

Blood cellular components in wild caught *Muraena helena* (Muraenidae)

by

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ABSTRACT. - Wild caught moray eels, *Muraena helena* L. 1758, were collected in Adriatic Sea near Dubrovnik, Croatia. Blood cells were evaluated by Natt-Hericks and MGG stain. Mean haematocrit was 23.22 ± 3.13 . RBC count was $4.007 \pm 1.60 \times 10^{11}/L$. Thrombocytes were present in four forms. Average WBC count was 3.37% of the total cell count. On average lymphocytes accounted for 15.05%, up to maximally 23% of WBC. Monocytes were least present (on average 4.59%). Basophiles or eosinophiles were not found and analogous to the reports in some kin species (*Anguilla anguilla*) they probably don't exist in spotted moray at all. Two types of granulocytes both with eccentric, round or bilobed nuclei were the most abundant leukocytes (49.13% and 31.23% of WBC respectively). The prevailing granulocyte was standard neutrophile (heterophile) found in all fish species. Second most abundant granulocyte type had the shape, size and cytoplasmic granules identical to the neutrophile but MGG stain revealed its high cytoplasmic affinity towards basophilic dye. No intermediate phases between two types were found indicating they are diverse cell types. This second granulocyte type may be a distinctive feature of innate immunity of spotted moray.

RÉSUMÉ. - Cellules sanguines chez la murène *Muraena helena* (Muraenidae) sauvage.

Des murènes *Muraena helena* L. 1758 ont été capturées en mer Adriatique près de Dubrovnik, Croatie. Les cellules sanguines ont été étudiées après coloration selon les techniques de Natt-Hericks et May-Grunwald/Giemsa (MGG). La valeur moyenne de l'hématocrite est $23,22 \pm 3,13$. Le nombre de globules rouges (RBC) est $4,007 \pm 1,60 \times 10^{11}/L$. Quatre formes de thrombocytes ont été identifiées. Le pourcentage moyen de globules blancs (WBC) est de 3,37%. Parmi ceux-ci les lymphocytes représentent 15-23%, et les monocytes sont en plus petit nombre (4,6%). Les basophiles et éosinophiles n'ont pas été identifiés et seraient probablement non présents comme chez une espèce proche (*Anguilla anguilla*). Deux sortes de granulocytes, toutes les deux avec des noyaux ronds ou bilobés, sont les leucocytes les plus abondants (49,1 et 31,2%, respectivement). La première correspond aux neutrophiles (hétérophiles) trouvés chez toutes les espèces de poissons. La deuxième présente la forme, la taille et des granules identiques aux neutrophiles mais leur cytoplasme est très basophile (coloration MGG). Aucune forme intermédiaire n'a été trouvée. La deuxième sorte de granulocyte pourrait être un trait caractéristique d'immunité innée chez la murène.

Key words. - Anguilliformes - Muraenidae - *Muraena helena* - Moray eels - Adriatic Sea - Granulocytes - Haematology.

Fish show wide diversity of haematological profile. Different cell types and structural heterogeneity is observed even between closely related species. No uniform blood cell classification was achieved until now (Hyder *et al.*, 1983; Parish *et al.*, 1986; Thuvander *et al.*, 1987). Piscine blood cells are generally less differentiated than their mammalian counterparts, making them more difficult to distinguish between species (Thrall *et al.*, 2004). Each fish species must be analysed separately for its distinctive specialities (Ainsworth, 1992). Identification of different piscine blood cells combined with other routine diagnostic methods indicates physiological health status of wild populations and assess the conditions that cause stress to the fish, as a consequence

of mishandling, disease, parasite infections, bioaccumulation and biomagnification of pollutants (Kakuta and Nakai 1992; Anderson and Zeeman, 1995; Sasal *et al.*, 1997; van Ginneken *et al.*, 2005; Bartoli and Gibson, 2007; Clauss *et al.*, 2008). Haematology data may mirror circannual ecology and provide comparative reference for captive kept specimens in potential aquaculture (Larsson *et al.*, 1976). For many fish species there is no haematological reference.

The description of blood cells in *Muraena helena*, one of the oldest described species of the moray eels, is not existing while some recent data on its genome (Pichiri *et al.*, 1995; Ronchetti *et al.*, 1995; Pichiri *et al.*, 2000) or on the structure of its jaws are available (Mehta and Wainwright, 2007).

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This could be due to the fact that blood sampling in wild population of *M. helena* is somewhat difficult considering the secretive life style, individual dispersion and aggressive behaviour.

Our work aspires to fill the gap on basic knowledge of morphologic and quantitative description of blood cells in *M. helena*, a commercially interesting and appreciated fish (Fishbase, 2010). Description of cells of circulating blood in *M. helena* revealed existing blood cell characteristics of this fish and append to the ongoing discussion on comparative fish haematology.

MATERIALS AND METHODS

Animals and environmental conditions

Morays were collected in summer (August) in Adriatic Sea, Elaphite Islands near Dubrovnik, Croatia. Environmental conditions: depth from 5–10 m, sea temperature $22.5 \pm 0.6^\circ\text{C}$ (four measurements at various depth at which fish have been caught). A total of 18 fish were analysed. Fish were caught by 200 m of long line hooks all at the same season to assure that fish have been analysed under approximately same environmental conditions and that the sample is representative by uniformity. Taking into account the nocturnal habits of the species the hooks were set at 03:00 in the morning and collected two hours later. All fish appeared healthy and very agile (active-aggressive).

Each fish was sedated individually for 15 minutes with MS222 (Sigma) in a separate 100 L plastic barrel in oxygenated seawater (MS222 dose = 250 mg L^{-1}). After sedation morphometric parameters (BL = body length, BM = body mass, VG = ventral girth, CG = caudal girth) were measured. Body mass index (BMI) was calculated from BM and BL ($\text{BMI} = \text{BM} / \text{BL}^2$). Age was estimated by analysing otoliths of each individual fish as described by Matić-Skoko *et al.* (2010). The age analysis showed that fish were in their 5 years (N = 5), 6 years (N = 6), 7 years (N = 2), 9 years (N = 2), 10 years (N = 3).

Blood analysis

Blood was collected from the heart with a 10 ml syringe with anticoagulant heparin (Sigma) and processed immediately to cell analysis. After blood collection all fish were sacrificed by instant decapitation. Detailed examinations by veterinarian on board (co-author A. Gavrilović) established absence of any external parasites or other pathological changes. No internal blood parasites or histopathological changes were present after inspection under microscope.

Erythrocyte, leukocyte and thrombocyte counts were performed from heparin-anticoagulated blood samples by Natt and Herrick's stain as described by Campbell and Murru (1990). All chemicals for blood analysis were

obtained from Sigma and Merck.

Samples were diluted 1:200 in stain immediately after sampling and counted under light microscope in the ship laboratory after cells become visible (approximately 10–15 minutes after blood collection) on Bürker-Türk hemocytometer. For each fish a duplicate was counted on upper and lower grid. Erythrocytes, leukocytes and thrombocytes were counted separately (three counts per grid).

Haematocrit was assessed on board by centrifugation of heparinised micro-haematocrit capillaries with the sample of blood at 115 g ($g = 118 \times 10^{-7} \times r \times n^2$; $n = 1400 \text{ rev min}^{-1}$, $r = 5 \text{ cm}$) for 5 minutes, room temperature in micro-centrifuge (Microfuge) immediately upon sampling. Haematocrit was determined by micro-haematocrit reader scale provided with the centrifuge.

Smears of blood film (four per animal) were made immediately after sampling, air dried for one day, taken to laboratory in Zagreb, stained with May-Grunwald/Giemsa (MGG) solutions for light microscopy and analysed for differential erythrocyte and leukocyte count. The slides were examined under oil-immersion at 1000 magnification. For each slide two cell counts have been carried out. First count was made to differentiate and classify various types of the erythrocytes and leucocytes. For this purpose 1000 RBC and 1000 WBC were counted randomly. The second count on 1000 randomly encountered cells was made to re-calculate the erythrocyte-thrombocyte-leucocytes ratio to recheck the results obtained on a hemocytometer at ship laboratory. This was necessary since some leukocytes and round thrombocytes under Natt and Herrick's stain may have similar appearance.

Slides with blood smear were also used for measuring size of individual cell types under a microscope with program Axiovision 4.8.2.0. (Carl-Zeiss Microimaging GmbH, Germany). Each size measurement was done on 100 cells of each type.

Statistical analysis

The computational program STATISTICA 9.1 (Statistica software, Tulsa USA) was used to determine descriptive statistics and data analysis. The statistical differences between measurements of various cells size were compared by Student t-test. Correlation analysis of log-transformed data of cell numbers and arcsine transformed data on percentages was performed to establish the connection between morphometric and age data and haematology parameters. The level of statistical significance was set to $p \leq 0.05$.

RESULTS

Morphometric data of *M. helena*

Measured and calculated morphometric parameters are shown in table I. There was a significant correlation

between body length (BL) and body weight (BW) of the fish ($r^2 = 0.796$, $p = 0.0102$).

Haematocrit and total number of blood cells in *M. helena*

On average there were 4.447×10^{11} cells per litre of blood (Tab. II). Haematocrit was 23.22% of the total blood volume. Haematocrit values were significantly correlated with age ($r^2 = 0.875$, $p = 0.00021$) but not significantly correlated with BL ($r^2 = 0.309$, $p = 0.112$) or BM ($r^2 = 0.303$, $p = 0.426$). Correlations between BMI and haematocrit

($r^2 = 0.222$, $p = 0.340$), BMI and total cell count ($r^2 = 0.007$, $p = 0.933$) and total cell count and age ($r^2 = 0.402$, $p = 0.06$) were also not significant.

Erythrocytes (RBC) and differential RBC count

In *M. helena* the RBC were elliptical cells with a central nucleus generally following the shape of the cell (Figure 1.A). RBC had a compact chromatin and acidophilic cytoplasm, which occupied most of the cell. No significant correlation between RBC number (Tab. II) and BMI have been found ($r^2 = 0.039$, $p = 0.802$) but correlation of age and RBC number showed $r^2 = 0.425$, $p = 0.049$. Different percentages of observed developmental stages of RBC are presented in table III.

Approximately 97.74% were mature RBC (cell size: $16.75 \pm 1.20 \mu\text{m}$ length, $10.70 \pm 1.24 \mu\text{m}$ width; nucleus size: $6.40 \pm 1.20 \mu\text{m}$ length, $4.23 \pm 1.06 \mu\text{m}$ width). On average, less than 1% from the total RBC number belonged to some developing stage (erythroblasts). Two types of juvenile erythrocytes were present. The first one (Fig. 1C), polychromatophilic erythrocytes (cell size: $13.86 \pm 0.98 \mu\text{m}$ length, $10.26 \pm 0.45 \mu\text{m}$ width), were significantly smaller than mature cells ($p \leq 0.05$) with more rounded cell shape and more rounded, centrally located nucleus (nucleus size: $6.94 \pm 1.36 \mu\text{m}$ length, $5.42 \pm 0.86 \mu\text{m}$ width) and with cytoplasm giving cell lightly bluish coloration. The second juvenile RBC was the basophilic erythroblasts (Fig. 1B) with grey blue-red cytoplasm. Nucleus of both types of juvenile RBC stained less intensely than in mature erythrocytes and their chromatin was not condensed as in mature RBC. Old erythrocytes (Fig. 1E-H) differed from mature ones by significantly bigger ($p \leq 0.05$) and more rounded cells (cell size: $19.13 \pm 1.20 \mu\text{m}$ length, $13.61 \pm 0.70 \mu\text{m}$ width) with weakly coloured, or almost colourless cytoplasm and round and reddish rounded nuclei (nucleus size: $6.78 \pm 1.20 \mu\text{m}$ length, $6.46 \pm 1.45 \mu\text{m}$ width). Senile RBC forms were less than 1% of the overall erythrocyte count.

Table I. - Morphometric values of wild caught *Muraena helena* L. BMI: body-mass index; SD: standard deviation; Min: minimum; Max: maximum.

Parameter	Mean	SD	Min	Max	Median
Body weight (g)	1296.33	804.45	751.00	3322.00	1043.00
Body length (cm)	72.37	13.41	60.20	93.20	64.70
Ventral girth (cm)	14.53	2.87	11.50	19.50	14.00
Caudal girth (cm)	11.38	2.19	8.50	15.00	11.00
BMI	0.23	0.06	0.18	0.38	0.21

Table II. - Cellularity of circulating blood in wild caught *Muraena helena* L. SD: standard deviation; RBC: red blood cells; WBC: white blood cells; Min: minimum; Max: maximum.

Parameter	Mean	SD	Min	Max	Median
Haematocrit (%/L)	23.22	3.13	28.00	36.00	28.00
Total cellularity ($\times 10^{11}/\text{L}$)	4.447	1.80	2.422	8.203	4.828
RBC count ($\times 10^{11}/\text{L}$)	4.007	1.60	2.137	7.490	4.312
WBC count ($\times 10^{10}/\text{L}$)	2.021	0.70	0.901	2.790	1.934
Thrombocyte count ($\times 10^{10}/\text{L}$)	2.629	1.20	1.384	4.710	2.190

Table III. - Differential erythrocyte count in wild caught *Muraena helena* L. SD: standard deviation; RBC: red blood cells; Min: minimum; Max: maximum; n: number of individual animals; N: total number of examined animals.

RBC subtype	Percentage of total RBC count				(n/N)x100
	Mean	SD	Min	Max	
Mature erythrocytes	97.74	1.35	95.50	98.90	100
Basophylic erythroblasts	0.13	0.17	0.00	0.49	55.56
Polychromatophilic erythrocytes (Proerythrocytes)	0.86	0.65	0.09	1.86	100
Old erythrocytes	0.17	0.21	0.00	0.57	100
Old achromatic erythrocytes	0.11	0.19	0.00	0.57	44.44
RBC Nuclei (Exocyttoplamic)	0.74	0.67	0.19	2.40	100
Round or swollen erythrocytes	0.16	0.46	0.00	1.38	22.22
Erythrocytes with eccentric nuclei	0.01	0.03	0.00	0.10	11.11
Microcytes	0.04	0.13	0.00	0.39	11.11
Erythrocytes with micronucleus	0.38	0.91	0.00	2.74	22.22

Leukocytes (WBC) and differential WBC count

WBC mean values (Tab.II) revealed that on average leukocytes encompass 3.37% of the total blood cell count. Differential WBC counts (Tab. IV) revealed absence of basophiles and eosinophiles and presence

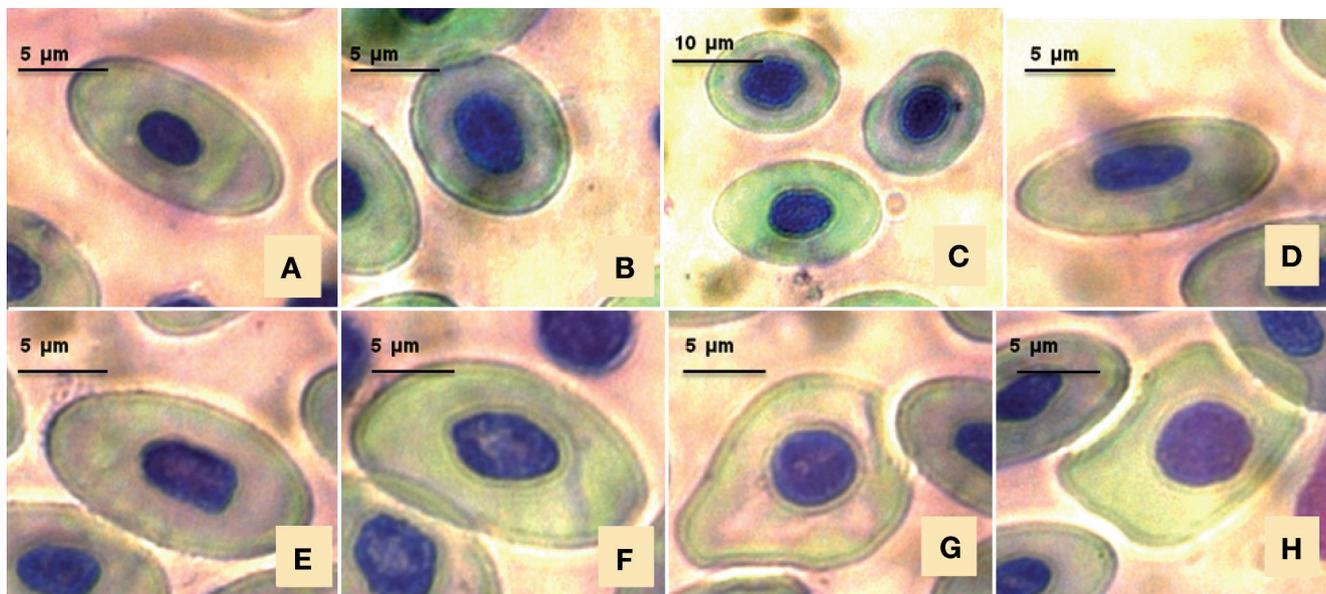


Figure 1. - Various forms of erythrocytes (RBC) found in circulating blood of *Muraena helena*. **A:** Normal (mature) erythrocyte; **B:** Basophilic erythroblast; **C:** Polychromatophilic erythrocyte (lower cell) with two basophilic erythroblasts; **D:** Erythrocyte with eccentrically positioned nucleus. **E-H:** Array of maturation stages of old, senile erythrocytes from swollen cell (E) to total degradation (H).

of monocytes, lymphocytes and granulocytes. Weak correlation between WBC count and BMI ($r^2 = 0.227$, $p = 0.337$) and age and WBC count ($r^2 = 0.088$, $p = 0.435$) were noted.

Neutrophiles (Heterophiles)

Two types of granulocytes dominated the total WBC count (Tab. IV, Fig. 2A-C). The prevailing one (average 49.13%), was identified as standard fish neutrophile (heterophile) (cell size: $14.86 \pm 1.56 \mu\text{m}$ length, $12.51 \pm 1.20 \mu\text{m}$ width; nucleus size: $7.47 \pm 0.65 \mu\text{m}$ length, $6.45 \pm 1.03 \mu\text{m}$ width). The second most abundant (average 31.23%) granulocyte type completely resembled the identified neutrophile (heterophile) by shape and size but showed high cytoplasmic

affinity towards basophilic dye of MGG stain. This second granulocyte type had spherically shaped cells, with average size (cell size: $14.82 \pm 1.75 \mu\text{m}$ length, $13.03 \pm 2.66 \mu\text{m}$ width; nucleus size: $6.81 \pm 1.40 \mu\text{m}$ length, $5.68 \pm 0.85 \mu\text{m}$ width) not significantly different ($p \geq 0.05$) from standard neutrophile (heterophile). In much of the cytoplasm small, deep violet/blue colour dots were present in high number, mainly aggregated near periphery of the cell. Eccentrically located rounded nucleus with patches of eu- and heterochromatin stained dark violet blue, giving nucleus rather granulated appearance. Because of the similarities in shape, size, occurrence and granules and single difference in stain affinity we marked this cell type as separate type of neutrophile (heterophile). Most importantly there were no intermediate forms between two cell types (Fig. 2C). Both types of cell were found in all fishes regardless of weight/length or age. Both described granulocyte types appeared with two forms of eccentric nuclei, round and bilobed (Tab. IV, Fig. 2D).

Table IV. - Differential leukocyte count in wild caught *Muraena helena* L. SD: standard deviation; WBC: white blood cells; Min: minimum; Max: maximum; n: number of individual animals; N: total number of examined animals.

WBC subtype	Percentage of total WBC count				(n/N)x100
	mean	SD	min	max	
Granulocytes					
Neutrophile (Heterophile)	45.03	13.77	25.00	66.00	100
Neutrophile (Heterophile)-bilobed	4.10	4.42	0.00	12.92	77.78
Neutrophile (Heterophile)-total	49.13	11.11	31.34	66.50	100
IBG Neutrophile (Heterophile)	30.00	11.98	0.00	52.07	100
IBG Neutrophile (Heterophile)-bilobed	1.23	1.19	0.00	3.33	77.78
IBG Neutrophile (Heterophile)-total	31.23	17.58	8.50	57.60	100
Agranulocytes					
Lymphocytes small	11.37	5.25	4.50	23.00	100
Lymphocytes large	3.68	3.65	0.00	11.11	100
Monocytes	4.59	2.88	0.50	8.50	100

Lymphocytes

M. helena lymphocytes (Tab. IV, Fig. 2F) were small round cells with large round nucleus which stained a dense deep red/violet colour. Nucleus occupied most of the cell and chromatin was compact and homogeneous. The basophilic-stained cytoplasm was

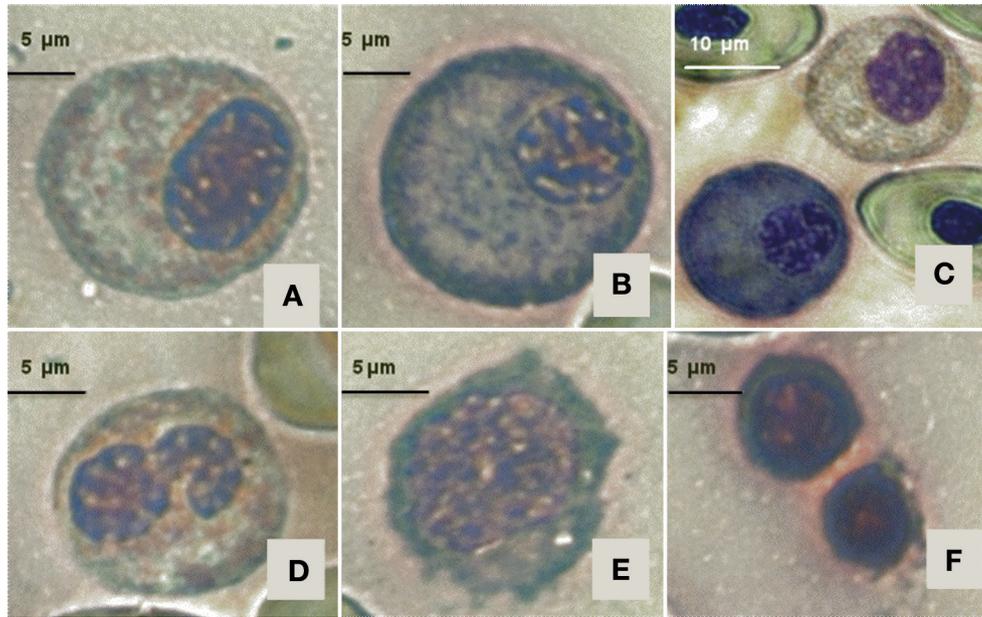


Figure 2. - Various types of leukocytes (WBC) found in circulating blood of *Muraena helena*. **A:** Neutrophile (Heterophile); **B:** Granulocyte with high cytoplasmic affinity towards basophilic dye (IBG); **C:** Comparison of both types of granulocytes; **D:** Neutrophile (Heterophile) with bilobed nuclei; **E:** Monocyte; **F:** Comparison of large lymphocyte (upper left) and small lymphocyte (lower right).

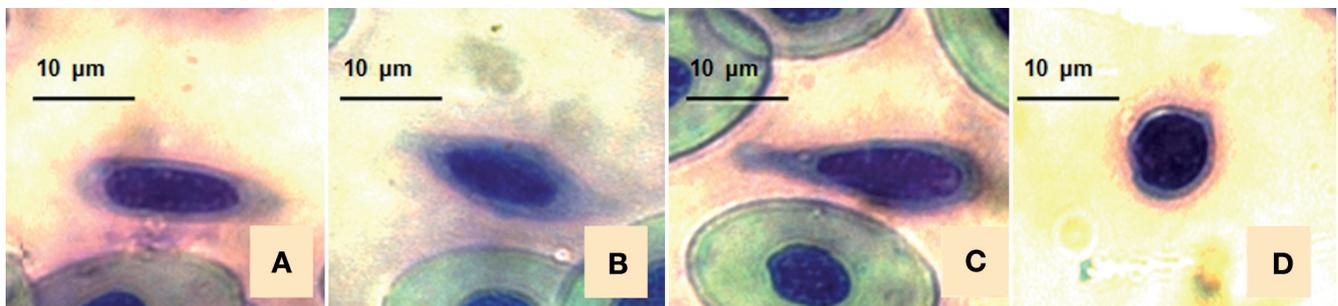


Figure 3. - Various types of thrombocytes found in circulating blood of *Muraena Helena*. **A:** Oval thrombocyte; **B:** Spindle shaped thrombocyte; **C:** Cone shaped thrombocyte; **D:** Round thrombocyte.

a tight dark blue ring close to nucleus. By size range lymphocytes were readily separated visually into two groups; the large (cell size: $12.87 \pm 1.94 \mu\text{m}$ length, $11.71 \pm 2.25 \mu\text{m}$ width; nucleus size: $9.29 \pm 1.06 \mu\text{m}$ length, $7.40 \pm 0.93 \mu\text{m}$ width) and the small lymphocytes (cell size: $8.34 \pm 0.75 \mu\text{m}$ length, $7.44 \pm 0.60 \mu\text{m}$ width; nucleus size: $7.82 \pm 0.50 \mu\text{m}$ length, $6.15 \pm 0.16 \mu\text{m}$ width). Large lymphocytes have been present as approximately one quarter (24.45%) of the total lymphocyte percentage (Tab. IV). On average lymphocytes (large and small) accounted for 15.05% of total WBC count, with up to maximally 23% of WBC count.

Monocytes

Monocytes in *M. helena* blood (cell size: $13.22 \pm 1.58 \mu\text{m}$ length, $11.59 \pm 1.49 \mu\text{m}$ width) were cells that mostly resembled large lymphocytes. However, these cells had darker basophilic violet blue nuclei (nucleus size: $9.27 \pm 1.65 \mu\text{m}$

length, $7.82 \pm 1.17 \mu\text{m}$ width) with distinguished granular formation of eu- and heterochromatin (Fig. 2E). Blue cytoplasm was darker than the cytoplasm in lymphocytes and broadly surrounded the nucleus giving cell rather uneven appearance. With approximately 5 % of the total WBC count these cell type have been the least present of all leukocytes (Tab. IV).

Thrombocytes

These cells appear in four forms: oval, round, elongated (cone) and spindle, separately from each other or in clusters. Oval form (Fig. 3A) had cell size: $14.99 \pm 1.58 \mu\text{m}$ length, $5.14 \pm 0.58 \mu\text{m}$ width and nucleus size: $9.30 \pm 0.71 \mu\text{m}$ length, $3.64 \pm 0.41 \mu\text{m}$ width). Round thrombocytes (Fig. 3D) had cell size: $7.33 \pm 1.22 \mu\text{m}$ length, $6.77 \pm 0.94 \mu\text{m}$ width and nucleus size: $6.06 \pm 0.86 \mu\text{m}$ length, $5.34 \pm 0.59 \mu\text{m}$ width. Other two less abundant forms were the cone and

spindle thrombocytes (Fig. 3B, C). Distinguishing round thrombocytes from small leucocytes was done on the basis that nucleus stained deep purple and the cytoplasm remained unstained around the cell in thrombocytes and the nucleus was lighter and cytoplasm visible in leukocytes. Thrombocyte count comprised on average 4.2% of total cell count (Tab. II). No significant correlation existed between thrombocytes count and BMI ($r^2 = 0.260$, $p = 0.725391$) or thrombocytes and age of the fish ($r^2 = 0.0077$, $p = 0.0802$).

DISCUSSION

Biology and life history of moray eels are still relatively unknown and remain to be understood. Except the description on haemoglobin composition in *M. helena* (Pellegrini *et al.*, 1995), even the basic data on haematology of *M. helena*, are not available in the literature and the present results are the first ones.

The weight-length range of the sampled fish was in accordance with reports of average weight/length recorded in the Adriatic sea and collected fishes shared morphometric features representative for the Adriatic population (Jardas, 1996; Matić-Skoko, 2010). *M. helena* blood parameters analysed in relation to the morphometric data showed low correlations except the correlation of age and haematocrit and age and RBC count. Measured hematologic parameters did not change with size of *M. helena* in the sampled range from 60.2 - 93.2 cm and 751 - 3322 g. While haematological values stay fairly constant over certain age (in this case 5-10 years) regardless of individual growth stage of the fish then haematological values were probably a reflection of environmental and seasonal conditions at the time of sampling. Similar conclusions appear in literature with the most comparable conclusions in related genus *Anguilla* (Johansson *et al.*, 1974). Total cell count, RBC and WBC count coincide with range of values reported in related species *Ghymnothorax funebris*. Furthermore, as in *Ghymnothorax funebris* erythrocytes in *M. helena* were larger in size (average = 16.75 μm) than in other teleosts (Francis-Floyd *et al.*, 1991). Interspecific comparison of RBC count and haematocrit recorded in *M. helena* fit in with the haematological frame of (semi) sedentary species. The results are in accordance with the findings of Filho *et al.* (1992) showing that active pelagic fish have higher haematology values (mostly RBC and haematocrit, WBC and thrombocytes depend on other factors), than sedentary and less active/sluggish species. Differentiating fish RBC aside usual leukocyte differentiation might be used in ecotoxicologic studies as done in other species (Strunjak-Perovic *et al.*, 2010) therefore a complete description of differential erythrocyte analysis was given in this work.

Following erythrocytes, the thrombocytes were the second most abundant blood cells in *M. helena*. Fish thrombo-

cytes exist in four different shapes (round, oval, cone and spindle) and frequently not all four types appear together in the same species (Campbell and Murru, 1990; Pastoret *et al.*, 1998). Good example of species-dependent occurrence of thrombocyte types is well presented by Pavlidis *et al.* (2007) among Sparidae. Our results let us to propose that *M. helena* is a species with all described forms of thrombocytes.

Fish leucocytes (WBC) are diverse in types and number with species and these differences are may be environmentally dependent. Neutrophils (heterophiles) are the most numerous granulocytes (20-60% of total WBC count) in individual species that may be occasionally further subdivided but the nomenclature of subpopulations is confusing and contradictory (Ainsworth, 1992; Hine, 1992; Suzuki and Iida, 1992). Specific differences of the species are sporadically found. In every analysed *M. Helena*, there were granulocytes similar to neutrophile (heterophile) taking into account their percentage ratio, their shape, their size and the cytoplasmic granules but with one prominent difference—the intensively basophilic cytoplasm. This cell type was hard to classify as either known granulocyte type (Campbell and Ellis, 2007). If this cell type was a different stage of development or activation of neutrophile (heterophile), then cells with characteristics of both types would appear on smears. There were no intermediary transitional stages between the two and it seems that the unidentified cell may be Type II neutrophile. In such cases the characterization of white blood cells on a simple morphological criterion requires additional studies for real identification of the cellular types. Further analysis might reveal that this cell is a Type II moray eel neutrophile. Until further analysis and for the purpose of expressing the percentage ratio of this cell type within limits of this descriptive study these distinctive cells have been nominated intensively basophilic granulocytes (IBG) of *M. helena*. Monocytes had a strong cytoplasmic affinity towards basophilic dye as well, however the monocytes were easily distinguishable from IBG by larger and irregular shape and larger centrally positioned nucleus and lack of granules in cytoplasm. Another feature that separates IBG from monocyte was its higher percentage in total WBC (Tab. IV). The percentage of identified monocytes did not digress from the values reported in literature for other fish, which rarely surpass 5%. Similarly IBG were distinguished from large lymphocytes by significantly bigger cell size. Morphologically IBG cell resembled avian or reptilian azurophiles (Pendl, 2006; Campbell and Ellis, 2007). No description on granulocytes was found on other moray species for comparison. In various *Anguilla* species descriptions of various cell types exist but with no report of similar cells (McArthur, 1977; Orecka-Grabida, 1986; Kusuda and Ikeda, 1987; van Ginniken *et al.*, 2005; Ponsen *et al.*, 2009).

Specialized characteristic cells of immune system are not uncommon in fish adapted to special biological and ecologi-

cal requirements. In *Salminus maxillosus* (Ranzani-Paiva *et al.*, 2003) for example authors report that beside standard heterophile they encountered a second similar granulocyte that didn't resemble any known fish granulocyte type (G1, G2, G3, Type I, II, III, etc.). Similar was a description of plasmocyte type of cell in *Maccullochella peelii peelii* (Shigdar *et al.*, 2009). Specialized granulocyte subtypes are common in many shark species as well (Ainsworth, 1992). All these species are predatory as is *M. helena*. Thus, specific cells types and higher granulocyte/leukocyte ratio might be a reflection of sporadic feeding on infected prey for it is generally accepted that the higher number of granulocytes than lymphocytes is clinically correlated with elevated bacterial exposure (Anderson and Zeeman, 1995; van Ginneken *et al.*, 2005; Clauss *et al.*, 2008). Besides, venomous properties of moray bites are attributed to populations of mouth bacteria (Erickson *et al.*, 1992). Nevertheless, high percentage ratio of neutrophiles and other cells with phagocytosis potential indicate the important physiological role of innate immune system in this fish.

Other two granulocytes, eosinophiles and basophiles were not detected in *M. helena*. Eosinophiles or basophiles are sporadically reported depending on species or environmental factors in range of 0-3% of total WBC count while in some fish species they don't appear at all. The physiological role of eosinophiles and even their presence in the piscine blood is disputed (Ellis, 1977; Cannon *et al.*, 1980; Hendrick *et al.*, 1986). Even between related species, one may lack these cell types, while other closely related species have it, for example various species of eel (McArthur, 1977; Orecka-Grabida, 1986; Kusuda and Ikeda, 1987; van Ginneken *et al.*, 2005; Ponsen *et al.*, 2009). Sometimes the lack is associated with the time of year at which the blood of certain species has been examined (Guijarro *et al.*, 2003). Lack of eosinophiles and basophiles in *M. helena* might be related to the season at which the fish were caught. Further sampling at other seasons or experimental exposure to pathogens might allow detection of the eosinophiles and basophiles in blood of *M. helena*. Further research by other assays might comprehend that described IBG cells compensate for their absence and partially take on their physiological role.

In conclusion, in *M. helena* the percentage and morphology of RBC, thrombocytes and agranular WBC does not diverge from general data recorded in other kin species such as *Gymnothorax funebris* (Francis-Floyd *et al.*, 1991) or other fish with a semi sedentary life style (Filho *et al.*, 1992). The high percentage of neutrophiles (heterophiles) in *M. helena* indicates the important role of innate immune defence in this fish. Detailed classification of WBC types in this species remains to be understood by use of other more discriminative methods.

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