Journal of Applied Animal Welfare Science

ISSN: 1088-8705 (Print) 1532-7604 (Online) Journal homepage: http://www.tandfonline.com/loi/haaw20

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To cite this article: Sonia S. Guadarrama, Hublester Domínguez-Vega, Hector M. Díaz-Albiter, Alejandro Quijano, Elizabeth Bastiaans, Porfirio Carrillo-Castilla, Javier Manjarrez, Yuriana Gómez-Ortíz & Victor Fajardo (2019): Hypoxia by Altitude and Welfare of Captive Beaded Lizards (*Heloderma Horridum*) in Mexico: Hematological Approaches, Journal of Applied Animal Welfare Science, DOI: 10.1080/10888705.2018.1562350

To link to this article: https://doi.org/10.1080/10888705.2018.1562350

Published online: 09 Jan 2019.

JOURNAL OF APPLIED ANIMAL WELFARE SCIENCE
https://doi.org/10.1080/10888705.2018.1562350

Routledge
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ARTICLE

Hypoxia by Altitude and Welfare of Captive Beaded Lizards (*Heloderma Horridum*) in Mexico: Hematological Approaches

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ABSTRACT

Heloderma horridum is one of the few known venomous lizards in the world. Their populations are in decline due to habitat destruction and capture for the pet trade. In México, many zoos have decided to take care of this species, most of them at altitudes greater than the natural altitudinal distribution. However, we know little about the capacity of the reptiles to face high-altitude environments. The objective of this study was to compare hematological traits of *H. horridum* in captivity in high and low altitude environments. Our findings show that *H. horridum* does not respond to hypoxic environments, at least in blood traits, and that the organisms appear to be in homeostasis. Although we cannot know if individual *H. horridum* housed in high-altitude environments are completely comfortable, it appears hypoxia can be avoid without modifications of blood parameters. We suggest that future work should address changes in metabolic rates and in behavioral aspects to understand how to maintain the health and comfort of the reptiles native to low altitude when they are housed in high-altitude environments.

KEYWORDS
Heloderma horridum; blood traits; captivity welfare; altitude

The beaded lizard *(Heloderma horridum)* is one of the few known venomous lizard species in the world (Reiserer, Schuett, & Beck, 2013). Their populations are in decline, and some of them are near extinction. The main causes of this decline include habitat destruction, the common local practice of killing venomous lizards, and the growing interest in the captive reptile trade within zoological and educational institutions (Beck, 2006; Domínguez-Vega, Monroy-Vilchis, Manjarrez, & Balderas-Valdivia, 2017).

Heloderma horridum ranges along the Pacific slope of southern Sonora, Mexico to Guatemala (Domínguez-Vega, Monroy-Vilchis, Balderas-Valdivia, & Ariano-Sánchez, 2012). It is found primarily in tropical deciduous forests but has also been recorded in temperate forests, coastal vegetation and foothill thorn scrub habitats. The highest elevation reported for the species is 1800 m, but this is considered an unusual habitat (Lemos-Espinal et al., 2003; Monroy-Vilchis, Hernández-Gallegos, & Rodríguez-Romero, 2005).

Due to the interest in conserving this species, many zoos have opted to keep them in captivity. However, they generally do not take into account the native altitudinal distribution of *H. horridum*,

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although we know little about how dramatic changes in altitude (see Ruiz, Rosenmann, & Nuñez, 1993), affect the welfare of captive reptiles.

Vertebrates that live at high altitude (above 2000 m) are subjected to hypoxic conditions that challenge aerobic metabolism. They usually exhibit functional and structural modifications that allow them to cope with the concomitant decrease in O_2 tension that potentially constrains aerobic life in such environments (González-Morales et al., 2015, 2017). Some of these adaptations include changes in respiratory surfaces, heart size, hemoglobin concentration, hematocrit, capillary density and myoglobin levels (Arredondo et al., 2017; González-Morales et al., 2015, 2017; Monge & Leon-Velarde, 1991, Samaja, Crespi, Guazzi, & Vandergriff, 2003; Weber, 2007).

Information in reptiles is still contradictory regarding how altitude affects blood physiology (see González-Morales et al., 2015, 2017; Ruiz et al., 1993). Some studies show little or no correlation between blood values for lizards and altitudinal distribution (Biswas, Patra, & Boral, 1981; Dawson & Poulson, 1962; Dessauer, 1970; He et al., 2013; Ruiz et al., 1993; Ruiz, Rosenmann, & Veloso, 1983; Weber, 2007). For example, Mexican lizards of the genus *Sceloporus* found in lowlands (61 m elevation.) and highlands (3100 m elevation.) showed no differences in lactate levels, which suggest that they both exhibit similar anaerobic activity patterns (Bennett & Ruben, 1975). Others have reported increased hematological values in highland lizards (Engbretson & Hutchison, 1976; González-Morales et al., 2015; Newlin & Ballinger, 1976; Vinegar & Hillyard, 1972; Weathers & White, 1972). However, most of the studies addressing hematological changes in reptiles have been made in wild animals whose natural distribution is associated with high altitude or natural altitudinal gradients (González-Morales et al., 2015, 2017; Ruiz et al., 1993), whereas studies of hematological traits with animals in captivity are scarce.

Some information exists on hematological traits in wild *Heloderma*. Espinosa-Aviles et al. (2008) studied *H. horridum* in wild-caught animals in Sinaloa, Mexico, and Cooper-Bailey et al. (2011) studied both captive and wild-caught *H. suspectum*, in Arizona, USA (360 m). However, the association between hematological traits and elevation in *Heloderma* is unknown. The objective of this study was to compare hematological traits in 25 blood samples of captive *H. horridum* from different altitudes and test for a relationship between blood traits and altitude. These results may indirectly measure the welfare of individuals that are taken from a low altitude to a higher altitude.

Material and methods

Ethics statement

All experimental procedures were carried out following the guidelines of the *Universidad Autónoma del Estado de México* (UAEM), as well as Mexican Federal Regulation for Animal Experimentation and Care (NOM-062-ZOO-2001; Governmental approval: SGPA/DGVS/02407/13).

Sampled animals

Twenty-five individuals of *H. horridum* were sampled from different sites, (Table 1). All animals had been in captivity for at least six months.

Collection of blood samples

First, we measured the animals' weight; after this, blood samples from individuals were obtained using the ventral tail vein (1 ml from each individual). According to Nardini, Leopardi, and Bielli (2013), most healthy reptiles tolerate an acute loss of up to 10% of the total blood volume, but usually lower volumes are sufficient for complete hematology. Animals were manually restrained and samples were obtained using syringes (20G and 2 ½ inches needle) with lithium heparin. The syringes with blood samples were transferred in a cooler with ice to the Laboratory of Behavioral

Table 1. Sites from which captive *H. horridum* were tested for this study.

Site Name	Sample Size	Location	Altitude (m)	Details
Barranca Honda	9	Municipality of Tlaltizapán, state of Morelos (−99.106944, 18.817778)	1107	Government-approved facility for breeding and commercialization.
CUCBA (Centro Universitario de Ciencias Biológicas y Agropecuarias), Universidad de Guadalajara	7	Municipality of Zapopan, state of Jalisco (−103.4, 20.7167)	1200	Animals are part of university reptile collection.
Universidad Mesoamericana and Africam Safari Zoo	4	Puebla City, state of Puebla (98.3933, 19.0133)	2109	Animals are part of the university and zoo reptile collections.
Zacango Zoo	5	Municipality of Calimaya, State of Mexico (99.646029900, 19.202276200)	2910	Animals are part of the zoo reptile collection.

Physiology at the *Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México*. Samples were used within 24 hours after obtaining them.

Blood traits

Using the blood samples, we measured hematocrit (Hct), erythrocyte count (Erc) and erythrocyte size (Ers) following the protocol used by Nardini et al. (2013) and González-Morales et al. (2015). We used a light microscope (model BX41TF; Olympus Corporation, Tokyo, Japan) to evaluate Ers, and a digital camera system with detail enhancement (model E-330; Olympus Corporation, Tokyo, Japan) to micro-photograph five fields of blood smears stained with Wright's stain to reveal erythrocytes. A minimum of 80 arbitrarily selected erythrocyte cells were measured per animal at each altitude, using the Sigma Scan Pro software version 4 for Windows (Systat Software Inc., San Jose, California, USA). We determined Hct as a percentage of packed cell volume by centrifuging blood samples in a microhematocrit tube for 7 minutes at 14,890 x g.

Additionally, we determined hemoglobin concentration ([Hb]), pH, serum electrolytes (Na^+, K^+, Ca^{2+}) blood gases (PO_2, PCO_2), glucose and lactate using an EPOC blood analysis system (ALERE company, USA) following the methodology used by Ding et al. (2014) and González-Morales et al. (2015, 2017).

Statistical analysis

We used a One-way ANOVA test with a Tukey *post-hoc* test to compare weight and Kruskal-Wallis ANOVA by ranks tests with Dunnett *post-hoc* tests to compare Erc, Ers, Hct, [Hb], pH, serum electrolytes, blood gases, glucose and lactate among altitudes, Additionally, we tested for correlations between altitude (treated as a quantitative variable) and Erc, Ers, Hct and [Hb] using a linear model. All tests were two-tailed, and we considered differences or correlations to be significant when $p < 0.05$. We used Statistica (version 12 for Windows, SAS Institute Inc., Cary, North Carolina, USA) for all statistical analysis. Descriptive statistics are given as mean ± SD.

Results

Weight of animals is summarized in Table 2.

Blood traits

Erc, Hct and [Hb] exhibited similar patterns of variation among our four altitudes; no differences were observed (Table 3). However Ers showed differences between altitudes, with the individuals from low

Table 2. Averages and standard deviation of *H. horridum* weight from different altitudes.

	Barranca Honda, Cuernavaca (1107 m)	CUCBA, Jalisco (1200 m)	U. M. & Africam Safari Zoo, Puebla (2109 m)	Zacango Zoo, Toluca (2910 m)	F-value	P
(Kg)	1.425 ± 0.219	**0.715 ± 0.312***	1.680 ± 0.898	1.424 ± 0.630	4.44	0.014

U. M. = Universidad Mesoamericana

Table 3. Summary of hematological traits of *H. horridum* from different altitudes.

	Barranca Honda, Cuernavaca (1107 m)	CUCBA, Jalisco (1200 m)	U. M. & Africam Safari Zoo, Puebla (2109 m)	Zacango Zoo, Toluca (2910 m)	KW-value	P
Erc (10^6 cells/mm^3)	1.03 ± 0.338	0.68 ± 0.377	0.9725 ± 0.338	1.168 ± 0.545	3.808	0.28
Hct (%)	0.29 ± 0.088	0.22 ± 0.124	0.385 ± 0.107	0.33 ± 0.074	6.254	0.09
[Hb] (g/L)	9.3 ± 1.283	8.1 ± 3.377	10.35 ± 2.333	8.88 ± 2.313	1.174	0.75
Ers (μ^2)	168.09 ± 23.275^a	144.31 ± 8.831^a	139.18 ± 7.433^b	126.71 ± 8.976^b	15.378	0.001

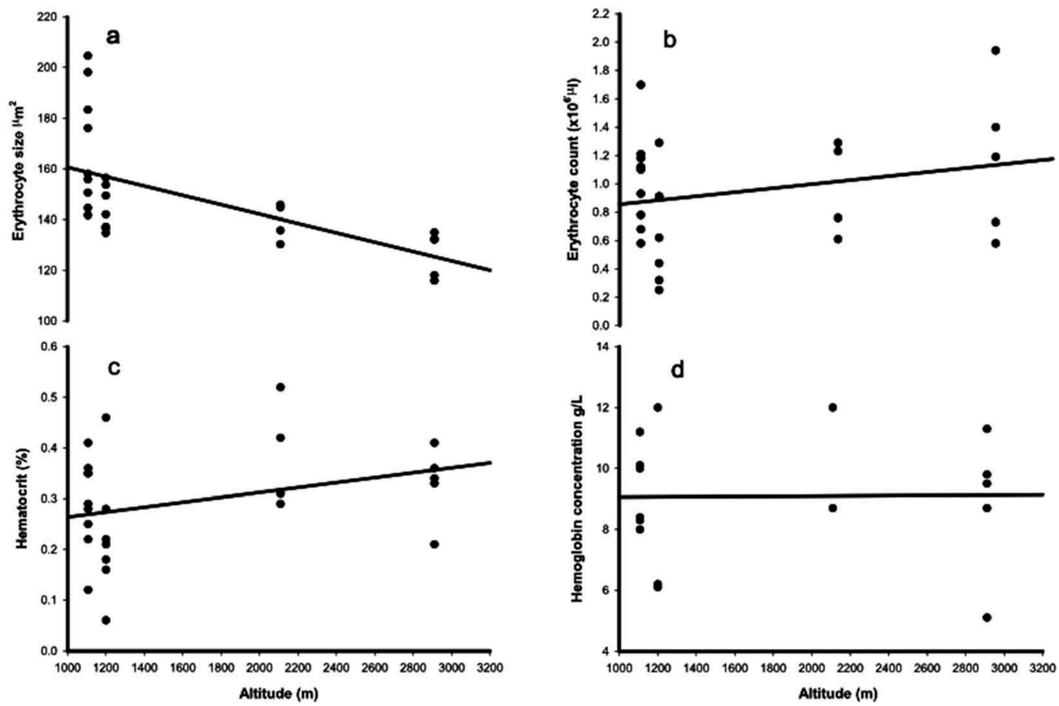

Figure 1. Correlations between Altitude and (a) Erythrocyte size, (b) Erythrocyte count, (c) Hematocrit and (d) Hemoglobin concentration.

altitude having the highest values (Table 3). Furthermore, we found a significant negative correlation between Ers and altitude (r = −79.4 p < 0.001; Figure 1a) but no significant relationships between the other blood traits (Erc, Hct, and [Hb]) and altitude (Figure 1b, c and d).

As with most of the other blood traits we measured, we did not observed differences in blood gases (PCO_2 and PO_2) (Table 4), nor in pH and serum electrolytes (Table 5) among groups from different altitudes.

Table 4. Blood gases of *H. horridum* from different altitudes.

	Barranca Honda, Cuernavaca (1107 m)	CUCBA, Jalisco (1200 m)	U. M. & Africam Safari Zoo, Puebla (2109 m)	Zacango Zoo, Toluca (2910 m)	KW-value	P
pCO_2 mmHg	34.917 ± 10.697 rank 20.8–53.2	25.140 ± 26.076 rank 5.3–69.0	34.150 ± 30.193 rank 12.8–55.5	27.860 ± 7.872 rank 18.0–39.1	2.288	0.514
pO_2 mmHg	84.1 ± 11.236 rank 30.0–135.7	87.950 ± 53.952 rank 43.9–99.4	67.080 ± 20.883 rank 49.8–126.1	97.967 ± 38.832 rank 67.8–98.9	3.184	0.363

Table 5. Metabolites and blood pH of *H. horridum* from different altitudes.

	Barranca Honda, Cuernavaca (1107 m)	CUCBA, Jalisco (1200 m)	U. M. & Africam Safari Zoo, Puebla (2109 m)	Zacango Zoo, Toluca (2910 m)	KW-value	P
Na (mmol/L)	114.800 ± 12.498	102.500 ± 17.678	104.667 ± 20.108	123.333 ± 13.155	4.470	0.214
K (mmol/L)	3.983 ± 0.747	3.120 ± 0.811	3.850 ± 1.202	4.000 ± 0.728	3.075	0.380
Ca (mmol/L)	1.037 ± 0.292	0.468 ± 0.330	0.625 ± 0.290	0.870 ± 0.225	7.572	0.055
Glu (mmol/L)	58.500 ± 9.290	36.000 ± 14.195	45.000 ± 8.485	56.400 ± 13.849	7.711	0.052
Lac (mmol/L)	7.530 ± 5.317	4.856 ± 3.153	8.700 ± 1.598	4.684 ± 3.453	4.769	0.189
pH	7.293 ± 0.0989	7.314 ± 0.241	7.243 ± 0.236	7.300 ± 0.0854	1.303	0.728

Discussion

This is the first study describing hematological traits in *H. horridum* from different altitudes. We showed that most of the blood traits measured in this study did not differ. The data obtained in our study are in accordance with Espinosa-Avilés et al. (2008). However and surprisingly, only Ers showed differences associated with altitude, with the individuals from high altitude showing the smallest erythrocyte size (Fig. 1a).

Reptiles are a diverse group (Dzal et al., 2015), nonetheless, in contrast with other taxa, comparative studies about reptiles' physiology are scarce. Furthermore, most of the hematological descriptions are done without considering population differences within species (Ruiz et al., 1993).

Increases in erythrocyte count, hemoglobin concentration and hematocrit are a common response to altitude in other vertebrate taxa (mammals: Monge & Leon-Velarde, 1991; amphibians: Ruiz et al., 1983), but in reptiles the relationship appears to be more complicated. Blood trait increases related to altitude have been reported in several species of the genus *Sceloporus*, including *S. jarrovi, S. poinsetti, S. virgatus*, and *S. scalaris* (Newlin & Ballinger, 1976; Vinegar & Hillyard, 1972). Increased blood trait values with altitude have also been reported in amphibians such as the toad *Bufo spinulosus* (Ruiz, Rosenmann, & Veloso, 1989) and in mammals such as the yak *Bos grunniens* (Ding et al., 2014). This pattern is somewhat similar to what was previously documented in our laboratory in four population of *S. torquatus*, where erythrocyte size, erythrocyte count, hemoglobin concentration, and hematocrit increased linearly with altitude (González-Morales et al., 2015), however in another closely related species, *S. grammicus*, the increase has a quadratic form, with blood traits leveling out or decreasing at extremely high altitudes (González-Morales et al., 2015, 2017).

In contrast, there was no change in hemoglobin concentration with altitude in *Urosaurus ornatus*, a phrynosomatid lizard species closely related to the genus *Sceloporus* (Newlin & Ballinger, 1976), nor in any blood traits in 27 species of South American lizards from the distantly related genus *Liolaemus* (Ruiz et al., 1993).

Transport of oxygen from the lungs to all tissues of the body is the major function of erythrocytes (Storz, Scott, & Cheviron, 2010). In response to hypoxic environments some organisms increase erythrocyte size and the amount of hemoglobin in their blood (González-Morales et al., 2015; Storz et al., 2010). Another strategy is to modify hemoglobin's affinity for oxygen by adjusting intra-erythrocytic conditions (for more details, see Bogdanova, Berenbrink, & Nikinmaa, 2009; Nikinmaa, 2013). However, most of the studies on erythrocyte size in hypoxic environments show an increase red blood cell area with altitude (see González-Morales et al., 2015, 2017; Mong and León-Velarde, 1991). In our results, we observed no changes in the majority of the blood traits we measured, except for the erythrocyte size, which decreased with altitude.

Differences in Ers have been observed in Andean mice (*Phyllotis xantophygus rupestris*; Ruiz, Rosenmann, & Cortes, 2004). Mice had significantly smaller erythrocytes during the winter than during the summer, but there were no differences in hematocrit and hemoglobin concentrations. The authors suggested that Ers may exhibit these differences due to photoperiodic cues in non-hibernating small mammals, such as reproductive activity, pelage characteristics, temperature regulation, food and changes in body fat. However, all of our results were obtained in summer, all individuals lived in environments where the temperature was regulated, and their diets were similar for at least 6 months before this study.

In birds, Gregory (2002) found that Ers appears to be related to metabolic activity; individuals with higher metabolism had smaller Ers. We did not evaluate any aspect of metabolism in the individuals we studied, but these tests are currently ongoing in our laboratory.

Baraquet et al. (2013) studied the morphology and size of erythrocytes of the frog *Hypsiboas cordobae* (Hylidae) and analyzed the geographic variation of this character along the distribution of the species, in relation to the latitudinal and altitudinal distribution. Erythrocyte size showed differences among populations, with the highest mean size corresponding to the population from the lowest altitude site and the lowest mean size to the highest altitude site. The authors hypothesized that the low concentration of oxygen causes a small Ers because small erythrocytes offer the possibility of greater rate of oxygen exchange than larger erythrocytes.

In vitro studies of erythrocyte oxygen uptake and ultrastructure performed in five amphibian species, found that the number of mitochondria and oxygen uptake were higher in smaller erythrocytes (see Goniakowska, 1970, 1973). Similarly, small erythrocytes offer a greater rate of exchange compared to larger ones (Wojtaszek and Adamowicz, 2003). Based on these works, smaller erythrocytes should be then selected in environments presenting lower levels of oxygen. Our study with *H. horridum* is the first to show a similar response in reptiles.

The altitudinal gradient we tested for *H. horridum* included higher elevations, and presumably greater hypoxia, than animals of this species would be exposed to in the wild. The highest elevation reported for the species is 1800 m, but this is considered an unusual habitat (Beck, 2006). Our findings show that *H. horridum* does not respond to hypoxic environments, at least in most of its blood traits.

However, animals at extremely high altitudes may exhibit more anaerobic metabolism, relative to aerobic metabolism, than animals in low altitude populations. At extreme altitudes, it may not be possible for animals to further increase the capture and consumption of oxygen, but suppressing metabolic oxygen demand may be an effective strategy as in the lizard *Sceloporus graciosus* (Sears, 2005). These physiological adjustments may manifest at multiple biological levels of organization, from cardiopulmonary organ systems to the molecular level of oxidative metabolism (Hochachka, 1986; Sears, 2005; Storz & Moriyama, 2008).

Our results show that *H. horridum* in captivity in high altitude do not change most of the blood parameters in which we expected to observe variation. Furthermore, the blood gases also do not show differences, and it appears the animals at high altitude are in homeostasis. This may be due to metabolic adjustment. On the other hand, it is well known that reptiles have cardiac shunting patterns (see Hicks, 2002). These cardiac adjustments are typically defined by their direction, either as left-to-right (L-R) or right-to-left (R-L). A L-R shunt represents the recirculation of pulmonary venous blood (oxygen rich) into the pulmonary circulation; this produces over-oxygenation of the blood. *Heloderma horridum* may use a L-R shunt to deal with high-altitude environments, which would explain the wide range of blood oxygen found in our study (49.8 to 126.1 for high and 30 to 135 for low environments, Table 4).

The L-R shunt in *H. horridum* in natural conditions may allow these animals to avoid hypoxia when inside burrows (see Birchard, Kilgore, & Boggs, 1984; Hillman, 2009; Rios, Rodriguez, Velazquez, & Hernández, 2013), The longest shelter occupancy period observed for *H. horridum* was during the dry season (November to May) and lasted for 62 days (Ariano-Sánchez & Salazar, 2015). The L-R cardiac shunt strategy may help wild animals to deal with natural hypoxia in their burrows and, in our study, may allow captive animals to avoid hypoxia due to high-altitude environments.

Our results cannot tell us directly if individuals of *H. horridum* housed in high-altitude environments are comfortable, but it appears that effects of hypoxia can be avoided without modifications of blood parameters. However, more detailed studies are necessary. For example, Study Ers throughout the year as it is known that the size of erythrocytes can change (Ruiz et al., 2004), or testing for changes in metabolic rates (using a respirometer) and in behavioral aspects (e.g., changes in respiratory frequency) will help assess the welfare of reptiles from low altitude when they are housed in high-altitude environments.

Acknowledgments

We are grateful to the *Consejo Nacional de Ciencia y Tecnología* (master degree scholarship SSG 745865) for financial support and the *Secretaría del Medio Ambiente y Recursos Naturales* for the permits to collect animals (SGPA/DGVS/02407/15). We thank L. Rodríguez, J. Quintana, T. Rubio, M. Sánchez and A. Reyna for field and technical assistance. We thank A. Alvarez-Trillo and Reptilium Herpetarium, M.V.Z. J. A. Díaz-Vallejo and M. in C. J. A. Hernández Díaz from African Safari and *Universidad Mesoamericana* Puebla, Biol. E. Fanti-Echegoyen fom *Centro Universitario de Ciencias Biológicas y Agropecuarias* (CUCBA) and Dr. M. A. Rebolledo-Rios from *UMA Barranca Honda*. We also thank the anonymous referees for their comments and suggestions, which significantly contributed to improving the quality of the publication.

Funding

This work was supported by the Consejo Nacional de Ciencia y Tecnología [SSG 745865].

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