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ORIGINAL ARTICLE

Haematology, morphology and blood cells characteristics of male and female Siamese fighting fish (*Betta splendens*)

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Abstract The aim of the present study was to obtain baseline data on blood cell size, morphology and haematological parameters in Siamese fighting fish *(Betta splendens)* since there is limited information in the published literature. Blood samples from the caudal vein of apparently healthy Siamese fighting fish (male: $n=40$ and female: $n=36$) were collected. Haematological values of the blood samples were determined using standard techniques. The morphological features of blood cells were described according to observations made by light microscopy. The various types of blood cells measurement were carried out with the help of a stage and an ocular micrometre at a magnification of ×1,000. Erythrocytes, thrombocytes and four types of leucocytes: lymphocytes, monocytes, heterophils and eosinophils, were distinguished and characterised. The average size of the erythrocyte cell and nucleus was 97.33 and 16.28 μm^2, respectively. Results showed a positive correlation between erythrocyte size and nucleus size for Siamese fighting fish ($r=0.470$, $p<0.01$). We also found sex-dependent differences for total white blood cell count, lymphocytes and heterophils in Siamese fighting fish ($p<0.05$). Statistical analysis revealed that differences in other haematological parameters and blood cell morphology, between male and female fish were not statistically significant ($p>0.05$).

Keywords Blood cells morphology · Blood cells size · Haematological parameters · Siamese fighting fish

Introduction

Haematological parameters that are used in veterinary and clinical medicine have been established as health indicators (Schutt et al. 1997). Blood biochemistry profiles and haematology are often used to assess the physiological status of lower vertebrate patients, such as fish, amphibians, reptiles and birds (Metin et al. 2008). Evaluation of the haemogram involves the determination of the total erythrocyte count, total white blood cell count (WBC), haematocrit (PCV), haemoglobin concentration (Hb), erythrocyte indices, WBC differential count and the evaluation of stained peripheral blood films (Campbell, 2004).

The Siamese fighting fish *(Betta splendens)* is a member of the Labyrinth fish family (Belonitiidae). *B. splendens* in Latin refers to brilliant warrior, indeed a suitable name, since males of the colourful species perform an elaborate aggressive display when provoked. The species' natural habitats are shallow freshwater ponds with muddy bottoms or flooded rice paddy fields

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(Jaroensutasinee and Jaroensutasinee 2000). They can breathe oxygen from the surface (due to their labyrinth organ), which enables the species to survive in low oxygen waters. Many papers have characterised the blood and haematology of fishes (Gulliver 1875; Cleland and Johnston 1912; Wintrobe 1933; Kisch 1949a, b, 1951; Hartman and Lessler 1964; Pedersen 1971; Conroy and Rodriguez 1966; Saunders 1966; Potter et al. 1982; Banerjee et al. 1988; Lewis 1996; Hrubec et al. 2000; Esteban et al. 2000; Bahmani et al. 2001; Ueda et al. 2001; Hardie and Hebert 2003; Beyea et al. 2005; Mohammadi Zarejabad et al. 2009; Zhou et al. 2009), however, the haematology and blood cell morphology of Siamese fighting fish *(B. splendens)* is currently unknown. The aim of the present study was to obtain a basic knowledge of blood cell size, morphology and haematological parameters in these fish.

Materials and methods

Seventy six, 6-month-old Siamese fighting fish from our fish breeding centre in Gorgan,Iran, were weighed and measured (40 males, 1.52±0.33 g in weight and 3.82±0.43 cm in length; 36 females, 1.69±0.48 g in weight and 3.63±1.17 cm in length) and kept in 0.5-L glass tanks at 28.5±1.3°C, pH 7.2, with 13-h light/11-h dark photoperiod for 1 week. In each glass tank, 100% of the water volume was renewed every day to assure proper water quality. Blood samples were taken by caudal venepuncture after anaesthetizing the fish with MS-222 (200 mg/L).

The PCV was determined after the blood had been transferred to microcapillary tubes and centrifuged at 4,000×*g* for 5 min and was expressed as percentage of the total volume (Snieszko 1960). Total red blood cell counts ($\times 10^6/mm^3$), WBC ($\times 10^3/mm^3$) and total thrombocyte counts ($\times 10^3/mm^3$) were determined manually with the improved Neubauer counting chamber after the blood was diluted with Daice diluting fluid solution. Hb was determined according to the cyanomethaemoglobin procedure (Blaxhall and Daisley 1973) and was expressed in g/dl. The haematological indices were calculated according to Seiverd (1964). The mean corpuscular volume (MCV) was expressed in femtolitres (fl), the mean corpuscular haemoglobin (MCH) resultant value is given in picograms (pg) and the mean corpuscular haemoglobin concentration was expressed in g/dl.

Blood smears were prepared immediately and air-dried, fixed in 95% methanol for 5 min then stained with Giemsa. One to two blood smears were prepared per individual and then photos were taken under light microscopy. Giemsa-stained blood smears were used for the measurement and assessment of blood cells. Blood cell dimensions were determined by using a stage and ocular micrometre. Cells were identified on the basis of morphology and cell ultrastructure as documented in previous fish leucocytes studies (Rowley 1990; V′azquez and Guerrero 2007). Erythrocytes in fish are elliptical, and as such, two different diameters are provided: erythrocyte lengths (EL) and erythrocyte widths (EW). Nucleus lengths (NL) and nucleus widths (NW) were obtained where possible. In both cases, the diameters were measured from dry smears. On each slide, EL and EW of mature erythrocytes and their nuclei (NL and NW), thrombocytes, heterophils, eosinophils, lymphocytes and monocytes were measured by an Olympus ocular micrometre at a magnification of ×1,000 (Olympus BX51, Japan). Erythrocyte and nuclear sizes (ES and NS) were calculated according to formulas $[(EL\times EW\times\pi)/4]$ and $[(NL\times NW\times\pi)/4]$, respectively (Metin et al. 2008).

Haematological indicators were summarised as mean±standard deviation (SD) and differences between male and female fish were statistically analysed using the Student's *t* test. Results were considered significant at $p<0.05$. Statistical analyses were carried out using SPSS version 11.5 (SPSS, Michigan Avenue, Chicago, IL, USA).

Results

Haematology

Summaries and comparison of haematological values in male and female Siamese fighting fish are shown in Table 1. The result showed that WBC and lymphocytes in females were significantly higher than males and there were significantly less heterophils in females than males. No significant differences were observed in other haematological parameter in relation with gender.

Blood cell size and morphology

Blood cell sizes and erythrocyte dimensions of male and female Siamese fighting fish are shown in Table 2. The mature erythrocytes are the predominant cell type found in blood and their size was not sex-dependent.

Mature erythrocytes in Siamese fighting fish were nucleated ellipsoidal cells with pink cytoplasm and a central hump corresponding to the position of their oval nucleus. This centrally located nucleus contains condensed chromatin and stained dark purple (Fig.1a).

Table 1 Haematological values of male and female Siamese fighting fish

Parameters	Male (n=40)	Female (n=36)
RBC($\times10^6$/mm^3)	1.84±0.13	1.82±0.08
max	2.21	2.04
min	1.70	1.70
PCV (%)	33.65±2.34	34.1±2.24
max	39	38
min	31	31
Hb (g/dl)	8.08±0.69	8.04±0.77
max	9.4	9.3
min	7.1	7.1
MCV (fl)	182.48±5.37	187.28±7.05
MCH (Pg)	43.81±2.55	44.15±3.05
MCHC (gdl^{-1})	240.00±9.13	235.56±9.62
Thrombocyte ($\times10^3$/mm^3)	96.32±6.75	98.0±7.79
WBC ($\times10^3$/mm^3)	5.22±0.75	5.53±3.13
max	5.98	6.93
min	3.52	4.32
Lymphocytes (%)	67.00±1.67	72.16±1.47
Heterophils (%)	26.66±2.16	20.83±2.31
Eosinophils (%)	3.00±1.41	3.66±1.36
Monocytes (%)	3.33±1.03	3.33±1.36

RBC Red blood cell; *PCV* Packed cell volume; *Hb* Haemoglobin concentration;

MCV Mean corpuscular volume; *MCH* Mean corpuscular haemoglobin;

MCHC Mean corpuscular haemoglobin concentration. *WBC* White blood cell;

Different superscripts in rows are significantly different (P<0.05)

Hb and *PCV* of fish blood has been shown to decrease after capture and transportation (Hattingh and Van Pletzen 1974). The fish used in this study were kept under laboratory conditions and anesthetised with MS222 before handling. The effect of stress resulting from handling was kept to a minimum

Thrombocytes often clump together in blood smears. The nucleus of thrombocytes was round or oval and dark. The cytoplasm was blue purple and positioned around the nucleus (Fig.1b). Four types of leucocytes were identified for Siamese fighting fish: heterophil, eosinophil, lymphocyte and monocyte (Fig.1). Descriptive statistics for the leucocytes are presented in Table 2. Lymphocytes had a compact, large, dark, centrally positioned nucleus. Light blue thin cytoplasm covered a narrow area around the nucleus (Fig.1c). The monocytes mostly had a kidney-shaped nucleus, which was less intensely chromatin stained than in lymphocytes. The monocyte cytoplasm was blue grey and covered a larger area (Fig.1d). Heterophils were easily identified by the presence of numerous, diffuse elongated pink red cytoplasmic granules. The nucleus was round, oval or mostly bilobed and eccentric (Fig.1e). Eosinophils are large, round, frequently irregularly outlined cells. They have an eccentric, round or sometimes bilobed nucleus. The cytoplasm is full of acidophilic granules that occasionally obscure the nucleus (Fig.1f).

Discussion

Data regarding the haematology of Siamese fighting fish are scarce; even simple morphological descriptions of the cell types to be found in the peripheral blood have not been reported. Morphological work, using light microscopy, on blood cells of other fish served as a basis for the present study. According to Wintrobe (1933) the erythrocyte size reflects the position of a species on the evolutionary scale: in lower vertebrates and those with a not so successful evolutionary past, i.e. in cyclostomes, elasmobranches and urodeles, the erythrocytes are large, but in higher vertebrates (mammals) the same cells are smaller and do not contain nuclei. Erythrocytes are the dominant cell type in the vast majority of fish species blood. One of the most important functions of erythrocytes is carrying oxygen and carbon dioxide and the ratio of size to surface area is also a determining factor in the tissues. Thus, a small erythrocyte offers the possibility of a higher rate of exchange than a larger one (Hartman and Lessler 1964; Sevinch et al. 2000). Table 3 shows the erythrocyte dimensions in different teleost species, the mature erythrocytes of *B. splendens* show an average size and ultra structural features similar to those described for mature erythrocytes of other fish species and, as in all the species examined so far, they are the predominant cell type found in blood (Banerjee et al. 1988; Lewis 1996; Hardie and Hebert, 2003; Hrubec et al. 2000; Esteban et al. 2000; Ueda et al. 2001). We found a positive correlation between erythrocyte size and nucleus size for individuals belonging to Siamese fighting fish (r=0.470, p<0.01).

Thrombocytes are the most abundant blood cells after erythrocytes, representing more than 50% of circulating leucocytes (Ueda et al. 1997). Thrombocytes were easily stained with Giemsa stain; the nucleus was stained dark purple and cytoplasm light purple. They were smaller than erythrocytes. In teleost thrombocytes, only a single population of granules has been reported under TEM (Cannon et al. 1980) and these have a clear space between the granule content and the bounding membrane which is characteristic of lysosomes (Daems et al. 1972). Giemsa stain was used for morphological evaluation of all types of

Table 2 Blood cell sizes and erythrocyte dimensions in microns of male and female Siamese fighting fish

Parameters	Male					Female					Overall				
	mean	max	min	S.D	n	mean	max	min	S.D	n	mean	max	min	S.D	n
EL	12.68	15.26	10.26	1.13	200	12.41	14.96	10.12	1.08	160	12.60	15.26	10.12	1.11	360
EW	9.52	11.06	7.37	0.75	200	10.52	12.59	8.74	0.93	160	9.84	12.59	7.37	0.92	360
NL	5.15	7.63	3.54	0.73	200	5.54	6.51	4.47	0.50	160	5.27	7.63	3.54	0.69	360
NW	3.75	4.68	2.83	0.45	200	4.30	5.10	3.46	0.46	160	3.92	5.10	2.83	0.52	360
ES (μm^2)	94.79	114.67	77.46	8.74	200	102.96	130.35	81.50	15.50	160	97.33	130.35	77.46	11.69	360
NS (μm^2)	15.22	23.00	8.80	3.06	200	18.85	23.86	15.26	2.64	160	16.28	23.86	8.80	3.36	360
EL/EW	1.34	1.96	0.99	0.20	200	1.18	1.40	0.98	0.11	160	1.29	1.96	0.98	0.19	360
NL/NW	1.39	2.16	1.03	0.23	200	1.30	1.67	0.89	0.19	160	1.36	2.16	0.89	0.22	360
NS/ES	6.55	12.00	4.72	1.46	200	5.53	7.40	4.42	0.72	160	6.24	12.00	4.42	1.35	360
Thromb L	9.79	12.13	8.53	1.64	85	9.65	11.94	8.29	1.46	72	9.72	12.13	8.29	1.38	157
Thromb W	3.98	5.87	2.85	0.63	85	4.05	5.62	3.71	0.65	72	4.01	5.87	2.85	0.75	157
Lymphocyte	5.26	5.8	4.8	0.34	66	5.31	5.9	4.8	0.33	52	5.28	5.9	4.8	0.33	118
Monocyte	6.94	7.8	5.7	0.73	47	6.76	7.8	5.7	0.74	33	6.85	7.8	5.7	0.72	80
Heterophil	7.81	9.7	5.3	1.65	62	7.54	9.7	5.5	1.44	57	7.67	9.7	5.3	1.51	119
Eosinophil	7.49	9.8	5.4	1.65	14	7.81	9.6	6.1	1.21	12	7.65	1.42	9.8	5.4	26

EL Erythrocyte length; *EW* Erythrocyte width; *NL* Nucleus length; *NW* Nucleus width; *ES* Erythrocyte size; *NS* Nucleus size; Thromb *L* Thrombocyte length; Thromb *W* Thrombocyte width

blood cells. Staining allowed the recognition of granulocytes (heterophil and eosinophil), agranulocytes (monocyte and lymphocyte) and thrombocyte. Lymphocytes are usually the most commonly present leucocyte type in some fish, accounting for as much as 85% of the total leucocyte population, excluding thrombocytes (Groff and Zinkl 1999). The most abundant types of leucocytes found in the peripheral blood of *B. splendens* were the lymphocytes and heterophils (Table 1), as demonstrated in *Pimelodus maculatus* (Ribeiro 1978), *Synbranchus mar-*

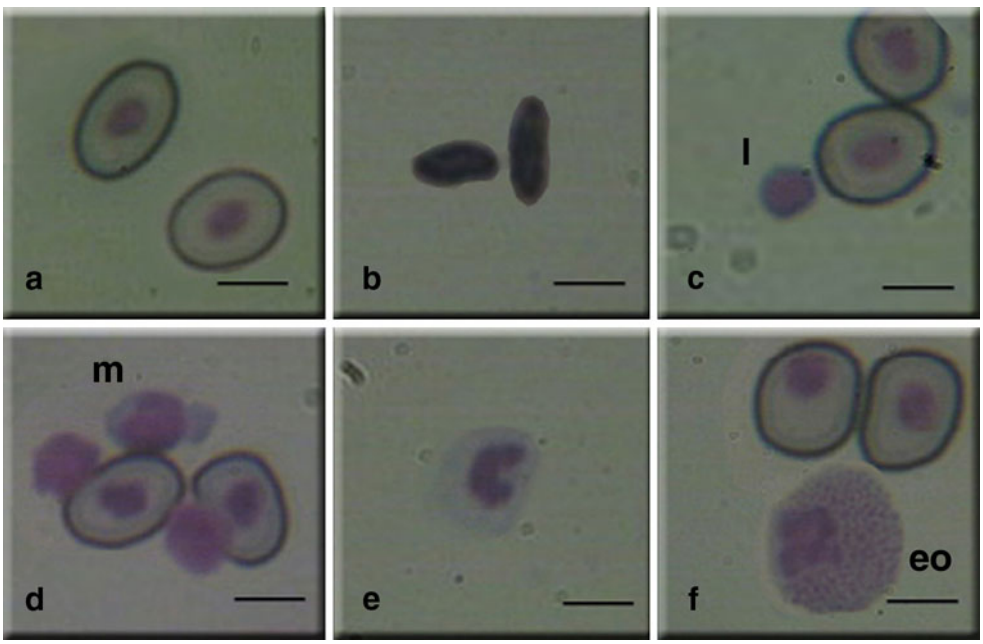

Fig. 1 Light microscopic micrographs of peripheral blood. **a** erythrocyte; **b** thrombocyte, **c** l, lymphocyte, **d** m, monocyte, **e** heterophilic granulocyte **f** eo, eosinophilic granulocyte. Scale bar=10 μm

Table 3 Erythrocyte dimensions in different teleost species

species	EL (μm)	EW (μm)	ES (μm^2)	NL (μm)	NW (μm)	NS (μm^2)	Reference
Petromyzon marinus	11.9	11.9	111.27	4.0	4.0	12.37	Gulliver 1875
Nematalosa erebi	12.2	8.0	76.65	4.8	2.3	8.67	Cleland & Johnston 1912
Carassius auratus auratus	12.5	8.5	83.45	5.5	4.0	17.28	Conroy & Rodriguez 1966
Lophius piscatorius	11.6	9.2	83.82	5.6	4.2	18.47	Lewis 1996
Xiphias gladius	11.7	8.3	76.27	4.0	3.4	10.68	Kisch 1949a
Salvelinus namaycush	16.0	11.3	142.00	6.5	4.4	22.46	Lewis 1996
Oncorhynchus keta	–	–	131.08	–	–	35.16	Hardie &Hebert 2003
Gadus morhua	–	–	76.51	–	–	17.33	Hardie &Hebert 2003

EL Erythrocyte length; *EW* Erythrocyte width; *ES* Erythrocyte size; *NL* Nucleus length; *NW* Nucleus width; *NS* Nucleus size

moratus (Nakamoto et al. 1991), *Mugil platanus* (Ranzani-Paiva 1995) and *Oncorhynchus mykiss* (Ranzani-Paiva et al. 1998). Lymphocytes are one of the most important cells to impact on a fish's immune response. These cells produce antibodies by specific immunity and increasing macrophages. An increase of such immune cells can promote a fish's defence to an adverse condition (Jalali et al. 2009). In the present study, lymphocytes in female Siamese fighting fish were significantly ($p<0.05$) higher than male. Based on the results of the current study, an investigation into the immune resistance of Siamese fighting fish in relation to gender is being considered for future research. Monocytes were larger than lymphocytes; they were round cells with a rough membrane. In fish, granulocytes are of three types: heterophils and eosinophils are the most common, while basophils are rare. Eosinophils and heterophils are granulocytic leukocytes present in the blood of most vertebrates. The heterophils were round with an irregular membrane surface. Eosinophils were round cells with many spherical spines protruding from their membrane surface. The blood cells size in different teleost species is shown in Table 4. *B. splendens'* cells size is similar to those described for other fish species.

Fish haematology is gaining increasing importance in fish culture because of its value in monitoring the health status of fish (Hrubec et al. 2000). Knowledge of the haematological characteristics is an important tool that can be used as an effective and sensitive index to monitor physiological and pathological changes in fish (Kori-Siakpere et al. 2005). Blood cells including WBCs are frequently used as indicators of health status in fish because WBCs are key components of innate immune defence and leukocytes are involved in regulation of immunological function in the organisms (Ballarin et al. 2004). In this study, WBC in female Siamese fighting fish was significantly higher than in males and is in agreement with the data on lymphocytes count. MCV is wet volume, whereas the diameters of erythrocytes were measured from dry smears and were used to calculate dry cell area (erythrocyte size, ES). These different measurements (between ES and MCV) should not be compared to each other. The erythrocyte indexes MCV and MCH have a wide range of physiological variation.

In summary, the results of our research provide a contribution to the knowledge of the characteristics of blood cells and haematological parameters of the Siamese fighting fish, *B. splendens*, under normal conditions employed in this study.

Table 4 Cell size in microns (mean±S.D. or reference interval) in some teleost fishes

Species	Thrombocyte	Lymphocyte	Heterophil	Monocyte	Eosinophil	References
Carassius auratus	4.7–5.6	7.4–8.4	10.2–12.1	7.0–17	7.4–8.4	Groff & Zinkl (1999)
Oreochomis hybrid	4.7–5.5	4.6–5×5.7–6.4	6.38±1.04	9.4–10.7	5.77–6.7	Hrubec et al. (2000)
Cyprynus carpio	4.6×7.7	6.6–11.8	10.0–15	10.0–16	13.8	Groff & Zinkl (1999)
Cichlasoma dimerus	8.0–9×2.1–2.7	3.4–4.7	5–9.5	4.0–7.1	4.8–9.5	V´azquez & Guerrero (2007)

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