Italian Journal of Zoology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tizo20

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Published online: 21 Jul 2014.

To cite this article: V. Arizza, D. Russo, F. Marrone, F. Sacco & M. Arculeo (2014): Morphological characterization of the blood cells in the endangered Sicilian endemic pond turtle, Emys trinacris (Testudines: Emydidae), Italian Journal of Zoology, DOI: 10.1080/11250003.2014.938371

To link to this article: http://dx.doi.org/10.1080/11250003.2014.938371

Italian Journal of Zoology, 2014, 1–10
http://dx.doi.org/10.1080/11250003.2014.938371

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Morphological characterization of the blood cells in the endangered Sicilian endemic pond turtle, *Emys trinacris* (Testudines: Emydidae)

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(Received 27 March 2014; accepted 13 June 2014)

Abstract
In this study, measurements of morphological parameters, sizes and frequencies of peripheral blood cells (erythrocytes, leukocytes, thrombocytes) on blood smear preparation devices stained with May-Grünwald stain were evaluated for both sexes in 20 *Emys trinacris* (Testudines: Emydidae) specimens. Erythrocytes were higher in male than in female specimens. The leukocyte of *E. trinacris* contains eosinophil, basophil, monocyte, heterophil and lymphocyte. The eosinophil was higher in males than in females whereas lymphocytes were higher in females than in males. The erythrocyte morphological parameters (EL [erythrocyte length], EW [erythrocyte width], L/W [length/width], ES [erythrocyte size]) were compared with the same data from *Emys orbicularis* s.l, and from species belonging to other chelonian genera. The erythrocyte size did not vary within the studied Palearctic *Emys* taxa, whereas it proved to differ from that observed in other chelonians.

Keywords: Emys trinacris, *blood smear, blood cell morphology,* Trachemys scripta elegans

Introduction

Emys trinacris (Fritz et al. 2005) (Testudines: Emydidae) is a Sicilian endemic pond turtle (Fritz et al. 2005); although it is morphologically close to *Emys orbicularis* s.l. (Fritz et al. 2006), molecular taxonomic studies have unambiguously revealed the presence of significant differences between the two species, which are adelphotaxa (Fritz et al. 2007; Pedall et al. 2011; Stuckas et al. 2014). Considering the biogeographical and evolutionary importance of this species and the drastic reduction of its populations caused by habitat destruction, pollution, and pathogens, in 2013, *E. trinacris* was listed in the International Union for Conservation of Nature (IUCN) Red List as "Endangered" (EN) (Rhodin et al. 2009).

To date, few studies have focused on the biology of this endemic species, and nothing is known about the hematologic blood characterization (HBC) of the species and on the health status of the wild populations of *E. trinacris*.

In the literature, HBC has been used successfully to diagnose chelonian diseases and to assess the physiological status of wild turtle populations (Duguy 1967; Dessauer 1970; Frye 1991; Campbell 1996; Stein 1996). This approach is widely used because the HBC is a minimally invasive tool that allows health evaluations, especially in relation to determining potential effects associated with stress factors such as pollution, disease, invasion by exotic species, etc. In order to be soundly usable, the reference evaluations have to be performed on healthy animals (Nagy & Medica 1986; Deem et al. 2006).

Blood cell parameters of reptiles may be influenced by several factors, such as age, sex, seasonality, reproduction, nutritional status and environmental parameters such as temperature, salinity, oxygen and light (Dessauer 1970; Duguy 1970; Frye 1991; Wilkinson 2003; Tavares-Dias et al. 2009; Yilmaz & Tosunoglu 2010; Gu et al. 2011; Scheelings & Jessop 2011; Tosunoglu et al. 2011; Scheelings & Rafferty 2012); these parameters can vary through the annual cycle and throughout the life of the individuals. Studies that describe chelonian

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blood cells are rare and the data are often in contradiction because of the lack of standard criteria used to categorize blood cells (Work et al. 1998). Various authors described circulating blood cells of different amphibian and reptile species; moreover, there are many chelonian species for which blood cell morphology and reference values are still unknown or imprecise. Descriptions of the morphologic characteristics of blood cells of pond turtles are limited and fail to standardize the parameters of the HBC (Metin et al. 2006; Rossini et al. 2012).

In order to perform conservation activities for the populations of *E. trinacris*, reference studies on biological parameters are necessary. Moreover, as a good practice, any action taken for the protection and the conservation of species in danger of extinction cannot ignore the full knowledge of their biology. For this reason, the blood cell parameters of the Sicilian pond turtle *E. trinacris* have been documented by analysing blood samples from free-living males and females. Moreover, blood cell parameter data obtained from *E. trinacris* were compared with those available from *E. orbicularis* s.l. Other comparisons were performed with those of the American emydid *Trachemys scripta elegans* (Wied, 1839).

The present study describes for the first time the blood cell parameters in *E. trinacris* obtained from the turtles under natural conditions, and aims to establish the blood cell parameter reference values necessary for the evaluation of the health status of individuals from wild populations.

Materials and methods

Sampling area

Collection was performed in four sites located in the Sicilian mainland as shown in Figure 1 and Table I.

The pond turtles were caught by hand or with hoop net traps (Ream & Ream 1966). Caught pond turtles were weighed and measured: measurements included the length and the width of the carapace, the length and the width of the plastron, the carapace height, the total and cloaca-apex tail length in mm

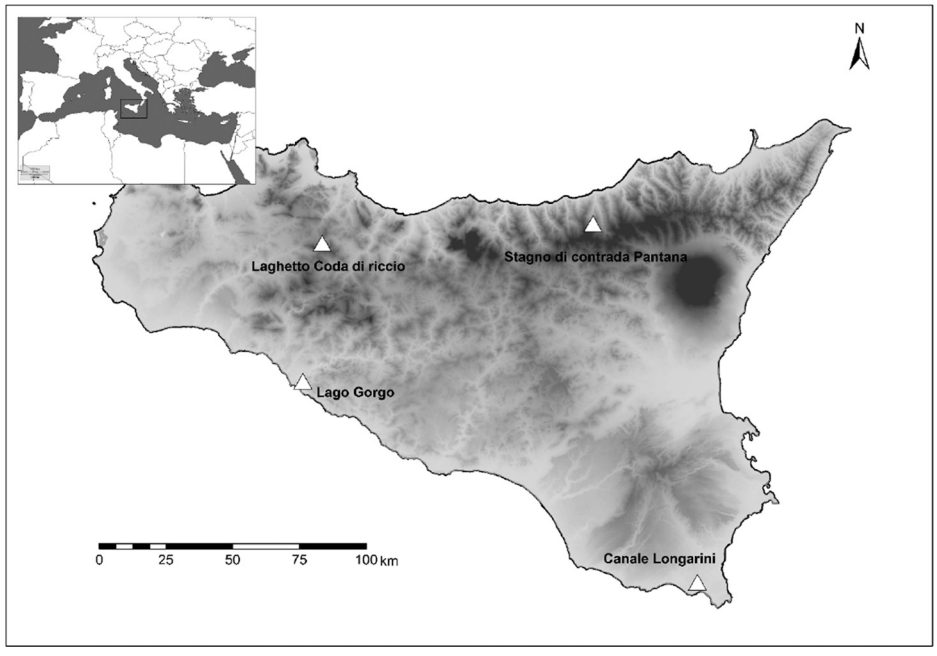

Figure 1. Locations of the sampled sites.

Table I. Geographical coordinates of the sampled sites and synopsis of the studied *Emys trinacris* specimens.

Sites	Coordinates (WGS84)	# Specimens	Males	Females
Laghetto "Coda di Riccio", Godrano (PA)	37.873333 N, 13.39845 E	7	7	0
Laghetto di Contrada Pantana, Caronia (ME)	37.949467 N, 14.551267 E	3	2	1
Lago Gorgo, Montallegro (AG)	37.405833 N, 13.327033 E	8	5	3
Canale Longarini, Ispica (RG)	36.73565 N, 14.996683 E	2	2	0

(with a caliper, to the nearest 0.5 mm) (Fritz et al. 2005), and the body mass in grams for each specimen (Zuffi & Gariboldi 1995; Zuffi et al. 2006). Ten blood samples of *Trachemys scripta elegans* were obtained in September 2013 from specimens housed in the Botanical Garden of the department of STEBICEF, University of Palermo.

Blood sampling, cell morphology and counts

Blood samples were obtained from the dorsal coccygeal vessel via heparinized glass capillaries (Hutchison & Szarski 1965; Szarski 1968). After obtaining blood samples, the animals were immediately released to their natural environments. For each individual, one to three capillaries were collected.

Blood smears were prepared *in situ*. A blood drop was smeared on a glass slide and air-dried. The sample was then fixed in methanol and stained by the Pappenheim method (May-Grünwald + Giemsa-Romanowsky staining diluted 1:10 in buffered water, pH 7) for 20 min, and washed in running tap water for 2 minutes. One hundred erythrocytes and 30 each of thrombocytes, eosinophils, basophils, lymphocytes and monocytes were measured under a microscope (Leica DMRE) equipped with a digital camera (Leica DCF420 C). In each smear were recorded lengths (L) and widths (W) of 100 randomly chosen erythrocytes as well as nuclear lengths (NL) and nuclear widths (NW). Erythrocyte sizes (ES) and their nucleus sizes (NS) were computed from the following equations (Arikan & Cicek 2010):

$$ES = (EL \times EW \times \pi)/4 \quad (1)$$

$$NS = (NL \times NW \times \pi)/4 \quad (2)$$

Cells and nuclear shapes were compared with EL/EW and NL/NW ratios, and nucleus/cytoplasm with NS/ES ratio. In addition, from the blood smears of each species, measurements of leucocytes (lymphocytes, monocytes, heterophils, eosinophils, basophils) and thrombocytes (TL, TW) were also taken to determine their sizes and computed from the following formula:

$$A = \pi r^2 \quad (3)$$

Statistical analyses

Hematological variables (number of cells or dimensions) were summarized as mean, standard deviation (SD), standard error of the mean (SE) and range. We used analysis of the t test for a comparison of the sexes.

Results

Sampling and measurements

A total of 20 wild pond turtles *Emys trinacris*, 16 male and four female, were sampled in Sicily from 2012 to 2013 (Table I). The average body weights for the male and female turtles used for the study were 355.31 ± 154.78 g and 504.33 ± 75.5 g, respectively. The measured carapace lengths were 12.77 ± 2.15 cm and 14.08 ± 1.11 cm for male and female specimens, respectively.

Those samples which showed wounds or epiphytes or possessed parasites in the blood were not included in the analyses.

Emys trinacris *blood cells*

Differential blood cell count of the peripheral blood of *E. trinacris* was carried out using blood smears stained with May-Grünwald Giemsa observed under a light microscope equipped with a digital camera. The leukocyte types recognized in *Emys trinacris* correspond with those found in other species of the family Emydidae (Table II). In particular, seven cell types were identified: (a) nucleated erythrocytes, (b) eosinophils, (c) basophils, (d) monocytes, (e) thrombocytes, (f) heterophils and (g) lymphocytes (Figure 2). Significant differences in cell size were not observed in either sex. Males possessed significantly higher numbers of red blood cells

Table II. Leukocytes in turtle species of the Emydidae family.

Species	Leukocyte type					Reference
	Lymphocyte	Heterophils	Eosinophils	Basophils	Monocyte	
Emys orbicularis galloitalica	+	+	+	+	+	(Metin et al. 2006)
	+	+	+	+	+	(Yilmaz & Tosunoglu 2010)
Graptemys gibbonsi	+	+	+	+	+	(Perpiñán et al. 2008)
Pseudemys rubriventris	+	+	+	+	+	(Innis et al. 2007)
Clemmys muhlenbergii	+	+	+	+	+	(Brenner et al. 2002)
Chrysemys picta	+	+	+	+	+	(Schwanz et al. 2011)

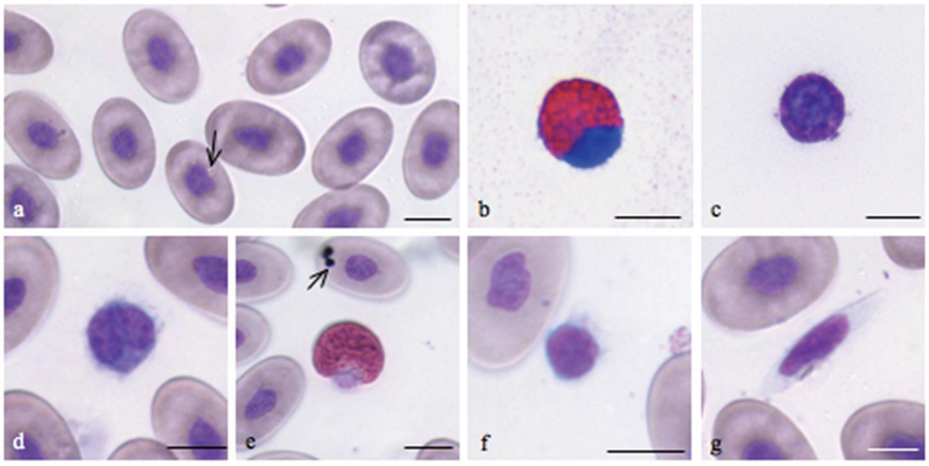

Figure 2. Blood smears of *Emys trinacris* stained with May-Grünwald Giemsa. Pictures were taken with an optical microscope equipped with a digital camera. **a**, erythrocyte; **b**, eosinophil; **c**, basophil; **d**, monocyte; **e**, heterophil; **f**, lymphocyte; **g**, thrombocyte. Scale bars: 10 μm.

($p < 0.05$) and eosinophils ($p < 0.01$), while females showed a significantly ($p < 0.05$) higher percentage of lymphocytes (Table I). For other cell types, although their number varied between the genders, no significant differences were found.

Erythrocyte morphology

Mature erythrocytes were homogeneous in size, shape and color (Figure 2a). They were nucleated cells with elliptical shape and abundant pale pink cytoplasm like those of the other turtle species (Kassab et al. 2009; Orós et al. 2010). The violet-blue oval or round nucleus with rounded or irregular poles was centrically positioned and had condensed deeply basophilic chromatin. Its major axis was parallel with the long diameter of the cell. Some erythrocytes had small intracytoplasmic inclusions (arrow Figure 2e) or might contain vacuoles (arrow Figure 2a). Although the erythrocytes of males compared with females were generally larger in size, a significant difference was not recorded. Parasites were not detected. The results of erythrocyte measurements are summarized in Table III.

White blood cell morphology

Eosinophils. The eosinophils (Figure 2b) were easily distinguished by their round eosinophilic cytoplasmic granules, which fill the whole cytoplasm. They were the smallest among the granulocytes, ranging between 13.5 ± 5.67 and 12.6 ± 1.3 μm for males and females respectively (Table III). The nucleus contains coarse, clumped chromatin and strongly stained blue. It was round to oval, single or bilobed and eccentrically placed near the membrane. The cytoplasm was filled with granules measuring approximately 1.15 ± 0.035 μm. Males had a significantly ($p < 0.01$) higher number of eosinophils (20.2 ± 1.2) than females (17.5 ± 1.09).

Table III. Differential blood cells count and size in peripheral blood of *Emys trinacris*. $^{(\star)}$ = Significant differences $p < 0.05$; $^{(\star\star)}$ = significant differences $p < 0.01$. The erythrocyte size was reported as ratio between measurements (μm) of length and width (L/W). The size of the thrombocyte was the length of the cells (L) expressed in μm.

Blood cell type		Male		Female	
		Count ± SD	Size ± SD (μm)	Count ± SD	Size ± SD (μm)
Erythrocyte		422.5 ± 12.6 (10^4/μL)	1.6 ± 0.12 (L/W)	379.4 ± 17.3 (10^4/μL)$^{(\star)}$	1.6 ± 0.14 (L/W)
Leukocyte	Eosinophils	20.2 ± 1.2 (%)	13.5 ± 5.67	17.3 ± 1.09 (%)$^{(\star\star)}$	12.6 ± 1.3
	Basophils	20.5 ± 2.58 (%)	9.5 ± 0.12	17.3 ± 1.58 (%)	9.3 ± 0.23
	Monocytes	4.1 ± 1.82 (%)	11.3 ± 4.81	4.3 ± 2.11 (%)	11.7 ± 1.75
	Heterophils	21.7 ± 0.8 (%)	13.9 ± 6.71	20.6 ± 1.2 (%)	13.3 ± 1.27
	Lymphocytes	31.6 ± 0.69 (%)	6.7 ± 2.12	41.3 ± 1.24 (%)$^{(\star)}$	6.5 ± 1.26
Thrombocytes		2.9 ± 1.25 (μL)	25.0 ± 1.23 (L)	2.8 ± 2.2 (μL)	22.9 ± 0.63 (L)

SD, standard deviation.

Basophils. The basophils (Figure 2c) were present with a percentage of 5.8 ± 0.45 and 5.4 ± 0.77 respectively for male and female turtles. They were small cells, about 9.49 μm, without significant differences between the genders. They are easily identified by their deeply stained cytoplasm filled with very dense, dark purple granules. Their large nuclei (7.09 ± 0.25) were round and centrically placed (Figure 2c).

Monocytes. The monocyte (Figure 2d) contained a large amount of light blue-gray, finely granular or vacuolated cytoplasm and an oval or kidney-shaped nucleus with a dense chromatin pattern near the membrane. The mean diameter in observed monocytes ranged between 11.3 ± 4.81 and 11.7 ± 1.75 μm and did not differ significantly between males and females (Table III). The presence of this cell in both males and females was the same.

Heterophils. Heterophils contained large, eosinophilic and fusiform cytoplasmic granules. The cytoplasm, which can be difficult to visualize, was light blue or clear (Figure 2e). The nucleus is segmented and frequently displaced toward the edge of the cell and appeared basophilic with dense chromatin. No significant differences were found between males and females for size; the diameter ranged from 13.9 ± 6.71 in males to 13.3 ± 1.27 in females, and the frequency was 15.4 ± 0.8% in males and 15.7 ± 1.2% in females.

Lymphocytes. The lymphocytes of *E. trinacris* were easily recognizable because they differed greatly from thrombocytes (Figure 2f). They were round cells with a diameter of 6.7 ± 2.12 μm in males and 6.5 ± 1.26 μm in females. They contained a small amount of blue-stained cytoplasm and a round nucleus with a fine reticular pattern. The lymphocyte showed a nuclear to cytoplasmic ratio greater than one.

Thrombocytes. The thrombocytes (Figure 2g) were observed as spindle-shaped cells (25.0 ± 1.23 × 5.0 ± 0.89 μm for males and 22.9 ± 0.63 × 4.8 ± 0.54 for females) that contained a central, ellipsoidal, densely stained nucleus of about 12.9 ± 1.3 × 4.11 ± 0.74 μm for both males and females. The cytoplasm was hyaline and had no granules.

Discussion

The comparison of the red blood cells of *E. trinacris* with those of *E. orbicularis* s.l. showed a high variability in size (Table IV). In particular, *E. orbicularis* data in the literature show mean values of L/W ranging from 1.6 to 1.8 μm, with cell average areas

Table IV. Erythrocyte dimensions from male and female individual of *Emys trinacris* compared with *E. orbicularis gallotalica* and *E. orbicularis hellenica* (μm ± standard deviation, SD). EL: erythrocyte length, EW: erythrocyte width, L/W: ratio between measurements (μm) of length and width, ES: erythrocyte size, NL: nucleus length, NW: nucleus width, NS: nucleus size. ES and NS were estimated with the respective formulas ELEWπ/4 and NLNWπ/4.

Species	Sex	EL	EW	L/W	ES (μm^2)	NL	NW	NL/NW	NS (μm^2)	NS/ES	Reference
E. trinacris	Male	22.7 ± 1.53	14.3 ± 0.56	1.6 ± 0.12	255.4 ± 22.74	6.5 ± 0.48	5.2 ± 0.24	1.3 ± 0.1	26.5 ± 3.3	0.1 ± 0.01	Present study
	Female	22.4 ± 0.93	13.9 ± 0.32	1.6 ± 0.16	244.4 ± 16.02	6.4 ± 0.32	5.0 ± 0.52	1.3 ± 0.06	25.1 ± 4.01	0.1 ± 0.01	
	Mean	22.5 ± 1.21	14.1 ± 0.44	1.6 ± 0.14	249.4 ± 21.58	6.3 ± 0.16	5.1 ± 0.34	1.2 ± 0.01	25.2 ± 2.48	0.1 ± 0.01	
E. orbicularis gallotalica	Male	14.9 ± 0.79	9.9 ± 0.54	1.5 ± 0.09	116.5 ± 10.17	4.9 ± 0.57	3.8 ± 0.03	1.3 ± 0.17	14.7 ± 2.3	0.13 ± 0.004	(Javanbakht et al. 2013)
	Female	15.4 ± 1.0	8.7 ± 0.65	1.8 ± 0.16	105.4 ± 11.63	4.7 ± 0.51	3.3 ± 0.38	1.5 ± 0.22	11.9 ± 0.19	0.11 ± 0.003	
	Mean	15.2 ± 0.18	9.3 ± 0.12	1.6 ± 0.12	110.9 ± 2.17	4.8 ± 0.42	3.51 ± 0.05	1.4 ± 0.03	13.3 ± 0.42	0.12 ± 0.003	
	Male	21.5 ± 1.37	12.9 ± 1.09	1.7 ± 0.18	217.8 ± 23.63	7 ± 0.73	5.4 ± 0.61	1.3 ± 0.18	29.5 ± 5.1	0.1 ± 0.02	(Metin et al. 2006)
	Female	21.8 ± 1.61	13.6 ± 1.05	1.6 ± 0.14	232.3 ± 22.55	6.8 ± 0.81	5.4 ± 0.69	1.3 ± 0.2	28.9 ± 5.64	0.1 ± 0.02	
	Mean	21.7 ± 1.27	13.2 ± 1.12	1.6 ± 0.16	225.1 ± 24.18	6.9 ± 0.78	5.4 ± 0.65	1.3 ± 0.19	29.2 ± 5.41	0.1 ± 0.02	
	Mean	19.9 ± 0.11	12.7 ± 0.09	1.6 ± 0.01	200.7 ± 1.88	7.1 ± 0.05	6.2 ± 0.19	1.2 ± 0.01	35.4 ± 1.1	0.18 ± 0.01	(Arikan & Cicek 2010)
	Mean	21.7 ± 1.51	11.5 ± 0.98	1.8 ± 0.14	195.8 ± 1.16	6.6 ± 0.75	5.7 ± 0.47	1.2 ± 0.15	29.5 ± 0.27	0.15 ± 0.023	(Colagar & Jafari 2007)
E. orbicularis hellenica	Mean	21.7 ± 1.01	12.5 ± 0.98	1.7 ± 0.12	214.0 ± 22.8	7.5 ± 0.54	5.7 ± 0.47	1.3 ± 0.15	33.6 ± 3.74	0.15 ± 0.01	(Ugurtas et al. 2003)

ranging from 110.9 to 225.1 μm^2. The red blood cells of *E. trinacris* were larger compared with the erythrocytes of the other two taxa. They were long on average, 22.5 μm and 14.1 μm wide, with a cell area of 249 μm^2. The nuclei of the red blood cells of *E. trinacris* were smaller when compared with those of the other two species (Table IV). A further comparison was made between the sizes of the erythrocytes of *E. trinacris* with those of *Trachemys scripta* (Schoepff, 1792) (Table V). The data show that the sizes of EL, EW, their ratio (L/W) and the cell areas were significantly larger than those of *T. scripta*. However, the size of the nuclei of the erythrocytes of *E. trinacris* did not differ significantly when compared with those of *T. scripta* (Table V).

Hematological and biochemical parameters are useful tools in measuring the physiological status of turtles because they may provide information on the health and general condition of individuals and populations (Campbell 1998; Oliveira-Júnior et al. 2009). Moreover, such tools have been used as physiological disturbance indicators of diseases, stress or exposure to contaminants, as well as to assess degrees of dehydration (Peterson 2002; Christopher et al. 2003; Tavares-Dias et al. 2008).

Since data on the hematology of the Sicilian endemic *Emys trinacris* are currently lacking, the main aim of this study was to characterize, for the first time, the blood cells of *E. trinacris* and, in particular, to classify its leukocytes. The classification criteria of chelonian leukocytes pose many problems, partly because these cells show morphological variation among the species and partly because several different nomenclatures have been used to describe them. Moreover, some cells are not easily identified based on their morphological differences, e.g.: small lymphocytes may be morphologically similar to thrombocytes. Most authors agree that reptiles do not have neutrophils, whereas they do have heterophils and eosinophils, which both show acidophilic granules (Canfield 1998). Some studies classify acidophils (i.e., heterophils and eosinophils) as a single cell type at different stages of maturation (Azevedo & Lunardi 2003). Neutrophils have been reported only in some reports (Wood & Ebanks 1984; Pitol et al. 2007). Some authors (Christopher et al. 1999; Dickinson et al. 2002; Knotková et al. 2002) refer to the presence of azurophils in the peripheral blood of chelonians, and the very existence of azurophils is still in dispute (Rosskopf 2000). Studies involving other species aiming to study leukocytes under light microscopy analysis showed, in the turtle species *Podocnemis expansa* (Schweigger, 1812) and *Emys orbicularis* s.l., the presence of basophiles, eosinophils, lymphocytes, monocytes and heterophils (Metin et al. 2006).

Table V. Erythrocyte dimensions of *Trachemys scripta elegans* (μm ± standard deviation, SD). EL: erythrocyte length, EW: erythrocyte width, L/W: ratio between measurements (μm) of length and width, ES: erythrocyte size, NL: nucleus length, NW: nucleus width, NS: nucleus size. ES and NS were esteemed with the respective formulas: ELEWπ/4 and NLNWπ/4. $\star = p < 0.05$.

Species	Sex	EL	EW	L/W	ES (μm^2)	NL	NW	NL/NW	NS (μm^2)	NS/ES
E. trinacris	Male	22.7 ± 1.53	14.3 ± 0.56	1.6 ± 0.12	255.4 ± 22.74	6.5 ± 0.48	5.2 ± 0.24	1.3 ± 0.1	26.5 ± 3.3	0.1 ± 0.01
	Female	22.4 ± 0.93	13.9 ± 0.32	1.6 ± 0.16	244.4 ± 16.02	6.4 ± 0.32	5 ± 0.52	1.3 ± 0.06	25.1 ± 4.01	0.1 ± 0.01
	Mean	22.5 ± 1.21	14.1 ± 0.44	1.6 ± 0.14	249.4 ± 21.58	6.3 ± 0.16	5.1 ± 0.34	1.2 ± 0.01	25.2 ± 2.48	0.1 ± 0.01
T. scripta elegans	Male	19.4 ± 0.45	13.8 ± 0.97	1.5 ± 0.20	206.6 ± 23.82	6.1 ± 0.56	5.4 ± 0.32	1.1 ± 0.03	26.0 ± 3.88	0.1 ± 0.005
	Female	19.0 ± 0.91	13.4 ± 0.45	1.4 ± 0.03	203.3 ± 11.78	5.9 ± 0.20	5.3 ± 0.18	1.1 ± 0.007	24.3 ± 1.7	0.1 ± 0.009
	Mean	19.2 ± 0.62$\star$	13.6 ± 0.61$\star$	1.4 ± 0.01$\star$	204.9 ± 15.46$\star$	6.0 ± 0.37	5.3 ± 0.25	1.1 ± 0.02	25.2 ± 2.8	0.1 ± 0.007

Furthermore, another difficulty lies in determining the proportions of these cells within a particular species since these vary with individual physiologic status and the method of investigation (Campbell & Ellis 2007).

Erythrocytes

Mature erythrocytes of *E. trinacris* proved to be morphologically similar to those of various species of turtles and tortoises and in particular to those of *E. orbicularis* s.l. (Ugurtas et al. 2003; Metin et al. 2006). They were ellipsoidal cells with a centrally positioned, ovoid nucleus and cytoplasmic inclusions, observed in over 30% of erythrocytes. For other reptiles, these inclusions have been reported to be degenerated organelles (Alleman et al. 1992; Clark et al. 2001; Chung et al. 2009) and may be related to the aging of erythrocytes (Heard et al. 2004). Others have postulated that the basophilic inclusions could be micronuclei and, therefore, they could be biomarkers for chromosomal damage from genotoxic environmental pollutants (Matson et al. 2005; Metin et al. 2006). The erythrocyte mean sizes did not differ significantly from those of *E. orbicularis* s.l. ($p > 0.05$), failing in an attempt to use the erythrocyte size as a discriminator between species of the same Emydinae subfamily. The higher number of red blood cells observed in males of *E. trinacris* than in females is similar to the findings in other turtles such as *E. orbicularis* (Duguy 1967) and *Kinixys erosa* (Schweigger, 1812) (Oyewale et al. 1998). The higher number of red blood cells found in males may depend on testosterone hormone levels. In fact, testosterone, when present in chelonians (Paitz & Bowden 2013), is able to increase the number of erythrocytes (Fried & Gurney 1965; Pati & Thapliyal 1984; Oyewale et al. 1998).

White blood cell morphology

Our findings conform to the basic morphological description for other Emydidae turtle species such as *Emys orbicularis*, *Graptemys gibbonsi* (Lovich & McCoy, 1992), *Pseudemys rubriventris* (LeConte, 1830) and *Clemmys muhlenbergii* (Schoepff, 1801) (Table III).

Eosinophils. In turtles, the same authors showed two types of eosinophils distinguishable by the shape of cytoplasmic granules. Azevedo and Lunardi (2003) observed in the blood of *Chrysemys dorbigni* (Duméril & Bibron, 1835) two types of granulocytes that exhibit eosinophilia, one of them with round cytoplasmic granules and the other with elongated cytoplasmic granules. It has been suggested that these cells may be eosinophils in different stages of maturation, but they also may be distinct cell types, i.e. eosinophils and heterophils (Azevedo & Lunardi 2003). In *E. trinacris*, most leukocytes were heterophils, basophils and eosinophils. Similar findings have also been reported by (Oliveira-Júnior et al. 2009) for *Podocnemis expansa*. This result was not confirmed for other species of turtles. In fact, only captive female *Clemmys muhlenbergii* had a higher absolute eosinophil count and a higher percentage of eosinophils compared with captive males; conversely, wild females were not significantly different from wild males (Brenner et al. 2002).

Basophils. The numbers of basophils in turtles vary greatly. In *Graptemys gibbonsi*, basophils were found to be the most abundant leukocyte type, about 40% of total leukocytes (Perpiñán et al. 2008). Higher percentages of basophils (50–63%) have been found in other chelonians, such as *Chelydra serpentina* (Linnaeus, 1758) (Mead et al. 1983). In contrast, moderate basophil percentages have been found in other species, such as 5.7% in *Gopherus polyphemus* (Daudin, 1802) (Taylor & Jacobson 1982) and 8% in *Geochelone radiata* (Shaw, 1802) (Marks & Citino 1990). Basophil numbers were almost nonexistent (~0.8 for both sexes) in *Clemmys muhlenbergii* (Brenner et al. 2002). However, care must be taken when analyzing published works; as an example, basophil counts in *E. orbicularis* varied widely: 0–4% was reported by Duguy (1970) and about 34% was reported by Javanbakht et al. (2013). This variation of basophil density in various species of turtles is difficult to explain. Many factors can affect the number of basophils and the leukocytic formula such as age, health status, ecological factors and the seasons (for a review see Duguy 1970). We found in *E. trinacris* that the percentage of basophils did not vary significantly between sexes, ranging between 20.5 ± 2.58 for males and 17.3 ± 1.58 for females.

Monocytes. This leukocyte type is not present in all species of turtles. Indeed, in *Chelonia mydas* (Linnaeus, 1758), authors did not identify monocytes (Wood & Ebanks 1984; Aguirre et al. 1995). Often, monocytes are not visible if the blood smears are performed with blood that was taken eight or more hours before (Work et al. 1998). Monocytes from *E. trinacris* were similar to the monocytes from *E. orbicularis* described by Metin et al. (2006) or *Ocadia sinensis* (Gray, 1870) described by Chung et al. (2009). In *E. trinacris*, monocytes were round cells and had a similar size in both males and females, (11.3 and 11.7 μm respectively). Also, their frequency was similar for both sexes (~4.2%).

Heterophils. The heterophils of chelonians are analogous to mammalian neutrophils (Montali 1988) and can be easily distinguished by the fusiform red granules contained in the cytoplasm. They had the same percentage for both sexes (~15%). These frequencies correspond with those found in *Graptemys gibbonsi* (Perpiñán et al. 2008), but differ from those in *Pseudemys rubriventris* (~26.9%) (Innis et al. 2007) and *Clemmys muhlenbergii* and *Chrysemys picta* (Schneider, 1783) (both about 9.3% (Brenner et al. 2002; Schwanz et al. 2011). Furthermore, the percentage value is included within the range indicated for *E. orbicularis* (Duguy 1970). The number and size of heterophils have been observed to be influenced by individual and seasonal factors (Duguy 1970).

Lymphocytes. The lymphocytes were the smallest cells, with a diameter on average about 6.6 μm for both sexes. Female had a significantly ($p < 0.05$) higher percentage of lymphocytes (27.3 ± 1.24) compared with males (22.5 ± 0.69). The same result was reported by Brenner et al. (2002) for *C. muhlenbergii*, where female and male lymphocyte percentages were 1.8 and 1.5%, respectively. The percentages of lymphocytes found in both genders of *E. trinacris* were coherent with values reported for other Emydidae turtles such as *G. gibbonsi* and *C. muhlenbergii* (Brenner et al. 2002; Perpiñán et al. 2008) but differ from those for *P. rubriventris*, in which these cells represent about 50% of the white blood cell differential count (Innis et al. 2007).

Thrombocytes. Although the similarity of thrombocytes and leukocytes in reptiles is known (Frye 1991), in the case of *E. trinacris*, thrombocytes differ greatly from those of other pond turtles. In *E. orbicularis*, the thrombocytes are round cells with a nucleus round to oval and dark (Metin et al. 2006), whereas in *Pseudemys rubriventris* these were elliptical, with central ovoid basophilic nuclei, lightly basophilic cytoplasm, and were often noted in small clusters (Innis et al. 2007). The thrombocytes of *E. trinacris* have an elongated cell shape with a central ovoid nucleus. The size and number do not differ between the sexes.

The comparison of the erythrocyte size parameters with those of *Emys orbicularis* s.l. showed no important differences even in comparison between the two sexes (Table IV). The data reported by Javanbakht et al. (2013) had the lowest values, which probably derived from different environmental conditions (e.g. temperature, air pressure) (Ruiz et al. 1983, 1989) or different activity levels (e.g. healthy, breeding, hibernating, foraging and daily activity) (Sykes & Klaphake 2008; Tosunoglu et al. 2011; Yu et al. 2013).

However, significant differences were found when the erythrocyte parameters (EL, EW, L/W, ES) of *E. trinacris* were compared with those of *T. scripta elegans*, a tortoise belonging to the same family but to a different genus (Table V). The morphology of *E. trinacris* erythrocytes was similar to that of *T. scripta elegans*, but the size was greater. The L/W ratio was about 1.6 for *E. trinacris* and about 1.4 for *T. scripta elegans*; consequently, erythrocyte shape was more ellipsoidal in *E. trinacris*. No significant differences were found in the nucleo-cytoplasmic ratio.

The results of our analysis show that the morphology of erythrocytes within the family of Emydidae does not change greatly and, in a comparison between species from different genera, only a few differences in size can be found.

The findings of this study present for the first time data on the cytomorphological structure and numbers of peripheral blood cells in both sexes of wild-caught, healthy *E. trinacris*. Since dates were derived from specimens in good health, the hematological profile here reported could be used as reference values for studies on *E. trinacris*, and could be beneficial to future clinical and conservation work on the endangered Sicilian pond turtle.

Acknowledgements

We thank the Director of Orto botanico of the University of Palermo for permission to sample the *Trachemys scripta elegans* specimens, the President of Parco dei Nebrodi and the Italian Ministry of the Environment and Protection of Land and Sea for granting the authorization U. prot. PNM-2011-0022035 25/10/2011 to sample *Emys*.

Research partially funded by the "Fondi di Ateneo" (60%) of the University of Palermo.

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