

## ORIGINAL ARTICLE

# Morphological characteristics of blood cells in monitor lizards: is erythrocyte size linked to actual body size?

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## Abstract

Blood cell morphology and count are not uniform across species. Recently, between-species comparisons revealed that the size of red blood cells is associated with body size in some lizard taxa, and this finding was interpreted in the context of the metabolic theory. In the present study, we examined the numbers and the size of blood cells in 2 species of monitor lizards, the mangrove-dwelling monitor (*Varanus indicus*) and the savannah monitor (*V. exanthematicus*), and we compared these traits in individuals of different body size. The results revealed that during the course of ontogeny, the size of red blood cells increases with body mass. Because the mass-specific metabolic rate decreases with body size and the cell volume-to-surface ratio decreases with the cell size, changes in the erythrocyte size might be the result of oxygen transport adjustment.

**Key words:** blood parameters, body size, erythrocyte size, metabolic theory, Varanidae

## INTRODUCTION

Comparative data on blood-cell dimensions were recently used for an evaluation of general ecomorphological and ecophysiological rules. Starostová *et al.* (2005) examined the relationship between erythrocyte size and body size among 14 taxa of eye-lid geckos of the family *Eublepharidae*, a taxon including species represent-

ing a wide scale of adult body sizes. Although the relationship was surprisingly close ( $r^2 = 0.80$ ), the slope of the allometric relationship of the erythrocyte area was relatively small (reduced major axis regression [RMA] slope = 0.118). In subsequent studies, Starostová *et al.* (2008, 2009) showed that in the eye-lid geckos, the mass-specific standard metabolic rates may be explained by erythrocyte size, but not by genome size. The authors interpret these results as a violation of the current metabolic theory of ecology (West *et al.* 1997, 2001; Brown *et al.* 2004), which is based on the models expecting cell-size invariance, and indirect support for alternative models considering the contribution of both cell sizes and numbers on metabolic rates (Kozłowski *et al.* 2003; Kozłowski & Konarzewski 2005).

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In the present paper, we paid attention to morphological characteristics (size) and counts of blood cells of monitor lizards (i.e. the group exhibiting extreme changes in body size from both phylogenetic and ontogenetic perspectives) (see Pianka 1995; Frýdlová & Frynta 2010; Frynta *et al.* 2010). In contrast to the above studies relying on between-species comparisons, we focused on the within-species relationships between blood cell size and body size assessed during the course of ontogeny. The mangrove-dwelling monitor (*Varanus indicus* Daudin, 1802) belongs to the subgenus *Euprepriosaurus* inhabiting New Guinea and surrounding areas (see Ast 2001; Böhme 2003). *V. indicus* is a representative of a closely related group of medium-to-large bodied species (Ziegler *et al.* 2007; Koch *et al.* 2009) exhibiting apparent sexual size dimorphism in adult body size (Frýdlová & Frynta 2010; Frynta *et al.* 2010; Frýdlová *et al.* 2011). These animals can live approximately 17 years and reach sexual maturity at 1.5 years old (Frynta *et al.* 2010). Considerable differences between body sizes of adult males and females of the same age provide an analytical tool for the separation of the effect of body size from that of age. We seized the possibility to collect samples from animals (siblings kept under identical conditions) whose growth, food intake, behavior and health condition were carefully monitored from hatching to adulthood as a part of another research project focusing on life histories and sexual size dimorphism (Frynta *et al.* 2010; Frýdlová *et al.* 2011). The timing of the sampling was performed to cover the period of ontogeny when body size differences between the sexes were already apparent, but both sexes still exhibited rapid growth.

In addition, to verify the generality of the uncovered relationship, we completed the data with the savannah monitor (*V. exanthematicus* Bosc, 1792), which belongs to the subgenus *Polydaedalus*, representing the basal-most branch of the phylogenetic tree of the monitor lizards (Ast 2001). In this species, we only compared the blood parameters of hatchlings and adults.

The aim of the present paper is: (i) to describe the size and counts of blood cells in 2 species of monitor lizards, and (ii) to assess the relationship between the size of blood cells and body size.

## MATERIALS AND METHODS

The present study included a total of 32 mangrove-dwelling monitors reared at Prague Zoo (21 males and 11 females; sexually mature approximately 2-year-

old adults) and 9 savannah monitors (2 males and 3 females; 4 hatchlings and 5 4-year-old adults). The mangrove-dwelling monitors were siblings from 1 mother belonging to 4 subsequent clutches hatched in November 2006 and January, February and June 2007. The genetic relatedness among studied individuals allowed us to reduce the between-individual component of variance in examined phenotypic traits and, therefore, to focus on the effects of sex and body size. Experimental animals were housed individually in terraria, which were gradually exchanged for larger terraria to satisfy the needs of the growing animals (ending with terraria 145 × 100 × 130 cm large). The ambient temperature in the breeding room varied from 28 to 30 °C, with hotter basking sites. Chicken livers and necks were provided weekly (*ad libitum*) and they were supplemented with vitamins and minerals (Nutri Mix, Trouw Nutrition Biofaktory, Prague, Czech Republic). Vitamins AD<sub>3</sub> and E were provided monthly.

On 14 December 2008, 15 February 2009 and 25 April 2009, we performed complex veterinary surveys, including the collection of blood samples from every individual of mangrove-dwelling monitor for the assessment of blood morphology and counts. Blood samples were taken from the ventral caudal tail vein. The blood was sampled by direct puncture with a 20-G needle and a 2-mL heparinized syringe. Approximately 0.8 to 1.5 mL of whole blood was aspirated and sent directly for biochemical analysis, blood smear and blood cell count.

The blood smears were stained with hematologic stains (May-Grunwald and Giemsa-Romanowski). The erythrocytes were photographed at the same magnification (40×), calibrated with a glass microscopic scale of a known length (1 mm). The area of red blood cells, white blood cells (heterophil) and blood platelets was measured using the UTHSCSA Image Tool (UTHSCSA 2002) and digital photographs taken by the Provis AX 70 microscope (Olympus). In each sample, we took the area of 30 randomly chosen red blood cells and a minimum of 10 heterophils. The number of red blood cells, white blood cells (heterophils, eosinophils, monocytes and lymphocytes) and blood platelets was counted. Basophils are degranulated by this staining method (Campbell & Ellis 2007) and, therefore, not recognized in our analysis. Prior to the collection of a small drop of blood, we recorded the body weight (W) of each individual.

We used the STATISTICA Analysis System (Version 6.0) for most calculations (SatSoft 2001). The variables were log-transformed prior to further analyses. Slopes of allometric relationships (not adjusted for repeat-

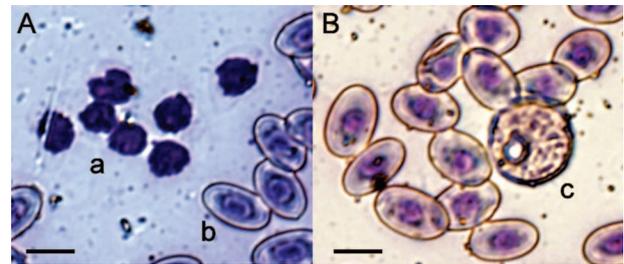
ed measures) were computed using both ordinary least squares (OLS) and RMA regression, which accounts for the errors in both axes (computed in RMA version 1.21; Bohonak & van der Linde 2004).

The models, including a random factor to account for repeated measures of the same individual, were performed in R Version 2.7.1 (R Foundation for Statistical Computing 2008). Body weight is traditionally used as a scaling variable in studies of allometric relationships and, therefore, we adopted this variable as an estimate of body size in further models. Snout–vent length may be viewed as a variable more accurately reflecting the structural size of the lizard body than body weight; however, these 2 variables were correlated enough ( $r = 0.9418$ ) to be mutually nearly equivalent. The effects of body weight, sex and weight  $\times$  sex interaction on individual blood parameters in *V. indicus* were examined using marginal models (package nlme: generalized least squares (GLS) function, normal distribution, link function = identity), with the individual as a random factor (random error terms; symmetric correlation structure corCompSymm). Nevertheless, inclusion of this random factor significantly improved only the models concerning the size of the red blood cells (L-ratio = 7.48,  $P < 0.0062$ ) and the number of white blood cells (L-ratio test = 10.66,  $P < 0.0011$ ). Thus, we removed this factor from the models and applied a linear model (function LM, normal distribution and link function = identity) when treating the other dependent variables. We further reduced the models according to the Akaike information criterion; however, this reduction had only a negligible effect on the results.

## RESULTS

We classified blood cells of both monitor lizard species as erythrocytes, leukocytes (heterophils, eosinophils, monocytes and lymphocytes) and thrombocytes (Fig. 1). Blood count and morphometric characterization of adults of both examined species are presented in Table 1.

In *V. indicus*, the area of erythrocytes (range from 92.30 to 157.82  $\mu\text{m}^2$ ) was positively correlated with body size ( $r = 0.63$ ), and the RMA slope of this allometric relationship was 0.2913 ( $N = 85$ , bootstrap 95% CI 0.2555–0.3306; OLS slope = 0.1835, 95% CI 0.1348–0.2322; (Fig. 2). In *V. exanthematicus*, the mean size of erythrocytes (range: 113.67 to 174.88  $\mu\text{m}^2$ ) was significantly higher in adults than in juveniles (166.28 and 119.29  $\mu\text{m}^2$ , respectively;  $t$ -test:  $t = 14.36$ ,  $P < 0.0001$ ) and the RMA slope of this allometric relationship was 0.0752 ( $r = 0.98$ ).

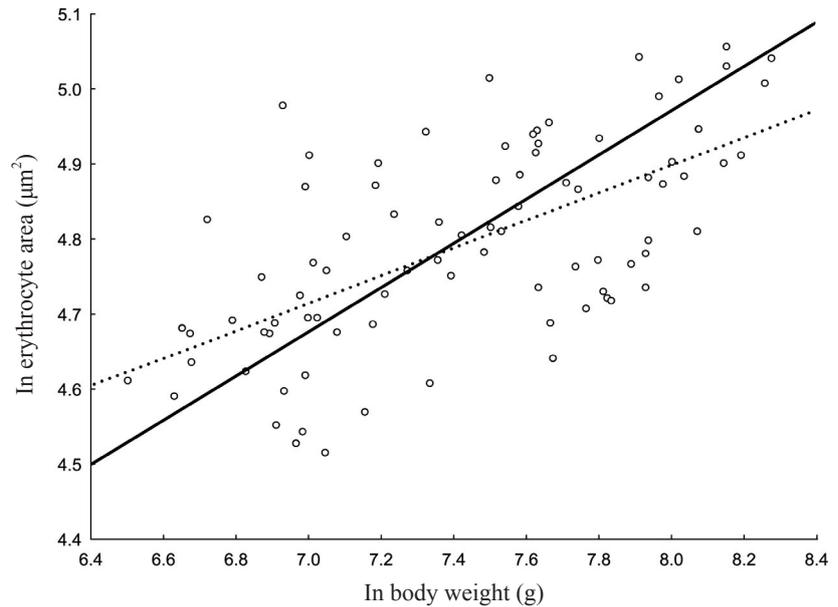


**Figure 1** Photomicrographs of various blood cells of the mangrove-dwelling monitor lizard (*Varanus indicus*): (A) (a) thrombocyte, (b) erythrocyte; (B) (c) heterophil. Bar = 10  $\mu\text{m}$ .

**Table 1** The means of blood counts and blood sizes (area in  $\mu\text{m}^2$ ) of 2 monitor lizards (*Varanus indicus* and *V. exanthematicus*)

	<i>V. indicus</i>				<i>V. exanthematicus</i>	
	Area ( $\mu\text{m}^2$ )	SE	Counts ( $10^9/\text{L}$ )	SE	Area ( $\mu\text{m}^2$ )	SE
	<i>N</i> individuals/cells	LQ–UQ	<i>N</i> cells	LQ–UQ	<i>N</i> individuals/cells	LQ–UQ
RBC	136.66	0.54	1131.00	0.06	144.77	1.67
	32/964	125.18–148.22	30	0.97–1.34	9/270	120.58–169.15
WBC	214.15	2.17	6.82	0.47	209.85	6.66
	32/361	181.95–241.83	30	4.50–9.00	5/51	170.56–251.08
TBC	48.38	1.58	431.00	18.53	54.49	1.72
	32/32	41.21–55.39	30	396–496	5/5	52.41–56.35

LQ, lower quartile;  $N$  = sample size (individuals/cells); RBC, red blood cells; SE, standard error; TBC, thrombocytes; UQ, upper quartile; WBC, number of white blood cells (heterophil, monocyte, eosinophil and lymphocyte).



**Figure 2** The relationship between the area of red blood cells and the body weight of mangrove-dwelling monitor (*Varanus indicus*). Variables were log-transformed.  $r^2 = 0.397$ ;  $r = 0.630$ ,  $P < 0.0001$ ; allometric equations OLS (dotted line):  $\ln \text{erythrocyte area} = 3.431 + 0.1835 \times \ln \text{body weight}$ . RMA (full line):  $\ln \text{erythrocyte area} = 2.629 + 0.2913 \times \ln \text{body weight}$

**Table 2** The results of analysis of variance (ANOVA) tests and coefficients of the models

Dependent variables	Predictors	ANOVA		Model parameters	
		$F_{1,81}$	$P$	coefficients	Standard error
Erythrocyte size	lnW	121.43	<0.0001	0.4489	0.0829
	sex	2.50	0.1179	0.4102	0.6634
	lnW $\times$ sex	0.56	0.4582	-0.0689	0.0924
Erythrocyte size	lnW	118.07	<0.0001	0.3867	0.0367
	sex	2.37	0.1279	-0.0788	0.0512
Leukocyte size	lnW	1.75	0.1899	-0.2760	0.1014
	sex	4.61	0.0348	-2.2075	0.7939
	lnW $\times$ sex	8.38	0.0049	0.3213	0.1110
RBC	lnW	0.00	0.8995		
	sex	3.70	0.0568		
	lnW $\times$ sex	0.10	0.7441		
WBC	lnW	1.01	0.3167	-1.2360	0.3572
	sex	2.22	0.1405	-8.0806	2.7033
	lnW $\times$ sex	9.35	0.0030	1.1628	0.3803
TBC	lnW	0.30	0.5786		
	sex	1.70	0.1978		
	lnW $\times$ sex	0.80	0.3639		

In the case of the erythrocyte size and the number of white blood cells, we applied a generalized least squares function to compute marginal models accounting for repeated measures, while the lm function appeared sufficient for modeling the remaining dependent variables. The coefficients belonging to the interaction refers to body size  $\times$  sex. lnW, log-transformed body weight; RBC, number of red blood cells; TBC, number of thrombocytes; WBC, number of white blood cells (heterophil, monocyte, eosinophil and lymphocyte).

The cell size of the heterophils was not correlated with that of erythrocytes ( $r = 0.004$ ,  $P = 0.974$ ) in *V. indicus*.

The GLS/LM revealed no effect of the examined predictors on the numbers of erythrocytes and thrombocytes in mangrove-dwelling monitors. The number of leukocytes was affected by body weight  $\times$  sex interaction (ANOVA:  $F_{1,81} = 9.35$ ,  $P = 0.0030$ ; for details, see Table 2).

Marginal models (GLS) confirmed that the body weight is a good predictor of the erythrocyte size (ANOVA:  $F_{1,81} = 121.43$ ,  $P < 0.0001$ ; Table 2). In contrast, the size of heterophils was affected by sex (ANOVA:  $F_{1,81} = 4.61$ ,  $P = 0.0348$ ) and body weight  $\times$  sex interaction (ANOVA:  $F_{1,81} = 8.38$ ,  $P = 0.0049$ ) in our linear model; Table 2). The size of heterophils was not significantly correlated with the body size in males ( $r = 0.127$ ,  $P = 0.347$ ), whereas this relationship was, in fact, negative in females ( $r = -0.522$ ,  $P = 0.004$ ).

## DISCUSSION

The blood parameters of the studied species of monitor lizards were quite comparable. Some of these parameters, however, appeared significantly dependent on the size of the animal. This should be further considered in veterinary surveys and comparative physiological and zoological studies.

Erythrocyte size is usually treated as a species-specific but size independent trait (see Schmidt-Nielsen 1984; Gregory 2002). Nevertheless, we demonstrated in the present paper that body weight is a good predictor of the erythrocyte size in both examined species of monitor lizards. The bigger the animal is, the larger its erythrocytes are. This surprising finding follows a clear physiological logic because smaller erythrocytes have relatively larger surfaces and, therefore, are more efficient in oxygen exchange. It is reasonable to expect that the erythrocyte size is adjusted to the actual mass-specific metabolic rate that gradually decreases along the ontogenetic growth trajectories (Smith *et al.* 2008; Clemente *et al.* 2009). In ectotherms, the most important factor potentially affecting the cell size is the temperature (e.g. Van Voorhies 1996). In our experiments, the examined animals were kept in the same room under identical thermal conditions. Although we cannot exclude the size specificity in thermal preferences and basking behavior, we recorded no such obvious differences in behavior of smaller and larger individuals.

On the between-species level, a close allometric relationship between the erythrocyte size and body size is reported in eye-lid geckos (Starostová *et al.* 2005, 2009). The authors consider studies reporting positive correlations among the size of different cell types (see Gregory 2002 for birds) and they interpret the erythrocyte size as a proxy of the typical cell size in a given individual and/or species. The allometric relationship between cell size and body size has fundamental consequences for allometry of metabolic rates because smaller cells (due to higher surface-to-volume ratios) are expected to exhibit higher mass-specific metabolic rates (see Starostová *et al.* 2009).

In contrast to cell size of erythrocytes, we failed to confirm that the size of heterophils is positively associated with body size in *V. indicus*. Moreover, the size of erythrocytes was totally uncorrelated with that of heterophils. This finding somewhat violates the view that the erythrocyte size is a reliable predictor of the cell size of the individual and that metabolic rates are linked to the erythrocyte size via the mean cell size of the whole organism. The specific association of a cell size with body size in the cell type functionally participating in the oxygen transfer and, thus, directly affecting metabolic rates rather suggests that the size of the erythrocytes is linked to the actual metabolic rate by a physiological feedback mechanism.

Erythrocyte (as well as thrombocyte) cell counts appeared independent of body size in our analyses and this simplifies the physiological interpretations of the above discussed ontogenetic allometry of the erythrocyte size. Body size invariance of erythrocyte counts conform to the findings of Troiano *et al.* (2008) in another large sized lizard (*Tupinambis merianae* Puméril & Bibrón, 1839). Nevertheless, a significant decrease in erythrocyte counts with age is demonstrated by Pejřilová *et al.* (2004) in green iguanas [*Iguana iguana rhinolopha* (Wiegmann, 1834)].

In conclusion, our results identify the need for further examination of ontogenetic changes in morphological and physiological parameters, which are seemingly species rather than age and/or size specific. This is especially important in the large reptiles exhibiting indeterminate and/or prolonged growth (see de Buffrénil *et al.* 2004, 2008) and multiplying their body size many times from hatching to senescence.

## ACKNOWLEDGMENTS

We thank Ivan Reháč for the encouragement to study mangrove-dwelling monitor lizards, Jaroslav Smrž for kind help with Provis AX 70 microscope (Olympus), Zuzana Starostová and Lukáš Kratochvíl for stimulating discussions during preparation of the preceding paper dealing with the erythrocyte size and Mr V. Straka, Medista, who kindly provided the equipment (Spotchem EZ analyzer) and material for biochemical analysis of the blood samples. We are grateful to Fritz Rickhoff and Silvie Lišková who kindly improved the English of the paper. The experiments were performed in accordance with the Czech law implementing all corresponding European Union regulations and were approved by the institutional animal care and use committee (8388/2011-30). The project was supported by grant SVV-2012-265 206 and by the Grant Agency of Charles University (No. 62910/2010).

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