



Fecal glucocorticoid metabolites and ectoparasites as biomarkers of heat stress close to roads in a Mediterranean lizard

R. Megía-Palma^{a,b,*}, I. Barja^{c,d}, R. Barrientos^e

^a Universidad de Alcalá (UAH), Parasitology Unit, Department of Biomedicine and Biotechnology, School of Pharmacy, Spain

^b CIBIO-InBIO: Research Center in Biodiversity and Genetic Resources, Portugal

^c Laboratory of Etho-Physiology, Department Biology (Unit Zoology), Autónoma University of Madrid, Spain

^d Research Centre in Biodiversity and Global Change (CIBC-UAM), Autónoma University of Madrid, Spain

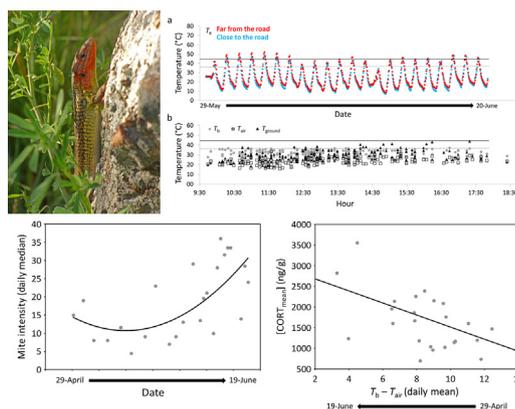
^e Road Ecology Lab, Department of Biodiversity, Ecology and Evolution, School of Biology, Complutense University of Madrid, Spain



HIGHLIGHTS

- Climate may impose thermal restrictions to ectotherms in Mediterranean habitats.
- Lizards increased thermoregulatory activity and glucocorticoids under heat stress.
- Coping responses were not influenced by the distance to a road.
- Seasonal increase of ectoparasites was explained by thermal restrictions.

GRAPHICAL ABSTRACT



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ABSTRACT

Differences between air and ground temperatures are expected to narrow with the advance of the season in temperate regions (*aka* seasonal restriction in the availability of thermal microhabitats), which may activate behavioral and physiological responses of ectotherm species adapted to temperate climates. However, according to cost-benefit models of ectotherm thermoregulation, we hypothesize that these responses may also carry some costs. We quantified seasonal shifts in thermoregulatory precision, concentration of fecal glucocorticoid metabolites, and load of ectoparasites in a Mediterranean lizard, *Psammotromus algerius*. We also tested whether the proximity to a road, a putative source of chronic stress, can facilitate the glucocorticoid-mediated response of lizards to heat stress. As expected, differences between body and environmental temperatures narrowed during the reproductive season and lizards responded by increasing their thermoregulatory precision and the secretion of glucocorticoids, as indicated by metabolites in feces. Interestingly, lizards tended to have higher glucocorticoid concentration when captured far from the road. This might reflect either a putative impairment of the glucocorticoid-mediated response of the lizards to heat stress close to the road or the plastic capability of *P. algerius* to acclimate to sources of moderate chronic stress. In the latter direction, the increase of both glucocorticoid metabolites and thermoregulatory precision supported that this Mediterranean species responds to environmental thermal restrictions with adaptive behavioral and physiological mechanisms. However, this was also associated with an increase in its susceptibility to ectoparasites, which represents an added cost to the current cost-benefit models of ectotherm thermoregulation.

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* Corresponding author at: Universidad de Alcalá (UAH), Parasitology Unit, Department of Biomedicine and Biotechnology, School of Pharmacy, Spain.
E-mail address: rodrigo.megia@uah.es (R. Megía-Palma).

1. Introduction

Roads have multiple impacts on wildlife populations, including their role as sources of chronic stress (e.g., Navarro-Castilla et al., 2014; Troianowski et al., 2017; Kechnebbou et al., 2019). These linear infrastructures can promote fragmentation of populations of species with low dispersal ability (Tellería et al., 2011), while also promoting a road effect zone, namely an increase in human activity due to easy access. This can lead to the degradation of the vegetation that surrounds roads, as well as habitat pollution and homogenization (Forman and Alexander, 1998; Eigenbrod et al., 2009; Shanley and Pyare, 2011). On the other hand, the rapid seasonal increase in warm temperatures in temperate regions can be a source of acute stress. Even in thermally restrictive environments, ectotherms must achieve body temperatures that allow them to optimize metabolic processes (Huey and Slatkin, 1976; Vickers et al., 2011). The classic cost-benefit model of ectotherm thermoregulation considers the case where organisms, particularly lizards, suffer costs derived from time invested to seek heat sources to attain body temperatures within their range of physiological competence (Huey and Slatkin, 1976). This model was based on the assumption that lizards usually find thermal microhabitats below their thermal optima. However, most temperate populations of lizards face the opposite challenge: seasonal thermal restrictions due to heat excess, a situation that may be aggravated under climate change (Dunlap and Wingfield, 1995; Vickers et al., 2011; Herrando-Pérez et al., 2020a). Heat stress may have dramatic consequences at the population level, especially if this occurs during reproductive periods (Sinervo et al., 2010; Dupoué et al., 2017). One of the predictions of Huey and Slatkin's (1976) model is that lizards facing high costs associated with thermoregulation in poor quality thermal habitats will decrease thermoregulation effort until they thermoconform (i.e. null thermoregulatory effort). However, Vickers et al. (2011) developed an extended version of this model where they also considered that temperate lizards face warm environments exceeding their physiological limits. Using a combination of field and laboratory data of temperatures recorded during different seasons, Vickers et al. (2011) demonstrated that, in the presence of microhabitat heterogeneity, active thermoregulators, rather than becoming thermoconformers, increase their thermoregulatory precision to avoid overheating in habitats with poor thermal quality. We are particularly interested in investigating if heat stress, which may occur before the summer in Mediterranean habitats, leads to detectable behavioral and physiological changes in lizards.

Ectotherms can respond to environmental stressors with behavioral and physiological coping mechanisms, which are adaptive to restore homeostasis (Wingfield and Ramenofsky, 1999; Romero, 2004). Likely, the most important and adaptive physiological response among vertebrates is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which elicits an increase in the secretion of glucocorticoids in the adrenal cortex (cortisol or corticosterone) (Harvey et al., 1984; Millspaugh and Washburn, 2004). Under life-risking situations (e.g. overheating hazard), the release of glucocorticoids activates alert mechanisms that include fat catabolism and energy allocation to locomotion (i.e. increased activity) (e.g. Moore and Jessop, 2003). Therefore, although glucocorticoids may likely have pleiotropic effects when attached to the multiple glucocorticoid receptors present in the body (MacDougall-Shackleton et al., 2019), their release facilitates behavioral and physiological shifts that are part of the body's coping mechanisms that minimize the negative effect of stress (Romero and Wingfield, 2015). This fact, while being adaptive to increase survival prospects (Cote et al., 2006), may also be harmful in the long-term in co-occurrence with other stressors (e.g. Josslerand et al., 2017). Indeed, circulating glucocorticoids acting at different levels can terminate the HPA response (reviewed by Hill and Tasker, 2012). This can occur when (i) glucocorticoid concentration reaches certain upper thresholds incompatible with life (Romero, 2004), and/or perhaps when (ii) stimuli exceed upper thresholds of physiological response (e.g. Dupoué et al.,

2018). In extreme situations, and as a complementary emergency mechanism, cessation of activity may occur as a behavioral coping response to acute anxious stimuli (e.g. extreme heat waves, Dupoué et al., 2018). We hypothesize that the increased activity associated with the release of glucocorticoids may also favor exposure of lizards to questing ectoparasites (Wieczorek et al., 2020; Barrientos and Megía-Palma, 2021; Smolinský et al., 2021). Thus, both glucocorticoids and parasites can be interpreted as biomarkers of increased activity associated with environmental stress in lizards (Oppliger et al., 1998; Josslerand et al., 2017; Kechnebbou et al., 2019; Tylan et al., 2020). Particularly, plasma glucocorticoids can reflect heat stress in lizards (Telemeco and Addis, 2014; Racic et al., 2020), and we hypothesize that fecal metabolites do as well.

There is mixed evidence on the capability of ectotherms to acclimate to environmental stress. Some studies revealed evidence of stress signatures, which suggests (i) a low acclimation capability of the stressed population, (ii) that the stimulus overtakes the response, or (iii) a combination of both (see Lucas and French, 2012; Cayuela et al., 2017; Kechnebbou et al., 2019; Megía-Palma et al., 2020a). Some stressors may even be partially beneficial (e.g. Graham et al., 2012), while other studies showed similar levels of glucocorticoids between stressed and naïve populations (Baxter-Gilbert et al., 2014; Owen et al., 2014; Megía-Palma et al., 2020b). However, the latter does not always indicate a plastic acclimation capability, but rather may be a symptom of a severe impact of stressors and impairment of this important coping response (Dupoué et al., 2018; Romero and Beattie, 2021). Moreover, acclimation can also motivate an enhanced stress response in the face of an acute stressor (i.e. facilitation hypothesis; Bhatnagar and Vining, 2003).

We hypothesize, in line with the extended cost-benefit model of ectotherm thermoregulation (i.e. Vickers et al., 2011), that Mediterranean climates are thermally restrictive to lizards during their reproductive season. Heat constrictions may commence throughout the summer months, constituting a source of seasonal acute stress. We analyze data collected during the mating season in a population of the Algerian sand racer, *Psammotromus algirus* (fam. Lacertidae). We predict that (i) lizards will reduce the variance of their body temperatures, which represents an increase in thermoregulatory precision and a behavioral coping response to restrictions in thermal microhabitats (Vickers et al., 2011); (ii) this constriction in the thermal breadth of the lizards will elicit the release of glucocorticoids, a physiological adaptive response in Mediterranean lizards that will be detectable in fecal metabolites; and (iii) these two coping responses will not be free of costs, with the load of ectoparasites increasing in lizard hosts because of an expectation that heat stress promotes an increase in shuttling rates among thermal microhabitats when lizards try to avoid overheating (Vickers et al., 2011). In the present study, we also investigated interactive effects of heat and anthropogenic sources of stress under the facilitation hypothesis (sensu Romero, 2004). The latter hypothesis predicts an enhanced response in individuals previously acclimated to a repeated or chronic stress source (Bhatnagar and Vining, 2003; Romero, 2004). Thus, we expected that (iv) under a closer influence of a road, lizards will show a stronger increase in both mite infestation and corticosterone metabolites in response to heat stress.

2. Material and methods

2.1. System description

The sampling area falls within the Mesomediterranean bioclimatic region, which is characterized by contrasting seasons (Rivas-Martínez et al., 2017). Monte de El Pardo (40.5063, -3.7662; ~680 m above sea level; Madrid, Spain) has temperate and rainy springs (average 15.0 °C; 54.0 mm) with hot and dry summers (23.4 °C; 14.8 mm) (Díaz et al., 2006). We studied lizards in two sampling plots that were located within a heterogeneous matrix of evergreen forest with sandy substrate resulting from the erosion of granite rock and dominated by

holm-oak trees (*Quercus ilex*) and rock rose bushes (*Cistus ladanifer*). One of the sampling plots, placed at the bottom of a valley, was an area of 6.6 ha ranging in distance between 0 and 150 m from a road with moderate intensity of traffic flow (9050 vehicles/day). This is a typical road-effect zone, with increased human pressure (high numbers of walkers, cyclers, pickers) with a lower cover of ground level vegetation, gramineous plants, and bushes (Supplementary Materials), transforming it into a low quality habitat for our study species (Carrascal et al., 1989). The second area, placed on the surrounding hills, was 7.9 ha and ranged between 350 and 550 m from the road (Barrientos and Megía-Palma, 2021). This left a 200-m band between both sampling plots. The size of the smallest of our sampling plots exceeded by approximately 75 times the maximum home range size described for the species, which is 0.09 ha (Díaz, 1993). Moreover, in a previous translocation capture-recapture experiment in the same population, control lizards (not translocated) travelled on average 18 m linear distance in a period of four weeks (Barrientos and Megía-Palma, 2021). Therefore, there was a high likelihood that only a few lizards, if any, moved between sampling plots and thus, ectoparasites and fecal glucocorticoid metabolites do reflect conditions of the plot where lizards were actually captured.

2.2. Sampling

P. algirus is a mid-sized lizard (adult snout-vent length range = 64–79 mm), and a precise thermoregulator with generalist habitat occupancy (Díaz, 1997). Its activity period in the study area ranges from late April to early October. We sampled lizards during 24 sampling days between April 29th and June 19th 2019, a period of maximum gonadal development and reproductive activity in this species (Díaz et al., 1994). Typically, sampling took place between 10:00 AM and 3:30 PM, but the earliest and latest lizards in our pool were sampled at 9:45 AM and 6:25 PM, respectively. We used a lasso to capture both adults and juveniles. To prevent re-sampling, we identified captured lizards by painting one spot on their back with a xylene-free white paint, which was visible prior to capture and that presumably lasted during the whole period of sampling as demonstrated by a previous mark-recapture experiment in the same population (Barrientos and Megía-Palma, 2021).

2.3. Quantification of individual thermal traits

Field body temperature (i.e. T_b ; Hertz et al., 1993) of active adult lizards ($N = 185$; 76 females and 109 males) was registered within five seconds of capture with an instant touchless infrared thermometer (0.1 °C precision in ~0.5 s; ThermoFlash®, LX-26 Evolution, NEXMED Technology Co. Ltd., NY, USA). We did this by pointing toward the cloacal area with the thermometer from less than 1 cm of distance while trying to minimize handling of the lizards to avoid heat transference. We also measured both the temperatures of the ground where the lizards were first spotted at 1 cm above the ground (hereafter, T_{ground}) and the air at the same location 1 m above the ground (hereafter, T_{air}). Prior to each of these three measurements, we set the modes 'body', 'surface', and 'room' of the thermometer, respectively. The time of day (GMT +02:00) was also registered. We validated the accuracy of the infrared thermometer by comparing its values for cloacal temperatures from 168 lizards with those taken with an instant contact thermometer (ETI 2001, Electronic Temperature Instruments Ltd., Sussex, UK), which was gently placed in contact with the cloaca of the lizards. These measures were highly correlated ($R^2 = 0.92$, $P < 0.0001$). We confirmed the sex of adult lizards by scrutiny of their femoral pores, which are enlarged in males. The relatively larger head size as compared to females, the longer tail, and the presence of orange coloration were also used as additional criteria. The snout-vent length of the lizards was measured with a ruler to the closest 1 mm.

The distribution of T_b s was left-skewed. Thus, to improve the residual distribution of the model, we calculated the inverse of the data by

subtracting to 100 the values of T_b . After this, the inverted scores of T_b were all positive and their distribution was right-skewed and hence, we were able to fit a Gaussian model after \log_{10} -transforming them. Sampling plot and sex were set as predictor factors. As continuous predictors we set the transformed time of day, Julian date, which was z-standardized to increase model stability, body size, and the temperatures of both air and ground measured at the same spots where the lizards were captured. We discarded model multicollinearity by checking variance inflation factor scores (all VIFs <2.0; Craney and Surlles, 2002). The sign of the beta-estimates reported by the model was inverted to reflect the correct relationships with the non-inverted original response.

2.4. Restrictions in thermal microhabitats

2.4.1. Daily hours of restriction

We deployed sixteen cylindrical copper models (50 × 10 mm; Díaz, 1997) at random spots within the matrix of habitat, but in places where lizards had been previously observed, between May 29th and June 20th 2019. We set eight of them in the sampling plot close to the road and another eight in the plot far away from it. We inserted in the copper models sixteen thermocouples (Hobo® U23-001A, Pro v2, Bourne, MA, USA, 0.01 °C of precision) that autonomously registered environmental (operative) temperature (i.e. T_e ; Black et al., 2019) every ten minutes. Since sampling took place in a forest, copper models were in the sun and shade at different times of day. Two of the copper models deployed in the plot close to the road disappeared during the sampling period, resulting in fourteen temperature datasets. Copper models are considered to have similar thermal properties to the body of lizards and are hence commonly used to obtain the null distribution of T_b s that a non-thermoregulating (passive) model would experience in the environment (Bakken, 1992; Bauwens et al., 1996; Díaz, 1997). We compared T_e data between the two sampling plots with a general mixed model (LMM) where the response was the \log_{10} -transformed-hourly averaged values of T_e from each of the two plots. We set plot as a fixed predictor, and we set the hour and the hour nested within the day as random terms.

Following Hertz et al. (1993) and previous authors, we used three indices of daily thermal restrictions, which used T_e , T_{set} , and CT_{max} . T_{set} represents the upper limit of 50% central boundaries of the thermal preference range of the species (Blouin-Demers and Nadeau, 2005; Zamora-Camacho et al., 2016). Based on previously published data on selected temperatures of *P. algirus* in artificial thermal gradients and sampled at a similar elevation, we adopted T_{set} as 36.33 °C (Zamora-Camacho et al., 2016). CT_{max} , or the critical thermal maximum, is a measure of thermal tolerance and quantifies the maximum body temperature at which lizards' performance, or fitness, is at a minimum (Huey and Kingsolver, 1993). We adopted the average CT_{max} of 43.1 ± 0.1 °C, which was experimentally assessed for males of *P. algirus* sampled at different localities (Herrando-Pérez et al., 2020b).

Using these parameters, (i) we calculated the percentage of daily hours between 9:00 AM and 8:00 PM that the absolute difference between T_{set} and T_e was between 3 and 5 °C. Environments within this thermal range are considered of good thermal quality for lizards (Hertz et al., 1993; Díaz and Cabezas-Díaz, 2004; Vickers et al., 2011). We also (ii) calculated the daily average of hours that the hourly mean T_e exceeded T_{set} ; and (iii) the daily average of hours that the hourly mean T_e exceeded CT_{max} . We also calculated an index of thermoregulatory precision as the hourly variation in T_b standard deviations (SD_h). We compared SD_h with the hourly mean of T_e s using a Spearman order correlation test weighed by the number of lizards captured per hour.

2.4.2. Seasonal restrictions

We calculated three indices of seasonal thermal restriction. These consisted of the daily ($N = 24$) arithmetic mean of both $\Delta T_b - T_{\text{air}}$ and $\Delta T_b - T_{\text{ground}}$, and the daily variation in standard deviations of T_b (SD_d).

We performed a multiple analysis of the variance (MANOVA) based on Pillai's test (Pillai, 1955) in Statistica 10.0 (StatSoft Inc., Tulsa, Ok, USA), where the responses were the daily scores of $\Delta T_b - T_{air}$, $\Delta T_b - T_{ground}$, and SD_d , while the date (z-standardized) was set as a predictor. This analysis was weighed by the number of lizards sampled per day.

2.5. Biomarkers of physiological stress indicators

2.5.1. Fecal glucocorticoid metabolites

We obtained fresh fecal samples from adults and juveniles ($N = 231$; average \pm SE daily sampling rate = 9.24 ± 0.82 lizards, range = 3–17) (Table 1). We gently massaged the belly of the lizards to obtain the fecal samples (Megía-Palma et al., 2016, 2020a), and avoided doing so with gravid females, or when individuals did not produce feces after a few tries. This was done in the field within a few minutes after capture. Fecal samples were directly collected in vials of 1.5 ml and deposited in a cooler with ice until they were stored at -20°C until processing. To extract the contents of fecal glucocorticoid metabolites, fecal samples were dried at 90°C for 4 h, weighed to the closest 0.01 g, and then approximately 0.05 g of dry sample was placed in an assay tube with 500 μl of 100% methanol and 500 μl of phosphate buffer. Tubes were vortexed at high speed in a shaker for 16 h. Subsequently, samples were centrifuged at 2500 rpm for 15 min. Finally, 100 μl of supernatant was stored at -20°C until analysis. A commercial corticosterone enzyme immunoassay kit (EIA, DEMEDITEC GmbH, Kiel, Germany) was used for quantification according to the manufacturer's instructions. The samples were randomly distributed in the assays and the recovery of fecal glucocorticoid metabolites was 90%. The between-assay and within-assay coefficients of variation were 10.1% and 11.4%, respectively. To analyze the individual variation in fecal glucocorticoid metabolite concentration, we fit a general linear model (LM) to the fecal glucocorticoid metabolite concentration (hereafter, $[\text{CORT}_{ind}]$). To comply with parametric assumptions, we had to \log_{10} -transform the $[\text{CORT}_{ind}]$ scores. The sex and age of the individuals was integrated in a single factor of three levels as follows: adult males, adult females, and juveniles. A second factor of two levels was set to group lizards captured in each of the sampling plots. We also included in the model the interaction between sex and sampling plot and between date and sampling plot. The latter aimed to test the hypothesis of road-mediated facilitation (i.e. Bhatnagar and Vining, 2003; Romero, 2004).

We analyzed the daily variation in fecal glucocorticoids. To do so, we fit a LM to the daily arithmetic means of the individual scores of fecal glucocorticoid metabolites (hereafter, $[\text{CORT}_{mean}]$) ($N = 24$ days). Date (z-standardized), the daily averaged scores of T_{air} and T_{ground} , and the daily median of ectoparasites were set as predictors. The number of fecal pellets sampled per day was included as a weighing term in the analysis.

2.5.2. Ectoparasites

We used in the field a 10 \times -magnifying glass to quantify ectoparasites in adult lizards ($N = 185$) (Table 1). We found hematophagous mites that were mostly located in the annuli of the tail close to the

Table 1

Sample size of lizards per sampling plot and variables analyzed. Mites and body temperatures (T_b) were quantified in 185 adult lizards. $[\text{CORT}_{ind}]$ refers to the fecal concentration of glucocorticoid metabolites and was quantified in adults and juveniles. 'Close' makes reference to lizards sampled from 0 to 150 m from a road, while 'Far' designates lizards sampled 350 to 550 m from the road.

	Females	Males	Juveniles		Females	Males
Close	48	56	22	Close	48	56
Far	28	53	24	Far	28	53
Subtotals	76	109	46	Subtotals	76	109
Total			231	Total Mites and T_b		185
$[\text{CORT}_{ind}]$						

cloaca. To analyze the individual variation in mite load, we fit a generalized linear model with a negative binomial distribution, which corrects the overdispersion parameter in parasite data (Pennycook, 1971). We included as predictors sex (we did not include juveniles), $[\text{CORT}_{ind}]$ (\log_{10} -transformed), sampling plot, body length, $T_b - T_{air}$ and $T_b - T_{ground}$, and the interactions between these two indices of thermal restriction with sex and with sampling plot. However, VIFs for $T_b - T_{air}$ main effect and its interactions were all > 8 and hence we removed them (Craney and Surlis, 2002). The new model without $T_b - T_{air}$ terms had the same Akaike score as the model that included them (i.e. $\Delta\text{AICc} = 0$), suggesting that $T_b - T_{air}$ was a non-informative predictor for mite intensity.

In addition, we analyzed the daily variation in mite intensity. We calculated daily mite medians, which better represent centrality for a typically aggregated distribution of parasite data (Rózsa et al., 2000; Megía-Palma et al., 2020a). We fit a LM to the median scores of mites, and we included as predictors date (z-standardized), daily averaged scores of $T_b - T_{air}$ and $T_b - T_{ground}$, and $[\text{CORT}_{mean}]$. Daily number of infested lizards was included as a weighing term in the analysis.

2.6. Model averaging and cross-validation of final models

To calculate the z-standardized coefficient of the effects analyzed, we used multimodel inference and model averaging implemented in R with the package 'MuMIn' (Barton, 2018). Model averaging was based on the models with $\Delta\text{AICc} \leq 4$ (Burnham and Anderson, 2004). The importance of each predictor was calculated by summing the weights of models where the variable appears. The estimate coefficients were z-standardized and the effects of significant predictors cross-validated using a k-fold split of 3 in the R package 'DAAG' implemented in R version 3.4.3 (Mairdonald et al., 2015; Megía-Palma et al., 2020a).

3. Results

3.1. Individual thermal traits

Mean \pm SE T_b obtained from active adult lizards with the infrared thermometer was $33.00 \pm 0.19^\circ\text{C}$ ($n = 185$, range = 20.7–36.6). The most important predictors of T_b were T_{air} and T_{ground} (both with similar $\beta = +0.003$ and importance = 1.00, r^2 between them = 0.187), followed by the time of the day ($\beta = -0.085$, importance = 0.97), and the body size of the lizards ($\beta = +0.002$, importance = 0.83). However, the cross-validation approach dropped off body length ($F_{1, 180} = 2.38$, $P = 0.12$) and hour ($F_{1, 180} = 0.82$, $P = 0.37$), confirming T_{air} ($F_{1, 180} = 45.42$, $P < 0.001$) and T_{ground} ($F_{1, 180} = 35.88$, $P < 0.001$) as significant predictors (Table 2). T_b was on average $4.0 \pm 0.4^\circ\text{C}$ higher than T_{ground} and $9.0 \pm 0.3^\circ\text{C}$ than T_{air} . Indeed, 77.8% of the lizards were warmer than the ground and 98.9% warmer than the air at the same spot where they were captured.

Table 2

Model averaging based on a general linear model analyzing predictors of T_b from adult lizards ($N = 185$). Significant effects confirmed by cross-validation are shown in bold (see Results).

	Importance	Estimate	Adj SE	z value	Pr(> z)
(Intercept)		4.420	0.074	59.34	<0.001
Time	0.97	-0.085	0.031	2.69	0.007
Body length	0.83	0.002	0.001	2.23	0.026
T_{air}	1.00	0.003	0.001	3.95	<0.001
T_{ground}	1.00	0.003	0.001	5.73	<0.001
Road proximity	0.40	-0.003	0.002	1.18	0.236
Sex	0.26	0.000	0.003	0.10	0.918
Date_stand	0.23	0.000	0.003	0.07	0.944

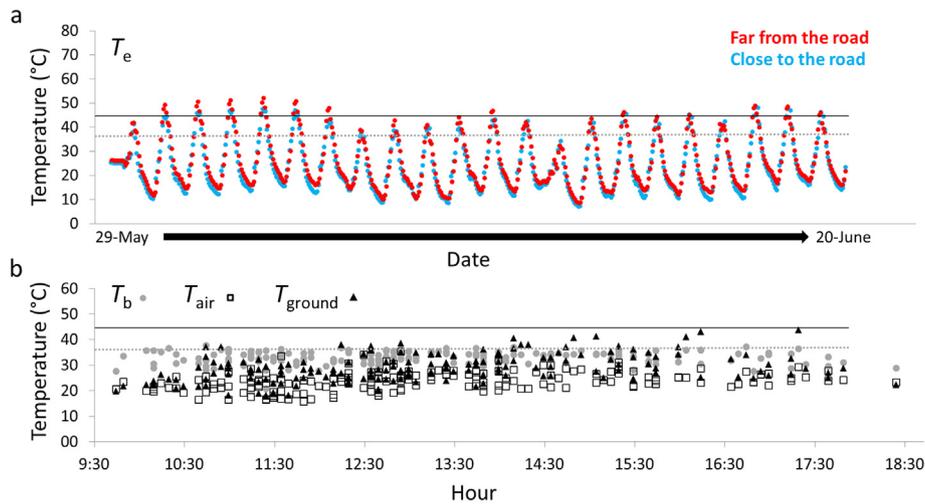


Fig. 1. (a) Hourly averaged T_e registered during 22 days at 14 random spots within the habitat of *P. algirus* represented in relation to the proximity to a road. (b) Accumulative hourly variation in T_b , T_{ground} , and T_{air} of free-ranging lizards in El Pardo. The grey dotted line in (a) and (b) indicates T_{set} of *P. algirus* (i.e. 36.33 °C; following Zamora-Camacho et al., 2016), while the black continuous line indicates CT_{max} (43.1 °C; Herrando-Pérez et al., 2020b). The graphs suggest that despite upper thermal limits in the environment are exceeded almost daily in central hours during the reproductive season, they are actively avoided by the lizards through the selection of cooler thermal microhabitats.

3.2. Environmental thermal restrictions

3.2.1. Daily hours of restrictions

T_e significantly differed between sampling plots (LMM, plot: $\chi_{1, 20} = 86.21, P < 0.001$). The daily mean difference in T_e between sampling plots was 0.69 ± 0.12 °C, being higher in the plot far from the road 86.4% (19/22) of the sampled days. In addition, only 13.6% of the hourly $|T_{set} - T_e|$ scores were within 3 and 5 °C. In this sense, daily scores of T_e exceeded T_{set} in both sampling plots on average 4.77 ± 0.38 h per day, 95.4% (21/22) of these days. Moreover, T_e exceeded CT_{max} 1.61 ± 0.07 h on average per day and 90.9% (20/22) of the days (Fig. 1a). However, only 4.3% (8/185) of the lizards' T_b s exceeded T_{set} , and none exceeded CT_{max} , indicating that lizards actively avoided exceeding T_{set} through selection of cooler thermal microhabitats (Fig. 1b). In addition, SD_h and hourly mean T_e were negatively correlated (Spearman's rho = $-0.72, P < 0.001$), suggesting an increase in thermoregulatory precision during the warmest hours of the day (Fig. 2).

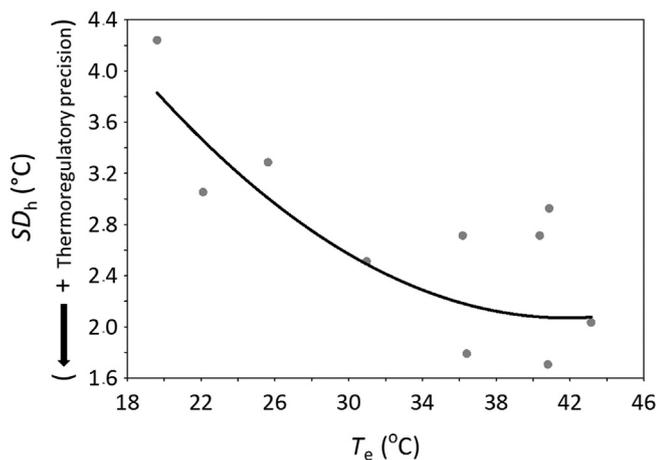


Fig. 2. Weighed relationship between hourly standard deviation of field body temperatures of active lizards (SD_h ; $N = 185$) during the ten-hour period of daily lizard activity and hourly mean operative temperatures of the environment. The analysis supports that lizards increase their thermoregulatory precision during hours of more heat stress.

3.2.2. Seasonal restrictions

The MANOVA performed to analyze SD_d and the two indices of daily thermal niche constriction was significant (Pillai's test: $F_{3, 209} = 12.43, P < 0.001$). The *a-posteriori* univariate tests supported the prediction that daily average differences between air and lizards' body temperatures narrowed with date ($\Delta T_b - T_{air}$: $\beta = -0.35; F_{1, 20} = 28.90, P < 0.0001$), and so did SD_d ($\beta = -0.16, F_{1, 20} = 5.63, P = 0.018$) (Fig. 3). Both results indicate that lizards suffer heat restrictions, are sensitive to them, and show a short-term response increasing their thermoregulatory precision. However, the variation with date of $\Delta T_b - T_{ground}$ was not significant ($\Delta T_b - T_{ground}$: $F_{1, 20} = 0.004, P = 0.94$), which suggested that lizards maintained $\Delta T_b - T_{ground}$ constant across the sampling period through the active selection of thermal microhabitats.

3.3. Biomarkers of physiological stress

3.3.1. Fecal glucocorticoid metabolites

Mean \pm SE individual concentration of fecal glucocorticoid metabolites was 1614.5 ± 173.6 ng/g. Date ($\beta = 0.08$, importance = 0.92) and road proximity (importance = 0.72) were the most important

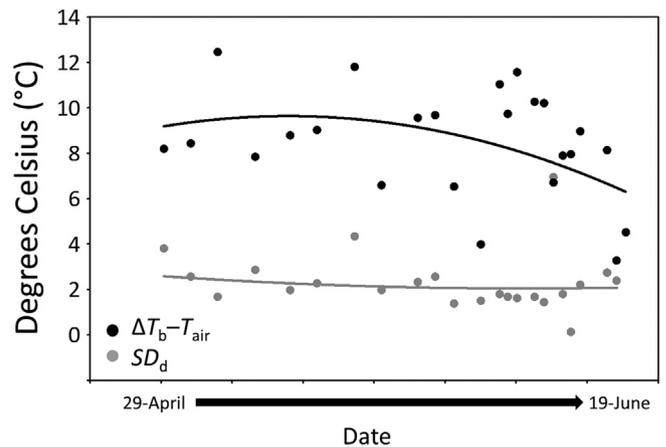


Fig. 3. Weighed significant relationships as indicated by MANOVA between date and both daily mean difference between body and air temperatures measured at the same spot where each lizard was captured (black line) and with daily standard deviations in body temperatures (grey line).

Table 3

Model averaging based on a general linear model analyzing predictors for the individual \log_{10} -transformed $[CORT_{ind}]$ ($N = 231$). 'Sex' represents males, females, and juveniles. Significant effects confirmed by cross-validation are shown in bold (see Results).

	Importance	Estimate	Adj SE	z value	Pr(> z)
(Intercept)		2.99	0.03	99.73	<0.001
Date_stand	0.98	0.08	0.03	2.65	0.008
Road proximity	0.70	-0.05	0.03	1.73	0.083
Road proximity:Date_stand	0.18	-0.01	0.03	0.25	0.800
Sex	0.15	0.02	0.05	0.50	0.620
Sex	0.15	0.00	0.06	0.03	0.979
Road proximity:Sex	0.02	-0.03	0.04	0.73	0.463
Road proximity:Sex	0.02	-0.02	0.05	0.39	0.696

predictors. No significant effect was found for sex ($P > 0.5$) (Table 3). Cross-validation confirmed the significant positive effect of date ($F_{1, 228} = 7.84, P = 0.006$), while indicating a non-significant trend ($F_{1, 228} = 2.99, P = 0.085$) in lizards captured far from the road to have higher $[CORT_{ind}]$, although the variance of $[CORT_{ind}]$ was higher close to the road (mean \pm SE: far away = 1806.33 ± 206.11 ng/g; close = 1694.35 ± 264.73 ng/g).

Daily mean $T_b - T_{air}$ was the most important predictor of $[CORT_{mean}]$ ($\beta = -149.76$, importance = 0.97) (Table 4). This was confirmed by cross-validation ($F_{1, 22} = 9.6, P = 0.005$) (Fig. 4).

3.3.2. Ectoparasites

Mites were morphologically and molecularly identified as *Ophionyssus* sp. (Acari: Macronyssidae). The prevalence of both adults and larvae of mites was 92.4% (171/185). The intensity of mites was best predicted by sex (importance = 0.99). Females had a mean \pm SE infestation of 18.03 ± 2.55 mites, and males had 24.88 ± 2.81 mites. Lizards had more mites when T_{ground} was closest to T_b ($\beta = -0.04$, importance = 0.89), and larger lizards had fewer mites ($\beta = -0.06$, importance = 0.75). The significance of these predictors was confirmed by cross-validation (Sex: $F_{1, 181} = 13.49, P < 0.001$; $T_b - T_{ground}$: $F_{1, 181} = 4.09, P = 0.045$; body length: $F_{1, 181} = 4.76, P = 0.030$) (Table 5).

In addition, infested individuals had a median intensity of 17.00 ± 1.86 mites (range = 1–123). The daily median of mite intensity was best predicted by date ($\beta = +5.31$, importance = 0.76) (Table 6), which was confirmed by cross-validation ($F_{1, 22} = 6.00, P = 0.023$) (Fig. 5).

4. Discussion

We tested general predictions of the extended cost-benefit model of ectotherm thermoregulation (i.e. Vickers et al., 2011) hypothesizing that *P. algirus*, a Mediterranean lizard, can suffer from and respond to heat stress during its reproductive season. This view was supported by the seasonal narrowing between body and air temperatures. Moreover, the increase in both thermoregulatory precision and concentration of fecal glucocorticoid metabolites following environmental heat restrictions supported that lizards increased their thermoregulatory activity in response to heat stress. However, this coping response had costs in terms of increased parasite load. Besides these effects of heat stress on

Table 4

Model averaging based on a weighted general linear model analyzing predictors for daily means ($N = 24$) of fecal glucocorticoids. 'Mites' refers to daily median of mites. Significant effects are shown in bold.

	Importance	Estimate	Adj SE	z value	Pr(> z)
(Intercept)		3011	602	5.00	<0.001
$\Delta T_b - T_{air}$	0.97	-149.76	57.24	2.62	0.009
$\Delta T_b - T_{ground}$	0.20	-32.48	55.02	0.59	0.555
Mites	0.19	-7.58	16.84	0.45	0.653
Date_stand	0.18	-52.73	142.56	0.37	0.711

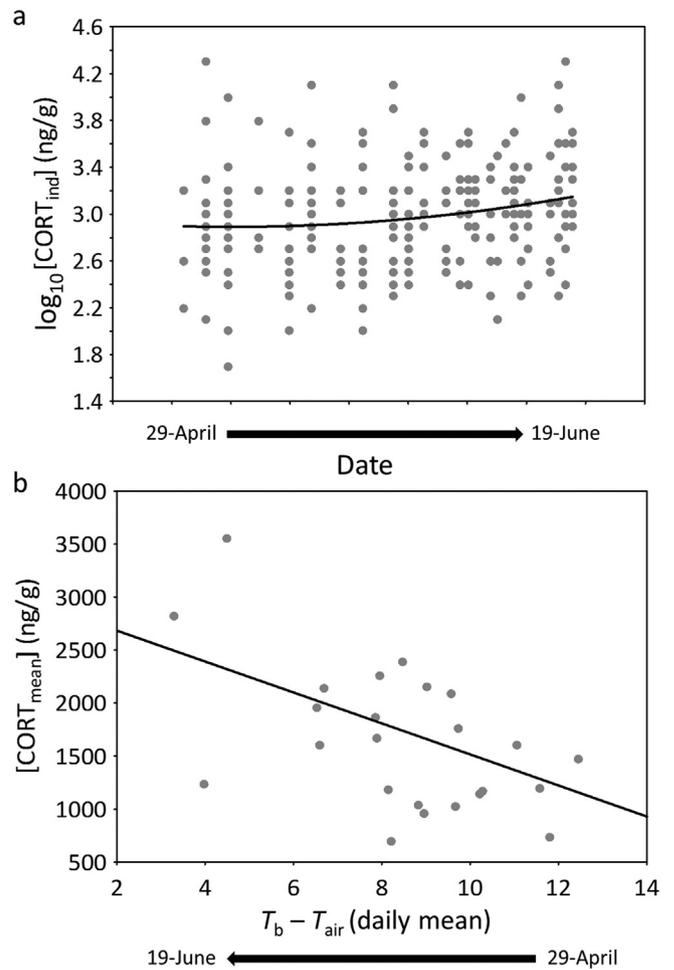


Fig. 4. (a) Relationship between date and the individual concentration of fecal glucocorticoid metabolites (\log_{10} -transformed) ($N = 231$). (b) Weighed negative relationship between $[CORT_{mean}]$ and Mean $T_b - T_{air}$ as suggested by model averaging and confirmed by cross-validation ($N = 24$). Therefore, the lowest the temperature difference between lizards and air, the highest glucocorticoid concentration found in their feces. Note that $\Delta T_b - T_{air}$ correlates with date (see Fig. 2b).

lizards, the results defied the facilitation hypothesis because concentrations of fecal glucocorticoid metabolites tended to be higher in the plot far from the road. The latter result, however, was consistent with the higher 0.69 ± 0.12 °C registered there, indicating that heat stress can elicit activation of the HPA axis in the lizards. The reasons for this difference in ambient temperature between sampling plots might be due to the road running through the valley floor, less exposed to the sun than the surrounding hills. The lower corticosterone concentration of lizards captured close to the road might reflect the plastic ecology of *P. algirus*, which is a generalist species that is distributed across Mediterranean

Table 5

Model averaging based on a generalized linear model fit with negative binomial distribution analyzing predictors for individual intensity of mites ($N = 185$). Significant effects confirmed by cross-validation are shown in bold (see Results).

	Importance	Estimate	Adj SE	z value	Pr(> z)
(Intercept)		6.56	2.63	2.50	0.013
Sex	0.99	-0.26	0.10	2.67	0.007
$\Delta T_b - T_{ground}$	0.89	-0.04	0.02	2.21	0.027
SVL	0.75	-0.06	0.03	2.12	0.034
Road proximity	0.64	0.17	0.10	1.62	0.105
$\log_{10}[CORT_{ind}]$	0.28	-0.12	0.18	0.66	0.508
Road proximity: $\Delta T_b - T_{ground}$	0.23	-0.02	0.02	1.25	0.210
$\Delta T_b - T_{ground}$: Sex	0.22	0.00	0.02	0.21	0.830

Table 6

Model averaging based on a weighted general linear model analyzing predictors for daily medians of mites (N = 24). Significant effects confirmed by cross-validation are shown in bold (see Results).

	Importance	Estimate	Adj SE	z value	Pr(> z)
(Intercept)		24.76	8.81	2.81	0.005
Date_stand	0.76	5.32	2.63	2.02	0.043
$T_b - T_{\text{air}}$	0.27	-1.19	1.19	1.00	0.317
$T_b - T_{\text{ground}}$	0.26	-1.23	1.27	0.97	0.331
[CORT _{mean}]	0.14	0.00	0.00	0.37	0.711

habitats from the southwest of France to the western Magreb, from sea level to 2400 m of elevation (e.g. Reguera et al., 2014). Nonetheless, in opposing view, recent research highlights that low glucocorticoid concentrations may sometimes be a sign of impaired coping capacity in stressed individuals (Romero and Beattie, 2021; see also Owen et al., 2014).

The results suggested that lizards experienced a restrictive thermal environment in both sampling plots, with only 1.63 h per day within 3–5 °C below the preferred upper thermal limits of *P. algirus*. This is considered a range of good thermal quality requiring low thermoregulatory effort (Hertz et al., 1993; Díaz and Cabezas-Díaz, 2004; Vickers et al., 2011). Moreover, thermal environments exceeded almost daily the upper limits of both thermal preference and critical thermal maxima of lizards during their reproductive season. This result is consistent with the seasonal increase in thermoregulatory precision, an adaptive

behavioral response that can reduce overheating risks when the thermal environment approaches lizards' critical thermal maximum (Clusella-Trullas and Chown, 2014; Cadena and Tattersall, 2009a, 2009b; Black et al., 2019).

Field body temperatures of the lizards were on average 4 and 9 °C warmer than ground and air temperatures, respectively. This suggests that the adopted critical thermal maximum of 43.1 °C (i.e. Herrando-Pérez et al., 2020b) could potentially be attained by the lizards at an operative temperature of 39.1 °C. This would increase the daily average hours of restriction up to 3.34 ± 0.20 during zenithal hours. Lizards are typically forced to retreat to underground microhabitats and cease their activity under extreme temperatures, even populations adapted to arid environments (e.g. Sinervo et al., 2010; Kirchhof et al., 2017). Moreover, the scarcity of drinking water in the prospected area might reduce the tolerance of lizards to heat stress (Rozen-Rechels et al., 2019; Dupoué et al., 2020; Herrando-Pérez et al., 2020a). However, despite the daily thermal restriction in El Pardo, lizards remained uninteruptedly active from the earliest hours in the morning to sunset, suggesting that they cope with the daily restrictions in thermal environment through the active selection of cooler thermal microhabitats during the warmest hours. This was indicated by the fact that only 4.3% of the lizards exceeded their preferred upper thermal limits despite the high operative temperatures attained at zenithal hours. Moreover, both the field body temperatures and thermal microhabitats selected by the lizards (ground and air) did not exceed the critical thermal maximum of *P. algirus* (Fig. 1b). Indeed, the strongly significant negative correlation between (i) the standard deviations of field body temperatures of the lizards and (ii) the operative temperatures registered with copper models deployed in the field suggested that thermoregulatory precision is facilitated by the heterogeneity of available thermal microhabitats in El Pardo. Indeed, even though lizards were observed to be active, they remained in the shade inside the bushes during zenithal hours. Díaz et al. (2006) demonstrated that *P. algirus* can increase its preferred thermal range by approximately 2 °C between May and July. However, the same authors suggested that this seasonal shift might not be enough to overcome heat excess during the hottest months (Díaz et al., 2006). Future research will also need to address how lizards cope with extreme temperatures of longer periods during the summer to fully understand the response of this population to heat stress.

Our analyses suggested that the increase in mite and glucocorticoid loads is complementary responses to heat stress, and not significant predictors of each other. Mean fecal glucocorticoid metabolites may reflect patterns of daily activity and the routine of the lizards (MacDougall-Shackleton et al., 2019). Thus, the seasonal increase in fecal glucocorticoid metabolites in the lizards matches the expected increase in the activity of lizards because they may struggle to maintain their body temperatures below their preferred upper thermal limits when optimal thermal microhabitats become a limiting resource due to heat excess (Dreisig, 1984; Black et al., 2019). Barrientos and Megía-Palma (2021) experimentally demonstrated in this population that mite load increase was explained by the distance travelled by the lizards. In the present study, the seasonal increase in mite loads was explained by differences between body and ground temperatures, supporting recent evidence of phenological variation of ectoparasite loads in lizards (Drechsler et al., 2021; Smolinský et al., 2021). However, complementary to the plausible effects of the warming environment on mite replication, our results suggest that lizards may increase exposure to ectoparasites when shuttling among thermal microhabitats during the warmest periods (Barrientos and Megía-Palma, 2021). Therefore, our results support that increased parasitism by mites is a cost added to the cost-benefit models of ectotherm thermoregulation of Huey and Slatkin (1976) and Vickers et al. (2011). This is because mites draw blood from hosts, can be competent vectors of blood parasites, inflict microwounds in the skin of hosts, and can reduce body mass gain in free-ranging lizards (Klukowski and Nelson, 2001; Álvarez-Ruiz et al., 2018). The fact that males had significantly more mites than females

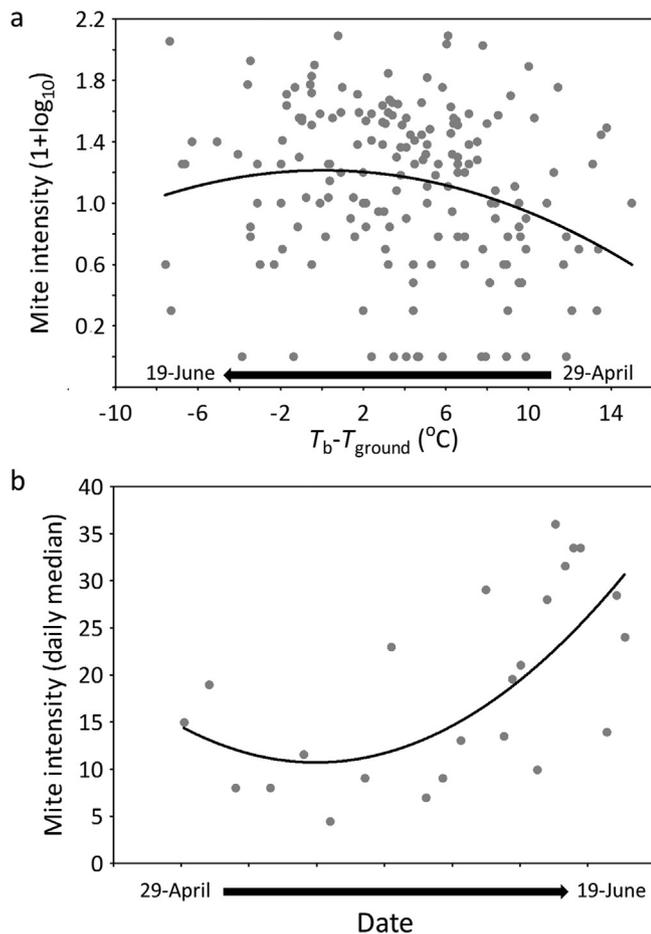


Fig. 5. (a) Relationships between the intensity of $1 + \log_{10}$ -transformed intensity of mites and the difference between the temperatures of the lizards' body and ground as confirmed by model averaging and cross-validation (N = 185). Note that $T_b - T_{\text{ground}}$ narrows with date in Monte de El Pardo and mite intensity tends to increase. (b) Weighted relationships between daily median mite intensity and date (N = 24).

also supported our interpretation, because males have larger home ranges than females (Díaz, 1993), which may favor more encounters with questing mites (Barrientos and Megía-Palma, 2021).

According to the extended cost-benefit model of thermoregulation proposed by Vickers et al. (2011), our data support that the thermal niche of *P. algirus* is constrained by heat excess during the mating season. This elicited both behavioral and physiological coping responses that were associated with increased loads of ectoparasites. However, the results defied the facilitation hypothesis because daily trends of glucocorticoid and ectoparasite increase were independent from road proximity. Previous studies suggested that low stress response close to roads may be indicative of acclimation, but this could also (i) indicate an impaired response to environmental stressors and/or (ii) result in increased road mortality if animals do not perceive roads as a potential threat (Owen et al., 2014). Plasma quantification of glucocorticoid response to a stressor following a capture and restraint standard protocol or the capacity to recover from such stress responses are complementary tests of stress vulnerability that could be performed in this population in the future (e.g. Tylan et al., 2020). More studies of similar nature analyzing ecological responses of ectotherm species to potentially interactive sources of stress are clearly needed. In the current scenario of global warming, aridification, and land homogenization, the coping responses of organisms to heat stress might be constrained (Megía-Palma et al., 2020c). The greater intensity and longer lasting heat waves forecasted for the Mediterranean basin for the next decades (Meehl and Tebaldi, 2004; Kuglitsch et al., 2010) are added adaptive challenges for lizards, drawing an uncertain future for an important proportion of lizard biodiversity (Sinervo et al., 2010; Quintero and Wiens, 2013).

CRediT authorship contribution statement

Rodrigo Megía-Palma: Conceptualization, Methodology, Investigation, Formal Analysis, Validation, Writing – Original draft preparation. **Isabel Barja:** Investigation, Biochemical Analyses, Hormonal validation, Resources (hormonal analyses). **Rafael Barrientos:** Resources, Project administration, Methodology, Investigation, Writing – Review Editing.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.149919>.

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