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

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Abstract

23 **Background**. Argus snakehead (*Ophiocephalus argus* Cantor) is a freshwater cultured bony fish

24 with high nutritional and economic value. Blood cells play a critical role in oxygen transport,

25 defensive and immunization, which are closely related to the health of fish. The purpose of this

26 study was to investigate the morphometry, microstructure and cytochemical characteristics of

27 peripheral blood cells in *O. argus*. The results could provide the necessary basic references for

28 the health monitoring in the large-scale cultivation of *O. argus*.

29 **Methods**. The number of blood cells in *O. argus* was counted on a blood count board, and the

30 size of which was measured by a micrometer under light microscope. The morphology and

31 classification of blood cells were studied by wright's staining, and the cytochemical

32 characteristics was investigated by seven chemical staining including peroxidase(POX), Sudan

33 black B(SBB), periodic acid-Schiff(PAS), acid phosphatase(ACP), alkaline phosphatase(ALP),

34 chloroacetic acid AS-D naphthol esterase(AS-D) and α-naphthol acetate esterase(α-NAE)

35 staining.

36 **Results**. The peripheral blood cells in *O. argus* could be divided into erythrocytes, leukocytes

37 and thrombocytes, the number of which was 2.9597 million /mm^3, 88,400 /mm^3 and 43,600

38   /mm³ respectively in females; 3.0105 million /mm³, 105,500 /mm³ and 34,000 /mm³ respectively

39   in males. Leukocytes consisted of neutrophils, monocytes, large lymphocytes and small

40   lymphocytes, and eosinophils and basophils were not found. Monocytes were the most numerous

41   leukocytes, followed by neutrophils and small lymphocytes, large lymphocytes were the fewest.

42   Cytochemical staining showed that erythrocytes were only positive for PAS staining. Neutrophils

43   were strongly positive for POX, SBB and ACP, and positive for all the other cytochemical

44   staining. Monocytes showed positive for PAS and α-NAE, and weakly positive for ACP and AS-

45   D staining. Large lymphocytes exhibited positive for PAS, and weakly positive for ALP, AS-D

46   and α-NAE staining. Small lymphocytes were positive for PAS, and weakly positive for AS-D

47   and α-NAE staining. Thrombocytes exhibited positive for PAS, and weakly positive for ACP and

48   AS-D, while negative for the remaining cytochemical staining. The morphology of peripheral

49   blood cells in *O. argus* was generally similar to that of other fish species, while the cytochemical

50   staining patterns have obvious species specificity.

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

52   blood cells

53

54   **Introduction**

55   As in other vertebrates, blood is an extremely important tissue in fish, consisting of plasma

56   and blood cells (Zhang et al., 2011). Fish blood cells can be divided into erythrocytes, leukocytes

57   and thrombocytes, which play a vital role in gas transportation, immune defense and coagulation

58   respectively in the body (Chen et al., 2019b; Palmer et al., 2015). Blood cells are very sensitive

59   to changes in internal physiological conditions and stimulus by external environmental factors

60   (Palmer et al., 2015). Variations in blood cell counts, morphology and various intracellular

61   functional components can be used as direct markers to judge the health status of fish (Fang et al.,

62   2014; Ishikawa, Ranzani-Paiva & Lombardi, 2008).

63   Traditional wright's staining can be used to study the microstructure and classification of

64   fish blood cells (Zhang et al., 2019). Cytochemical staining is widely used to detect the content

65   of biological macromolecules and the activity of enzymes, so as to understand the function and

66   physiological state of blood cells and further understand the cell lineages (Massar et al., 2012;

67   Shigdar, Harford & Ward, 2009). At present, there have been many reports on the classification,

68   microstructure and cytochemical characteristics of peripheral blood cells in fish, especially in

69   commercially cultured fish. Tripathi, Latimer & Burnley (2004) determined the hematological

70   reference intervals for koi (*Cyprinus carpio*), including blood cell morphology, cytochemistry,

71   and ultrastructure. Tavares-dias & Moraes (2006) described the morphology, cytochemistry and

72   ultrastructure of thrombocytes and leukocytes in neotropical fish (*Brycon orbignyanus*). Tavares-

73   dias (2006) studied the morphology and cytochemistry of erythrocytes, thrombocytes and

74   leukocytes in four freshwater teleosts: big head carp (*Aristichthys nobilis*), oscar (*Astrootus*

75   *ocellatus*), traíra (*Hoplias malabarus*) and lambari (*Astyanax bimaculatus*). Fang et al. (2014)

76   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

97   number, microstructure and cytochemical characteristics of peripheral blood cells in *O. argus*

98   were investigated by the cell counts, wright's staining and cytochemical methods under the light

99   microscope, which could enrich the basic data of fish hematology, provide reference for health

100   monitoring in artificial breeding of *O. argus*, and provide basic information for the further

101   studies on physiology and immunology of this species.

102

Materials & Methods

Animals and blood smears preparation

105   Thirty healthy adult *O. argus* (15 males and 15 females) were selected, with an average

106   body length of 30.56-42.78 cm and weight of 960.20-1850.32 g. All the fish were obtained from

107   wuhu local aquaculture farm from May to September 2019. Blood samples were collected by

108   caudal vein puncture, and about 2 ml of blood was taken from each fish using a sterile 5 ml

109   syringe and 22 G needle. The K_2-EDTA was used as an anticoagulant to avoid blood coagulation

110   and blood smears were prepared immediately after blood collection. This work was approved by

111   the ethics committee of Anhui Normal University (permit no. 20190312). All the handling and

112   sampling were carried out in accordance with standard vertebrate procedures and veterinary

113   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.

134 SBB staining: the dried blood smears were placed in Sudan Black B (Sangon, 4197-25-5)

135 staining solution for 60 min at 37℃, then rinsed in 70% ethanol solution and distilled water for

136 1-2 min.

137 PAS staining: blood smears were oxidized with 10 mg/ml periodate for 18-20 min, and

138 rinsed in distilled water for 2 min. Then they were placed in Schiff's solution for 60-90 min at

139 37℃. After rinsing in sulfuric acid solution (0.6 g sodium bisulfite (Sangon, 7681-57-4)

140 dissolved in 5 ml 1mol/l hydrochloric acid and 100 ml distilled water) for 3-4 times, the smears

141 were washed with distilled water for 2-3 min.

142 ACP staining: blood smears were stained with the reaction solution (0.1 g lead nitrate

143 (Xilong, 10099-74-8) and 0.128 g β-sodium glycerophosphate (Sangon, 819-83-0) dissolved in

144 74 ml distilled water and 12 ml pH4.7 acetic acid buffer) for 4-4.5 hours at 37°C. After washed

145 with distilled water for 5 min, they were immersed in 2% ammonium sulfide solution (Aladdin,

146 12135-76-1) for 30 min.

147 ALP staining: the smears were immersed in substrate incubation solution (10 mg naphthol

148 AS-BI phosphate (Sangon, 1919-91-1) dissolved in 10 ml 0.05 mol/l propanediol buffer, mixed

149 with 10 mg fast blue B salt (Yuanye, 14263-94-6), and then filtered) for 45-60 min at 37℃, and

150 rinsing in distilled water for 2min.

151 AS-D staining: blood smears were stained with the incubation solution (10 mg chloroacetic

152 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

173   male and female individuals, and the cell sizes(the length and width of various cells and nuclei)

174   were obtained by measuring 20 randomly selected cells for each cell type from male and female

175   individuals with an ocular micrometer scale. The hemoglobin (Hb), hematoceit (HCT) and

176   erythrocyte sedimentation rate (ESR) were determined according to the methods described

177   previously (Peng, et al., 2018). The mean corpuscular volume (MCV), mean corpuscular

178   hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated

179   from RBC, HCT and Hb according to the formulae below (Gao et al., 2007a):

180   MCV (fl) = HCT/RBC

181   MCH (pg) = Hb /RBC

182   MCHC (g/dl) = Hb/HCT

183   **Statistical analysis**

184   The experimental data were represented by Mean ± SD. The significant differences in

185   morphometric values among different cell types or between sexes were compared by one-way

186   ANOVA analysis in SPSS 21.0 software (SPSS Inc, Chicago, USA), A P-value less than 0.05

187   was significant difference, and P-value less than 0.01 was extremely significant difference.

188

189   **Results**

190 **Classification and counting of peripheral blood cells**

191   The blood smears stained by wright's staining were observed under light microscope with

192 oil-immersion at 1000× magnification. According to the morphology and size of cells and nuclei,

193 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

213   males (P < 0.01). A small number of immature erythrocytes (Fig. 1A) were also observed on the

214   blood smears stained by wright's staining, which were round and smaller than mature

215   erythrocytes, with round or elliptic, dark purplish-stained nuclei.

216   **Neutrophils**

217   Neutrophils (Fig. 1B, C) were spherical or round in shape, with purplish stained nuclei. The

218   nuclei had a variety of shapes, including bilobate, trilobed, kidney-shaped or non-bilobed, and

219   bilobed nuclei were most frequently observed. The cytoplasm was rich and stained light blue,

220   containing numerous fine mauve and reddish particles.

221   **Monocytes**

222   Monocytes (Fig. 1D) were the largest leukocytes in *O. argus* (Table 2). Most of them were

223   round and oval, and a few were irregular. The nuclei were oval, pear-shaped or horseshoe-shaped,

224   and generally stained purple. The most obvious morphological feature of monocytes was that the

225   cytoplasm contained a large number of vacuoles of different sizes with pseudopodia

226   protuberances at the cell edges.

227   **Large lymphocytes**

228   Lymphocytes could be divided into large lymphocytes and small lymphocytes. Large

229   lymphocytes (Fig. 1E) were generally round or irregularly round, with large, oval nuclei on one

230   side of the cells, and the purplish nucleus occupied almost the entire cytoplasm. Some large

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

232   the cells.

233   **Small lymphocytes**

234   Small lymphocytes (Fig. 1F) were oval in shape with an eccentric, purple, rounded, gapped

235   nucleus that occupied almost the entire cell, and contained a thin rim of pale blue cytoplasm.

236   Small lymphocytes were characterized by numerous microvilli protuberances from the

237   cytoplasmic margins.

238   **Thrombocytes**

239   Thrombocytes showed different forms in the smears, including round, oval, long ovoid and

240   spindle. Spindle-shaped thrombocytes (Fig. 1G) were usually isolated and their nuclei were

241   consistent with the shape of the cells, most of which are centered and purplish, and the cytoplasm

242   was approximately colorless. Round thrombocytes (Fig. 1H) usually appeared in clusters with

243   multiple cells, and they had round, dark purple-stained nuclei, and less cytoplasm was flocculent

244   around the nucleus.

245   **The cytochemical staining characteristics of peripheral blood cells**

246   **POX staining**

247   Neutrophils (Fig. 2AB) showed strongly positive with blue-black coarse and rod-shaped

248   granules in the cytoplasm. The cytoplasm of erythrocytes (Fig. 2AA), monocytes (Fig. 2AC),

249   large lymphocytes (Fig. 2AD), small lymphocytes (Fig. 2AE) and thrombocytes (Fig. 2AF) was

250   light blue without granules, and all of these cells were negative.

251   **SBB staining**

252   Neutrophils (Fig. 2BC) were covered with a large number of diffusely distributed dark

253   black granules in the cytoplasm, and which exhibited strongly positive. The cytoplasm of

254   erythrocytes(Fig. 2BB), monocytes (Fig. 2BD), large lymphocytes (Fig. 2BE), small

255   lymphocytes (Fig. 2BF) and thrombocytes (Fig. 2BG) was pale purple without granules, all of

256   these cells were negative.

257   **PAS staining**

258   The cytoplasm of erythrocytes, neutrophils, monocytes, large lymphocytes, small

259   lymphocytes and thrombocytes (Fig. 2CC-CH) was purple or dark purple with diffusely granular

260   matter, and all of these cells were positive.

261   **ACP staining**

262   Neutrophils (Fig. 2DE) contained a large number of brown-black granules or tablets in the

263   cytoplasm, and showed strongly positive. Monocytes (Fig. 2DF) and thrombocytes (Fig. 2DI)

264   were weakly positive, with small amounts of brown granules in the cytoplasm. The cytoplasm of

265 erythrocytes (Fig. 2DD), large lymphocytes (Fig. 2DG) and small lymphocytes (Fig. 2DH) was

266 pale purple without granules, and all of these cells were negative.

ALP staining

268 Both neutrophils (Fig. 2EF) and large lymphocytes (Fig. 2EH) exhibited weakly positive,

269 with many fine uniformly distributed purple granules in the cytoplasm. The cytoplasm of

270 erythrocytes (Fig. 2EE), monocytes (Fig. 2EG), small lymphocytes (Fig. 2EI), and thrombocytes

271 (Fig. 2EJ) was pale yellow without stained granules, and all of these cells were negative.

AS-D staining

273 Neutrophils (Fig. 2FG) were with many uniformly distributed red granules, and which

274 showed positive. The cytoplasm of monocytes (Fig. 2FH), large lymphocytes (Fig. 2FI), small

275 lymphocytes (Fig. 2FJ) and thrombocytes (Fig. 2FK) was pale red with fine granules, and all of

276 these cells were weakly positive. Erythrocytes (Fig. 2FF) were negative with pale pink

277 cytoplasm.

α-NAE staining

279 Monocytes' (Fig. 2GI) cytoplasm was filled with gray-black diffused or granular deposits,

280 and which were positive. The cytoplasm of neutrophils (Fig. 2GH), large lymphocytes (Fig. 2GJ)

281 and small lymphocytes (Fig. 2GK) was purple with dark brown or purple granules, and all of

282 these cells were weakly positive. Erythrocytes (Fig. 2GG) and thrombocytes (Fig. 2GL) were

283 negative, their cytoplasm was purplish without granules.

284   **The cytochemical staining patterns of peripheral blood cells**

285   The cytochemical staining patterns of peripheral blood cells in *O.argus* were summarized in

286   Table 3. Erythrocytes were positive for PAS, and negative for POX, SBB, ACP, ALP, AS-D and

287   α-NAE staining. Neutrophils exhibited strongly positive reaction for POX, SBB and ACP,

288   positive for PAS and AS-D, and weakly positive for ALP and α-NAE staining. Monocytes

289   showed positive for PAS and α-NAE, and weakly positive for ACP and AS-D, while negative for

290   POX, SBB and ALP staining. Large lymphocytes exhibited positive for PAS, and weakly

291   positive for ALP, AS-D and α-NAE, while negative for POX, SBB and ACP staining. Small

292   lymphocytes were positive for PAS, and weakly positive for AS-D and α-NAE, while negative

293   for POX, SBB, ACP and ALP staining. Thrombocytes showed positive reaction for PAS, and

294   weakly positive for ACP and AS-D, while negative for all the other cytochemical staining.

295

296   **Discussion**

297   **The number and morphology of erythrocytes in *O. argus***

298   Transporting oxygen and carbon dioxide through intracellular hemoglobin is the primary

299   function of erythrocytes (Minasyan, 2014). Erythrocytes are the predominant blood cell type in

300   the vast majority of fish (Satheeshknmar et al., 2011; Satheeshknmar et al., 2012), while the

301   erythrocyte counts were significantly different among different fish (Table 4). The number of

302   erythrocytes in *O. argus* was comparable to that of spotted rose snapper (*Lutjanus guttatus*) and

303   cichlid fish (*Cichlasoma dimerus*), and lower than that of Persian sturgeon (*Acipenser persicus*)

304   and shovelnose catfish (*Sorubim cuspicaudus*), and higher than that of Siamese fighting fish

305   (*Betta splendens*), Asian sun catfish (*Horabagrus brachysoma*) and piebald naked carp. The

306   values of Hb and HCT in *O. argus* were comparable to those of spotted rose snapper, and higher

307   than those of cichlid fish, Persian sturgeon, Siamese fighting fish and piebald naked carp. The

308   value of MCV in *O. argus* was comparable to that of spotted rose snapper, cichlid fish, Asian sun

309   catfish and piebald naked carp, and lower than that of Persian sturgeon and Siamese fighting fish.

310   RBC and Hb values are related to the ability of the blood to carry dissolved oxygen (Fazio et al.,

311   2012; Tavares-dias & Moraes, 2004).The values of RBC, Hb, HCT and MCV in fish are related

312   to various factors, such as diet habits, fish body length, age, sex, water temperature, salinity and

313   living environment (Jawad, Al-Mukhtar & Ahmed, 2004; Kori-Siakpere, Ake & Idoge, 2005;

314   Martins et al., 2011; Milad et al., 2016). The above fish with higher values of RBC, Hb, HCT

315   and MCV were mostly carnivorous, with wide range of motion and high activity, which were

316   consistent with previous reports that the carnivorous fish with high activity needed to consume

317   more oxygen and had a correspondingly higher values of RBC, Hb and HCT (Engel & Davis,

318   1964; Molnár & Tamássy, 1970; Rambhaskar & Rao, 1987). This study showed that there was

319   no significant difference in the values of RBC, Hb and HCT between males and females, which

320   was consistent with most of fish, such as Persian sturgeon (Milad et al., 2016), Siamese fighting

321   fish (Motlagh et al., 2012), shovelnose catfish (Negrete et al., 2010) and cichlid fish (Vázquez &

322   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

343   in certain species (Zhou et al., 2006). Few of fish have both eosinophils and basophils, such as

344   Tilapia (*Oreochromis niloticus*) (Ueda et al., 2001), most of the fish have only eosinophils,

345   without basophils (Gao et al., 2007b; Milad et al., 2016; Motlagh et al., 2012; Rio-Zaragoza et al.,

346   2011;Vázquez & Guerrero, 2007; Zheng et al., 2016), few of fish have only basophils,without

347   eosinophils (Shigdar, Harford & Ward, 2009; Zhang et al., 2019), and some fishes have neither

348   eosinophils nor basophils (Chen et al., 2019a; Fang et al., 2014; Tavares-dias & Moraes, 2006;

349   Silva et al., 2011; Zhang et al., 2011). In this study, eosinophils and basophils were also not

350   found in the peripheral blood of *O. argus*.

351   The percentages of leukocytes were different among different fish species. Lymphocytes

352   were the most abundant leukocytes in most of the fish, such as sovel-nosed catfish (Bianchi et al.,

353   2014), turbot (*Psetta maxima*) (Burrows, Fletcher & Manning, 2001), Siamese fighting fish

354   (Motlagh et al., 2012), shovelnose catfish (Negrete et al., 2010), spotted rose snapper (Rio-

355   Zaragoza et al., 2011), piebald naked carp (Tang et al., 2015), cichlid fish (Vázquez & Guerrero,

356   2007) and sisorid catfish (Zhang et al., 2011). Neutrophils were the most common in some fish,

357   such as Persian sturgeon (Milad et al., 2016), *S. prenanti* (Fang et al., 2014). In this study,

358   monocytes were the most abundant leukocytes in *O. argus*, which was consistent with the

359   reported Chinese sturgeon (Gao et al., 2007b).

360   Fish monocytes are mostly round and oval, and a few are irregular in shape, which have

361   phagocytic function and extremely sensitive to the environmental variations(Zheng et al., 2016).

362   Mononcytes were the largest leukocytes in *O. argus*, which were significantly larger than those

363   of *S. prenanti* (Fang et al., 2014), spotted rose snapper (Rio-Zaragoza et al., 2011), piebald naked

364   carp (Tang et al., 2015) and neotropical fish (Tavares-Dias & Moraes, 2006). The most obvious

365   morphological characteristics of monocytes in *O. argus* was that their cytoplasm contained

366   vacuoles of different sizes and pseudopodia protuberances on the cell edge, which was consistent

367   with the monocytes reported in other fish (Tavares-Dias, 2006; Tripathi, Latimer & Burnley,

368   2004; Zheng et al., 2016) and may be related to their phagocytic function (Zheng et al., 2016).

369   Lymphocytes belong to agranulocytes and play an important role in both innate and

370   acquired immunity (Shigdar, Harford & Ward, 2009). Compared with the lymphocytes in some

371   other fish (Tavares-dias, 2006; Zheng et al., 2016), the lymphocytes in *O. argus* were varible in

372   size, which can be divided into large lymphocytes and small lymphocytes. Most of large

373   lymphocytes had small fingerlike protuberances on the cell surface. Many microvilli

374   protuberances can also be found in the cytoplasmic edges of small lymphocytes. These features

375   have also been reported in other fishes (Burrows, Fletcher & Manning, 2001; Rio-Zaragoza et al.,

376   2011; Vázquez & Guerrero, 2007;Silva et al., 2011), and the protuberances on the surface of

377   lymphocytes may be related to immune function of antigen binding receptor molecules

378   (Scapigliati, 2013).

379   Neutrophils in *O. argus* were usually round or spherical with a bilobate nucleus, their

380   cytoplasm contained numerous fine mauve and reddish granules, these morphological

381   characteristics were somewhat different from the reports in some other fish. For instance, the

382   neutrophils of *S. prenanti* (Fang et al., 2014) were round or irregular-shaped, their nuclei were

383 usually kidney-shaped or trilobed, and their cytoplasm contained a large number of light blue or

384 pink granules, neutrophils of shovelnose catfish (Negrete et al., 2010) had an eccentric and round

385 nucleus with light blue granules in the cytoplasm, neutrophils of neotropical fish (Tavares-Dias

386 & Moraes., 2006) were round with an oval-shaped eccentric nucleus, their cytoplasm contained

387 many purple granules of different sizes, the heterophils of sisorid catfish (Zhang et al., 2011)

388 were round and regular in shape with kidney-shaped or round nuclei, the cytoplasm contained

389 pale blue granules.

390 **The morphology and number of thrombocytes in *O. argus***

391 Thrombocytes of *O. argus* were observed in different forms, including round, oval, oblong

392 and spindle-shaped, which was consistent with the reports in other fish (Fang et al., 2014; Gao et

393 al., 2007b; Micha et al., 2019; Rio-zaragoza et al., 2011; Zhang et al., 2011; Zheng et al.,

394 2016).The spindle-shaped thrombocytes often existed alone with some vacuoles in the cytoplasm,

395 which were related to cell phagocytosis (Nagasawa, Somamoto & Nakao, 2015; Stosik et al.,

396 2002). The round thrombocytes usually appeared in clusters with 2-8 cells, which may be related

397 to their hemostatic function (Chen et al., 2019b; Peng et al., 2018).

398 The number of thrombocytes in *O. argus* was lower than that of Siamese fighting fish

399 (Motlagh et al., 2012), shovelnose catfish (Negrete et al., 2010) and spotted rose snapper (Rio-

400 Zaragoza et al., 2011), higher than that of Chinese sturgeon (Gao et al., 2007b) and piebald

401 naked carp (Tang et al., 2015), and similar to grass carp, blunt snout bream (*Megalobrama*

402 *amblycephala*), yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al., 2019) and cichlid fish

403   (Vázquez & Guerrero, 2007). The differences in the number of thrombocytes in different species

404   of fish may be related to biotic and abiotic factors and their adaptability to the environment

405   (Pavlidis et al., 2007; Prasad & Charles, 2010).

406   **The cytochemical staining patterns of blood cells in *O. argus***

407   In this study, the cytochemical characteristics of peripheral blood cells of *O. argus* were

408   studied by seven staining methods of POX, SBB, PAS, ACP, ALP, AS-D and α-NAE for the

409   first time. POX is an enzyme specific to neutrophils in mammals and participates in the defense

410   mechanism of bacterial infection (Tavares-Dias, 2006). SBB and PAS staining were used to

411   detect intracellular glycogen and lipids, which could provide energy for phagocytosis (Ueda et

412   al., 2001). ACP and ALP are lysosomal enzymes involved in phagocytosis and degradation

413   (Shigdar, Harford & Ward, 2009; Silva et al, 2011). AS-D is a specific esterase,which is

414   associated with cellular defense and phagocytic material processing (Casaletti-Rosa & Lunardi,

415   1997; Tavares-Dias et al., 2007). As a non-specific esterase, α-NAE plays an important role in

416   phagocytosis and antigen presentation (Fang et al., 2014).

417   Erythrocytes of *O. argus* in this study were only positive for PAS, which was different from

418   that described in other fish, such as *S. prenanti* (Fang et al., 2014), Tilapia (Ueda et al., 2001),

419   crucian carp and grass carp (Zhang et al., 2019) and piebald naked carp (Zheng et al., 2016),

420   whose erythrocytes showed negative for PAS. The erythrocytes in *O. argus* were negative for

421   POX, SBB, ACP, ALP, AS-D and α-NAE staining, which was consistent with above fish. PAS

422 positivity and SBB negativity indicated that glycogen is the main energy source of erythrocytes

423 in *O. argus*.

424 Neutrophils of *O. argus* exhibited strongly positive reaction for POX, SBB and ACP,

425 positive for PAS and AS-D, and weakly positive for ALP and α-NAE staining, which was

426 generally similar to the staining results of Murray cod (*Maccullochella peelii peelii*) (Shigdar,

427 Harford & Ward, 2009), but different from reports in some other fish. For instance, neutrophils

428 of fat snook (Centropomus parallelus) (Silva et al., 2011) were positive for PAS, SBB, ACP and

429 NAE, while negative for ALP staining, American paddlefish (*Polyodon spathula*) (Petrie-Hanson

430 & Peterman, 2005) and piebald naked carp (*Gymnocypris eckloni*) (Zheng et al., 2016) were

431 positive for ACP, while negative for SBB staining, and Asian sun catfish (*Horabagrus*

432 *brachysoma*) (Prasad & Charles., 2010) were negative for ALP, NAE and ASD staining.

433 Neutrophils in human and mammals are mainly involved in the phogocytosis and degradation of

434 invading microorganisms (Azevedo and Lunardi, 2003; Rieger and Barreda, 2011; Wang et al.,

435 2019). The strongly positive reactions for POX, SBB and ACP and the positive reactions for

436 PAS, ACP, AS-D and α-NAE indicated that the neutrophils of *O. argus* were similar to those of

437 mammals, which had strong phagocytic and bactericidal abilities.

438 Monocytes of *O. argus* showed positive for PAS and α-NAE, and weakly positive for ACP

439 and AS-D, while negative for SBB, POX and ALP staining, which was basically consistent with

440 those of other fish, such as *S. prenanti* (Fang et al., 2014), American paddlefish (Petrie-hanson &

441 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, ACP 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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Haematological parameters of female and male argus snakehead.

Each data point represents the mean of three replicates ± SD and the range. *indicates 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 significant differences between leukoyte types (P < 0.05). Coefficient of Variation (CV) (%) = (Standard Deviation / Mean)×100%. TBC: total blood cell counts.

1 **Table 1: Haematological parameters of female and male argus snakehead.**

parameters	Female (N = 15)		Male (N = 15)	
	Mean ± SD	Range	Mean ± SD	Range
RBC ($\times 10^6$/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 ($\times 10^4$/mm^3)	8.84 ± 0.47	8.29−9.44	10.55 ± 0.82	9.55−11.56
Neutrophil($\times 10^4$/mm^3)	1.82 ± 0.10[c]	1.71−1.94	2.03 ± 0.16[b]	1.83−2.22
Monnocyte ($\times 10^4$/mm^3)	5.11 ± 0.27[d]	4.79−5.46	6.09 ± 0.47[c]	5.51−6.67
Large lymphocyte ($\times 10^4$/mm^3)	0.60 ± 0.03[a]*	0.56−0.64	0.77 ± 0.06[a]*	0.70−0.84
Small lymphocyte ($\times 10^4$/mm^3)	1.31 ± 0.07[b]*	1.23−1.40	1.67 ± 0.13[b]*	1.51−1.83
TC ($\times 10^4$/mm^3)	4.36 ± 0.23*	4.09−4.65	3.40 ± 0.27*	3.08−3.73
TBC ($\times 10^6$/mm^3)	3.09 ± 0.21	2.90−3.30	3.15 ± 0.24	2.85−3.45
CV of TBC (%)	6.95		7.70	

2 Notes: Each data point represents the mean of three replicates ± SD and the range. *indicates

3 significant differences in blood cell counts and haematological parameters between males and

4 females (P < 0.05); different letters (a, b, c, d) in the same column indicate significant differences

5 between leukoyte types (P < 0.05). Coefficient of Variation (CV) (%) = (Standard Deviation /

6 Mean)×100%. TBC: total blood cell counts.

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).

1 **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

2 Notes: ** indicates that extremely significant differences between males and females (P < 0.01);

3 different letters (a, b, c, d) in the same column indicate significant differences among different

4 cells (P < 0.05).

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.

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)

2   Notes: RBC: red blood cell counts; Hb: hemoglobin; HCT: hematoceit; MCV: mean corpuscular volume.

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.
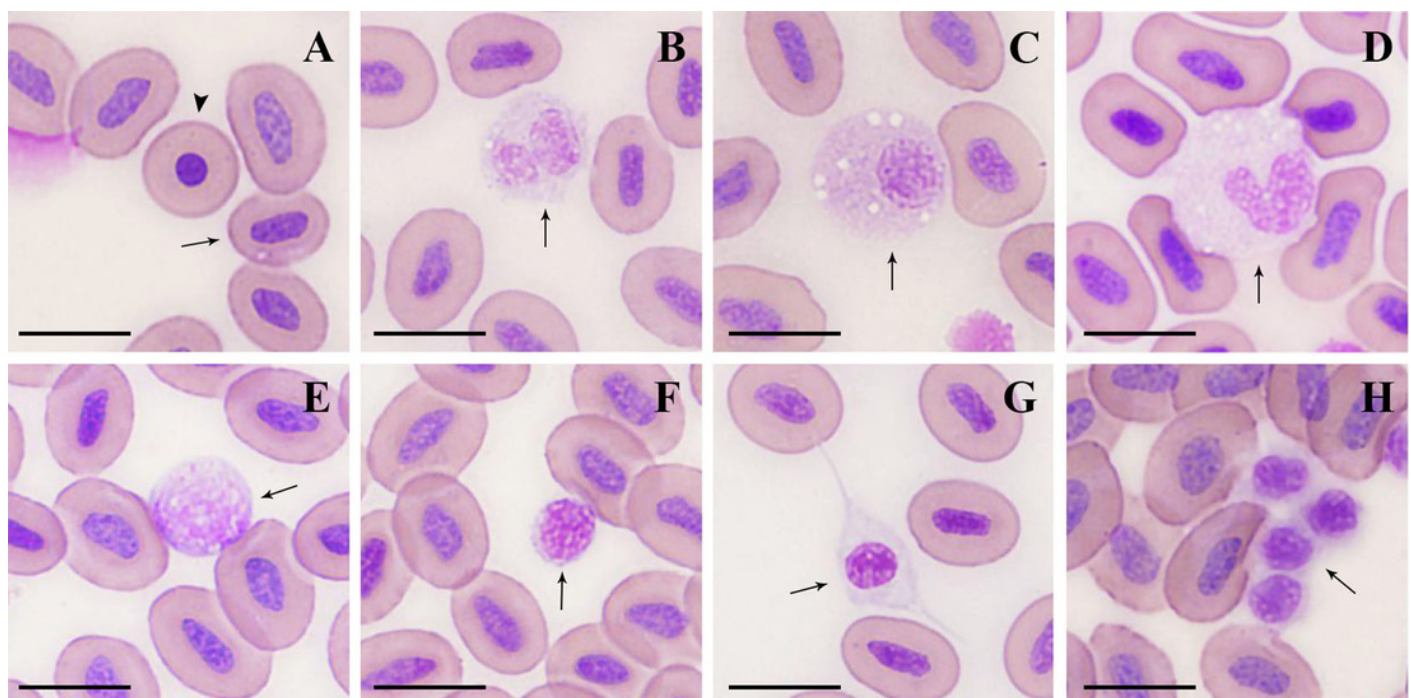
Figure 2

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	AA	AB	AC	AD	AE	AF
SBB	BB	BC	BD	BE	BF	BG
PAS	CC	CD	CE	CF	CG	CH
ACP	DD	DE	DF	DG	DH	DI
ALP	EE	EF	EG	EH	EI	EJ
AS-D	FF	FG	FH	FI	FJ	FK
α-NAE	GG	GH	GI	GJ	GK	GL