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Harmless parasites? Infections with *Hemolivia mauritanica* (Apicomplexa: Adeleorina: Karyolysidae) and *Haemocystidium* spp. (Apicomplexa: Haemosporida: Haemoproteidae) have a negligible impact on white cell counts in tortoise hosts

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Abstract: The pathogenicity of haemogregarines and their effects on the health status of ectothermic hosts remain largely unexplored. In this study, we examined the impact of *Hemolivia mauritanica* (Sergent et Sergent, 1904) infection on the differential leukocyte count (DLC) as a measurable indicator of health in tortoise hosts. A total of 206 blood smears were analysed, including 181 from spur-thighed tortoises (*Testudo graeca* Linnaeus) and 25 from marginated tortoises (*Testudo marginata* Schoepff). Light microscopy was used to identify infected individuals, determine DLC, and quantify parasitaemia levels. Overall, *H. mauritanica* was detected in 125 of 181 (69%) *T. graeca* samples and 21 of 25 (84%) *T. marginata* samples. To assess whether infection influenced DLC, we statistically compared leukocyte profiles between infected and uninfected individuals. Additionally, we evaluated the effects of other factors, including host species, parasitaemia intensity, sex, age, and the month and year of blood collection. Wilcoxon rank-sum tests revealed that parasitaemia and age had a statistically significant effect on DLC in *T. graeca*. Further analysis using linear models showed a significant association between parasitaemia and DLC, specifically affecting azurophils in *T. graeca* and basophils in *T. marginata*. Nine *T. graeca* tortoises positive for *H. mauritanica* were co-infected with haemosporidian parasites of the genus *Haemocystidium* Castellani et Willey, 1904, specifically three with *Haemocystidium anatolicum* (Orkun et Güven, 2013) and six with *Haemocystidium caucasicum* (Krasilnikov, 1965). Although co-infection itself was not statistically significant, a separate analysis of *Haemocystidium* parasitaemia revealed a significant effect on lymphocyte DLC. Furthermore, the frequent presence of mitotic and polychromatophilous erythrocytes in *H. mauritanica*-infected tortoises suggests a potential increase in erythrocyte regeneration.

Keywords: blood parasites, haemogregarine, haemosporidia, differential leucocyte count, mitotic erythrocyte, polychromatophil, reptiles

The very definition of parasitism implies a negative effect of parasitic species on their hosts. Compared to domestic animals, whose host-parasite interactions are well studied, our understanding of the impact of parasites on wildlife remains limited, primarily to a few model or iconic species (Bower et al. 2019). Research on haemoparasite infections in reptiles has largely focused on describing their diversity, while their clinical effects remain poorly understood (Apache et al. 2023, Cavalcante et al. 2024). Among vertebrates, reptiles are among the least studied in terms of parasite impact on their host. Moreover, significant clinical symptoms of parasitic disease in reptiles typically manifest only in cases of severe damage (Bower et al. 2019, Jacobson and Garner 2021).

Haemogregarines and haemosporidians are the predominant blood-parasitic protists in reptiles (Telford 2009). *Hemolivia mauritanica* (Sergent et Sergent, 1904), which infects western Palaearctic tortoises *Testudo graeca* Linnaeus and *Testudo marginata* Schoepff, is one of the most extensively studied haemogregarines (e.g., Sergent and Sergent 1904, Brumpt 1938, Michel 1973, Landau and Paperna 1997, Paperna 2006, Široký et al. 2007a, Laghzaoui et al. 2020, Barradas et al. 2021).

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This intracellular apicomplexan parasite has a heteroxenous life-cycle, with tortoises serving as intermediate hosts and *Hyalomma aegyptium* (Linnaeus, 1758) ticks acting as definitive hosts and vectors (Široký et al. 2007a). Infected tortoises can carry the parasite for many years (Široký et al. 2004) without showing any signs of disease. However, how these tortoises manage to coexist with their parasites over such prolonged periods – despite infection prevalence exceeding 80% among adults in some localities – remains unknown (Široký et al. 2005, 2009, Javanbakht et al. 2015b). Despite numerous studies on this haemogregarine species, the key drivers underlying the long-term *Hemolivia-Testudo* coexistence have yet to be identified.

So far, two haemosporidian parasites have been described from *T. graeca* – *Haemocystidium caucasicum* (Krasilnikov, 1965) and *Haemocystidium anatolicum* (Orkun et Güven, 2013). Both species were originally described as members of the genus *Haemoproteus* Kruse, 1890 (Krasilnikov 1965, Telford 2009, Orkun and Güven 2013). However, later studies reassigned the reptilian species mostly to the genus *Haemocystidium* Castellani et Willey, 1904 (Pineda-Catalan et al. 2013, Galen et al. 2018). The biology and life cycles of these parasites remain poorly understood. To date, only blood stages observed in tortoise hosts have been described (Krasilnikov 1965, Orkun and Güven 2013, Javanbakht et al. 2015a).

The geographic distributions of *Hc. anatolicum* and *Hc. caucasicum* partially overlap in Iran: the former occurs from Iran westwards into Turkey, while the latter ranges from Georgia and Iran eastwards to Afghanistan, where infects another tortoise species, *Testudo horsfieldii* Gray (Javanbakht et al. 2015a). In some localities, the prevalence of infection can exceed 85% in both species (Javanbakht et al. 2015a). The definitive hosts and competent vectors of both *Haemocystidium* species remain unknown. Biting midges *Leptoconops bezzii* (Noé, 1905), known to feed on *T. graeca* (Široký et al. 2007b), have been discussed as possible vectors, but no vector has yet been confirmed for either species (Javanbakht et al. 2015a).

Assessing the veterinary significance of reptilian blood protists presents several challenges. Our limited understanding of their pathogenicity, virulence, or even their potential effects on reptilian hosts is based on only a few pioneering studies. The most notable findings describe morphological changes in host blood cells (Strik et al. 2007). Mihalca et al. (2002) reported that severe haemogregarine infections may increase lymphocyte counts while decreasing eosinophil counts. However, Salakij et al. (2002) found no significant differences in differential leucocyte counts (DLC) in *Homalopsis buccata* (Linnaeus) snakes infected by haemoparasites. Despite high levels of haemogregarine parasitaemia, pathogenicity is generally low, manifesting primarily as anaemia, immunosuppression, or minor pathological changes in visceral organs such as the liver and kidneys (Telford 1984, 2009, Široký et al. 2007a, Halla et al. 2014).

The pathological effects of *Haemocystidium* on tortoise hosts have not been observed or remain unknown (Telford 2009). Under optimal husbandry conditions and proper care, tortoises may not exhibit clear clinical symptoms. However, the likelihood of disease development due to parasitic infection can be influenced by various factors, including stress, environmental temperature, coexisting illnesses, vector presence, and age. In captive specimens, additional factors such as hygiene in the breeding facilities and nutritional quality may also play a role (Rataj et al. 2011, Ali et al. 2018). Consequently, the clinical significance of haemoparasitic infections may become more pronounced in rescue centres and *ex-situ* breeding programmes.

Due to the virtual absence of clinical signs in haemogregarine-infected reptiles, haematological analysis may serve as a valuable proxy for assessing their health status and determining whether these parasites have harmful effects on their hosts (Stacy et al. 2011, Ali et al. 2018). In this study, we utilised samples from previous research (Široký et al. 2005, 2009, Javanbakht et al. 2015a,b) to investigate whether, and to what extent, the intensity of blood parasite infection influences DLC in tortoise hosts. Additionally, we examined the correlation between parasitaemia levels and measurable changes in DLC.

MATERIALS AND METHODS

Samples

We analysed 206 Giemsa-stained blood smears from the parasite collection of the Department of Biology and Wildlife Diseases, University of Veterinary Sciences Brno, Czech Republic. These smears, collected from two tortoise species, the marginated tortoise (*Testudo marginata*, N = 25) and the spur-thighed tortoise (*Testudo graeca*, N = 181), originated from previous studies focusing on the taxonomy of the genus *Testudo* Linnaeus (e.g. Fritz et al. 2007, Mikulíček et al. 2013, Javanbakht et al. 2017), and the biology of *Hemolivia mauritanica* (Fig. 1A) and *Haemocystidium* spp. (Fig. 1B) (Široký et al. 2005, 2009, Javanbakht et al. 2015a,b). Only smears for which host species, sex and estimated age were available were included. Samples were obtained from free-living tortoises through the dorsal coccygeal vein punctures using insulin syringes with fine needles. These samples originated from Greece (N = 25), Turkey (N = 58), Syria (N = 89), and Iran (N = 34) (Fig. 2). Sampling took place between April and June in the years 2004, 2007, 2011, and 2012.

Microscopy

Blood smears were examined for parasites using light microscopy. The intensity of parasitaemia in positive smears was determined by calculating the percentage of infected erythrocytes within 10,000 observed cells (Široký et al. 2007a). Differential leukocyte counts (DLC) were assessed using an Olympus BX53 microscope under a 100× magnification lens with immersion oil. Leukocytes were identified and classified (Fig. 1C–G) by counting 100 leukocytes per smear, with results recorded as percentages (Eatwell et al. 2014).

Statistical analyses

We conducted a series of Wilcoxon rank-sum tests for non-parametric data to assess whether there were statistically significant differences in DLC percentages based on sex (males *vs.* females),

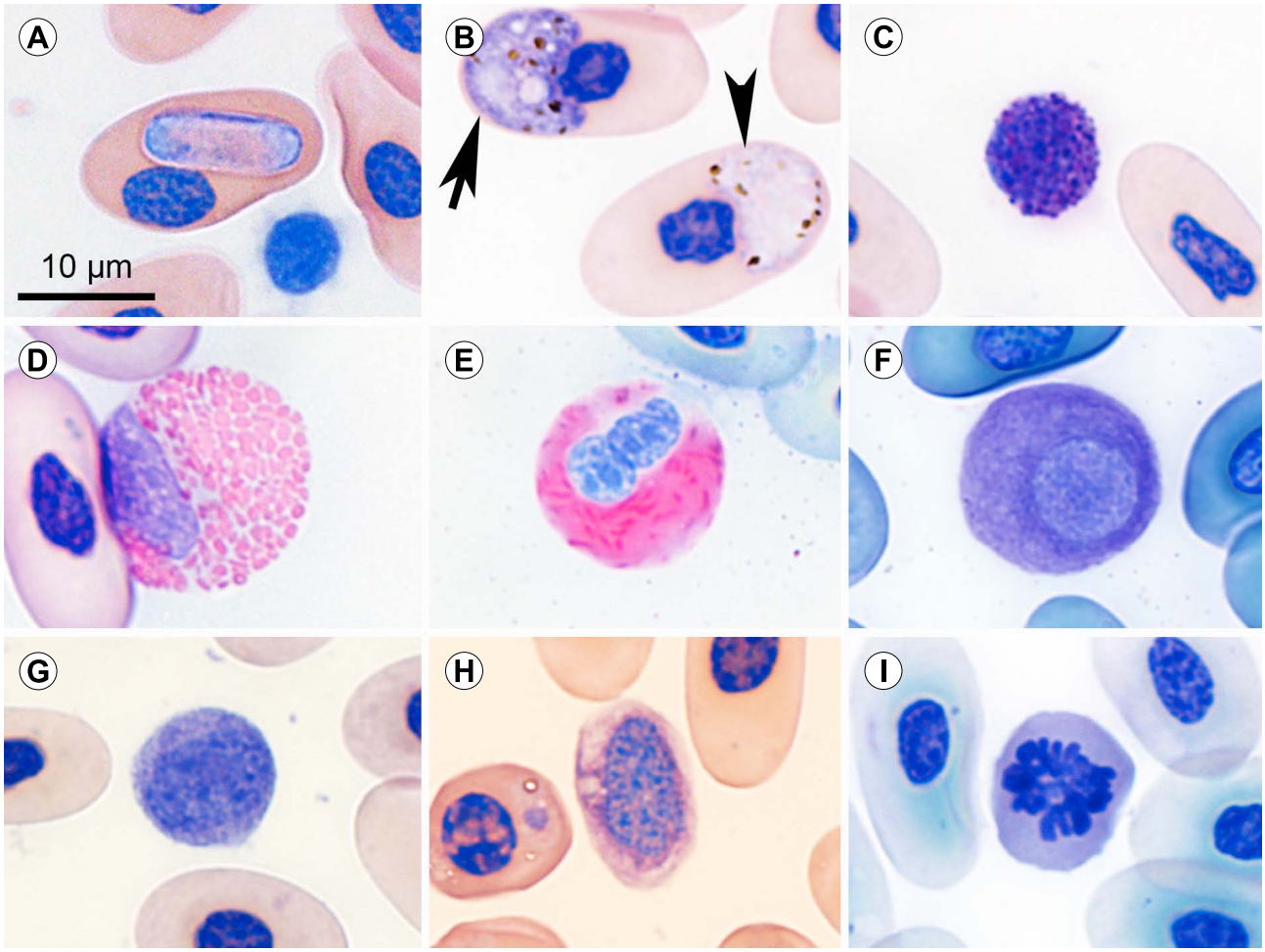

Fig. 1. Photomicrographs of blood protists and blood cells analysed in the present study, all shown at the same scale. **A** – mature gamont of *Hemolivia mauritanica* (Sergent et Sergent, 1904); **B** – *Haemocystidium caucasicum* (Krasilnikov, 1965), arrow indicates macrogametocyte, arrowhead indicates microgametocyte; **C** – basophil; **D** – eosinophil; **E** – heterophil; **F** – azurophil; **G** – lymphocyte; **H** – polychromatophilic erythrocyte; **I** – mitotic erythrocyte.

age group (juveniles *vs.* adults), and infection status (infected *vs.* non-infected individuals). These analyses were performed separately for *T. graeca* and *T. marginata*. However, due to limited data (only one juvenile sample), the effect of age could not be assessed in *T. marginata*. For statistically significant leukocyte groups, we visualised the results using boxplots. We further performed a Wilcoxon rank-sum test to assess possible differences in parasitaemia and a Fisher's exact test to assess prevalence between age groups in *T. graeca.*

To model the effects of parasitaemia, age, and the month of blood collection on leukocyte abundance, we applied generalised linear mixed models (GLMMs) with a negative binomial distribution using the 'glmmTMB' package (Mollie et al. 2017) in RStudio (Posit Team 2023). Sex was excluded as an explanatory variable since no significant differences in leukocyte counts were observed between males and females. A negative binomial distribution was chosen due to the presence of zero values in the response variable. For *T. graeca*, the year of sampling was included as a random factor to account for inter-annual variations. However, for *T. marginata*, the year and month were excluded from the models since all samples were collected within two weeks in 2004. Consequently, these data were analysed using a generalised linear model (GLM) with a negative binomial distribution via the 'MASS' package (Venables and Ripley 2002), as no random effects were included.

Additionally, a subset of *T. graeca* samples from Iran contained parasitaemia data for *Haemocystidium* spp. To explore potential co-infection effects on DLC, we conducted an additional series of models incorporating interaction terms between *H. mauritanica* and *Haemocystidium* spp. parasitaemia. Since we obtained only three specimens infected with *Hc. anatolicum* and six with *Hc. caucasicum*, we pooled the data into a single group labelled *Haemocystidium* spp. Separate analyses for each species would not have allowed for meaningful statistical analysis due to the low sample sizes. These data were also fit to a GLM with a negative binomial distribution in the "MASS" package, without random effects.

Model estimates were obtained by averaging the best-supported models with Akaike Information Criterion (ΔAICs) of less than two, using the "dredge" function in the MuMIn package (Barton et al. 2020) (Table 1). For *T. marginata*, model selection and averaging were not performed, as only a single explanatory variable (parasitaemia) was tested for its effect on basophil counts. However, we ran a null model to assess whether including parasitaemia as an explanatory variable improved the model fit. All statistical tests were conducted with an $\alpha = 0.05$, and mean values are presented as ± standard deviation (SD) unless stated otherwise.

Table 1. Linear mixed models (GLMMs) and linear models (GLMs) for two species of *Testudo* tortoises.

Species	Model	Formula	df	logLik	AICc	ΔAICc	weight
Testudo graeca	% Ly	Age + (1|Year)	4	-750.84	1509.9	0.00	0.45
		Age + Month + (1|Year)	5	-750.38	1511.1	1.18	0.25
		Age + Paras + (1|Year)	5	-750.72	1511.8	1.87	0.18
	% He	Age + (1|Year)	4	-654.52	1317.3	0.00	0.49
		Age + Month + (1|Year)	5	-654.14	1318.6	1.36	0.25
	% Eo	Age + (1|Year)	4	-568.45	1145.1	0.13	0.34
		Month + (1|Year)	4	-569.17	1146.6	1.57	0.22
		Age + Month + (1|Year)	5	-568.23	1146.8	1.81	0.10
		Age + Paras + (1|Year)	5	-568.26	1146.9	1.86	0.10
		Paraz + (1|Year)	4	-569.34	1146.9	1.90	0.10
	% Az	Paras + (1|Year)	4	-476.86	961.9	0.00	0.34
		Age + Paras + (1|Year)	5	-476.64	963.6	1.68	0.20
		Month + Paras + (1|Year)	5	-476.65	963.6	1.71	0.18
	% Ba	Age + Month + (1|Year)	5	-398.19	806.7	0.00	0.26
		Age + (1|Year)	4	-399.25	806.7	0.01	0.26
		Age + Month + Paras + (1|Year)	6	-397.50	807.5	0.77	0.17
		Age + Paras + (1|Year)	5	-398.64	807.6	0.99	0.16
T. marginata	% Ba	Paras	3	-24.38	56.1	0.00	-
		Null model	2	-31.19	67.0	9.90	-
T. graeca (Iran)	% Ly	Age	3	-134.34	275.5	0.00	0.43
		Age + Paras 2	4	-133.346	276.1	0.61	0.32
	% He	Age	3	-140.98	288.8	0.00	0.38
		Age + Paras 2	4	-139.77	288.9	0.16	0.35
	% Eo	Age	3	-72.78	152.4	0.00	0.41
		Age + Paras 1	4	-72.03	153.4	1.07	0.24
	% Az	Age	3	-106.90	219.6	1.37	0.14
		Paras 1	3	-106.89	219.6	1.36	0.14
		Paras 2	3	-106.92	219.6	1.41	0.13
	% Ba	Paras 1	3	-81.55	169.9	1.99	0.15
		Paras 2	3	-81.49	169.8	1.87	0.16

Abbreviations: % Az – percentage of azurophils, % Ba – percentage of basophils, % Eo – percentage of eosinophils, % He – percentage of heterophils, % Ly – percentage of lymphocytes, AICc – The Akaike information criterion, df – degrees of freedom, logLik – log-likelihood, Paras – parasitaemia, Paras 1 – parasitaemia by *Hemolivia*, Paras 2 – parasitaemia by *Haemocystidium*, ΔAICc – delta Akaike information criterion.

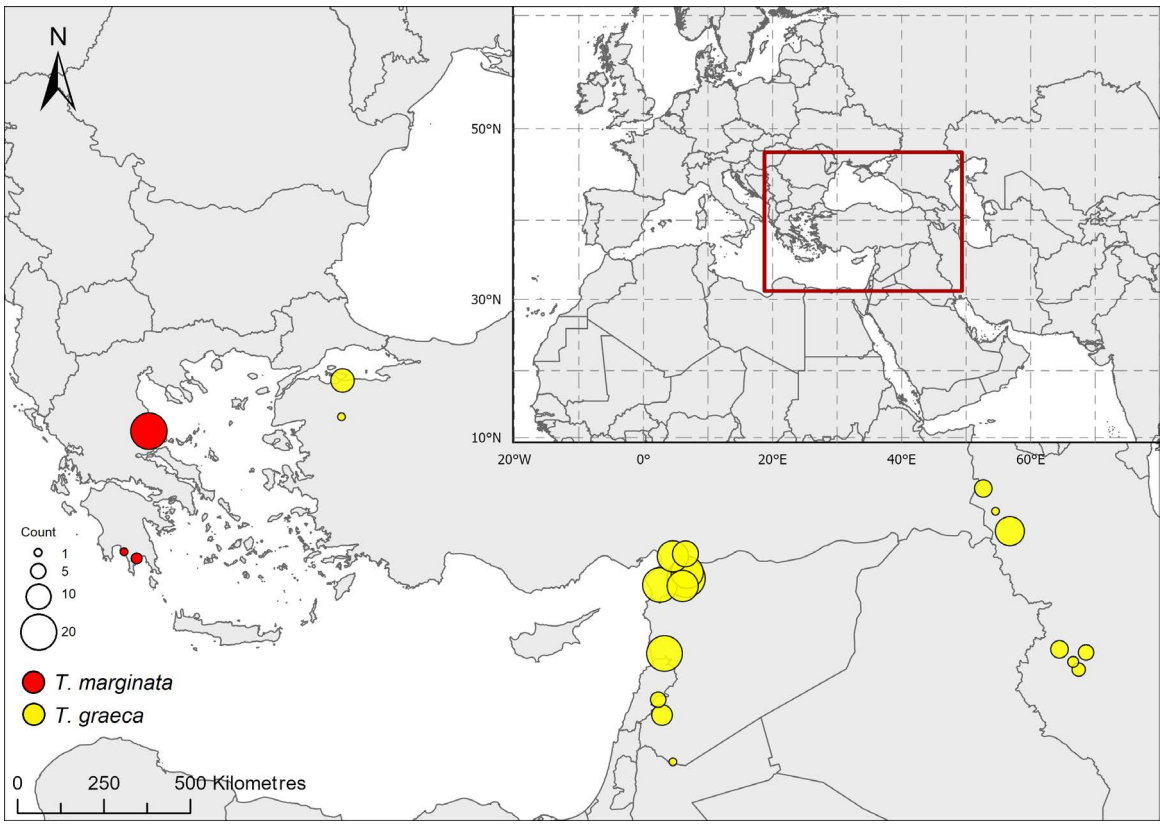

Fig. 2. Map showing the geographic distribution of sampling localities.

Table 2. Results of Wilcoxon rank-sum tests. Numbers of individuals are presented in brackets. Values are presented as mean ± standard deviation. Statistically significant variables are in bold.

Species	Variable	Status				Sex				Age			
		Noninf. (56)	Inf. (125)	W	p	Female (90)	Male (74)	W	p	Juvenile (19)	Adult (162)	W	p
T. graeca **(n =181)**	% Ly	70 ± 16.6	64.5 ± 17.3	4238	**0.02**	65.2 ± 17.9	64.6 ± 17.2	3389	0.85	77.4 ± 7.5	64.8 ± 17.6	852	**0.00**
	% He	15 ± 15.6	16.8 ± 12.9	2978	0.11	15.3 ± 11.9	19.1 ± 16.4	2936	0.19	8.7 ± 6.0	17.1 ± 14.2	2144	**0.00**
	% Eo	5.1 ± 5.0	5.6 ± 4.4	4187	**0.03**	5.2 ± 4.2	6.0 ± 5.2	3796	0.12	4.8 ± 3.4	5.5 ± 4.7	945	**0.00**
	% Ba	5.9 ± 8.0	10.2 ± 10.5	2388	**0.00**	11.0 ± 11.6	7.5 ± 8.1	3835	0.10	3.7 ± 2.7	9.5 ± 10.3	2110	**0.01**
	% Az	4.0 ± 3.3	2.9 ± 2.6	3128	0.25	3.3 ± 2.6	2.7 ± 2.6	3090	0.43	5.2 ± 4.1	3.0 ± 2.6	1619	0.71
		(4)	(19)			(8)	(15)						
T. marginata **(n = 25)**	% Ly	57.3 ± 17.2	64.2 ± 12.0	27.0	0.39	66.3 ± 14.2	61.3 ± 12.3	70.0	0.53	-	-	-	-
	% He	29.5 ± 16.5	25.1 ± 11.5	45.5	0.57	22.8 ± 11.6	27.5 ± 12.5	46.5	0.40	-	-	-	-
	% Eo	7.0 ± 5.8	7.5 ± 5.3	37.0	0.96	6.6 ± 6.4	7.8 ± 4.7	50.0	0.53	-	-	-	-
	% Ba	3.8 ± 3.3	0.5 ± 1.0	62.0	**0.03**	1.4 ± 2.8	0.9 ± 1.5	60.0	0.91	-	-	-	-
	% Az	2.5 ± 2.1	2.4 ± 1.8	40.0	0.22	3.0 ± 1.2	2.1 ± 2.1	79.0	0.92	-	-	-	-

Abbreviations: % Az – percentage of azurophils, % Ba – percentage of basophils, % Eo – percentage of eosinophils, % He – percentage of heterophils, % Ly – percentage of lymphocytes, Inf. – infected tortoises, Noninf. – not infected tortoises, p – value of significance, W – Wilcoxon rank-sum test statistic.

Table 3. Results of GLMM and GLM models. Statistically significant variables are in bold.

Species	Model	Variable	Estimate	SE	z	p
Testudo graeca	% Ly	(Intercept)	4.20	0.13	32.77	**0.00**
		AgeJuv	0.16	0.05	3.03	0.00
		Month6	-0.19	0.18	1.05	0.30
		Paras	0.00	0.01	0.50	0.62
	% He	(Intercept)	2.67	0.32	8.33	**0.00**
		AgeJuv	-0.61	0.19	3.29	**0.00**
		Month6	0.42	0.44	0.94	0.35
	% Eo	(Intercept)	2.10	0.30	6.88	**0.00**
		AgeJuv	-0.32	0.24	1.33	0.18
		Month6	0.34	0.48	0.71	0.48
		Paras	-0.02	0.03	0.51	0.61
	% Az	(Intercept)	1.61	0.19	8.54	**0.00**
		Paras	0.05	0.02	2.06	**0.04**
		AgeJuv	-0.11	0.17	0.65	0.52
		Month6	0.20	0.30	0.68	0.50
	% Ba	(Intercept)	1.11	0.26	4.32	**0.00**
		AgeJuv	0.41	0.17	2.43	**0.02**
		Month6	-0.22	0.17	0.79	0.43
		Paras	0.01	0.02	0.55	0.58
Testudo marginata	% Ba	(Intercept)	1.09	0.37	2.96	**0.00**
		Paras	-5.79	2.35	-2.46	**0.01**
T. graeca (Iran)	% Ly	(Intercept)	3.89	0.06	65.22	**0.00**
		AgeJuv	0.45	0.11	4.06	**0.00**
		Paras2	0.15	0.06	2.36	**0.02**
	% He	(Intercept)	3.51	0.12	28.82	**0.00**
		AgeJuv	-1.07	0.36	2.89	**0.00**
		Paras2	-0.25	0.19	1.30	0.19
	% Eo	(Intercept)	0.97	0.17	5.56	**0.00**
		AgeJuv	0.76	0.27	2.73	**0.01**
		Paras1	0.09	0.07	1.26	0.21
	% Az	(Intercept)	2.20	0.13	16.22	**0.00**
		AgeJuv	0.06	0.29	0.19	0.85
		Paras1	0.02	0.07	0.23	0.82
		Paras2	-0.00	0.19	0.02	0.99
	% Ba	(Intercept)	1.31	0.16	7.96	**0.00**
		Paras1	0.05	0.08	0.65	0.51
		Paras2	0.14	0.18	0.77	0.44

Abbreviations: % Az – percentage of azurophils, % Ba – percentage of basophils, % Eo – percentage of eosinophils, % He – percentage of heterophils, % Ly – percentage of lymphocytes, AgeJuv – juvenile individuals with adults used as the reference category, p – value of significance, Paras – parasitaemia, Paras 1 – *Hemolivia* parasitaemia, Paras 2 – *Haemocystidium* parasitaemia, SE – standard error, z – Wald test statistic.

RESULTS

Among the 206 examined samples, 146 (70.9%) were found to be positive for apicomplexan parasites, specifically *Hemolivia mauritanica* and the genus *Haemocystidium. Hemolivia mauritanica* was detected in 125 out of 181 *Testudo graeca* samples (69%), and in 21 out of 25 *Testudo marginata* samples (84%). Haemosporidians of the genus *Haemocystidium* were found exclusively as co-infections with *H. mauritanica* in 9 out of 181 (5%) *T. graeca* samples, all from Iran (Fig. 1A,B). Specifically, six *T. graeca* were infected with *Haemocystidium caucasicum* and three with *Haemocystidium anatolicum*.

In both tortoise species, lymphocytes represented the most abundant leucocyte type, followed by heterophils. Our analysis revealed statistically significant differences in the DLC within both tortoise species (Table 2, Fig. 3). In *T. marginata* infected with *H. mauritanica*, significant differences were observed only in basophil percentages between infected and non-infected samples. In contrast, *T. graeca* samples exhibited significant differences in lymphocyte, eosinophil and basophil counts between infected and non-infected tortoises (Table 2, Fig. 3). Additionally, differences between adults and juveniles were observed in nearly all leucocyte types, except for azurophils (Fig. 3).

Of the 181 *T. graeca* samples, 162 were from adults (121 positive, 41 negative) and 19 from juveniles (4 positive, 15 negative). The mean parasitaemia was 1.1 ± 1.9 in adults and 0.2 ± 0.4 in juveniles. Statistical analyses revealed significant differences between the age groups in both parasitaemia ($W = 2340$, $p < 0.01$) and infection prevalence (OR = 0.09, 95% CI = 0.02–0.31; Fig. 4).

For *T. graeca*, GLMM models revealed a statistically significant effect of parasitaemia on the differential count of azurophils (Table 3), with increasing parasitaemia positively correlating with higher azurophil counts (Fig. 5A). Regarding other leukocytes, the models indicated a positive effect of age on the differential counts of lymphocytes and basophils, with older individuals exhibiting higher counts of both cell types. Conversely, a negative effect was observed in heterophils, as adults had significantly lower heterophil counts than juveniles (Table 3).

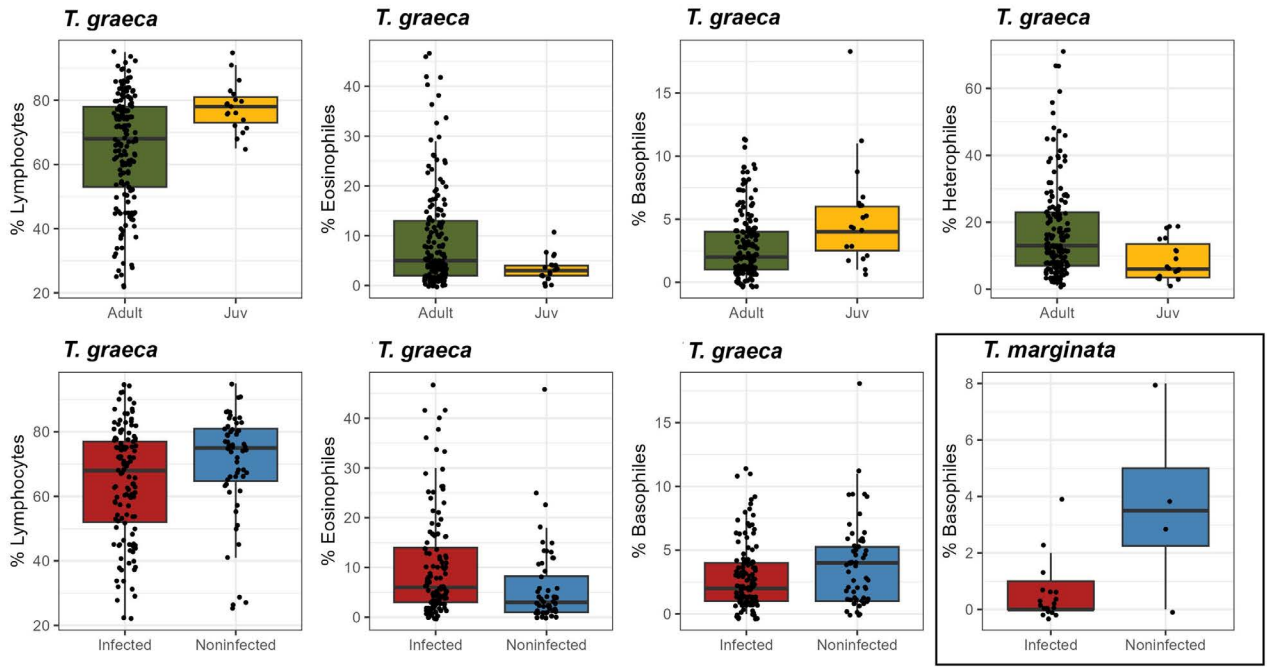

Fig. 3. Boxplots showing the relationship between differential leukocyte counts (DLC) of selected leukocyte types and variables such as age and infection in *Testudo graeca* and *T. marginata*.

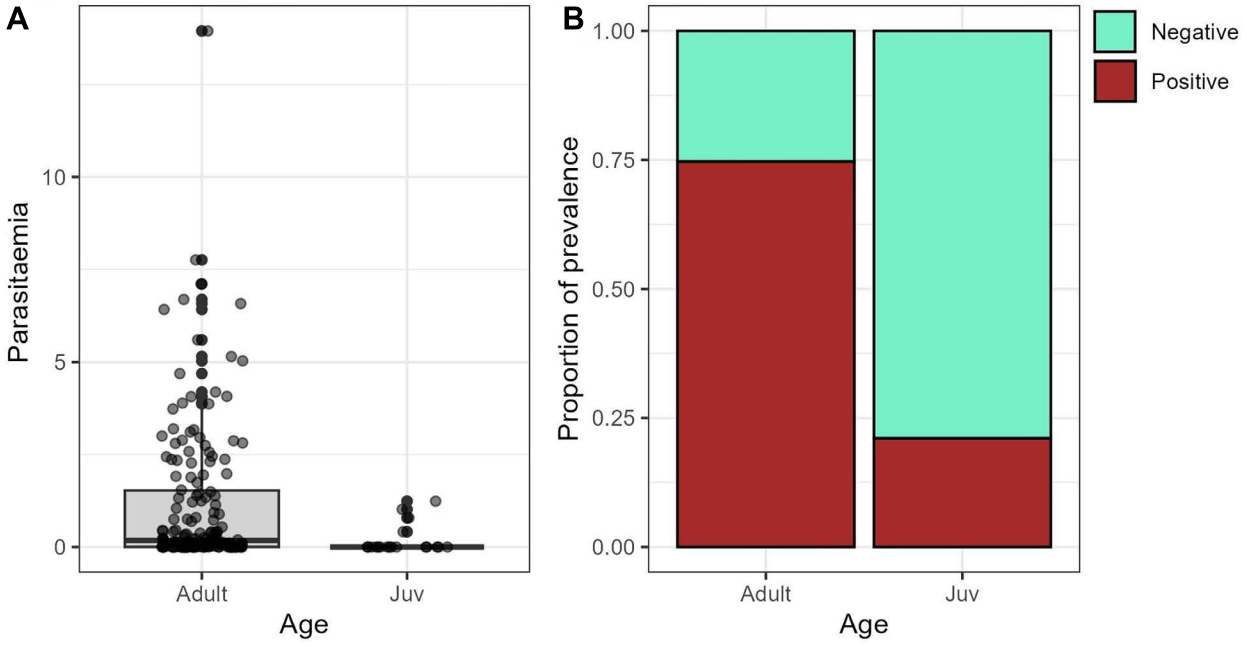

Fig. 4. Age-related differences in blood parasite infection in *Testudo graeca.* **A** – parasitaemia levels are significantly higher in adults compared to juveniles; **B** – prevalence of infection is also higher in adults, with a greater proportion of positive cases relative to juveniles.

In Iranian samples, where we compared single infections of *H. mauritanica* with coinfections involving *H. mauritanica* and *Haemocystidium* spp., we found that *Haemocystidium* parasitaemia was associated with statistically significant higher lymphocyte counts (Table 3, Fig. 5B). For *T. marginata*, the GLM model revealed a statistically significant negative effect of parasitaemia on the differential count of basophils (Table 3, Fig. 5C).

DISCUSSION

In this study, we examined the impact of blood protists from the genera *Hemolivia* Petit, Landau, Baccam et Lainson, 1990 and *Haemocystidium* on specific differential leukocyte count (DLC) parameters in *Testudo* tortoises. Our findings highlight that these parasites can significantly affect certain DLC values, emphasising the importance of investigating blood parasites in reptiles and other ectother-

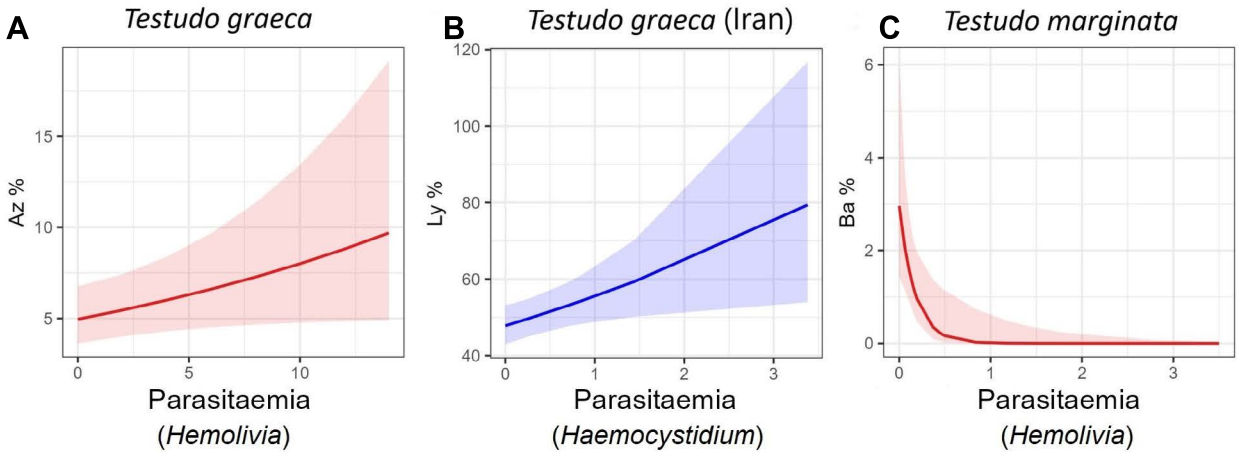

Fig. 5. Graphs showing the influence of *Hemolivia mauritanica* parasitaemia on differential counts of azurophils in *T. graeca* (A), the influence of *Haemocystidium* parasitaemia on differential counts of lymphocytes in *T. graeca* from Iran (B), and the influence of *H. mauritanica* parasitaemia on differential counts of basophils in *T. marginata* (C).

mic vertebrates, an area often overshadowed by mammalian research. This imbalance has resulted in a scarcity of data for meaningful evaluation and comparison, a gap our study aims to address. Another challenge lies in the taxonomic fragmentation of existing studies, which examine a wide range of host and parasite species. From this perspective, we focus on well-studied and widely distributed model taxa: *Hemolivia* (Michel 1973, Landau and Paperna 1997, Široký et al. 2007a, 2009, Kvičerová et al. 2014); and *Testudo* (Fritz 2001, Fritz et al. 2007, 2009, Mikulíček et al. 2013, Javanbakht et al. 2017), supplemented in nine cases by co-infection with two *Haemocystidium* species (Krasilnikov 1965, Orkun and Güven 2013, Javanbakht et al. 2015a), facilitating future comparative and follow-up studies.

It is well documented that the sex of turtles can influence certain haematological parameters, such as packed cell volume, haemoglobin levels, red blood cell count, and mean cell volume (Stacy et al. 2011, Bielli et al. 2015, Apache et al. 2023). However, our results did not reveal any statistically significant differences in DLC between males and females. Similar findings were reported by Martínez-Silvestre et al. (2001) in captive *Testudo marginata*, and by Hernández et al. (2017) in their study on yellow-bellied slider turtles, *Trachemys scripta scripta* (Schoepff). The authors also examined seasonal variations in blood parameters and found that DLC exhibited significant seasonality. In our study, this aspect could not be assessed, as sampling was limited to only two months of the year (April and June). Consequently, the month of collection did not have a statistically significant influence on our results.

Significant differences were found between juvenile and adult *Testudo graeca* tortoises, with adults showing higher prevalence of infection and greater parasitaemia than juveniles. Statistically significant differences were found in almost all examined leukocyte types, including lymphocytes, eosinophils, heterophils, and basophils. Adults had a higher percentage of eosinophils and heterophils, whereas juveniles exhibited a higher count of basophils and lymphocytes (Fig. 3). For *T. marginata* tortoises, we lacked sufficient samples to compare age classes, as only one juvenile specimen was analysed.

In a study on eastern box turtles, *Terrapene carolina carolina* (Linnaeus), juveniles consistently had higher white blood cell counts (WBC) than adults (Winter et al. 2019). Similarly, juvenile loggerhead sea turtles, *Caretta caretta* (Linnaeus), had significantly higher WBC than adults, with heterophils being the most abundant leukocyte type (Casal et al. 2009). Similarly, Martínez-Silvestre et al. (1999) reported heterophils as the most abundant leukocyte type in *T. graeca*, which is inconsistent with our results and requires further investigation. Notably, Martínez-Silvestre et al. (2001) reported no effect of age cohort on haematological parameters in a captive group of *T. marginata*, in contrast to our findings, which clearly indicate that DLC is age-dependent and should be considered in future studies.

Our study revealed an association between the presence of *Hemolivia mauritanica* and specific changes in DLC in tortoises. Infected *T. graeca* exhibited higher DLC of eosinophils but lower DLC of lymphocytes and basophils (Fig. 3). Similarly, infected *T. marginata* tortoises also showed a reduced DLC of basophils (Fig. 3). When assessing the influence of parasitaemia levels on DLC, we observed distinct effects on different leukocyte types. In *T. graeca*, parasitaemia was linked to changes in azurophils (Fig. 5A), a unique leukocyte type in reptilian blood. In snakes, azurophils are thought to serve a function similar to that of neutrophils in mammals. Increased numbers of these cells are often associated with inflammation and infection during acute stages (Stacy et al. 2011, Arikan and Çiçek 2014, Martins et al. 2016).

In *T. marginata*, basophils appeared to respond to parasitaemia, with their percentage decreasing as parasitaemia increased (Fig. 5C). The function of basophils in reptiles remains poorly understood, but their percentage has been reported to rise in response to certain haemoparasite and viral infections (Stacy et al. 2011). Most infectious agents in chelonians – including haemoparasites, bacteria, fungi, and viruses – can elicit inflammatory responses in affected tissues, potentially leading to significant changes in periph-

eral blood. For instance, erythrocytic parasites have been associated with anaemia, reduced haemoglobin, basophilia, eosinophilia, heterophilia, and azurophilia (Knotkova et al. 2005, Martins et al. 2016).

Although blood parasites are commonly found in reptiles, tortoises often do not exhibit clinical signs of infection. Research over the past two decades generally suggests that these parasites have low pathogenicity (Knotkova et al. 2005, Široký et al. 2007a, Rossow et al. 2013, Picelli et al. 2015, Apache et al. 2023). Additionally, we observed the presence of polychromatophilic and mitotic erythrocytes in the bloodstream of infected tortoises (Fig. 1H,I). This phenomenon has been documented in several studies on other reptiles and amphibians (Schall 1990, Martínez-Silvestre et al. 2011, Martínez-Silvestre and Arribas 2014, González et al. 2021). The increase in young erythrocytes may result from blood regeneration stimulated by parasitic infection. The life cycle of the parasite often leads to the destruction of infected blood cells, prompting blood regeneration, which is characterised by a high frequency of young erythrocytes in the peripheral blood (González et al. 2021).

Coinfections with various blood parasites are common in studies examining the influence of individual parasite species on the host organisms. Coinfecting agents can exhibit a range of interactions, from synergy to antagonism (Thumbi et al. 2013, Okon et al. 2023). Clear diagnostic differences between *H. mauritanica* and *Haemocystidium* spp. eliminate the possibility of diagnostic errors and prevent the misinterpretation of results, enabling differentiation between individuals infected solely with *H. mauritanica* and those with mixed infections of both parasites.

We found that nine out of 34 Iranian *T. graeca* were infected with *Haemocystidium* parasites, three with *Haemocystidium anatolicum* and six with *Haemocystidium caucasicum*, always in coinfection with *H. mauritanica*. Coinfections with various blood parasites can have significant implications for the health and fitness of infected hosts. They may increase pathogenicity, alter host immune responses, and induce more severe clinical symptoms (Hananeh et al. 2022). In our study, coinfection did not significantly influence the DLC of hosts. However, in terms of parasitaemia level, *Haemocystidium* impacted differential counts of lymphocytes (Fig. 5B). It is important to note that the individual contributions of each *Haemocystidium* species could not be determined due to pooling their data together. One sample was so heavily infected with *Hc. caucasicum* that it skewed the curve in the graph. Since only nine tortoises were co-infected by both studied parasites, further investigation with a larger sample size is needed to better understand the dynamics and potential impacts of these parasites, including their mixed infections, on tortoise DLC and overall health.

We can conclude that *Hemolivia*-infected tortoises exhibited higher erythrocyte regeneration, and the level of parasitaemia influenced the DLC of azurophils in *T. graeca* and the DLC of basophils in *T. marginata*. Similarly, the parasitaemia level of *Haemocystidium* infection affected the DLC of lymphocytes in *T. graeca*. These findings suggest a possible interplay between parasitic infection, leukocyte profiles, and erythrogenesis in infected tortoises. A larger sample size, additional tortoise species models, and experimental infections under controlled laboratory conditions could provide further insights into the interaction between blood protists and their tortoise hosts.

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