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Oxidative stress decreases with elevation in the lizard *Psammodromus algirus*

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ABSTRACT

Oxidative stress is considered one of the main ecological and evolutionary forces. Several environmental stressors vary geographically and thus organisms inhabiting different sites face different oxidant environments. Nevertheless, there is scarce information about how oxidative damage and antioxidant defences vary geographically in animals. Here we study how oxidative stress varies from lowlands (300–700 m asl) to highlands (2200–2500 m asl) in the lizard *Psammodromus algirus*. To accomplish this, antioxidant enzymatic activity (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, DT-diaphorase) and lipid peroxidation were assayed in tissue samples from the lizards' tail. Lipid peroxidation was higher in individuals from lowlands than from highlands, indicating higher oxidative stress in lowland lizards. These results suggest that environmental conditions are less oxidant at high elevations with respect to low ones. Therefore, our study shows that oxidative stress varies geographically, which should have important consequences for our understanding of geographic variation in physiology and life-history of organisms.

1. Introduction

Oxidative stress, the unbalance between the production of pro-oxidant substances and antioxidant defences (Halliwell, 2007), is considered one of the most important ecological and evolutionary forces (von Schantz et al., 1999; Costantini, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009; Costantini et al., 2010; Metcalfe and Alonso-Alvarez, 2010). Aerobic metabolism implies the production of pro-oxidant substances (reactive oxygen/nitrogen species, RONS; Finkel and Holbrook, 2000), which may react with molecular components of the cell such as lipids, proteins and nucleid acids, producing damages in cells' machinery (Halliwell and Gutteridge, 1995; Sies, 1997; Halliwell, 2007). Organisms are protected against oxidative damage by enzymatic and non-enzymatic antioxidant defences, which work to maintain RONS levels at equilibrium and minimize RONS damages in the organism (Sies, 1997; Finkel and Holbrook, 2000; Blokhina et al., 2003). Production and maintenance of antioxidants implies energy and resources consumption and therefore it is costly (Halliwell, 2007). When this balance is lost and antioxidant mechanisms cannot face RONS, oxidative stress occurs in cells (Sies, 1997; Jones, 2008).

In the wild, oxidative stress is induced by a wide range of environmental factors including changes in oxygen availability (Storey, 1996; Hermes-Lima and Zenteno-Savín, 2002; Buttemer et al., 2010), high or low temperatures (Hermes-Lima and Storey, 1993; Voituron et al., 2006), contaminants (Regoli, 2000; Prevodnik et al., 2007; Labrada-Martagón et al., 2011), and ultraviolet (UV) radiation (Dahms et al., 2011). These environmental factors vary geographically, and thus levels of oxidative stress and antioxidant defences should vary along a cline of these environmental factors. Nevertheless, there is a lack of studies examining geographic variation in oxidative stress in animals (Prevodnik et al., 2007; Costantini et al., 2010).

Sunlight radiation, whose UV-B wavelength component is the most harmful (Dahms et al., 2011), is an important causes of oxidative stress (Chang and Zheng, 2003; Chuang and Chen, 2013). UV-radiation initiates a series of redox reactions ending in free radical formation and leading to oxidative stress in cells (Dahms and Lee, 2010). Moreover, reduction of O_2 by photolytic reactions results in negative effects on oxidative balance, increasing oxygen radicals and producing lipid peroxidation as well as changes in antioxidant enzyme activities (Dahms and Lee, 2010). Damages produced by UV-radiation negatively affect organisms' fitness, by reducing sperm motility, hatching success and growth rates, as well as by increasing embryo malformation and

Abbreviations: BCI, Body Condition Index; CAT, catalase; DTD, DT-diaphorase; MDA, malondialdehyde; m asl, metres above sea level; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione transferase; DCPIP, 2,6-dichlorophenol indophenol; RONS, reactive oxygen/nitrogen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reacting substance; UV-radiation, Ultraviolet radiation.

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mortality (e.g. Blaustein et al., 1998; Pahkala et al., 2002; Marquis et al., 2008; Hylander et al., 2014). As a consequence, UV-radiation is an important abiotic selective agent shaping physiology and life histories of organisms (Merilä et al., 2000). Because UV-radiation increases with elevation (Blumthaler et al., 1997; Sola et al., 2008), we expect that organisms from high elevations will be more prone to suffer oxidative stress than organisms from low elevations.

However, along an elevation gradient there are other environmental factors that gradually vary, such as temperature and oxygen partial pressure (PO_2; Körner, 2007; Graae et al., 2012). Both temperature and PO_2 are involved in biochemical reactions and both decrease along elevation gradient. These environmental factors may have antagonistic effects with solar radiation regarding to oxidative stress generation. Oxygen plays an important role in oxidative metabolism and oxidative damages can be reduced in the presence of low PO_2 (Buttemer et al., 2010). Low temperatures slow down biochemical reactions in ectotherms (but do the reverse in endotherms), which lead to low oxidants production and, for instance, low oxidative damage in cold environments (Jena et al., 2013). Moreover, in cold environments many animals hibernate, a period in which animals decrease metabolism to the minimum. Considering that PO_2 and temperature decrease with elevation, organisms from highlands might show less oxidative stress levels than organisms from lowlands.

In this work, we studied how oxidative stress damage and antioxidant enzymatic activity vary in the lizard *Psammodromus algirus* along an elevation gradient of 2200 m. *P. algirus* is an abundant lizard in Mediterranean landscapes of south-western Europe and north-western Africa, inhabiting along a wide elevation gradient (0–2700 m above sea level; m asl), therefore, facing a wide range of environmental conditions of temperature, PO_2 and UV-radiation (Salvador, 2011).

Oxidative stress processes combine various components such as free radical production, antioxidant defences, oxidative damage, and repair mechanisms (Monaghan et al., 2009). In this study, two of these components were assayed, lipid peroxidation (as an indicator of oxidative stress; Del Rio et al., 2005; Monaghan et al., 2009; Hõrak and Cohen, 2010), and activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione transferase (GST), DT-diaphorase (DTD) (as indicators of antioxidant defences), to guess possible variation in oxidative stress of lizards along a strong environmental gradient.

2. Material and methods

2.1. Study area and field procedures

Samplings were performed in Sierra Nevada Mountain (SE Spain), during the second half of July, in 2011. Sampling was concentrated in a short time period in order to avoid possible biases due to seasonal changes in oxidative stress (Hermes-Lima et al., 2012). Two sample sites were located at lowlands (36°53′N, 3°24′W, 300 m asl and 36°55′N, 3°26′W, 700 m asl) and two at highlands (36°58′N, 3°19′W, 2200 m asl and 37°01′N, 3°19′W, 2500 m asl). In Sierra Nevada, mean annual temperature goes from 3.5 to 17.6 °C (in the highest and lowest elevation respectively; REDIAM; http://www.juntadeandalucia.es/medioambiente/site/rediam), and in the study area, environmental temperature during *P. algirus* activity season (March to October) differs 8 °C on average between the lowest (300 m asl; mean ± sd = 25.0 ± 5.09 °C) and the highest sampling plot (2500 m asl; 17.2 ± 4.87 °C; Zamora-Camacho et al., 2013). Relative irradiance also varies with elevation, increasing on average 6–8% km^{-1} for UV-A and 7–11% km^{-1} for UV-B radiations (Sola et al., 2008). In the study area, UV-B radiation went from 1.28 ± 0.03 to 4.8 ± 0.37 μW cm^{-2} nm^{-1} between the lowest and the highest sampling plot (measured with the 305 nm wavelength channel of a BIC compact 4-channel radiometer, Biospherical Inc., CA, USA).

We captured 19 individuals (9 from highlands and 10 from lowlands) to measure oxidative stress under natural conditions at different elevations. Only adult males were used in this study, in order to avoid confounding effects due to age or sex (Olsson et al., 2012). Specimens were captured by hand. We measured snout-vent length (SVL) with a metal ruler (accuracy 1 mm) and weighted with an electronic balance (Model Radwag WTB200, accuracy of 0.01 g). To avoid killing specimens, tissue samples were taken from the tail because lizards can regenerate it. Taking a tissue sample from tail has small or null impact on lizard survival (Niewiarowski et al., 1997). We took tail sample in situ, preserving samples in liquid nitrogen until carried to the laboratory facilities, where they were keep in a freezer at −80 °C.

2.2. Biochemical analyses

Tissue samples were homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA and 0.1% Triton X-100 (v/v), pH 7.8) at a ratio of 1:9 (w/v). Homogenates were centrifuged at 30,000 *g* for 30 min in a Centrikon H-401 centrifuge. After centrifugation, the supernatant was collected and frozen at −80 °C until analysed. All enzymatic assays were carried out at 25 ± 0.5 °C using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). The enzymatic reactions were started by the addition of the tissue extract, except for SOD, where xanthine oxidase was used. The specific assay conditions were as follows.

Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 concentration at 240 nm according to Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture was consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU mL^{-1} xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm (McCord and Fridovich, 1969).

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). A freshly prepared glutathione reductase solution (2.4 U mL^{-1} in 0.1 M potassium phosphate buffer, pH 7.0) was added to a 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH and 0.15 mM cumene hydroperoxide. After the addition of 1 mM GSH (reduced glutathione), the NADPH-consumption rate was monitored at 340 nm.

Glutathione reductase (GR; EC 1.6.4.2) activity was assayed as described by Carlberg and Mannervik (1975) with some modifications, by measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.15 mM GSSG.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined by the method of Habig et al. (1974) adapted to microplate. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH and 1.23 mM solution of 1-chloro-2,4-dinitrobenzene in ethanol were prepared just before the assay. GST activity was monitored at 340 nm by the formation of glutathione-CDNB-conjugate.

DT-diaphorase (NADPH): quinone oxidoreductase; EC 1.6.99.2) activity was measured according to Sturve et al. (2005) and adapted by Sanz et al. (2010). The reaction mixture contained 50 mM Tris–HCl (pH 7.3), 50 μM DCPIP (2,6-dichlorophenol indophenol) and 0.5 mM NADH. Control reaction was measured with the addition of distilled water instead of sample extract. DTD activity was defined as the difference between sample and control DCPIP reduction.

Except for SOD, for which the arbitrary units have already been mentioned, for other enzymatic activities, one unit of activity is defined as

Table 1
Repeatability of biochemical analyses. The value of every variable was estimated twice, from different aliquots.

Variable	$F_{18,19}$	*P*-value	Repeatability
MDA	155.52	<0.001	0.987
SOD	6.91	<0.001	0.747
CAT	4.14	<0.001	0.611
GPX	60.94	<0.001	0.968
GR	45.09	<0.001	0.957
GST	37.80	<0.001	0.948
DTD	164.31	<0.001	0.988

MDA = malondialdehyde, CAT = catalase, SOD = superoxide dismutase, GPX = glutathione peroxidase, GR = glutathione reductase, GST = glutathione transferase, DTD = DT-diaphorase.

the amount of enzyme required to transform 1 μmol of substrate/min under the above assay conditions. To estimate the enzymatic specific activity, soluble protein of the extracts was determined by bicinchoninic acid protein assay reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as the standard.

Lipid-peroxidation levels were determined according to Buege and Aust (1978), based on malondialdehyde levels generated as product of lipid peroxide degradation. In the presence of thiobarbituric acid, MDA reacts producing coloured thiobarbituric acid-reacting substances (TBARS) that were measured at 535 nm expressed as MDA per g tissue. Despite this method for lipid peroxidation assay is not so accurate as HPLC analysis (Halliwell and Gutteridge, 2007), evaluation of MDA levels as TBARS determination has been widely considered as a suitable indicator of tissue oxidation in different species of insects, fish or birds (Sanz et al., 2010; Moreno-Rueda et al., 2012; Sanz et al., 2013). Although TBARS reacts with other aldehydes, most of the chromogen formed can be ascribed to the complex MDA-TBARS even when little MDA is present, because lipid peroxides break down to release MDA during the test conditions (Gutteridge and Quinlan, 1983).

For all the reagent variables, two measurements were taken from each aliquot, and the average was used in statistical analyses. Except for SOD and CAT, repeatabilities (estimated according to Nakagawa and Schielzeth, 2010) of biochemical measurements were high (Table 1).

All reagents, including substrates, coenzymes, and purified enzymes, were obtained from Roche (Mannheim, Germany), Sigma Aldrich Chemical Co. (USA) or Merck (Darmstadt, Germany).

2.3. Statistical analyses

We performed a Mann–Whitney *U*-test to examine differences in oxidative stress biomarkers (lipid peroxidation [MDA] and antioxidant enzymatic activity [SOD, CAT, GPX, GR, GST, DTD]), between lizards from low and high elevation. Statistical power (1-β) was estimated for these comparisons. Because SVL and mass were highly correlated ($r = 0.93$, $P < 0.01$), both variables were combined in a factor (called "size") by using a Principal Components Analysis. In addition, we obtained Body Condition Index (BCI), calculated from residuals of regression of log body mass on log SVL. Finally, we explored relationships among oxidative stress biomarkers themselves and with body size and BCI of lizards with Spearman's correlations. All analyses were performed by using software R 2.15.2 (R Development Core Team, 2012).

3. Results

Lizards from highlands were significantly larger than lizards from lowlands (Table 2). However, BCI was similar between both elevations (Table 2). All enzymes but GST had lower activity in highland than in lowland lizards, although any difference was significant (Table 2). By contrast, lipid peroxidation was significantly lower in highland than in lowland lizards (Table 2). Therefore, lowland lizards showed higher levels of oxidative stress than highland lizards.

Lipid peroxidation (MDA concentration) was negatively correlated with body size (Table 3). However, considering each elevation separately, MDA and body size were not significantly correlated (highlands, $r_s = 0.04$, $P = 0.93$; lowlands, $r_s = -0.43$, $P = 0.34$). By contrast, the activity of most of enzymes was not significantly correlated with size, excepting CAT and GR, which were negatively correlated (Table 3). Moreover, lipid peroxidation was negatively correlated with activity of several of the antioxidant enzymes, although the correlation was only significant with GST activity (Table 3). BCI did not correlate with any oxidative stress biomarker but GR (Table 3).

4. Discussion

Our results show that lipid peroxidation (as biomarker of oxidative stress damage) decreases with elevation in the lizard *P. algirus*. The question arises why oxidative stress level is lower at high elevation. Sunlight (including UV-B and UV-A radiation) is an important stressor for organisms (Ortonne, 2002; Chang and Zheng, 2003; Dahms et al., 2011). At high elevations, where UV-radiation is more intense than at low elevations, we could expect that individuals of *P. algirus* would present higher levels of oxidative stress. The effect of UV-radiation is exacerbated because, as an ectothermic organism, *P. algirus* expends long periods of time sunbathing to get suitable body temperatures (Díaz, 1997), which makes it more susceptible to solar radiation damages. However, our results contrast with this expectation: lizards showed lower lipid peroxidation levels at high elevations.

One possible explanation for this result is that the environment is less pro-oxidant at high elevations. At low elevations, PO_2 and environmental temperature are higher, which increases metabolic rates and therefore free radical production. In reptiles, volume of consumed O_2

Table 2
Body size (factor made up of snout-vent length and mass variables), BCI (residuals of regression of log body mass on log snout-vent length), lipid peroxidation levels (MDA) and antioxidant enzymatic activity for lowland and highland lizards. Table shows mean values and standard error (±se), Mann–Whitney *U* test results, signification value (*P*-value), and statistical power (1-β) for biochemical variables.

	Highlands mean ± se	Lowlands mean ± se	*U*	*P*-value	1-β
Size	**1.04 ± 0.26**	**−0.96 ± 0.22**	**53**	**<0.01**	**0.90**
BCI	0.03 ± 0.02	−0.01 ± 0.02	38	0.27	0.05
MDA (nmol/g tissue)	**140.96 ± 50.74**	**423.04 ± 83.23**	**13**	**0.02**	**0.62**
SOD (U/mg protein)	42.27 ± 2.45	42.79 ± 3.71	51	0.66	0.05
CAT (U/mg protein)	5.33 ± 0.47	6.35 ± 0.63	31	0.27	0.21
GPX (U/mg protein)	3.53 ± 0.53	4.25 ± 0.66	28	0.48	0.10
GR (mU/mg protein)	9.92 ± 0.58	12.44 ± 1.12	23	0.07	0.37
GST (mU/mg protein)	35.48 ± 2.94	28.54 ± 2.46	63	0.15	0.35
DTD (mU/mg protein)	71.17 ± 7.26	72.19 ± 9.98	48	0.84	0.05

MDA = malondialdehyde, CAT = catalase, SOD = superoxide dismutase, GPX = glutathione peroxidase, GR = glutathione reductase, GST = glutathione transferase, DTD = DT-diaphorase. Significant differences between altitudes are in bold. Sample size in the experiment was 19 (9 lizards from highlands and 10 lizards from lowlands) and samples were taken in situ to measure basal state of the parameters.

Table 3
Spearman's correlation matrix among body size (factor of mass and snout-vent length), BCI (residuals of regression of log body mass on log snout-vent length), lipid peroxidation (MDA), and antioxidant enzymatic activity. Table contains *rho*-values for pairwise Spearman's correlations and sample size (*in italic*).

	Size	BCI	MDA (nmol/g tissue)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	GR (mU/mg protein)	GST (mU/mg protein)
MDA	−0.58*	0.05						
(nmol/g tissue)	*15*	*15*						
SOD	0.01	−0.31	−0.39					
(mU/mg protein)	*15*	*15*	*18*					
CAT	−0.54*	−0.47	0.03	0.66**				
(mU/mg protein)	*15*	*15*	*18*	*19*				
GPX	−0.45	−0.15	−0.05	0.03	0.15			
(mU/mg protein)	*15*	*13*	*16*	*17*	*17*			
GR	−0.61*	−0.71*	0.21	0.43	0.68**	0.18		
(mU/mg protein)	*15*	*15*	*18*	*19*	*19*	*17*		
GST	−0.03	−0.08	−0.52*	0.62	0.44	0.17	0.23	
(mU/mg protein)	*15*	*15*	*18*	*19*	*19*	*17*	*19*	
DTD	−0.29	−0.37	−0.03	0.52*	0.54*	0.3	0.52*	0.56*
(mU/mg protein)	*15*	*15*	*18*	*19*	*19*	*17*	*19*	*19*

MDA = Malondialdehyde, CAT = catalase, SOD = superoxide dismutase, GPX = glutathione peroxidase, GR = glutathione reductase, GST = glutathione S-transferase, DTD = DT-diaphorase.
* $P < 0.05$ is the significant value.
** $P < 0.01$ is the significant value.

is usually proportional to environmental PO_2 (Snyder and Weathers, 1977), and metabolism increases with elevated temperature (Shine, 2005). Indeed, that oxidative damage increases with temperature in ectotherms has already proved (Jena et al., 2013). In our study system, lizards from lowlands have a body temperature 1.5 °C on average higher than lizards from highlands (Zamora-Camacho et al., 2013). Higher metabolism and free radical production in lowland lizards would lead to higher oxidative stress levels ("the rate-of-living hypothesis"; Sohal and Weindruch, 1996; Speakman, 2005).

In addition, lizards from low elevations were significantly smaller. Metabolic rates and, in consequence, RONS production rates, are higher in relatively smaller individuals (Speakman, 2005), which would lead to higher levels of oxidative damage. Because body size increased with elevation, and MDA decreased with body size, is difficult to disentangle whether oxidative damage changed with elevation per se, or such a variation is a consequence of variation in body size. To analyse this issue, we tested separately in both elevations the relationship between body size and oxidative stress. When each elevation is considered separately, no relationship between body size and oxidative stress emerges, which suggests that variation in oxidative stress with elevation is not due to variation in body size.

Furthermore, lizards from highlands show a longer hibernation period, thus undergoing a more restricted period of feeding (Zamora-Camacho et al., 2013). According to the "oxidative damage attenuation hypothesis" (Noguera et al., 2011), short periods of restricted feeding could help to prevent oxidant production, which could contribute to explain why lizards at highlands have low tissue oxidation levels. Additionally, individuals of *P. algirus* from highlands are darker than individuals from mid and lowlands (Reguera et al., 2014). Higher concentration of melanins in highland lizards might be protecting from UV-radiation (Ortonne, 2002), contributing to reduce oxidative stress.

Finally, besides higher temperatures, the lowest sampling plot is characterised by the presence of fruit trees and olive crops. These kinds of habitats usually have higher presence of contaminants and heavy metals which are known as important stressor agents (Amaral et al., 2012). More contaminants in lowlands could contribute to the fact that lizards in lowlands showed higher oxidative stress levels.

We can discard that the differences in oxidative stress between lizards from highland and lowland are due to higher enzymatic antioxidant defences in highlands than in lowlands, given that we did not find significant differences in the activity of antioxidant enzymes with altitude. Consequently, differences in oxidative stress between highland and lowland lizards cannot be explained as a consequence of differences in enzymatic antioxidant capacity, although we cannot rule out the effect of non-enzymatic antioxidant defences.

Our findings contrast with studies in humans, which find that oxidative stress increases with elevation (review in Askew, 2002). One of the main variables affecting oxidative stress with elevation is temperature, but temperature differentially affects metabolism (and thus RONS production) in endotherms and in ectotherms. Endotherms increase metabolism as temperature decreases (Beamonte-Barrientos and Verhulst, 2013), which might explain higher oxidative stress in humans living at higher elevations. However, ectotherms reduce metabolism as temperature decreases, and consequently oxidant molecules production (Jena et al., 2013).

In conclusion, although one may naively think that alpine habitats, being more extreme, generate more oxidative stress in lizards, our findings show that *P. algirus* lizards from high elevation have less oxidative stress levels than those from low elevation. Therefore, our findings suggest that highland environments are less stressful than lowlands. Differences in stressful conditions probably have importantly influenced the evolution of life-history in animals (Pérez-Campo et al., 1998; Alonso-Alvarez et al., 2007; Buttemer et al., 2010). Consequently, our results may explain why highland ectotherms (exposed to less oxidative conditions) show higher longevity (e.g. Zhang and Lu, 2012) and increased growth rate (e.g. Iraeta et al., 2006). Our study, therefore, highlights the ecological importance of geographic variation in pro-oxidant stressors in order to cast animal evolution.

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