The European Zoological Journal

ISSN: (Print) 2475-0263 (Online) Journal homepage: http://www.tandfonline.com/loi/tizo21

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To cite this article: V. Parrino, T. Cappello, G. Costa, C. Cannavà, M. Sanfilippo, F. Fazio & S. Fasulo (2018) Comparative study of haematology of two teleost fish (Mugil cephalus and Carassius auratus) from different environments and feeding habits, The European Zoological Journal, 85:1, 194-200, DOI: 10.1080/24750263.2018.1460694

To link to this article: https://doi.org/10.1080/24750263.2018.1460694

Published online: 02 May 2018.

The European Zoological Journal, 2018, 194–200
Vol. 85, No. 1, https://doi.org/10.1080/24750263.2018.1460694

Comparative study of haematology of two teleost fish (*Mugil cephalus* and *Carassius auratus*) from different environments and feeding habits

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(Received 2 November 2017; accepted 29 March 2018)

Abstract
Haematological parameters are valuable indicators of fish health status. This study is aimed to provide 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* Linnaeus, 1758, a marine herbivorous fish, and the goldfish *Carassius auratus* (Linnaeus, 1758), a freshwater omnivorous fish. Using an automated system coupled with flow cytometry and light microscopy, significant variations were found between *M. cephalus* and *C. auratus* blood parameters, except for haemoglobin concentration (Hgb). A significant increase in red blood cell count (RBC) and haematocrit (Hct) levels, associated with reduced mean corpuscular volume (MCV), was revealed in mullets in respect to goldfish. These data may be attributable to differences in fish species, or to their divergent physiological activeness as high RBC values are associated with fast movement and high activity with streamlined bodies, or to environmental factors such as water salinity, an increase in which may lead to erythropoiesis as an adaptive process in seawater fish. Additionally, lower values of white blood cell count (WBC) and thrombocyte count (TC) were recorded in mullets with respect to goldfish, and these changes may be due to divergent feeding habits of the two fish species, or to their different environments since increased salinity may inversely affect WBC. Overall, findings from this study provide a better understanding of the influences of divergent environmental conditions and feeding habits on fish blood parameters. The combined use of an automatic haematological count with flow cytometry was demonstrated to be effective for an early assessment of blood parameters in different fish species.

Keywords: *Blood parameters*, mullet *(Mugil cephalus)*, goldfish *(Carassius auratus)*, *flow cytometry*, *light microscopy*

Introduction

Haematological parameters are commonly used as valuable indicators for the assessment of fish health status (Gabriel et al. 2004). Variations in blood parameters depend upon the fish species, aquatic biotope, health and nutritional status, age and sexual maturity (Blaxhall 1972; Chaudhuri et al. 1986; Wilhem et al. 1992; Hrubec et al. 2001; Fazio et al. 2016). Moreover, blood parameters of fish are highly sensitive to environmental changes. Quality of water, oxygen, temperature and salinity are directly reflected in blood parameters (LeaMaster et al. 1990; Luskovav 1997; Sheikh & Ahmed 2016), as well as basic ecological factors such as feeding regime and stocking density (Což-Rakovac et al. 2005; Ferri et al. 2011). A correct interpretation of fish haematology depends on the availability of reference values, helpful in understanding the relationship of blood characteristics to the phylogeny, activity, habitat and adaptability of the species to the environment (Blaxhall 1972; Wilhem et al. 1992). This study is therefore aimed at providing baseline data of the haematological profile of two teleost fish species living in different aquatic environments, namely the flathead grey mullet *Mugil*

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Published online 02 May 2018

cephalus Linnaeus, 1758, a marine herbivorous fish, and the goldfish *Carassius auratus* (Linnaeus, 1758), a freshwater omnivorous fish.

Grey mullets (Osteichthyes: Mugilidae) have a worldwide distribution, commonly inhabiting tropical and warm-temperate estuaries. Even if spawning occurs in the sea, grey mullets are highly euryhaline as they are capable of living in a wide range of salinities, from marine to estuarine and freshwater environments (Cardona 2006; Brandão et al. 2015; Cappello et al. 2016a,b). Although *M. cephalus* larvae are planktivorous, juveniles and adults undergo a change in diet and feed mainly on detritus and benthic microalgae, especially diatoms (Whitfield et al. 2012). *Mugil cephalus* is also a species of considerable commercial value as it is widely exploited worldwide, being an important fishing resource as well as aquaculture species (Cardona 2006; Whitfield et al. 2012).

Goldfish (Cypriniformes: Cyprinidae) primarily inhabit freshwater environments and are distributed widely in and around the Eurasian continent (Takada et al. 2010). Goldfish are ominvorous, and their diet includes planktonic crustaceans, phytoplankton, insect larvae, fish eggs and fry, benthic vegetation and detritus (Specziár et al. 1998). Goldfish can tolerate low oxygen levels, temperature fluctuations and high levels of anthropogenic pollution, and for these reasons they are frequently used as sentinel species in various laboratory studies (Fan et al. 2013; Maisano et al. 2013; Zhelev et al. 2016). Also, *C. auratus* is a domesticated ornamental fish of economic relevance in the freshwater aquarium fish industry.

The haematological profiles of the grey mullet *M. cephalus* and the goldfish *C. auratus* were herein determined by the use of an automated system, coupled with flow cytometry and light microscopy.

Material and methods

Fish collection and acclimation

Twenty flathead grey mullets *M. cephalus* were caught from Faro Lake, a coastal brackish system located in north-eastern Sicily, Italy (Fazio et al. 2013; D'Agata et al. 2014; Maisano et al. 2016b). All fishes (fork length: 21.25 ± 2.72 cm; weight: 75.40 ± 12.35 g) were considered healthy on the basis of an external examination for any signs of abnormalities or infestation. After collection, all animals were transported to the laboratory to be acclimated for 3 weeks in 400-L tanks each containing pond water (physico-chemical characteristics are reported in Table I), equipped with filter and oxygenation systems. According to a previous study conducted in the laboratory with mullets (Faggio et al. 2013), for the first 3 days of the acclimatisation period, fish were fasted and then fed twice per day with commercial floating pelleted feed (0.45 cm diameter), the proximate composition of which was, on a wet basis, 8.9% moisture, 51.1% protein, 8.0% lipid and 11% ash.

Additionally, 20 goldfish *C. auratus* (fork length: 18.13 ± 1.45 cm; weight: 70.50 ± 7.20 g) were purchased from a commercial supplier. Upon arrival, fish were transferred to circular tanks supplied with aerated and dechloraminated water, the physico-chemical features of which are reported in Table I. The fish were held in aquaria for acclimation for 3 weeks, and fed with commercial pellets (0.3 cm size) 2 times a day.

For the entire acclimation period, both the mullet tanks and goldfish tanks were maintained under a 12:12-h light:dark photoperiod. Water temperature, pH, salinity, dissolved oxygen (DO_2) and ammonia (NH_3/NH_4) levels, as reported in Table I, were checked daily in each tank using a multi-parametric probe C 203 (Hanna-Instruments, United Kingdom). No mortality was recorded during the entire acclimation period.

Fish blood collection

For both mullets and goldfish, feeding was stopped 24 h prior to blood sampling. The fish were anaesthetised using tricaine methane sulfonate (MS-222; 0.3 g/L) immediately before blood collection. Blood samples were drawn from the caudal vein using a sterile plastic syringe (2.5 mL), and transferred into microtubes (Miniplast 0.6 mL, LP Italiana Spa, Milano) containing ethylenediamine tetraacetic acid (EDTA, 1.26 mg/0.6 mL) as the anticoagulant agent. Animal maintenance and experimental procedures were in accordance with the ethical guidelines

Table I. Water physico-chemical characteristics for acclimation of *Mugil cephalus* and *Carassius auratus*.

Fish species	Temperature	pH	Conductivity	Dissolved oxygen (DO_2)	Salinity	(NH_3/NH_4)
M. cephalus	18.2°C	8.23	47.6 mS/cm	5.7 mg/L	38°/oo	0.20 mg/L
C. auratus	18.1°C	8.01	342.1 µS/cm	5.3 mg/L	0.03°/oo	0.23 mg/L

of the European Union Council (Guide for Care and Use of Laboratory Animals, Directive 2010/63/EU).

Automatic haematological analysis

The haematological profile was determined immediately after collection of mullet and goldfish whole-blood samples using an automated haematology analyser (HeCo Vet C; SEAC, Florence, Italy). This apparatus uses an impedance analysis system that was already used and validated by comparative manual tests in the veterinary field to investigate haematological profiles in various fish species (Faggio et al. 2013; Fazio et al. 2013, 2016). Evaluation of the haemogram involved the determination of the red blood cell count (RBC), haematocrit (Hct), haemoglobin concentration (Hgb), white blood cell count (WBC), thrombocyte count (TC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). All fish blood samples were analysed in duplicate by the same operator.

Flow cytometric analysis

Flow cytometric analysis was performed within 5 h of drawing mullet and goldfish whole-blood samples, using ImageStream^X (Amnis, Seattle, WA), a multispectral flow cytometer combining standard microscopy with flow cytometry. It can acquire up to 100 cells/s, with simultaneous acquisition of six images of each cell, including bright field, scatter and multiple fluorescent images. For the present study, the integrated software INSPIRE, running on the ImageStream^X Mark II, was applied. Samples were always left on ice before being injected into the flow cell. Then, the cells were allowed to form a single core stream before acquisition. Images were analysed using IDEAS image-analysis software (Amnis).

Morphological analysis

Morphological analysis of the grey mullet *M. cephalus* and goldfish *C. auratus* erythrocytes was carried out by smearing heparinised whole blood on a glass slide. The slides were air-dried overnight and then fixed in absolute methanol for 20 min before staining with 10% Giemsa solution for 15 min. Blood smears were observed using a 63 × oil-immersion objective with a motorised Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany).

Statistical analysis

Data obtained for haematological parameters were tested for normality using Kolmogorov–Smirnov test, using $P < 0.05$ as the threshold for significance. Unpaired *t*-tests were used to determine statistical differences between the two fish species in all parameters measured. Data were considered statistically significant at $P < 0.05$. All calculations were carried out using the statistical software Prism v. 5.00 (Graph Pad software Ldt., USA, 2003).

Results

Automatic haematology

The haematological parameters of the two teleost fish species, *M. cephalus* and *C. auratus*, are reported as mean values ± standard error of the mean (SEM) in Table II. In detail, a statistically significant increase in the levels of RBC and Hct was recorded in grey mullets with respect to goldfish. In contrast, the values of MCV, MCH, MCHC, WBC and TC were statistically significantly lower in mullets than goldfish. Additionally, no statistical differences between the two teleost fish were found in the level of Hgb.

Table II. Mean values ± standard error of the mean (SEM) of haematological parameters recorded in the two teleost species *Mugil cephalus* and *Carassius auratus* (*$P < 0.05$).

Haematological parameters	*Mugil cephalus* (n = 20)	*Carassius auratus* (n = 20)
RBC (×10^6/μL)	2.08 ± 0.16*	0.50 ± 0.01
Hct (%)	19.55 ± 1.86*	7.56 ± 0.12
Hgb (g/dL)	4.45 ± 0.33	4.18 ± 0.18
MCV (fL)	92.11 ± 2.10*	1152.80 ± 3.70
MCH (pg)	21.58 ± 0.36*	84.54 ± 3.79
MCHC (g/dL)	23.63 ± 0.61*	55.93 ± 2.72
WBC (× 10^3/μL)	30.08 ± 2.28*	66.35 ± 2.46
TC (× 10^3/μL)	27.96 ± 3.58*	83.75 ± 4.19

RBC, red blood cell count; Hct, haematocrit; Hgb, haemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; WBC, white blood cell count; TC, thrombocyte count.

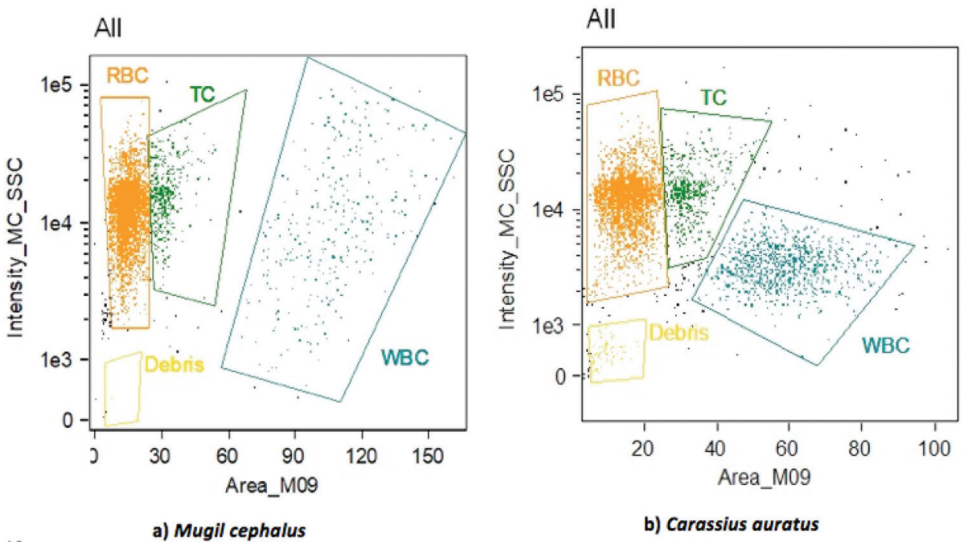

Figure 1. Flow cytometry performed on blood samples of (a) the grey mullet *Mugil cephalus* and (b) the goldfish *Carassius auratus*. The score plots show a clear grouping of red blood cells (RBC; in orange), white blood cells (WBC; in blue), thrombocytes (TC; in green) and debris (in yellow).

Flow cytometry

The results obtained by the flow cytometric analysis performed on the blood samples of the two teleost fish are shown in Figure 1. A clear grouping of the three main blood cell populations, namely RBC, WBC and TC, is depicted in the score plots, as well as appreciable variations between the two fish species, with higher RBC and lower WBC and TC in the grey mullet *M. cephalus* (Figure 1(a)) than in goldfish *C. auratus* (Figure 1(b)).

Red blood cell morphology

Microscopic examination of fish blood samples revealed the presence of a high number of erythrocytes of reduced size in the grey mullet *M. cephalus* (Figure 2(a)). Conversely, the red blood cells of the goldfish *C. auratus* appeared lower in number but much greater in size in comparison with those observed in mullets (Figure 2(b)).

Discussion

Fish are known to live in a very intimate contact with their environment, and therefore they are extremely dependent upon it (Guerriero et al. 2003; Acharya & Mohanty 2014; Giannetto et al. 2014; Maisano et al. 2016a). It is well documented that ambient changes influence blood cell number, morphology and distribution (Srivastava & Choudhary 2010). Haematological parameters are therefore widely used as an early signal of changes in fish health status, and have proven to be a valuable approach also for monitoring the effects of

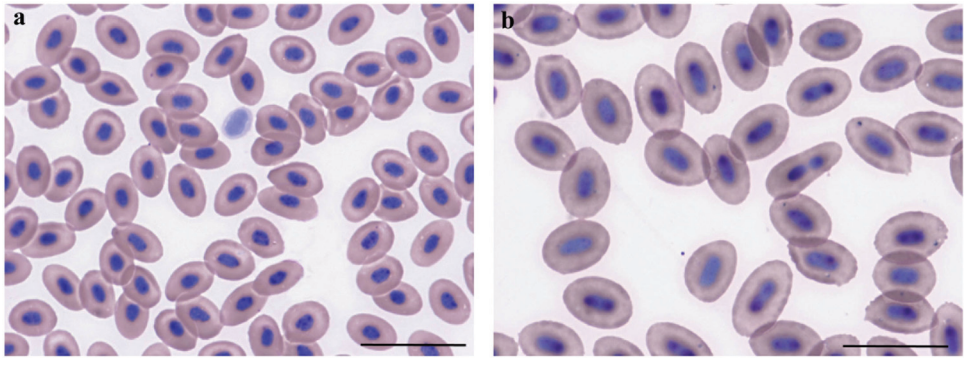

Figure 2. Erythrocytes from (a) the grey mullet *Mugil cephalus* and (b) the goldfish *Carassius auratus*. Scale bars: 20 μm.

habitat changes on fish biology (Gabriel et al. 2004; Fazio et al. 2012a, 2012b, 2012c, 2013; Sheikh & Ahmed 2016).

In order to use blood parameters as biomarkers, it is necessary to know their standard values and reference interval for a given fish species. However, the ranges of normal values of the key haematological parameters are still undefined for some fish species living in different habitats. The evaluation of the haematological parameters of mullets and goldfish was herein conducted using an automated system, together with flow cytometry and light microscopy, which provided comparable results.

Except for the Hgb value that proved to be nearly the same in the two fish species, a significant increase in RBC and Hct levels, coupled with reduced MCV (and therefore directly linked also to decreased MCH and MCHC), were revealed and microscopically observed in *M. cephalus* with respect to *C. auratus*. It is known that the RBC of an organism determines the carrying capacity of dissolved oxygen (Al 2000). Therefore, the differences herein observed may be attributable to the divergent physiological activeness of the examined fish species. As previously reported by Svobodova and collaborators (Svobodova et al. 2008), active species present higher values of haematological parameters compared to less active forms. Indeed, high RBC values are usually associated with fast movement and high activity with streamlined bodies, as already documented in various studies conducted on wild and farmed species, including grey mullets (Fazio et al. 2012a, 2012b, 2013, 2016). Additionally, environmental factors such as water salinity have a direct effect on various blood parameters such as RBC and Hct through their effect on the haemoglobin oxygen-binding properties and thus on oxygen transport (Witeska 2013). The increased number of erythrocytes and concomitant reduction in their volume recorded herein in mullets is due to an adaptive process to salinity of seawater habitat. Oxygen transportation in salt water moves faster than in fresh water, and this implies a degeneration of part of the red cells resulting in an increased erythropoiesis. This leads to an augmented production of new erythrocytes with a decreased volume unit. Similar data were provided by Izergina et al. (2007), after investigating the influence of water salinity on the physiological status of juvenile chum salmon.

Interestingly, lower values of WBC and TC were herein recorded in mullets with respect to goldfish. It is known that WBC play a major role in the immune defensive system of fish, whereas TC are blood cells that serve mainly to form protective barriers (Magnadottir 2006). The numbers of WBC and TC may change in relation to various environmental parameters or stimuli such as infection, as well as multiple other factors, from the age of fish to species characteristics or nutritional differences (Romano et al. 2017), which may explain the variations in WBC and TC values observed herein in mullets compared to goldfish, probably reflecting the different feeding habits of the two fish species under study. Notably, in a previous study it was reported that *M. cephalus* showed the lowest WBC and TC values with respect to two other seawater carnivores species, namely *Sparus aurata* and *Dicentrarcus labrax* (Fazio et al. 2016). Also, increased salinity may inversely affect the values of WBC as documented for *M. cephalus* collected from two habitats with different physico-chemical features (Fazio et al. 2012c).

The results of this preliminary study provide basic knowledge of the blood profile of *M. cephalus* and *C. auratus*, two teleost species of ecological and economic importance, allowing better comprehension of the influences of divergent environmental conditions and feeding habits on fish blood parameters. Additionally, findings from this study demonstrate the effectiveness of coupling an automatic haematological count with flow cytometry as diagnostic tools for an early understanding of the variability of blood cells in different fish species.

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical approval

All sampling, animal maintenance and experimental procedures of this study involving fishes were performed in accordance with the ethical guidelines of the European Union Council, namely the Guide for the Care and Use of Laboratory Animals, Directive 2010/63/EU.

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