sodium glycerophosphate (Sangon, 819-83-0) dissolved in 74 ml distilled water and 12 ml pH4.7 acetic acid buffer) for 4-4.5 hours at 37°C. After washed with distilled water for 5 min, they were immersed in 2% ammonium sulfide solution (Aladdin, 12135-76-1) for 30 min.

ALP staining: the smears were immersed in substrate incubation solution (10 mg naphthol AS-BI phosphate (Sangon, 1919-91-1) dissolved in 10 ml 0.05 mol/l propanediol buffer, mixed with 10 mg fast blue B salt (Yuanye, 14263-94-6), and then filtered) for 45-60 min at 37℃, and rinsing in distilled water for 2min.

AS-D staining: blood smears were stained with the incubation solution (10 mg chloroacetic acid AS-D naphthol (Sangon, 528-66-5) dissolved in 0.5 ml acetone solution, then added 5 ml

153 distilled water, 5 ml pH7.5 Veronal acetic acid buffer and 10 mg fast blue B salt (Yuanye,
154 14263-94-6)) for 60-80 min at 37℃, and washed with distilled water.

155 α-NAE staining: the smears were placed in the reaction solution (100 ml phosphate buffer
156 mixed with 1 ml 4mg/ml α-naphthol acetate (Sangon, 90-15-3), then added 100 mg fast blue B
157 salt (Yuanye, 14263-94-6), and filtered by oscillation) for 45-60 min at 37℃, and washed with
158 distilled water for 3-4 times.

159 After cytochemical staining, the smears were counterstained with wright's reagent for POX
160 and SBB, 20 mg/ml methyl green (Sangon, 7114-03-6) for PAS, ACP and α-NAE, and 1 mg/ml
161 hematoxylin (Sangon, 517-28-2) for ALP and AS-D.

162 **Evaluation of cytochemical staining results**

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

167 **Blood cell counts and measurements**

168 The total number of blood cells was calculated through Neubauer counter board under an
169 Olympus BX61 microscope (Tokyo, Japan). The number of erythrocytes (RBC), leukocytes
170 (WBC) and thrombocytes (TC) were calculated according to the proportions of these cells
171 counted on the wright's blood smears (total number × the percentage of cells). The percentages of
172 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. The hemoglobin (Hb), hematoceit (HCT) and erythrocyte sedimentation rate (ESR) were determined according to the methods described previously (Peng, et al., 2018). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from RBC, HCT and Hb according to the formulae below (Gao et al., 2007a):

MCV (fl) = HCT/RBC

MCH (pg) = Hb /RBC

MCHC (g/dl) = Hb/HCT

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 (SPSS Inc, Chicago, USA), 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

194 cytoplasm, the peripheral blood cells of *O. argus* could be divided into erythrocytes, leukocytes
195 and thrombocytes, and the leukocytes could be subdivided into neutrophils, monocytes, large
196 lymphocytes and small lymphocytes.

197 The number of erythrocytes, leukocytes, thrombocytes and various hematological
198 parameters in *O. argus* were calculated and shown in Table 1. There was no significant
199 difference in the number of erythrocytes and total leukocytes between sexes ($P > 0.05$), while the
200 number of thrombocytes in females was significantly higher than that in males ($P < 0.05$). The
201 number of different leukocytes were also shown in Table 1. Monocytes were the most abundant
202 leukocytes in *O. argus*, followed by neutrophils and small lymphocytes, and the large
203 lymphocytes were the least numerous(one-way ANOVA: $F_{4,11}=354.476.\ P < 0.01$). The number
204 of large lymphocytes and small lymphocytes in females was significantly lower than that in
205 males ($P < 0.05$). No statistically significant differences in the Hb, HCT, ESR, MCV, MCH and
206 MCHC were found between females and males ($P > 0.05$).

207 **The microstructure of peripheral blood cells**

208 **Erythrocytes**

209 Mature erythrocytes (Fig. 1A) were oval in shape, surface smooth, and contained an ovoid
210 or rod-shaped purple nucleus in the center of the cell, with light brown or yellowish cytoplasm.
211 The size of the mature erythrocytes and their nuclei were shown in Table 2. The cell length and
212 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.

Neutrophils

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

Monocytes

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

Large lymphocytes

Lymphocytes could be divided into large lymphocytes and small lymphocytes. Large lymphocytes (Fig. 1E) were generally round or irregularly round, with large, oval nuclei on one side of the cells, and the purplish nucleus occupied almost the entire cytoplasm. Some large

lymphocytes had smooth surfaces and some had small fingerlike protuberances on the surface of the cells.

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. 2BB) showed strongly positive with blue-black coarse and rod-shaped granules in the cytoplasm. The cytoplasm of erythrocytes (Fig. 2AA), monocytes (Fig. 2CC),

large lymphocytes (Fig. 2DD), small lymphocytes (Fig. 2EE) and thrombocytes (Fig. 2FF) was light blue without granules, and all of these cells were negative.

SBB staining

Neutrophils (Fig. 2BC) were covered with a large number of diffusely distributed dark black granules in the cytoplasm, and which exhibited strongly positive. The cytoplasm of erythrocytes(Fig. 2AB), monocytes (Fig. 2CD), large lymphocytes (Fig. 2DE), small lymphocytes (Fig. 2EF) and thrombocytes (Fig. 2FG) was pale purple without granules, all of these cells were negative.

PAS staining

The cytoplasm of erythrocytes, neutrophils, monocytes, large lymphocytes, small lymphocytes and thrombocytes (Fig. 2AC-FH) was purple or dark purple with diffusely granular matter, and all of these cells were positive.

ACP staining

Neutrophils (Fig. 2BE) contained a large number of brown-black granules or tablets in the cytoplasm, and showed strongly positive. Monocytes (Fig. 2CF) and thrombocytes (Fig. 2FI) were weakly positive, with small amounts of brown granules in the cytoplasm. The cytoplasm of erythrocytes (Fig. 2AD), large lymphocytes (Fig. 2DG) and small lymphocytes (Fig. 2EH) was pale purple without granules, and all of these cells were negative.

ALP staining

Both neutrophils (Fig. 2BF) and large lymphocytes (Fig. 2DH) exhibited weakly positive, with many fine uniformly distributed purple granules in the cytoplasm. The cytoplasm of erythrocytes (Fig. 2AE), monocytes (Fig. 2CG), small lymphocytes (Fig. 2EI), and thrombocytes (Fig. 2FJ) was pale yellow without stained granules, and all of these cells were negative.

AS-D staining

Neutrophils (Fig. 2BG) were with many uniformly distributed red granules, and which showed positive.. The cytoplasm of monocytes (Fig. 2CH), large lymphocytes (Fig. 2DI), small lymphocytes (Fig. 2EJ) and thrombocytes (Fig. 2FK) was pale red with fine granules, and all of these cells were weakly positive. Erythrocytes (Fig. 2AF) were negative with pale pink cytoplasm.

α-NAE staining

Monocytes' (Fig. 2CI) cytoplasm was filled with gray-black diffused or granular deposits, and which were positive. The cytoplasm of neutrophils (Fig. 2BH), large lymphocytes (Fig. 2DJ) and small lymphocytes (Fig. 2EK) was purple with dark brown or purple granules, and all of these cells were weakly positive. Erythrocytes (Fig. 2AG) and thrombocytes (Fig. 2FL) 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 rection for POX, SBB and ACP, positive for PAS and AS-D, and weakly positive for ALP and α-NAE staining. Monocytes showed positive for PAS and α-NAE, and weakly positive for ACP and 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 AS-D and α-NAE, while negative for POX, SBB, ACP and ALP staining. Thrombocytes showed positive rection for PAS, and weakly positive for ACP 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 (Table 4). The number of erythrocytes in *O. argus* was comparable to that of spotted rose snapper (*Lutjanus guttatus*) and cichlid fish (*Cichlasoma dimerus*), and lower than that of Persian sturgeon (*Acipenser persicus*)

and shovelnose catfish (*Sorubim cuspicaudus*), and higher than that of Siamese fighting fish (*Betta splendens*), Asian sun catfish (*Horabagrus brachysoma*) and piebald naked carp. The values of Hb and HCT in *O. argus* were comparable to those of spotted rose snapper, and higher than those of cichlid fish, Persian sturgeon, Siamese fighting fish and piebald naked carp. The value of MCV in *O. argus* was comparable to that of spotted rose snapper, cichlid fish, Asian sun catfish and piebald naked carp, and lower than that of Persian sturgeon and Siamese fighting fish. RBC and Hb values are related to the ability of the blood to carry dissolved oxygen (Fazio et al., 2012; Tavares-dias & Moraes, 2004).The values of RBC, Hb, HCT and MCV in fish are 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; Milad et al., 2016). The above fish with higher values of RBC, Hb, HCT and MCV were mostly carnivorous, with wide range of motion and high activity, which were consistent with previous reports that the carnivorous fish with high activity needed to consume more oxygen and had a correspondingly higher values of RBC, Hb and HCT (Engel & Davis, 1964; Molnár & Tamássy, 1970; Rambhaskar & Rao, 1987). This study showed that there was no significant difference in the values of RBC, Hb and HCT 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), shovelnose catfish (Negrete et al., 2010) and cichlid fish (Vázquez & Guerrero, 2007).

323 The morphological characteristics of mature erythrocytes of *O. argus* were similar to those
324 of other fish (Ahmed & Sheikh., 2020; Chen et al., 2019a; Negrete et al., 2010; Vázquez &
325 Guerrero, 2007), which were usually oval in shape with an oval or long oval nucleus. The size of
326 erythrocytes in *O. argus* was smaller than Chinese sturgeon (*Acipenser sinensis*) and sisorid
327 catfish (*Glyptosternum maculatum*), and larger than siamese fighting fish and cichlid fish, and
328 similar to the piebald naked carp (Table 4). The erythrocyte size reflects the capacity of
329 transporting oxygen, small erythrocytes have a strong ability to transport oxygen (Fang et al.,
330 2014).

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

338 **The proportion and morphology of leukocytes in *O. argus***

339 Four types of leukocytes: neutrophils, monocytes, large lymphocytes and small
340 lymphocytes were found in the peripheral blood of *O. argus*. There were three types of
341 granulocytes in vertebrates: neutrophils, eosinophils and basophils (Fang et al., 2014). Almost all
342 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., 2007b; 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., 2019a; Fang et al., 2014; Tavares-dias & Moraes, 2006; Silva et al., 2011; Zhang et al., 2011). In this study, eosinophils and basophils were also not found in the peripheral blood of *O. argus*.

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

Fish monocytes are mostly round and oval, and a few are irregular in shape, which have phagocytic function and extremely sensitive to the environmental variations(Zheng et al., 2016). Mononcytes were the largest leukocytes in *O. argus*, which were significantly larger than those

of *S. prenanti* (Fang et al., 2014), spotted rose snapper (Rio-Zaragoza et al., 2011), piebald naked carp (Tang et al., 2015) and neotropical fish (Tavares-Dias & Moraes, 2006). The most obvious morphological characteristics of monocytes in *O. argus* was that their cytoplasm contained vacuoles of different sizes and pseudopodia protuberances on the cell edge, which was consistent with the monocytes reported in other fish (Tavares-Dias, 2006; Tripathi, Latimer & Burnley, 2004; Zheng et al., 2016) and may be related to their phagocytic function (Zheng et al., 2016).

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

Neutrophils in *O. argus* were usually round or spherical with a bilobate nucleus, their cytoplasm contained numerous fine mauve and reddish granules, these morphological characteristics were somewhat different from the reports in some other fish. For instance, the neutrophils of *S. prenanti* (Fang et al., 2014) were round or irregular-shaped, their nuclei were

usually kidney-shaped or trilobed, and their cytoplasm contained a large number of light blue or pink granules, neutrophils of shovelnose catfish (Negrete et al., 2010) had an eccentric and round nucleus with light blue granules in the cytoplasm, neutrophils of neotropical fish (Tavares-Dias & Moraes., 2006) were round with an oval-shaped eccentric nucleus, their cytoplasm contained 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., 2007b; 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., 2019b; Peng et al., 2018).

The number of thrombocytes in *O. argus* was lower than that of Siamese fighting fish (Motlagh et al., 2012), shovelnose catfish (Negrete et al., 2010) and spotted rose snapper (Rio-Zaragoza et al., 2011), higher than that of Chinese sturgeon (Gao et al., 2007b) and piebald naked carp (Tang et al., 2015), and similar to grass carp, blunt snout bream (*Megalobrama amblycephala*), yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al., 2019) and cichlid fish

(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*.

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

Monocytes of *O. argus* showed positive for PAS and α-NAE, and weakly positive for ACP and AS-D, while negative for SBB, POX and ALP staining, which was basically consistent with those of other fish, such as *S. prenanti* (Fang et al., 2014), American paddlefish (Petrie-hanson & Peterman, 2005), Murray cod (Shigdar, Harford & Ward, 2009), Tilapia(Ueda et al., 2001), fat

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

446 Lymphocytes of *O. argus* were positive for PAS, which was different from those of sovel-
447 nosed catfish (Bianchi et al., 2014), four freshwater teleosts（Tavares-Dias, 2006）, channel
448 catfish (*Ictalurus punctactus*) (Tavares-Dias & Moraes, 2007) and fat snook (Silva et al., 2011),
449 whose lymphocyte were negative for PAS, while consistent with *S. prenanti* (Fang et al., 2014),
450 Tilapia (Ueda et al., 2001) and piebald naked carp (Zheng et al., 2016), suggesting that there
451 were certain glycogen in the lymphocytes of *O. argus*.

452 Thrombocytes were positive for PAS, and weakly positive for ACP and AS-D, while
453 negative for all other cytochemical staining, and the similar results were also found in Murray
454 cod (Shigdar, Harford & Ward, 2009), Tilapia (Ueda et al., 2001) and piebald naked carp (Zheng
455 et al., 2016).The thrombocytes of lower vertebrates are functionally similar to platelets of
456 mammals (Micha et al., 2019), playing an important role in the process of hemostasis and
457 coagulation (Chen et al., 2018; Peng et al., 2018). Studies have considered that thrombocytes in
458 some fish have phagocytosis (Michał et al., 2019; Shigdar et al., 2007; Shigdar, Harford & Ward,
459 2009; Silva et al, 2011; Zhang et al., 2019). In this study, thrombocytes of *O. argus* were positive
460 for PAS and AS-D staining, indicating that they may have some phagocytic and material
461 processing functions.

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

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Table 1(on next page)

Haematological parameters of female and male argus snakehead.

Each data point represents the mean of three replicates ± SD except the range. The N number of female and male argus snakehead is 15. *indicatesthe significant differences in blood cell counts and haematological parameters 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: Haematological parameters of female and male argus snakehead.

	Female (N = 15)		Male (N = 15)	
parameters	Mean ± SD	Range	Mean ± SD	Range
RBC ($\times10^6$/mm^3)	2.96 ± 0.16	2.78–3.16	3.01 ± 0.24	2.72–3.30
Hb (g/dl)	10.53± 0.37	10.17−11.03	10.60 ± 0.29	10.30−11.0
HCT (%)	42.41 ± 2.38	39.45−45.27	43.21 ± 1.77	41.52−45.66
ESR (mm/h)	1.52 ± 0.22	1.23−1.76	1.49 ± 0.10	1.42−1.63
MCV (fl)	143.51 ± 13.54	124.84−162.85	144.86 ± 14.15	125.83−167.87
MCH (pg)	35.72 ± 2.57	32.17−39.69	35.46 ± 3.03	31.21−40.44
MCHC (g/dl)	24.56 ± 2.59	21.95−29.44	24.73 ± 2.03	22.50−27.56
WBC ($\times10^4$/mm^3)	8.84 ± 0.47	8.29–9.44	10.55 ± 0.82	9.55–11.56
Neutrophil($\times10^4$/mm^3)	1.82 ± 0.10^c	1.71−1.94	2.03 ± 0.16^b	1.83−2.22
Monnocyte ($\times10^4$/mm^3)	5.11 ± 0.27^d	4.79−5.46	6.09 ± 0.47^c	5.51−6.67
Large lymphocyte ($\times10^4$/mm^3)	0.60 ± 0.03^a*	0.56−0.64	0.77 ± 0.06^a*	0.70−0.84
Small lymphocyte ($\times10^4$/mm^3)	1.31 ± 0.07^b*	1.23−1.40	1.67 ± 0.13^b*	1.51−1.83
TC ($\times10^4$/mm^3)	4.36 ± 0.23*	4.09−4.65	3.40 ± 0.27*	3.08−3.73
Coefficient of Variation (CV) (%)	9.34	1.80−20.91	8.46	3.13−21.16

Notes: Each data point represents the mean of three replicates ± SD except the range. The N number of female and male argus snakehead is 15. *indicatesthe significant differences in blood cell counts and haematological parameters 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 = 20).

** 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 = 20).

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

Table 4(on next page)

The values of RBC, Erythrocyte sizes, Hb, HCT and MCV in argus snakehead and some other fish species.

The values of RBC, Erythrocyte sizes, Hb, HCT and MCV in argus snakehead were compared with those of some other fish.

1 **Table 4: The values of RBC, Erythrocyte sizes, Hb, HCT and MCV in argus snakehead and some other fish species.**

Species	RBC (10^6 /mm^3)	Erythrocyte sizes (μm)	**Hb (g/dl)**	**HCT(%)**	**MCV (fl)**	References
Ophiocephalus argus	2.72–3.30	14.33 ± 1.16 × 10.43 ± 1.05	10.30−11.0	41.52−45.66	125.83−167.87	This study
Lutjanus guttatus	0.75–3.71	11.04 ± 0.85 (10–13)	7.29−17.03	33.53−71.14	135.66−369.80	Rio-Zaragoza et al. (2011)
Cichlasoma dimerus	1.68–4.27	9.4–10 × 6.2–7.3	5.23−8.33	22.5−39.12	70.14–198	Vázquez and Guerrero (2007)
Acipenser persicus	4.8–7.9		8.60−9.87	29.58−31.72	412.20−621.70	Milad et al. (2016)
Sorubim cuspicaudus	3.5–14.0	10.5×8.8	10.5 ± 2.3	25.5 ± 5.6		Negrete et al. (2010)
Betta splendens	1.70–2.21	10.12–15.26 × 7.37–12.59	7.1−9.4	31-39	187.28 ± 7.05	Motlagh et al. (2012)
Horabagrus brachysoma	1.66–2.43		7.2−9.9	21.40−55.61	88.07−335.0	Prasad and Charles (2010)
Gymnocypris eckloni	1.49–1.78	14.88 ± 0.76 × 10.02 ± 0.42	5.21−7.93	22.42−36.92	150.98–207.42	Tang et al. (2015)
Acipenser sinensis	0.85 ± 0.10	17.98 ± 0.96 × 12.65 ± 0.87				Gao et al. (2007)
Ctenopharyngodon idella	1.76 ± 0.23	12.31 ± 0.78 × 8.27 ± 0.72			4.08 ± 0.12	
Megalobrama amblycephala	1.53 ± 0.12	13.61 ± 0.85 × 7.47 ± 0.55			3.06 ± 0.10	Chen et al. (2019a)
Pelteobagrus fulvidraco	1.41 ± 0.10	12.22 ± 0.92 × 9.98 ± 0.83			3.02 ± 1.14	
Glyptosternum maculatum		19.39 ± 2.48 × 15.15 ± 1.91				Zhang et al. (2011)

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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 tiny mauve and reddish particles. (C) neutrophil: round, with an eccentric and oval shape nucleus, cytoplasm contained a large number of fine light purple particles and vacuoles of different sizes. (D) monocyte: oval with a horseshoe-shaped nucleus, cytoplasm contained many small vacuoles. (E) large lymphocyte: round or irregularly round, with many projections 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. The magnification is 1000X.

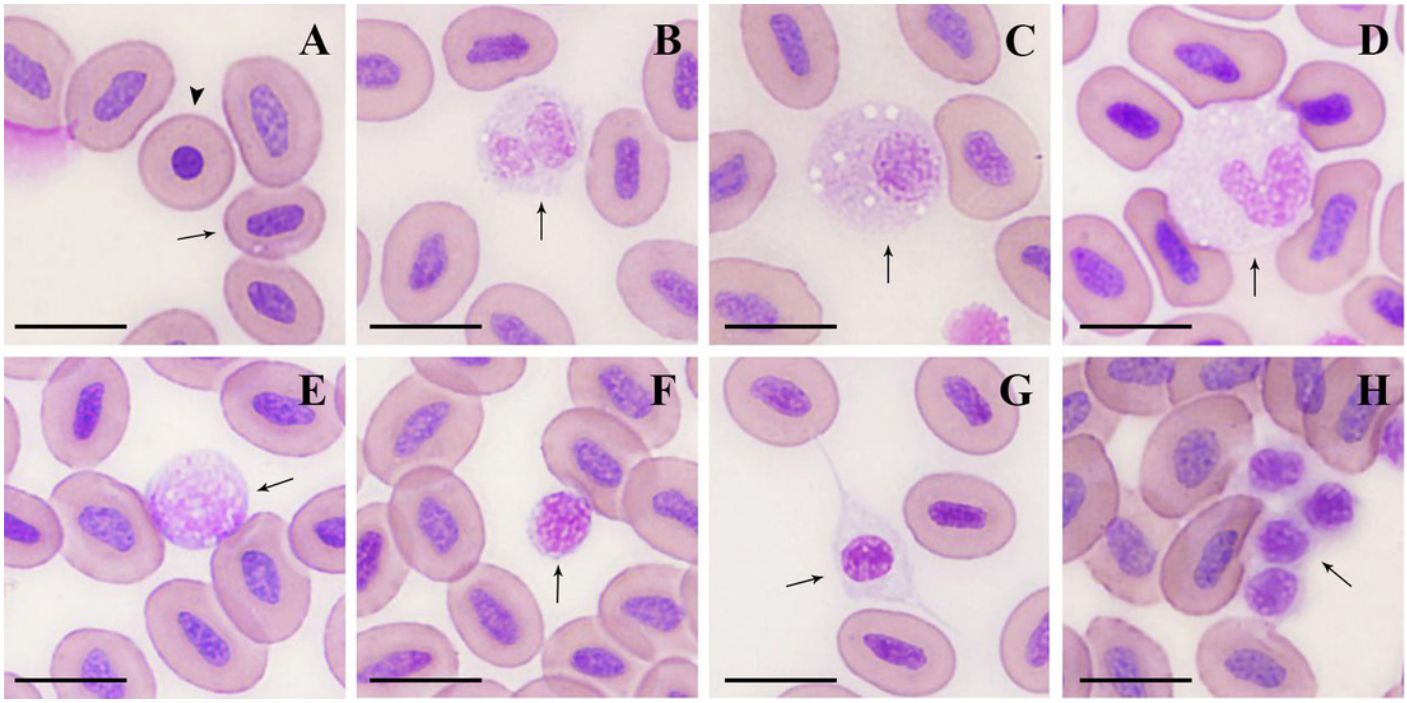
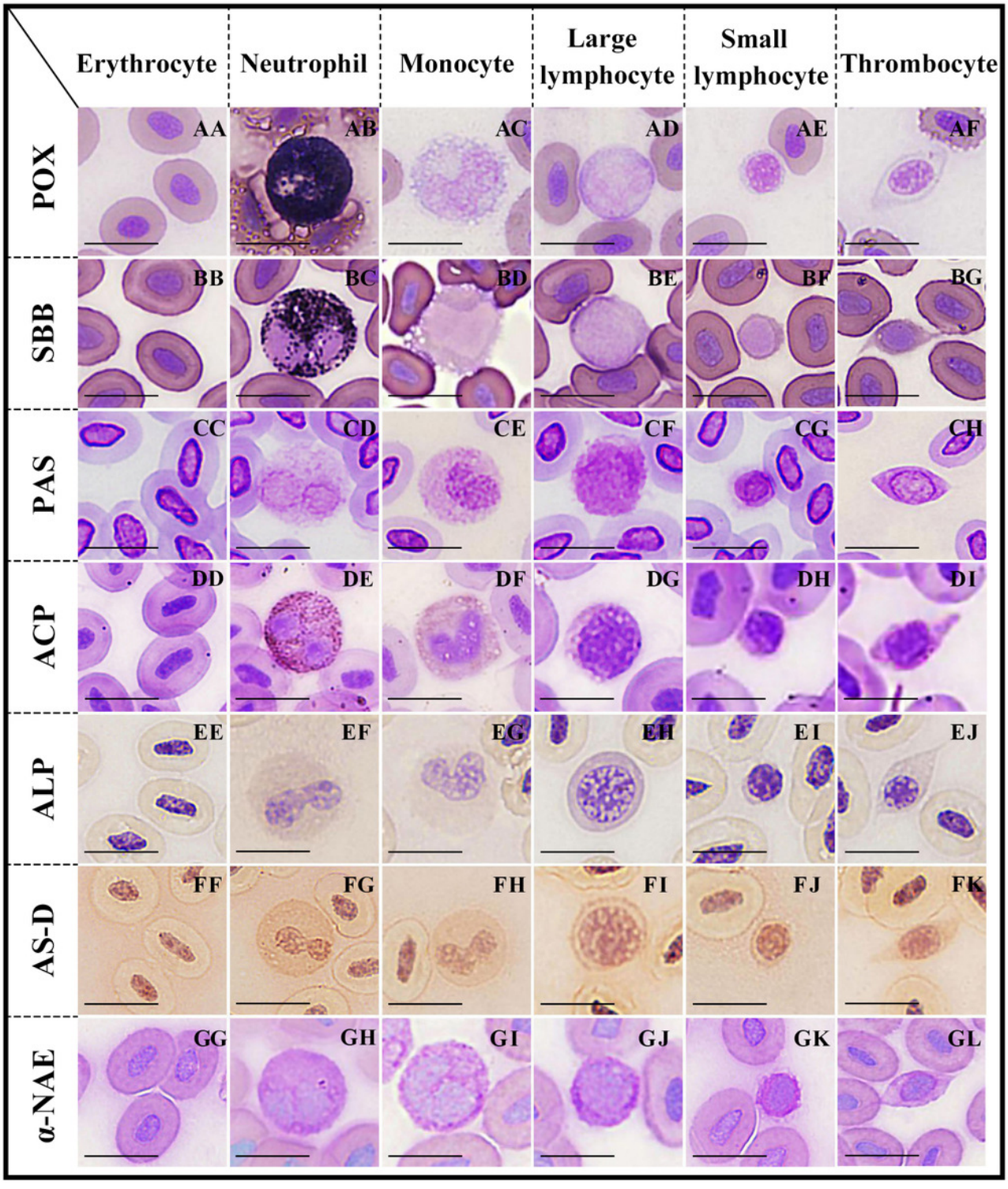


Figure 2

Cytochemical staining of peripheral blood cells in argus snakehead.

Erythrocytes were positive for PAS (CC), and negative for POX, SBB, ACP, ALP, AS-D and α-NAE staining (AA, BB, DD, EE, FF, GG); neutrophils exhibited strongly positive rection for POX, SBB and ACP (AB, BC, DE), positive for PAS and AS-D (CD, FG), and weakly positive for ALP and α-NAE staining (EF, GH); monocytes showed positive for PAS and α-NAE (CE, GI), and weakly positive for ACP and AS-D (DF, FH), while negative for POX, SBB and ALP staining (AC, BD, EG); large lymphocytes exhibited positive for PAS (CF), and weakly positive for ALP, AS-D and α-NAE (EH, FI, GJ), while negative for POX, SBB and ACP staining (AD, BE, DG); small lymphocytes were positive for PAS (CG), and weakly positive for AS-D and α-NAE (FJ, GK), while negative for POX, SBB, ACP and ALP staining (AE, BF, DH, EI); thrombocytes showed positive rection for PAS (CH), and weakly positive for ACP and AS-D (DI, FK), while negative for POX, SBB, ALP and α-NAE staining (AF, BG, EJ, GL). Scale bars=10 μm. The magnification is 1000×.


Erythrocyte
Neutrophil
Monocyte
Large lymphocyte
Small lymphocyte
Thrombocyte
POX
SBB
PAS
ACP
ALP
AS-D
α-NAE
AA
AB
AC
AD
AE
AF
BB
BC
BD
BE
BF
BG
CC
CD
CE
CF
CG
CH
DD
DE
DF
DG
DH
DI
EE
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GJ
GK
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