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Haemoparasites of the genus *Haemogregarina* in a population of european pond turtles *(Emys orbicularis)* from Drăgăşani, Vâlcea county, Romania

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Introduction

Genus *Haemogregarina* Danilewsky, 1885 is included in phylum *Apicomplexa*, class *Sporozoasida*, order *Adeleina*, suborder *Adeleorina*, family *Haemogregarinidae*. Suborder *Adeleorina* includes seven families with fertilization and sporogony usually taking place in the definitive invertebrate host and merogony in the intermediate vertebrate host. Family *Haemogregarinidae* includes three genera with heteroxenous life-cycles, gamonts within erthrocytes and/or other blood cells, most with merogony in erythrocytes of vertebrate host; sporozoites excyst within invertebrate host, undergo merogony, and merozoites transmitted by bite of a leech vector. Genus *Haemogregarina* infects lower vertebrates (fish and reptiles) as intermediate hosts and leeches (as definitive hosts) (Wilford, 1977; Siddall and Desser, 1992).

Among reptiles, species of the genus *Haemogregarina* were recorded in turtles (Wilford, 1977; Siddall & Desser, 1992; Paterson & Desser, 1976; Acholonu, 1974), lizards (Elwasila, 1989) and crocodiles (Khan et all., 1980).

In this study, peripheral blood from eight European pond turtles (*Emys orbicularis*) was checked for haemoparasites.

Materials and methods

Turtles were collected in August 2002, from an irrigation channel, near Dragaşani, Vâlcea County, Romania. They were killed by decapitation, after general analgesia with ketamine hydrochloride (Calypsol®, Gedeon Richter, Budapest, Hungary), in a dose of 100 mg/kg b.w. Euthanasia was necessary because we studied also helminth parasites (data not published yet). Blood was collected on heparin, using a fine needle attached to a syringe. Blood smears were stained using DIA PANOPTIC ® (Reagens Kft; Diagon Kft, Hungary) and examined with the immersion objective and an Olympus BX41® microscope. Pictures of certain intra-erythrocytic forms were taken with an Olympus DP10 ® digital camera and processed using Adobe Photoshop 6.0. Intensivity of haemogragarine infection was assessed by counting erythrocytes from 30 microscope fields with the immersion objective and then reported in percents of infected erythrocytes from total counted erythrocytes. Percent of parasitic forms were determined for 50 parasites. Correlation coeficient was calculated between various parameters using Microsoft Excel 8.0

Results

Turtles were submitted to a complete parasitological examination. This paper presents only the results regarding the *Haemogregarina* sp. infection. *Haemogregarina* sp. stages found by us in *Emys orbicularis* were localized intraerythocitary, in the peripheral blood (Figure 1). No morphometric parameters were determined, because this criterion has little taxonomic value (Paterson & Desser, 1976; Siddall & Desser, 1992).

Epidemiology and statistics

Turtles were infested with *Placobdella costata* Müller, 1846 (phylum *Annelida*, subphylum *Clitellata*, class *Hirudinea*, order *Rhynchobdellida*, family *Glossiphoniidae*). The prevalence of leech infestation could not be determined, because after capture, turtles were kept together. Seven leeches were collected from the basin were turtles were kept.

Haemogregarina sp. infection prevalence in our study was 100%, all of the eight examined turtles being infected with various parasite stages. Intensity of infection (Table 1) varied between 0.08 and 2.20% (percent of infected erythrocytes). Correlation coefficient determined between the carapace length and infection intensity was of -0.63 (negative correlation), meaning that infection intensivity is higher in younger animals.

Table 1
Carapace length, sex, and haemogregarine infection intensity in *Emys orbicularis*

Turtle	Carapace length (mm)	Sex	*Haemogregarina* infection intensity (% of infected erythrocytes)
Emo/1	110	F	0.38
Emo/2	136	M	0.24
Emo/3	131	M	1.03
Emo/4	104	F	1.11
Emo/5	125	M	0.08
Emo/6	106	M	2.20
Emo/7	142	M	0.08
Emo/8	120	M	0.87

Results for differential parasitic forms count for each individual are presented in Table 2. The value of the correlation coefficient determined for carapace length and infection intensity, respectively for each parasitic form percentage is shown in Table 3.

The values shown in the Table 3 show that: the percent of trophozoites is higher in bigger turtles than in the smaller turtles; the percent of schizonts and the percent of gametocytes are higher in smaller turtles than in bigger turtles; when the intensity of infection is higher, we can expect to find more schizonts and gametocytes than trophozoites and vice versa.

Correlation between *Haemogregarina* sp. infection and differential white blood cell count

Results of the differential white blood cell count are shown in Table 4. Calculating the correlation coeficient for values shown in Tables 2 and 4, but only for six individuals (Emo/5 and Emo/7 were excluded because of the low number of parasites found) and excluding the basophiles, we have reached the results shown in Table 5.

Analysing Table 5, we can observe that: a higher percent of gametogonic forms is correlated with an increased relative eosinophile number and a lower relative number of monocytes; a higher infection intensity is correlated with a higher relative number of lymphocites and a lower relative number of heterophiles; infection intensity is not correlated with the relativ number of eosinophiles.

Morphology and tinctorial characteristics of infected erythocytes

Infected erythrocytes showed shape alteration, marginal and atrophic nucleus and were larger than non-parasitized erythrocytes. The shape alterations consisted in legthening of the red blood cells and the presence of various abnormal shapes: pear-shaped, curved and intermediary forms. We have also noticed tinctorial alteration of the infected erythrocytes. Thus, the atrophied nuclei of the infected cells were darker than nuclei of uninfected erythorocytes. Similarly, cytoplasm of infected erythrocytes was darker than of uninfected erythtocytes.

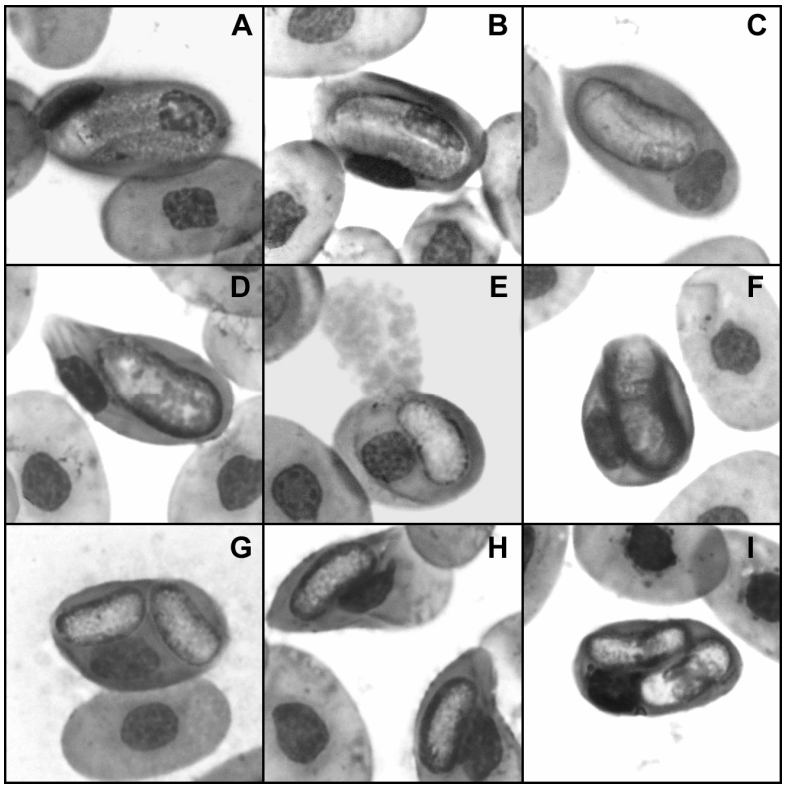

Figure 1
Different parasitic stages of *Haemogregarina stepanowi* in *Emys orbicularis*. A,B – Immature trophozoite; C – Mature trophozoite; D – Macroschizont (note the shape alteration of the infected erythrocyte); E – Microgametocyte (note the attached eosinophile); F – Macrogametocyte; G – Erythrocyte infected with two microgametocytes; H – Two altered erythrocytes infected with microgametocytes; I – An erythrocyte infected with a micro- (above) and macrogametocyte (below).

Table 2
Percentages of *Haemogregarina* sp. forms found in each individual

Turtle	Trophozoites	Merogonic forms	Gametogonic forms
	(%)		
Emo/1	68	4	28
Emo/2	96	0	4
Emo/3	92	4	4
Emo/4	18	8	74
Emo/5	Not relevant		
Emo/6	12	10	78
Emo/7	Not relevant		
Emo/8	32	4	64

Table 3. Correlation coefficient determined for carapace length and infection intensity respectively for each parasitic form percentage

Compared values	Carapace length	Infection intensivity
Infection intensivity	-0.63	1
Percent of trophozoites	0.84	-0.70
Percent of schizonts	-0.91	0.74
Percent of merozoites	0.48	0.04
Percent of gametocytes	-0.83	0.67

Table 4. Differential white blood cell count for each individual

Turtle	Lymphocytes	Monocytes	Heterophils	Eosinophils	Basophils
	(%)				
Emo/1	26	7	34	33	0
Emo/2	39	24	18	18	0
Emo/3	57	21	12	10	0
Emo/4	54	5	10	31	0
Emo/5	40	27	11	22	0
Emo/6	57	10	3	30	0
Emo/7	71	14	7	8	0
Emo/8	51	14	12	21	2

Table 5. Correlation coeficients between percent of parasitic forms and differential blood count

Compared values	Limphocytes	Monocytes	Heterophiles	Eosinophiles
Trophozoites	-0.44	0.75	0.52	-0.65
Merogonic forms	0.54	-0.72	-0.55	0.56
Gametogonic forms	0.42	-0.75	-0.51	0.65
Infection intensity	0.73	-0.33	-0.78	0.22

Discussions

Species identification within genus *Haemogregarina*, based on the morphology of intraerythrocytic stages in the vertebrate host, was characterized as non-"accurate" (Wozniak et all., 1994). According to Wilford, the specific identification should be based on the intermediate host (the vertebrate host), definitive host(s) (the leech) and localisation within the vertebrate and invertebrate host (Wilford, 1977). With the view of the forementioned aspects, we consider the species found belonging to *Haemogregarina stepanowi* Danilewsky, 1885. More recent studies identify the species based on molecular studies (Wozniak & McLaughlin, 1993; Wozniak et all., 1994).

Siddall and Desser studied prevalence and intensity of *Haemogregarina balli* infection in three turtle species from Ontario, Canada. They found a 100% prevalence in all adults of C*helydra serpentina serpentina* but for the other two species the prevalence varied depending on the season between 39 and 47% in *Chrysemys picta marginata* and between 67 and 100% in *Clemys insculpta*. In *Chelydra serpentina serpentina*, prevalence was lower in juveniles. Age differences for the other to species were not presented. According to the same study, the higher prevalence for all the three species was recorded in the midsummer (July) as compared to May and June (Siddall & Desser, 1992). In our study, as mentioned above, we found an infection prevalence of 100%, similar to that found by Siddall and Desser in the adults of C*helydra serpentina serpentina*. Our studies were performed in turtles captured in August so further studies need to be done in order to determine the seasonal prevalence variation. Prevalence in other turtle species its impossibile to determine, as *Emys orbicularis* is the only aquatic turtle from Romania.

The same authors determined infection intensity for *Haemogregarina balli* in *Chelydra serpentina serpentina*. This varied between 0.4 and 1.0 % infected erythrocytes, depending on season. The maximum intensity in was recorded for June and July as compared to May (Siddall & Desser, 1992). In our study we found an infection intensity varying from 0.08 to 2.20 % (in August). We did not perform yet any seasonal variation studies, as mentioned above.

Regarding the percentage of different parasitic forms (reported to total parasitic forms), we found only one study dealing with it (Siddall & Desser, 1992), but for *Haemogregarina balli*. Because of the differences in the development between *Haemogregarina stepanowi* and *Haemogregarina balli* we will not compare our results with the ones obtained by Siddall and Desser.

The positive correlation between the infection intensity and the percentage of merogonic and gametogonic forms can be explained by knowing that in *Haemogregarina stepanowi* one infectant sporozoite produces 13-14 macromerozoites and each of the later produces 6 micromerozoites. So, if the time passed from the infection is longer, the turtle will have a higher percent of merogonic and gametogonic forms and a lower percent of trophozoites, but an increased number of infected erythrocytes. If the time from infection is shorter, the turtle will have a higher percent of trophozoites and a lower percent of merogonic and gametogonic forms, and a low number of infected erythrocytes.

An interesting correlation was found between the infection intensity and the relative number of lymphocytes, also the values are within the normal limits for this turtle species (Stein, 1996). We can assume, that a high infection intensity may produce relative lymphocytosis and heteropenia. Another interesting assumption would be that a higher percent of gametogonic forms may induce eosinophilia. An explanation could be the extra-erythrocitic stages before gametogony occurs, exposing the parasites directly to the host's cellular immune system. Unfortunately, WBC was not determined in order to assess a presumed lecocitosis during the merogonic phase. Of course, a more complete study would be required, considering the seasonal variance and other factors that influence the hematologic values of reptiles.

The fact that eosinophiles could be implicated in the immune response of the turtles against *Haemogregarina*, is sustained by another interesting observation. While we were examining the smears, we found a lot of infected erythrocytes (especially with gametogonic forms), with eosinophiles attached to them (figure 1E). This is of course a hypothesis, regardindg that that attachement could be an artefact resulted in the process of blood smearing.

We have found no studies dealing with influence of various *Haemogregarina* infective stages on the differential white blood cell count or studies dealing with the turtle immune response against this hemoparasite. A study performed in Australia showed that there are no signifficat changes of the blood chemistry between snakes infected with hemogregarines and uninfected snakes (Caudell, 2001).

REZUMAT

Hemoparaziții din genul *Haemogragarina* la o populație de țestoase europene *(Emys orbicularis)* din Drăgăşani, județul Vâlcea, România

Studiul a fost efectuat pe opt țestoase din specia *Emys orbicularis*, capturate în august 2002 lângă localitatea Drăgăşani, județul Vâlcea, România. S-a determinat o prevalență a infecției cu hemoparaziți din genul *Haemogregarina* de 100%, cu o intensitate ce a variat între 0,8 şi 2,2 %. În urma calculului statistic s-au găsit corelații pozitive între intensitatea infecției şi procentul de forme gametogonice, între intensitatea infecției şi numărul de limfocite şi între procentul de forme gametogonice şi numărul relativ de eozinofile precum şi corelații negative între intensitatea infecției şi lungimea carapacei şi intensitatea infecției şi numărul relativ de heterofile. În lucrare se fac şi aprecieri referitoare la efectul infecției asupra morfologiei eritrocitelor.

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