

Morphological studies of peripheral blood cells of the Chinese sturgeon, *Acipenser sinensis*

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Abstract The peripheral blood cells of one-year-old Chinese sturgeon (*Acipenser sinensis*) have been studied by light microscopy and transmission electron microscopy. The erythrocyte count was 84.86×10^4 cell mm^{-3} in the peripheral blood of the fish and that of leukocytes was 2.24×10^4 cell mm^{-3} . The erythrocytes and four main types of leucocyte—thrombocytes, lymphocytes, granulocytes (including neutrophils and eosinophils), and monocytes, were identified in

the peripheral blood. In addition to normal erythrocytes, reticulocytes and division of erythrocytes were observed. Thrombocytes were the most numerous among the leukocytes, and the number of neutrophils with lobated nuclei was larger than for other fish. The structures of the erythrocytes, lymphocytes, monocytes, granulocytes, and thrombocytes of the fish were studied. The erythrocytes were almost completely devoid of organelles, except for some mitochondria and granules. A large number of vacuoles and a few organelles were observed in cytoplasm of the monocytes. There were many microvilli on the membrane and pseudopodia-like cytoplasm bulge in the lymphocytes. The neutrophils were round or oval in shape with bilobed, trilobed, or multilobed nuclei whereas the eosinophils had big special granules, dark stained. There were many vesicles in some thrombocytes, which were related to its phagocytosis; some thrombocytes had almost no cytoplasm or organelles.

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Introduction

In aquaculture, the control of fish disease has generated growing interest in the hematology of

both marine and freshwater teleosts, e.g. goldfish *Carassius auratus* (Weinreb 1963), plaice *Pleuronectes platessa* (Ellis 1976), the Australian bream *Acanthopagrus australis* (Roubal 1986), and the catfish *Ictalurus punctatus* (Fijan 2002a). Among hematological indices, blood cells are one of the best indicators of body health. They are vitally important in the immune system. Despite of recent interest in the immune systems of fish species regarded as important in aquaculture, very little attention has been devoted to the structural features of fish peripheral blood cells. Among Acipenseridae fish, the characteristics and classification of the blood cells have been studied for the huso huso *Huso dauricus*, the starlet *Acipenser stellatus*, and the Siberian sturgeon *Acipenser baerii* (Palikova et al. 1999), the pallid sturgeon *Scaphirhynchus albus* (Jenkins 2003), the Amur sturgeon *Acipenser schrencki* (Liu et al. 2006), and the kalugaa *Huso dauricus* (Zhou et al. 2006). Little work has been done on the microstructure and ultrastructure of Chinese sturgeon, however.

The Chinese sturgeon, a type of cartilage ganoid, has a history of over 1 billion years and is called the living fossil of aquatic biology because it retains some evolutionary characteristics (Liao and Zhu 2004). The anadromous Chinese sturgeon is included in the IUCN Red List of Threatened Species (http://www.wcmc.org.uk/species/animals/animals_redlist.html), and is also under Category I State protection in China (the highest level of protection) (Birstein et al. 1997; Chang and Cao 1999). Historically, the Chinese sturgeon had a wide range of distribution that included water bodies in Korea and Japan, the Yellow River, the Pearl River, and the Yangtze River in China, and the continental shelf of the Yellow Sea, South China Sea, and East China Sea (Li 1987). In recent decades, however, as a result of damming and overfishing, populations of the Chinese sturgeon have declined substantially in abundance (Yu et al. 1986; Zen 1990; Zhuang 1994; Zhuang et al. 1997; Wei et al. 1997; Chang 1999) and it is now largely restricted to the Yangtze River (Zhuang et al. 2002). All commercial fishing has been banned since 1983 and a series of protective measures have been taken, including setting up protection stations

along the river and an institute at Yichang for artificial propagation and research. As a result the number of Chinese sturgeon has started to increase.

Research on Chinese sturgeon had focused on artificial culture, spawning, population dynamics, ontogenetic behavior, and migration. In hematological studies, only blood biochemistry has been studied (Shi et al. 2006). The purpose of this study was to investigate the morphological characteristics of peripheral blood cells of one-year-old Chinese sturgeon by light microscopy and electron microscopy, to provide information for domestication and disease cure and to accumulate basic data for hematology and comparative cytology of the fish.

Materials and methods

Materials

Ten male and ten female specimens of the Chinese sturgeon (all one year old) with average body length 50.32 cm and average body weight 439.80 g were studied. The fish were produced by artificial reproduction at the Yangtze River Fisheries Research Institute of the Chinese Academy of Fishery Sciences, Jingzhou City, Hubei Province, China. The fish were reared in an outdoor aquaculture facility in the College of Fisheries Sciences, Huazhong Agricultural University, under ambient light and temperature. The specimens were anaesthetized with MS222 (Sandoz; 100 mg L⁻¹), and the peripheral blood was collected under sterile conditions by puncture of the caudal vein with a heparin-coated 23-gauge needle attached to a 2.5 mL syringe.

Methods

Blood cell count

The blood was diluted 200 times with 0.85% NaCl and put in a Neubauer hemocytometer. Erythrocytes were then counted under microscope. To count leucocytes, the blood was diluted 20 times with acetic acid containing 1% crystal violet.

Light microscopy

Blood smears, four for each fish, were prepared as described by Humason (1979). Air-dried smears were fixed for 1 min in absolute methanol, stained with Wright-Giemsa (WG) fluid (Ellsaesser et al. 1984) for 8 min, and washed twice in distilled water for 1 min, then air-dried. The stained smears were studied and photos were taken under a Motic light microscope with a video camera linked to computer image-analysis software (Motic Images Advanced 3.2, USA). Counting of different leukocytes was performed simultaneously and a total of 2,902 cells (RBC + WBC) were counted. One hundred cells of each type were measured with computer image-analysis software, including the lengths (a) and widths (b) of the cells and nuclei. The volume (V) of both the cell and its nucleus were computed by using the formula for ellipsoids or oblate spheroids (Benfey and Sutterlin 1984): $V = 4/3 \times \pi \times (a/2) \times (b/2)^2$. Data are presented as mean \pm standard deviation.

Electron microscopy

Blood was centrifuged at 5,000 rpm and the serum was removed. Glutaraldehyde (2.5%) was added to the centrifuge tube on the surface of the deposited blood cells. A small (approx. 1 mm³) clot was detached from the surface of the deposit and washed twice with phosphate buffer. The clot was then post-fixed in 1% OsO₄, dehydrated through a graded series to absolute ethanol and embedded in Epon812. Ultrathin sections (50–80 nm) were cut with a Nova ultramicrotome (after semi-thin sectioning to find the best location) and stained with uranyl acetate and lead

citrate before examination under a transmission electron microscope (Hitachi H-7000, Japan).

Results and discussion

Blood-cell counts and sizes of all types of blood cell

The numbers of erythrocytes and lymphocytes were $84.86 \pm 10.43 \times 10^4$ cell mm⁻³ and $2.24 \pm 0.96 \times 10^4$ cell mm⁻³, respectively. Results from differential leukocyte cell counts (DLC), and sizes of different blood cells are listed in Table 1.

According to existing records of piscine blood cells, the cells of Chinese sturgeon were bigger than their counterparts in other Acipenseridae fish. This fits its chromosome number of 264, because there have been reports of a direct relationship between cell size and number of chromosomes (Palikova et al. 1999).

Structural and ultrastructural characteristics of the cells

Erythrocytes were the most abundant cells in smears of the peripheral blood of the Chinese sturgeon; smaller numbers of leucocytes (lymphocytes, heterophilic and acidophilic granulocytes, monocytes, and thrombocytes) were distributed diffusely among the dense erythrocytes, either singly or as clumps.

Erythrocytes

Two kinds of erythrocyte were found in Chinese sturgeon blood. Under the light microscope the

Table 1 Sizes of different blood cells (length \times width) and differential leukocyte cell counts (DLC) for *A. sinensis*

Cells	Percentage of the different leukocytes	Cell size (length \times width; μm^2)	Nucleus size (length \times width; μm^2)
Erythrocytes		$(17.98 \pm 0.96) \times (12.65 \pm 0.87)$	$(7.66 \pm 0.57) \times (5.28 \pm 0.23)$
Thrombocytes	60.78	$(10.88 \pm 1.85) \times (4.52 \pm 1.78)$	$(9.87 \pm 1.19) \times (4.11 \pm 1.55)$
Monocytes	12.47	$(18.10 \pm 1.96) \times (17.78 \pm 1.53)$	$(11.19 \pm 2.34) \times (9.40 \pm 1.96)$
Lymphocytes	12.10	$(12.80 \pm 1.29) \times (11.20 \pm 1.63)$	$(10.12 \pm 1.58) \times (9.53 \pm 1.87)$
Neutrophils	10.43	$(16.59 \pm 2.25) \times (14.88 \pm 1.76)$	$(10.65 \pm 1.76) \times (7.46 \pm 2.14)$
Eosinophils	4.22	$(15.93 \pm 2.53) \times (13.55 \pm 1.64)$	$(9.62 \pm 2.01) \times (6.37 \pm 1.55)$

Note: Data in the table are means \pm SD except for the percentages of the different leukocytes

predominant shape of the erythrocytes was elliptical with an oval, central or nearly central, dark purple-stained nucleus and blue–gray-stained cytoplasm (Fig. 1a). The surface of the cells was smooth. The other kind of erythrocyte was the immature erythrocyte, which could be larger or smaller than the mature variety (Fig. 1a). They were oval to round in shape and fewer in number, and are called reticulocytes. They had a magenta-stained chromatin of reticular appearance and a blue-stained cytoplasm, darker than that of the mature erythrocyte. The special form (Fig. 1b) and division of the erythrocyte (Fig. 1c) could both be observed occasionally. Under the electron microscope the erythrocyte was a long ellipse, a long olive-shaped or irregular shaped

cell with an ellipse or long ellipse nucleus. The nucleus contained heavily clumped chromatin. The cytoplasm was uniform and electronlucent with few mitochondria. Some dense granules and small round vesicles were also observed. The nuclear membrane of the erythrocytes was clear and there was a nuclear pore between the cytoplasm and the nucleus (Fig. 2a).

In fish blood the presence of reticulocytes indicates the erythrocytes of the fish are primitive and can proliferate in peripheral blood (Fijan 2002a). Chinese sturgeon blood contained a larger number of mature erythrocytes than immature erythrocytes, in direct proportion to their stage of maturity; these proportions can therefore be used as erythropoietic activity change indicators (Fijan

Fig. 1 The microstructure of peripheral blood cells of *A. sinensis* ($\times 500$) (a) immature erythrocyte (*Ime*) and mature erythrocyte (*Me*); (b) special erythrocyte (*Er*) and single thrombocyte (*Th*); (c) dividing erythrocyte (*De*); (d) lymphocyte (*Ly*); (e) plasma cell (*Pc*); (f) neutrophil showing horseshoe-shaped nucleus (*Ne1*); (g) neutrophil showing tetra-segmented nucleus (*Ne2*); (h) eosinophil showing round nucleus (*Eo1*); (i) eosinophil showing tetra-segmented nucleus (*Eo2*); (j) monocyte showing vesicles (*Mo*); (k) thrombocytes in clusters (*Th1*); (l) round thrombocyte (*Th2*); magnifying power: $500\times$

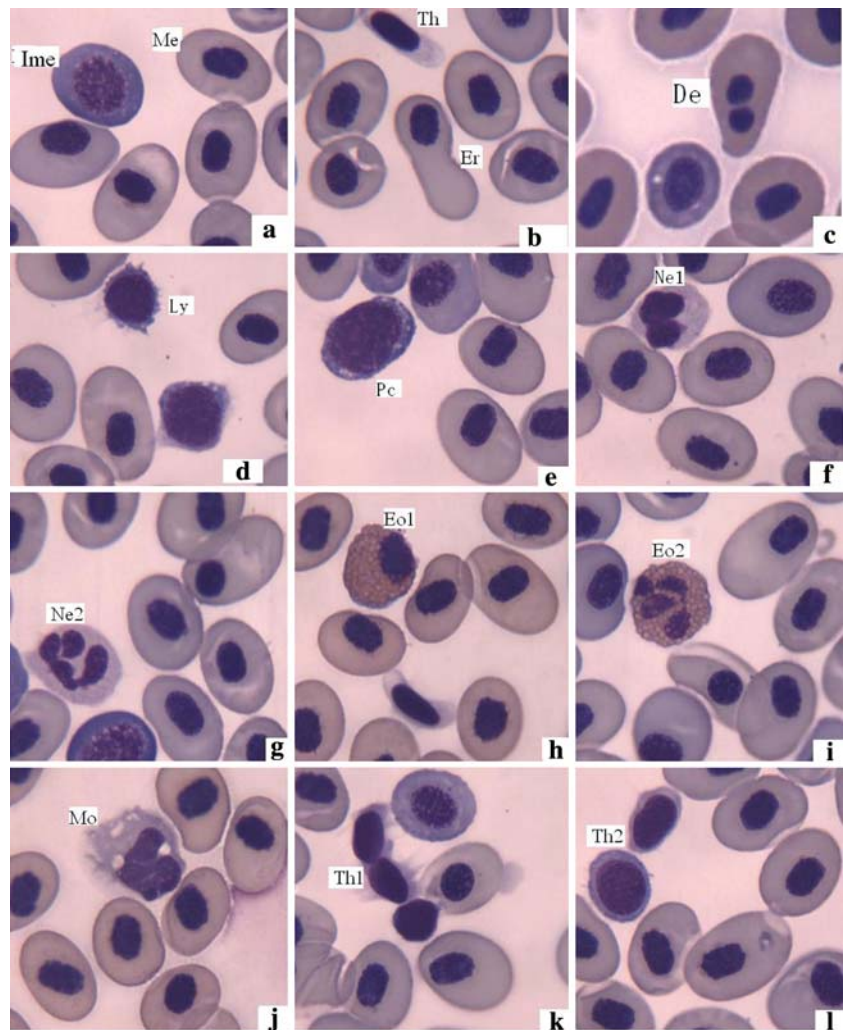
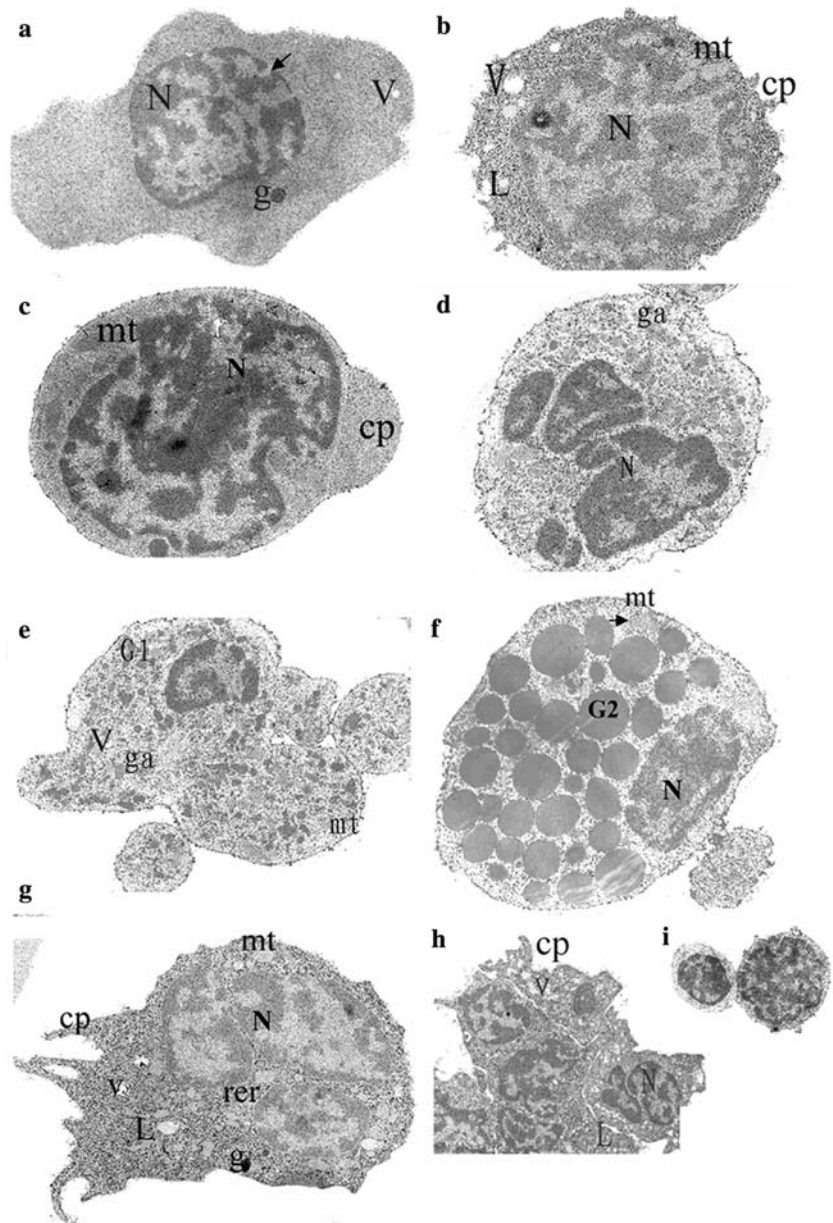


Fig. 2 The ultrastructure of peripheral blood cells of *A. sinensis* (a) erythrocyte, the arrowhead indicates the nuclear pore ($\times 6,000$); (b) lymphocyte ($\times 8,000$); (c) lymphocyte ($\times 9,000$); (d) neutrophil showing tetra-segmented nucleus ($\times 5,400$); (e) neutrophil showing band nucleus and G1 granules ($\times 4,200$); (f) eosinophil showing G2 granules ($\times 8,400$); (g) monocyte ($\times 4,800$); (h) thrombocytes in clusters ($\times 4,000$); (i) round thrombocytes with a little or nearly no cytoplasm ($\times 3,000$); Abbreviations: cp, cell processes; g, granules; ga, Golgi apparatus; L, lysosome; mt, mitochondria; N, nucleus; rer, rough endoplasmic reticulum; V, vesicle



2002b). Because erythropoiesis in fishes can be affected by anemia, temperature, seasonality (Lecklin and Nikinmaa 1998), and bleeding (Fijan 2002b), this could affect the number of circulating reticulocytes, which occupied almost 11.31% of whole erythrocytes in our study. The reticulocyte count may be an indicator of a regenerative response that can be modulated by anemic processes and environmental factors, for example temperature and oxygen availability. Erythrocytes

which are splitting can occasionally be found in the Chinese sturgeon, but not all fish have that kind of blood cell. Fan et al. (2000) reported no erythrocytes splitting in *Sciaenops ocellatus* in fresh water or in sea water. Splitting of erythrocytes has been found in the Mandarin fish *Siniperca chuatsi* and the European eel *Anguilla anguilla* (Zhou et al. 2002; Yuan et al. 1998), however. The difference may be specific to the species or the biogeography of the fish.

Leucocytes

It is difficult to classify the leukocytes of fish. In addition to the numerous species of fish, the basis or standards of classification are not the same in different studies. Leukocytes at different stages of development occur in fish blood and they are difficult to identify (Hibiya 1983). In our study there was sometimes slight difficulty classifying the lymphocytes and thrombocytes by light microscopy; it was, however, easy by electron microscopy. The other cells were easily classified.

Lymphocyte

The lymphocyte was spherical or irregularly spherical, and the round, or horseshoe shape, or slightly irregular nucleus was heavily stained purple. The size varied (Fig. 1d). In the smaller lymphocytes the nucleus occupied most of the space in the cells whereas the large lymphocytes had more cytoplasm which, sometimes, is seen extended into pseudopodia or apophysis on its surface. Lymphocytes (plasma cells) (Fig. 1e) were oval and had a round purple-stained nucleus and a very dark blue-stained cytoplasm. Under the electron microscope the round to slightly irregular nucleus of the lymphocyte was nearly occupied by heavily clumped heterochromatin and was surrounded by a small region of cytoplasm containing mitochondria and free ribosomes. Vacuoles could often be seen in the cytoplasm. The nucleus-to-cytoplasm ratio was large (Fig. 2b, c).

The lymphocytes observed in our study took the general form reported by other workers and were morphologically similar their mammalian counterparts. In some work the lymphocytes have been classified into “large” or “small” groups (Zhou et al. 2002; Lin 1996). Barber et al. (1981), however, remarked that lymphocyte volumes changed continuously and there was no evidence that the function of a lymphocyte was related to its volume. Ellis (1976), Roubal (1986), and Yuan et al. (1998) described lymphocytes as one group. In our study it was difficult to assign the epithets “large” and “small” to many lymphocytes—a continuum of sizes was evident. The small lymphocytes could be differentiated from round

thrombocytes in good, well-stained preparations by their bluish cytoplasm and a few pseudopodia, in contrast with the ragged pale gray cytoplasmic remnant of the round thrombocyte. Under the electron microscope lymphocytes could be differentiated from thrombocytes on the basis of different nuclear chromatin patterns. The lymphocytes lacked the dense bands of heterochromatin seen in the nucleus of thrombocytes and, instead, had a nucleus with a less dense rim and scattered patches of heterochromatin.

Granulocytes

Two kinds of granulocyte, neutrophils and eosinophils, were observed in the peripheral blood of the Chinese sturgeon. The neutrophils were of several sizes, but usually larger than erythrocytes in volume, and of several shapes, for example spherical, pear-shaped, or irregular ellipse. The nucleus stained purple, usually ovoid and eccentric, with pale blue cytoplasm that may be extended into a blunt ended pseudopodium. Occasionally the nucleus may be observed as a ribbon-like structure across the diameter of the cell—horseshoe-shaped, band-shaped, two-segmented in shape, or several-segmented in shape and centrally or eccentrically located (Figs. 1f, g). The nucleus-to-cytoplasm ratio of granulocytes was smaller than that of monocytes and lymphocytes. The cytoplasm was stained light blue and the nucleus was also stained lighter than that of the monocyte and lymphocyte. The eosinophil was observed only rarely in the peripheral blood, being round or nearly round. There were large bilobed, trilobed, and tetralobed, eccentric nuclei stained dark purple in the cell. The cytoplasm was light red and full of large, spherical, bright red-stained granules (Figs. 1h, i).

Neutrophils and eosinophils were observed under an electron microscope. Neutrophils were irregularly outlined because of the presence of short cell processes. They had an eccentric, bilobed, or trilobed nucleus, with large peripheral heterochromatin blocks, and the nucleus-to-cytoplasm ratio was small (Figs. 2d, e). The mitochondria were round and large with clear crista. Some neutrophils contained small granules (Fig. 2d) whereas some contained many granules

(Fig. 2e) with a manifest membrane packed and located on one side of the nucleus. The cytoplasm was rich with free ribosomes. The rough endoplasmic reticulum, smooth endoplasmic reticulum, and Golgi apparatus were visible. Several vacuoles could be seen in the cell. Eosinophils were round or oval cells with a band or several-segmented nucleus (Fig. 2f). The nucleus-to-cytoplasm ratio was smaller than in neutrophils and slightly less heterochromatin was present. There were so many large granules in the cytoplasm that they almost occupied all the cytoplasm. These granules were round and of different sizes and of different electronic density from those in neutrophils (Fig. 2f). Besides some mitochondria, few other organelles were observed.

Available information about fish blood granulocytes is very confusing (Rowley et al. 1988). It is assumed that the three types of granulocyte described in higher vertebrates (heterophilic/neutrophilic, acidophilic, and basophilic) are also present in fish (Doggett and Harris 1989; Meseguer et al. 1990), although sometimes more than three (Mainwaring and Rowley 1985a, b), or only one or two (Savage 1983) circulating types of granulocyte have been described for some fish species. In our study we found two kinds of granulocyte—neutrophils and eosinophils. Cell like basophils were not observed. Palikova et al. (1999) reported that compared with other bony fishes the sturgeons studied so far have fewer myelocytes or metamyelocytes and more eosinophilic and neutrophilic granulocytes with band or segmented nuclei. They reported that neutrophilic myelocytes and metamyelocytes were smaller than neutrophilic granulocytes with band or segmented nuclei; this is in agreement with the results obtained in this study for the Chinese sturgeon. In the blood of the Chinese sturgeon most neutrophils have lobated nuclei and the number of segments is usually 3–4, more than in other fish. The eosinophils also have two or three segments and were easy to identify by light microscopy, because they have light red cytoplasm and bright red granules. The granules in granulocytes are variable. They differ in electron density, structure, and membrane, but there is no uniform standard for classification of granules in neutrophils (Zhou et al. 2002). In Chinese stur-

geon granulocytes, the granules are of different electron density but all have a readily apparent membrane. In eosinophils there were many large granules; these have not been found in other fish. Golgi apparatus was observed in eosinophils which has not been reported for the Amur sturgeon (Liu et al. 2006). The presence of basophil-type cells in the peripheral blood of fish has not been widely reported, and most workers believe them to be absent. Pitombeira and Martins (1970) reported the presence of a PAS (periodic acid Schiff)-positive basophil in the blood of the Spanish mackerel *Scomberomorus niphonius*, and Lin (1979) reported it was visible under a light microscope; these have rarely been observed, however. These cell types were not observed in our study, possible because of their scarcity, if, indeed, they are present. The presence of blood basophils and eosinophils in teleost blood is extremely rare, and is not because of the failure of the basophil granules to be revealed by classical stains (Tavares-Dias 2006), as was once believed. This study corroborates these findings. Environmental pressure during evolution has probably induced adaptations in tissue distribution of cells involved in host immunity (Reite and Evensen 2006). It thus seems reasonable to postulate that granulocytes have been evolving similarly in fishes.

Monocytes

In this cell, the nucleus stained dark blue and reticulated, and was eccentric and usually ovoid, kidney-shaped, or horseshoe-shape, but can be more irregularly shaped or lobed, with vacuolated cytoplasm which may be extended into pseudopodia. The nucleus-to-cytoplasm ratio for the cell was in between that of lymphocytes and granulocytes. The plasma of the cell stained slightly darker than that of neutrophils (Fig. 1j).

The volume of the cell was nearly the same as, or slightly larger than, that of erythrocyte, but larger than that of granulocyte. Under electron microscope heavily stained heterochromatin clumped in the nucleus was observed mainly along the inside of the nuclear membrane. Mitochondria have many clear crista stacked in the cytoplasm and were rich in free ribosomes.

Endosome could be seen in the cytoplasm. The monocyte/macrophages could be distinguished by the presence of large cytoplasmic vesicles of heterogeneous content corresponding to lysosomes (Fig. 2g).

Monocytes have been reported to be present in many fish species. It has, however, been reported that monocytes are absent from the blood of brown trout *Salmo trutta* (Blaxhall and Daisley 1973) and goldfish *Carassius auratus* (Weinreb and Weinreb 1969). It may be that in these two fish confusion occurred because monocytes were identified as large lymphocytes, a problem reported by Roubal (1986) when examining the blood of *Acanthopagrus australis*. In this study, monocytes could easily be distinguished from neutrophils under the electron microscope because of the absence of typical granules; under the light microscope, however, they were difficult to distinguish. The term monocyte/macrophage is widely used to refer to a fish blood cell type (Barber et al. 1981; Savage 1983) with phagocytic activity (Morrow and Pulsford 1980) or morphophysiological features comparable with those of the monocytes of higher vertebrates (Rowley et al. 1988). In Chinese sturgeon blood, we observed monocytes and monocyte/macrophages; the latter had larger and more numerous lysosomes than the former. This seems to indicate that, in contrast with those observed in higher vertebrates, some circulating forms of the fish mononuclear phagocytic series could be functionally active cells.

Thrombocytes

Thrombocytes appeared as round, oval, spike-shaped, or fusiform cells. The oval and spike-shaped thrombocytes were the most frequent; in these the nucleus followed the shape of the cell. They occurred as single cells (Fig. 1b) or in clusters (Fig. 1k) and had a large central dark-purple-stained nucleus with chromatin clusters which were more abundant in the spike-shaped and fusiform thrombocytes. There was little blue-gray-stained cytoplasm surrounding the nucleus. Another kind of thrombocyte found in the blood of Chinese sturgeon had more cytoplasm and was much larger than the others, was (Fig. 1l). Under

the electron microscope it was a small cell with a serrated membrane. The elliptical nucleus contained fine sand-like chromatin and blocky heterochromatin. There was almost no cytoplasm in the cell and few mitochondria; some phagocytic vacuoles and lysosomes could occasionally be seen outside the nucleus (Fig. 2h). Another kind of thrombocyte with some cytoplasm but no organelle (Fig. 2i) was also found in the blood of the Chinese sturgeon.

The thrombocytes of fish are not the same as those of mammals. In fish, they often have intact cell structures and, in addition to a role in blood coagulation, phagocytosis has been reported. This phagocytosis is doubted by Ellis (1976), however. In our study, under electron microscope, we found many vesicles which may be related to its phagocytosis. Although fish blood thrombocytes have been described as the most abundant blood cells after erythrocytes, very little is known about their morphological features and functional properties (Rowley et al. 1988), the data available on the percentage of thrombocytes in fish peripheral blood being very confused. Different reports have suggested they account for 70% and 3–13% of total blood leukocyte count, whereas Catton (1951) observed no thrombocytes in *Perca fluviatilis* blood. These differences between numbers of circulating thrombocytes may be because of difficulties identifying them by light microscopy, because Chinese sturgeon thrombocytes are highly variable in shape, ranging from round to spindle cells. In different fish species round, oval, spindle, and spike forms have also been described (Doggett and Harris 1989; López-Ruiz et al. 1992). In our study, thrombocytes accounted for 60.78% of the total blood leukocyte count, which is more than in most fish and different from other sturgeons.

In summary, the Chinese sturgeon basically follows the hematological pattern of other vertebrates, although with significant differences, for example the presence of circulating immature erythrocytes, multi-segment nuclei of granulocytes, and different forms of thrombocyte. The morphological characteristics of the leukocytes are similar to those of other Acipenseridae species. According to our data, the blood cells of the Chinese sturgeon are larger than their

counterparts in other fish. Future studies will focus on the development of blood cells in Acipenseridae fish.

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