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BLOOD FLOW CYTOMETRY IN MUGIL CEPHALUS AND CARASSIUS AURATUS: A COMPARATIVE STUDY

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ABSTRACT. Blood and its parameters can give specific indications on the welfare of fishes. Many endogenous and exogenous factors exert influences on the characteristics of blood. The correct interpretation of fish hematology for a given species depends on the availability of reference values. The purpose of the present study was therefore to build a database with data on haematological profiles of *Mugil cephalus* (Linnaeus 1758) and goldfish *Carassius auratus* (Linnaeus 1758), in particular of some blood cells, namely red blood cells (RBC), white blood cells (WBC) and thrombocytes (TC). All the blood parameters studied showed significant differences in the two fish species considered, using flow cytometry and optical microscopy coupled with an automated system. In particular, RBC showed an increase in mullets in respect to goldfish, while WBC and TC decreased. The differences found may be due to the environmental conditions and the different eating habits of the two species. The results of this research will allow to better understand how the different dietary habits and environmental conditions can influence the haematological parameters of fishes. Flow cytometry represents a modern diagnostic technique in human. Moreover, the technique used by combining flow cytometry with automated haematological counting, has proved very effective in the early evaluation of haematological parameters of various fish species.

1. Introduction

Studies of fish blood cells made to date presented numerous problems derived from different type of techniques used. A combination of quantitative and morphological methods is needed if the classification of fish blood cells is to advance from its present provisional state. Blood and its parameters are generally used to give specific indications on the welfare of fishes. It is known that a multitude of endogenous and exogenous factors exert some influences on the haematological characteristics (Blaxhall 1972; Chaudhuri *et al.* 1986; Filho *et al.* 1992; Hrubec *et al.* 2001; Gabriel *et al.* 2004; Fazio *et al.* 2016). Moreover, blood cells of fish are highly sensitive to environmental changes (quality of water, oxygen, temperature and salinity) that influence the number of blood cells (LeaMaster *et al.* 1990; Luskovà 1997; Sheikh and Ahmed 2016). For these reasons, the evaluation of haematological parameters represents an effective approach for monitoring the health condition of fish

and for investigation of ecological stress, physiological homeostasis and aquatic pollution (Anderson *et al.* 2010; Fazio *et al.* 2012b, 2013; Zhelev 2016). In order to correctly interpret the haematological parameters of fish, a reference database is necessary, with ecological and physiological studies that are useful for understanding the relationship and influence that environmental parameters have on the haematological characteristics of fish (Blaxhall 1972; Filho *et al.* 1992). However, few studies have been done regarding the feeding behavior of fishes that live in different environments. Therefore, this study aims to cover this gap on the blood cells (RBC, WBC and TC) of the two species studied that live in two totally different environments and with different dietary habits: the grey mullet Mugil cephalus (marine herbivorous fish), and the goldfish Carassius auratus (omnivorous freshwater fish). Grey mullets *M. cephalus* (Osteichthyes: Mugilidae) is a species cosmopolitan in coastal waters of the tropical, subtropical and temperate zones of all seas, frequently found in estuaries and freshwater environments. Adult mullet have been found in waters ranging from zero salinity to 75%, while juveniles can only tolerate such wide salinity ranges after they reach lengths of 4–7 cm (Cardona 2006; Brandao *et al.* 2015; Cappello *et al.* 2016). *M. cephalus* is a diurnal feeder, consuming mainly zooplankton, dead plant matter, benthic microalgae and detritus (Whitfield *et al.* 2012). Due to its eurytopic characteristics and foraging at the bottom of the food web, this species plays a crucial ecological role. Additionally, the flathead grey mullet is among the innovative species in aquaculture and of high commercial value, usually cultured in extensive and semi-intensive pond systems(Cardona 2006; Whitfield *et al.* 2012). *Goldfish C. auratus* (Cypriniformes: Cyprinidae) *C. auratus* is distributed principally in central Asia and China and Japan.Typical habitat includes the quiet backwaters of streams and pools, eutrophic waters, ponds and well-vegetated canals, rivers, lakes, and they live better in cold water. In the wild, goldfish are omnivores (Specziàr *et al.* 1998). They feed mainly on plankton, benthic invertebrates, fish eggs, phytoplankton, plant material and debris (Takada *et al.* 2010). C. auratus adapts to temperature fluctuations and tolerate high levels of anthropogenic pollution and also low amounts of oxygen, so it is an excellent sentinel organism for laboratory experiments (Fan *et al.* 2013; Maisano *et al.* 2016; Zhelev 2016). Moreover, it is a very important fish from the commercial point of view as it is sold as ornamental fish for aquariums. In order to obtain a basic knowledge of the haematology of the two selected fish species and perform a comparative evaluation of their blood cells in relation to divergent environmental conditions and feeding behaviour of the fish, in this study we utilized flow cytometry with automated haematological counting and light microscopy, to examinate the blood cells of the grey mullet *M. cephalus* and the goldfish *C. auratus*. Flow cytometry represents a modern diagnostic technique in human and veterinary medicine. It has been largely employed in fish, mainly for qualitative and quantitative analysis of blood and immune system cells (Esteban *et al.* 2000; Stosik *et al.* 2001; Inoue *et al.* 2002), as well as in immunopathological research (Chilmonczyk and Monge 1999)

2. Material and methods

Sixty fish (thirty flathead grey mullets *M. cephalus* and thirthy goldfish C. *auratus*) were used for the research. The flathead grey mullets were fished in Faro Lake, a coastal quagmire that is part of the lagoon of Capo peloro, in the north-eastern part of Messina (Sicily, Italy) (Fazio *et al.* 2013; D'Agata *et al.* 2014; Maisano *et al.* 2016) and immediately

transferred to the laboratories for acclimatization in 400L tanks containing pond water, equipped with filter and oxygenation systems. The acclimatization lasted three weeks and the physico-chemical characteristics of the water were measured with multi-parametric probe C 203 (Hanna-Instruments, United Kingdom) (Table2). The fishes were fed twice a day with commercial feed (0.45 cm diameter), whose raw composition was given by: 8.9% moisture, 51.1% protein, 8% lipids and 11% ash. The specimens of goldfish were purchased from a specific retailer and then transferred to the laboratories in circular tanks equipped with aerator and filled with de-chlorinated water, which physico-chemical parameters are reported in Table2. Acclimatization, also in this case, lasted three weeks and the organisms were fed twice a day with commercial food pellet (0.3 cm size). For all fish biometric values are shown in Table1. No specimens died during the acclimatization period.

Fish Species	**Biometric parameters**	
	Fork length (cm)	**Weight (gr)**
Mugil cephalus	22.00±1.50	76.20±11.00
Carassius auratus	20.00±1.00	72.50±5.50

TABLE 1. Mean values±SD of biometric parameters recorded in the two teleost species Mugil cephalus and Carassius auratus.

Both for mullets and goldfish, feeding was stopped 24 h prior blood sampling. The anaesthetic used before the blood sample was tricaine methane sulfonate (MS-222; 0.3 g/L). The samples were taken from the caudal vein with a sterile plastic syringe (2.5 mL) and the blood transferred into micro tubes (Miniplast 0.6 mL, LP Italiana Spa, Milano) containing EDTA (ethylenediamine tetraacetic acid, 1.26 mg/0.6 mL), as anticoagulant. Blood sampling was performed in the morning for all fishes.

Fish species	**Temperature**	**pH**	**Dissolved Oxygen (DO_2)**	**Salinity**
M. cephalus	18.2°C	8.23	5.7 mg/l	38%
C. auratus	18.1°C	8.01	5.3 mg/l	0.03%

TABLE 2. Physico-chemical characteristics of the water during acclimatizaton period of *Mugil cephalus* and *Carassius auratus*.

All specimen-handling procedures have been compliant with the guidelines of the European Union Council (Guide for Care and Use of Laboratory Animals; Directive 2010/63/EU). Blood cells were analyzed immediately after collection by determining the number of red blood cells (RBC), white blood cells (WBC) and thrombocytes (CT), using an automatic blood analyzer (HeCo Vet C; SEAC, Florence, Italy), already tested in previous experiments (Fazio *et al.* 2013, 2016). All fish blood samples were tested in duplicate by the same person to minimize errors. Flow cytometric analysis was performed within 5 hours of blood sampling of the two species, using the multispectral flow cytometer ImageStream^X (Amnis, Seattle, WA). This instrument can acquire up to 100 cells / s, acquiring at the same time six images of each cell. The integrated software INSPIRE, running on the ImageStreamX

Blood cells	*Mugil cephalus* (n=30)	*Carassius auratus* (n=30)
RBC (x10^6/*mu*L)	2.30±0.20*	0.60±0.02
WBC (x10^3/μL)	32.10±1.50*	68.40±2.00
TC (x10^3/μl)	28.02±2.40*	85.00±3.20

TABLE 3. Mean values±SEM of blood cells (RBC, WBC and TC) obteined in the two teleost species *M. cephalus* and *C. auratus* (*P<0.05).

Mark II, was used for this research. Before being injected, the samples were kept on ice. The IDEAS software (Amnis) was used for image analysis. The morphological analysis of the blood samples of the two species was carried out by the spread of the heparinized blood on slides, kept on the air overnight and then fixed in absolute methanol (20 minutes) before being colored with the 10% Giemsa solution. (15 minutes). The slides were observed using a Zeiss Axio Imager Z1 powered microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with a 63x immersion objective, and with an AxioCam digital camera (Zeiss, Jena, Germany). The results were validated using the Kolmogorov-Smirnov test ($P < 0.05$ as positive significance). The statistical differences in the parameters investigated in the two species were evaluated by applying unpaired t-tests (P <0.05 as positive significance). For all statistical analyses, the Prism v. 5.00 software (Graph Pad software Ldt., USA, 2003) was used.

3. Results

The results of analyses carried out on blood cells blood cells of M. cephalus and C. auratus, target species of this study, are reported in Table3 as mean values *pm* error standard (SEM).

As reported, in particular, RBC showed an increase in mullets in respect to goldfish, while WBC and TC decreased. The results of the flow cytometric analyses are presented in Figure1. As shown in the score plots, there is a grouping of the three main blood cell populations, red blood cells (RBC; in orange), white blood cells (WBC; in blue), thrombocytes (TC; in green) and debris (in yellow). Furthermore, there are appreciable differences between the two target species, with lower WBC and CT values and higher RBC values in M. cephalus (Figure1 a) compared to C. auratus (Figure1 b). Furthermore, microscopic analysis shown that grey mullet has a high number of erythrocytes of small size (Figure1 a), while goldfish has a lower number of erythrocytes but much larger in size compared to grey mullet (Figure1 b). Other flow cytometric analysis characteristics are shown in Figure1.

4. Discussion

Use of haematological status in diagnosing fish welfare and diseases is possible only when information about ranges of physiological variation of hematological parameters exists, as well as knowledge on cause and effect relationship between changes in external and internal environment with changes in fish blood. In the aquatic habitat, the fish homeostatic

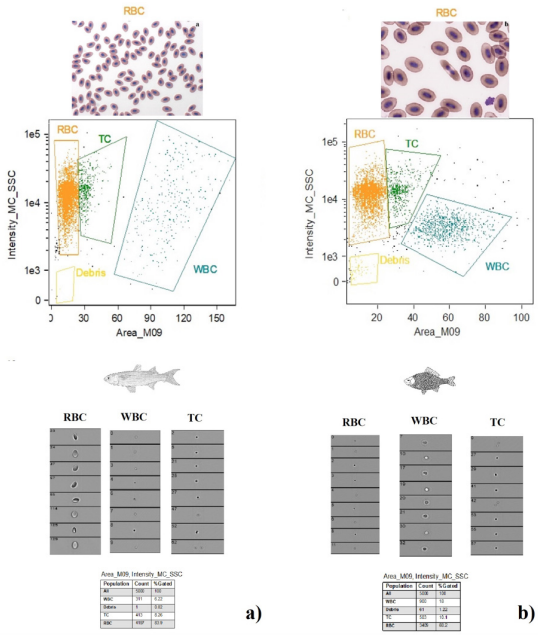

FIGURE 1. Flow cytometry and specific blood cells erythrocytes scale bar: 20 μm, of (a) *Mugil cephalus* and (b) *Carassius auratus*.

system is continuously affected by fluctuations in the level of salinity, temperature, pH and oxygen (Arthanari and Dhanapalan 2016), and the influence of environmental changes on the number, morphology and distribution of blood cells is clear from the literature (Giaquinta and Saija 2010). Hence, an early detection of changes in these parameters could allow an early identification of any disorder in fish, long before the appearance of any visible manifestation of disease. Blood parameters are therefore often used as indices to assess the welfare of fish and the changes in their biology according to the environmental changes (Gabriel *et al.* 2004; Fazio *et al.* 2012a,b,c, 2013; Sheikh and Ahmed 2016). The growing interest in the use and validation of haematological parameters as fish health monitoring tools is mainly due to aquacultural purposes and comparative physiology (Suljevic *et al.* 2016). However, in order to use the haematological parameters as a biomarker it is necessary a thorough knowledge of the standard and reference values for the various fish species. However, these values are not yet well defined for some species that live in different habitats. The purpose of the present study was therefore to provide a baseline data of the blood profile of two teleost fish species living in different environments and with divergent feeding behaviour, namely the flathead grey mullet *Mugil cephalus* (Fazio *et al.* 2016), a marine herbivorous fish, and the goldfish *Carassius auratus* (Fazio *et al.* 2012c), a freshwater omnivorous fish. These species were selected because of their ecological and economical relevance, being an aquaculture species and a common aquarium fish, respectively. Significant variations in almost all the haematological parameters investigated

were found between *M. cephalus* and *C. auratus* for all blood cells. The increase in RBC was revealed and microscopically observed in *M. cephalus* in respect to *C. auratus*. It is well known that the RBC of an organism determines its ability to transport dissolved oxygen. The differences observed between the two species can be attributed both to the specific difference of species and to their different physiology. As already reported in the literature (Svobodova et al., 2008), more active species have higher RBCs. In fact the RBC values increase in the species that make rapid movements and are very active, as already seen in other studies (Fazio *et al.* 2012a,b, 2013, 2016). Furthermore, environmental factors, such as salinity, directly influence haematological parameters, such as RBC. In fact, the increase in the number of red blood cells and the reduction of their volume found in the mullet is linked to an adaptation of the species to the typical salinity of the sea. In fact, the transport of oxygen in the salty water is much faster than in fresh water, this leads to a degeneration of erythrocytes that are replaced by cells of reduced volume (Izergina *et al.* 2007). As for the white blood cells, they have a very important role for the immune defences, while the thrombocytes mainly serve to form protective barriers with phagocytosis (Magnadòttir 2006). In our analyzes, the mullet has lower WBC and TC values than the goldfish. These two parameters vary mainly according to the environmental parameters, the characteristics of the fish, their eating habits, but also external stimuli, such as an infection (Parrino *et al.* 2018). In this case it is mainly the alimentary habit to change the parameters,in fact the mullet that is a purely herbivorous species has lower values than the red fish that is omnivorous. Similar data have been found in other marine carnivorous species (Sparus aurata and Dicentrarcus labrax) with higher WBC and TC values than M. cephalus (Fazio *et al.* 2016). In addition, the salinity, among the environmental factors, inversely affects the WBC trend of the grey mullet, as seen in a study conducted in two environments with different physical and chemical characteristics in comparison (Fazio *et al.* 2012c). The results obtained from this research will allow to build a data base on the blood profile of two fish species of significant importance from the commercial and ecological point of view (M. cephalus and C. auratus), in order to better understand the effects exerted by the different environmental factors and the diversity of eating habits on the haematological parameters of fish. The application of these methods is a valuable teaching aid useful for the complete evaluation of blood cells in teleosts whose role of bioindicators is increasingly emerging. These different methods of analysis represent new approaches for teaching and assessment of blood cells in different teleost species. Future researches will be aimed at improving the application of flow cytometry as monitoring tool of fish health status, and investigate other fish species of interest for biomonitoring in order to expand the basic knowledge on their blood characteristics and adaptability to the environment.

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