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Inference of lizard preferred temperatures differs substantially among experimental methods

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Abstract. Experimentally assessing the preferred body temperature (T_{pref}) of ectothermic animals is important to understand thermal adaptation. In lizards, this variable is usually estimated by measuring body temperature in thermal gradients. To quantify the extent to which different experimental setups influence the inferred T_{pref} values we submitted 65 individual lizards of three species to randomized tests using six different experimental setups at Oukaimeden, Morocco, including setup variants similar to those that have been most often used in lacertids. Among-treatment differences were substantial. Using an infrared bulb as heat source in combination with artificial cold lighting yielded about 5°C lower T_{pref} estimates than photothermal treatments with an incandescent bulb as heat and light source, possibly because lizards thermoregulated differently without a visual cue related to the heat source, or due to the absence of a natural photoperiod. Photothermal assays in which T_{pref} was assessed by hourly cloacal measurements over a 10h period yielded 2.2°C lower T_{pref} estimates than 2h treatments where body temperature was measured every minute with a thermocouple attached to the belly. This probably reflects that the 2h treatments targeted lizards in the initial warming-up phase, whereas the 10h treatment attempts to capture the preferred temperature of a lizard over its entire daily cycle including phases of inactivity. Lastly, we observed large differences among treatments with contact thermometers versus infrared laser thermometer measurements, calling for caution when the latter are used with artificial heat sources. Our data do not provide thorough tests of the physical, behavioural or physiological causes underlying the observed differences between treatments, but illustrate that for meta-analyses where detailed comparisons are needed, a rigorous consideration of the optimal experimental setup and its consistent use will remain necessary.

Key words. Squamata, Lacertidae, *Podarcis*, *Atlantolacerta*, *Scelarcis*, Morocco, thermoregulation, preferred temperature, field body temperature.

Introduction

Assessing the thermal preferences of ectothermic animals is crucial to numerous questions in evolutionary biology, ecology and conservation, and depends on robust experimental methods. This applies for instance to lizards, which have served as a model group to develop and test the hypothesis of faster adaptive processes to cold than to heat

† Co-author Barry Sinervo passed away while this study was in the proofing stage. Driven by his enormous passion for science, he continued contributing to this paper until a few days before his death.

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(Araújo et al. 2013, Muñoz et al. 2014), because their capacity for thermoregulatory behaviour may serve as a buffer in the case of cooling. Macroecological studies have elucidated that environmental temperatures predict lizard life history better than their body temperatures (Meiri et al. 2013). However, in one recent study, estimations of occurrence based on activity outperformed those based on temperature alone, which suggests other environmental factors (e.g. humidity) are also involved (Caetano et al. 2020). Among lizards, forest-dwelling species are mostly thermoconformers and cold-adapted species are usually thermoregulators, and both groups might suffer from thermal and/or hydric stress, and undergo declines, with global warming and aridification (Sinervo et al. 2010, Diele-Viegas et al. 2018, Garcia-Porta et al. 2019). Analyzing these ecological, evolutionary and conservational questions depends on inferring a series of physiological traits that inform on the interactions of lizards with their thermal environment (Clusella-Trullas & Chown 2014). These encompass thermal limits for survival (critical temperatures: CT_{min}, CT_{max}) but especially for activity (voluntary thermal maximum and minimum: VT_{max}, VT_{min}; Camacho et al. 2018), field body temperatures measured in active lizards in the wild (T_b), optimal performance temperature (T_{opt}), and preferred body temperature (T_{pref}). Especially this latter variable, T_{pref}, has played a prominent role in understanding thermal adaptations (reviewed in Clusella-Trullas & Chown 2014), and is a crucial component of models of climate change responses of lizards (e.g., Sinervo et al. 2010, García-Muñoz & Carretero 2013). In many lizards, T_{pref} is also highly correlated with other physiological optima such as running speed or digestion (Bauwens et al. 1995).

Experimentally, T_{pref} is measured by exposing lizards to a temperature gradient in the supposed absence of costs and constraints of operative environmental temperatures that are present in field conditions (Bakken et al. 1985; but see Sannolo & Carretero 2019 for hydric costs), and recording their body temperature over time (Camacho & Rusch 2017). Traditionally, lizard body temperature is measured as cloacal temperature (Huey & Webster 1975, 1976, Huey & Pianka 1977), an approach also used in T_{pref} experiments by many researchers (e.g., Carretero & Llorente 1995, Marquez et al. 1997, Veríssimo & Carretero 2009, Carretero 2012, Osojnik et al. 2013, Zamora-Camacho et al. 2014, Kapsalas et al. 2016). Other studies monitored body temperatures with a thermocouple attached to the outside of the body (e.g., Calsbeek & Sinervo 2002, Aguado & Braña 2014, Moreno Azocar et al. 2016, Garcia-Porta et al. 2019), implanted into the body cavity or underneath the skin (e.g., Licht 1968, Cowgell & Underwood 1979), or measured temperature of the body surface using a laser thermometer (Carretero 2012, Bouazza et al. 2016). Remarkably, Carretero (2012) found that lizard body temperatures measured by infrared vs. contact thermometers were only poorly correlated and with a biased relation. In contrast, Barroso et al. (2016) obtained a good correlation and minimum bias in the comparison of thermographic images with cloacal temperature measured by contact thermometers. As a further potentially confounding factor, even in small lizards regional heterothermy occurs, i.e., different body parts can have different temperatures (Sannolo et al. 2014, Barroso et al. 2016, 2020).

The kind of thermal gradient used also differs substantially between studies (reviewed in Camacho & Rusch 2017). Some researchers use incandescent light bulbs as heat source to create a photothermal gradient (e.g., Licht 1968, Gvoždík 2002, Aguado & Braña 2014, Kirchhof et al. 2017, Garcia-Porta et al. 2019), while others advocate the use of uniformly distributed (natural) lighting originating from windows, and use infrared (IR) bulbs to create a purely thermal gradient (e.g., Bowker and Johnson 1980, Angilletta 2001, Lailvaux et al. 2003, Veríssimo & Carretero 2009, Carneiro et al. 2015). It is likely that such differences between heat and light sources lead to behavioural differences of the lizards in the gradient, mirroring differences in thermoregulatory behaviour in shaded vs. exposed environments (e.g., Huey 1974).

A further difference between studies refers to the time during which lizards are exposed to a temperature gradient. Individuals can be kept full days up to weeks in the gradient with occasional temperature measurements (which often involves catching the lizard out of the gradient thereby influencing its behaviour and body temperature via convection and removal from the original temperature source) (Licht et al. 1966, Licht 1968, Sievert & Hutchison 1988, 1989, Carneiro et al. 2015, Ortega et al. 2016). Alternatively, lizard body temperature is measured over a relatively short period of a few hours, randomized throughout the day, mostly with continuous temperature measurements, to minimize the possible effect of diel variation in T_{pref} (e.g., Paranjpe et al. 2013, Yang et al. 2008, Li et al. 2009, Wang et al. 2013, Kirchhof et al. 2017, Garcia-Porta et al. 2019).

Given the importance of cross-taxon comparisons and meta-analyses to understand evolutionary and macroecological patterns of lizard thermal physiology (Sinervo et al. 2010; Meiri et al. 2013, Diele-Viegas et al. 2018, Garcia-Porta et al. 2019), it is of high importance to assess how these methodological differences influence the inferred values of T_{pref}. While the reviews of Clusella-Trullas & Chown (2014) and Camacho & Rusch (2017) highlighted the wide variation of methods used, a rigorous experimental approach is necessary to quantitatively assess impacts of using different methods (e.g., Carretero 2012, Barroso et al. 2016).

Thus far, methodological studies on lizard thermal physiology have (i) demonstrated differences in thermal preference at different times of the day (e.g. Cowgell & Underwood 1979, Rismiller & Heldmaier 1982, Sievert & Hutchinson 1988, Firth & Belan 1998), (ii) shown differences in preferred temperature depending on the relative position of heat vs. light source in diurnal and nocturnal lizards (Sievert & Hutchinson 1988, 1989), (iii) compared measurements of infrared laser vs. contact thermometers (Carretero 2012) and thermographic images vs. contact

thermometers (Barroso et al. 2016), and (iv) compared temperature measurements at different lizard body parts (Sannolo et al. 2014, Barroso et al. 2016, 2020). However, experimental comparisons of approaches to T_{pref} inference from long vs. short segments of the diel activity period, or from trials with infrared vs. incandescent bulbs as heat source, have not been carried out yet. Ideally, such comparisons should be carried out in the same study period and lizard species, and if possible the same lizard individuals.

Here, we empirically quantify differences among T_{pref} measurements obtained from different experimental approaches on the same organisms. Our study focuses on three species of the family Lacertidae, an Old World clade that has been intensively explored for thermal ecology. Carretero (2012) and Garcia-Porta et al. (2019) have highlighted differences in T_b and T_{pref} values of lacertids obtained using different experimental approaches. We expand these data by assessing T_{pref} of the same individual lizards in six different settings reflecting the most commonly used approaches in these lizards, mainly to understand the impact of (a) full day hourly measurements of cloacal temperature vs. short-term measurements by ventrally attached thermocouples, and (b) usage of a photothermal gradient vs. thermal gradient with uniform lighting; and furthermore, (c) the difference between contact and laser thermometers, as previously assessed by Carretero (2012). The main goal of our study is to describe the differences between various experimental approaches that have often been applied to estimate T_{pref} in lacertids and/or other lizards. We provide hypotheses that could explain the observed differences among methods but do not aim at providing functional explanations within a thorough hypothesis-testing framework.

Methods

Experiments were carried out in Oukaimeden, Morocco (approximate geographical coordinates 31.2075°, -7.854°, ca. 2600 m above sea level), with lizards of three sympatric species: *Atlantolacerta andreanskyi* (Werner, 1929), *Podarcis vaucheri* (Boulenger, 1905), and *Scelarcis perspicillata* (Duméril & Bibron, 1839). Active lizards were directly captured in the field in the breeding season, April and May, corresponding to the lizards' main activity period. Overall, 30 *A. andreanskyi*, 23 *P. vaucheri,* and 12 *S. perspicillata,* all adult males to exclude biases due to sex, reproduction or body condition in thermal preferences, were included in the experiments (S'khifa et al. 2020). Active specimens were captured in the field on sunny days, opportunistically during random searches between 9–17 h local time. Field body temperatures were measured immediately upon capture as (i) cloacal temperatures as described in treatments 3 and 4 below, and (ii) as dorsal surface temperatures as described in treatments 5 and 6 below.

Our methodological approach relies on determining selected body temperature in a thermal gradient as a proxy for preferred body temperature (T_{pref}). All experiments were conducted in an experimental setup consisting of five individual tracks, each of which ca. 120 cm in length and 20 cm in width, corresponding to a surface of ca. 2400 cm^2. Experiments started at 8:30 h in the morning, and again at 14:30 h in the afternoon for the 2 h treatments, to mirror the lizards' regular activity period. Before and after the experiments, lizards were kept for several hours at an air temperature of 10–15°C (corresponding to natural air temperatures outside of the sun), providing water and food ad libitum. In all treatments, the gradient ranged from surface and air temperatures of ca. 10–15°C in its coldest part to very hot temperatures (> 40°C) in the immediacy of the (infrared or incandescent) bulbs used as heating source, and this also approximately represents the minimum and maximum potential operative temperatures that these small lizards could attain in the gradient if spending sufficient time in the extreme parts of the gradient. After completion of the experiments, lizards were released at the site of initial capture. When lizards showed signs of fatigue or non-natural behaviour, or when completion of all treatments was logistically not feasible within five days, they were released back at the capture sites. Each lizard was subjected to each of the six treatments following a randomized order. Altogether, 65 lizards were studied, and for 21 *A. andreanskyi,* 20 *P. vaucheri,* and 11 *S. perspicillata* complete data sets for each of the following six treatments were obtained:

(1) photothermal-2h: an incandescent 100 W light bulb (full spectrum) was suspended 30 cm above one end of a track, to create a photothermal gradient of approximately 15 to 55°C at ground level. Body temperature was determined every minute by ultra-thin T-type thermocouples (OMEGA 5SCTT-T-40-72, diameter = 0.076 mm, Norwalk, Connecticut, USA) affixed with medical tape to the lizards' venter and connected to an 8-Channel USB Thermocouple Data Acquisition Module (OMEGA TC-08; resolution < 0.1°C). Lizards were allowed unrestricted movement within their individual gradient. Lizard body temperature was measured over a period of up to 2.5 hours. Lizards were randomly assigned to either morning or afternoon treatments. The initial 30 minutes of the experiment were discarded as acclimation time, resulting in a maximum of 120 individual measurements (= 2 hours). Individuals that remained inactive at the cold end of the gradient for more than 50 minutes were not included in the final analysis assuming they were not thermoregulating. Temporarily entangled or detached thermocouples were fixed and reattached, the respective data points were discarded, and the experiment was continued. This treatment corresponds to the one used in Garcia-Porta et al. (2019).

(2) thermal-2h: as in the previous treatment, but instead of an incandescent light bulb, a 75 W IR bulb was used and, separate, uniform cold lighting provided for the entire gradient and for the entire time of the experiment.

(3) photothermal-10h: as in photothermal-2h (incandescent bulb), but experiment was run for 10 hours, lizards caught once per hour, and their cloacal temperature measured within 20 s with a digital quick-reading thermometer (GHM-Greisinger: GTH 1170, GHM, Regenstauf, Ger-

many), introducing the thin external probe (K-type thermocouple) ca. 2 mm into the lizard's cloaca within 10 s after capture. Temperatures were recorded with a resolution of 0.1°C.

(4) thermal-10h: as in photothermal-10 h, but with a 75 W IR bulb and uniform cold lighting. This treatment largely corresponds to the one extensively used in previous studies of lacertid thermophysiology (e.g., Carretero et al. 2006, Veríssimo & Carretero 2009, García-Muñoz & Carretero 2013) except for the use of artificial rather than natural light as in the quoted studies, and narrower gradient setups (30 cm in the quoted studies).

(5) photothermal-10hL: as in photothermal-10h, but temperatures measured each hour with an infrared laser thermometer (Fluke 62; Fluke Europe B.V., Eindhoven, The Netherlands) dorsally near the tail base in line with the body axis from approximately 300 mm distance (Carretero 2012, Aguado & Braña 2014, Lara-Resendiz et al. 2015). The Fluke 62 measures with a resolution of 0.1°C and an accuracy of +1.0°C or +1.0% of reading with a sensor diameter of 38 mm (at 300 mm distance).

(6) thermal-10hL: as in photothermal-10h, but with an IR bulb and uniform lighting, and temperatures measured each hour with an infrared laser thermometer as in photothermal-10hL.

In the 2h treatments, the lizards were observed during the entire time of the experiment, and all time periods in which thermocouples were detached or entangled were noted, and later excluded from analysis. We also excluded all obvious outliers corresponding to erroneous measurements from the raw data set. We then calculated individual medians, yielding one datapoint per individual and treatment (i.e., typically six data points per individual).

As usual in these kinds of data, they were skewed towards higher values and therefore not normally distributed (Shapiro-Wilk tests, $p > 0.05$ in comparisons of the full data set and subsets by method), even after log-transformation. We therefore used Poisson-distributed Generalized Linear Mixed Models (GLMMs) which are robust against non-normally distributed data, as implemented in JMP 13.0 (SAS Institute) with the GLMM add-in (https://community.jmp.com/t5/JMP-Add-Ins/Generalized-Linear-Mixed-Model-Add-in/ta-p/284627) to assess the influences of various predictors on inferred T_{pref}. Specifically, the following models were calculated:

Model 1: To understand if time period (morning or afternoon) played a role in explaining T_{pref} inferred from the photothermal-2h and thermal-2h treatments, an initial GLMM was conducted with T_{pref} as response variable, experimental method (treatment), species and time period as predictor variables, and lizard individual as random factor (no interactions specified), only including data from the two 2h treatments. A total of N=106 T_{pref} values were included in this model.

Model 2: A GLMM with T_{pref} as response variable, experimental method and species as predictor variables, lizard individual as random factor (no interactions specified), for all treatments. A total of N = 346 T_{pref} values were included in this model.

Model 3: A variant of Model 2 with the same factors and specifications but excluding the deviant treatments with an infrared laser thermometer. A total of N = 228 T_{pref} values were included in this model.

Model 4: A more complex variant of Model 2, also considering body size and body mass of lizards and the trial order of experiments as predictors. A total of N = 346 T_{pref} values were included in this model.

We furthermore performed non-parametric Kruskal-Wallis Analyses of Variance (K-W ANOVA) with post-hoc tests in Statistica version 7 (Statsoft), to detect differences in inferred T_{pref} among species and treatments.

Results

After excluding experiments with outliers due to erroneous measurements, data for a total of 346 experiments with 65 individual lizards were available. For each experiment and individual lizard, the medians of all measurements of body temperature were defined as inferred T_{pref} and used as data points for further analysis. For the photothermal-2h and thermal-2h treatments which were carried out either in the morning or in the afternoon (see Methods), we assessed with a GLMM (Model 1) that time period had no significant effect on T_{pref} (Table 1), justifying the merging of morning and afternoon trials in all subsequent statistics.

To obtain a first overview of thermal preferences among individual lizards, and to exclude that such preferences would substantially influence our results, we carried out rank correlation analyses among the different methods and field body temperatures for each individual lizard, separately by species. These analyses revealed only sporadic significant correlations which lost significance after Bonferroni correction (Supplementary Tables S1–S3), suggesting that individual thermal preferences across treatments were poorly expressed.

A GLMM (Model 2) for all 346 available T_{pref} measurements revealed a highly significant influence of method, and an only weakly significant influence of species (Table 1). A further GLMM (Model 3) excluding the treatments with an infrared laser thermometer confirmed a highly significant effect of method whereas the effect of species was not significant (Table 1). A more complex GLMM (Model 4) also considering body size and body mass of lizards and the trial order of experiments yielded similar results, with only the effects of experimental method and species being statistically significant (Table 1).

Given the relatively weak or absent effect of species in these models, we pooled the data of all three species in several of the analyses reported in the following. This was done in an effort to increase sample size in these simpler statistical analyses that were carried out to better understand the differences between treatments.

Averaged over all three species, the 2h and 10h treatments with contact thermometers (cloacal measurements or thermocouples attached to venter) revealed an obvious difference between incandescent and IR bulbs, the latter

Table 1. Fixed effects of various predictor variables in general linear mixed models (GLMMs) on T_{pref}. See Methods for detailed descriptions of Model 1–4. In brief, in all models T_{pref} was specified as response variable and lizard individual as random factor. Model 1 was performed only for 2h treatments, and Model 3 excluded treatments with laser thermometer measurements. Model 4 is a variant of Model 2 with additional predictor variables. Abbreviations: DF, degrees of freedom (i.e., number of fixed factors -1); DFDen, denominator degrees of freedom for the effect test (the degrees of freedom for error); F ratio, computed F ratio for testing that the effect is zero; Prob > F, P-value of the effect test.

Source	DF	DFDen	F Ratio	Prob > F
Model 1				
time (morning/afternoon)	1	58.9	0.10	0.7523
treatment	1	48.6	114.64	<.0001
species	2	47.7	2.09	0.1344
Model 2				
treatment	5	278.4	62.47	<0.0001
species	2	52.0	4.99	0.0104
Model 3				
treatment	3	172.5	79.06	<0.0001
species	2	56.7	1.24	0.2977
Model 4				
treatment	5	277.4	62.24	<0.0001
species	2	52.5	3.98	0.0247
trial order	1	286.9	2.54	0.1123
body mass	1	49.6	1.10	0.3002
SVL	1	48.6	2.87	0.0967

yielding about 5°C lower T_{pref} estimates (IR vs. incandescent bulb medians of 27.9°C vs. 33.9°C for the 2 h treatments; and 26.9°C vs. 32.2°C for the 10h treatments). For the photothermal approach, the T_{pref} estimates obtained in the 2h treatment were on average 2.2°C higher than in the 10h treatment (33.8°C vs. 31.6°C). The differences between all treatments except thermal-2h vs. thermal-10h were highly significant (K-W ANOVA; $P < 0.001$) with pairwise post-hoc tests (see Fig. 1 for test details). When comparing results separately for each species the pattern was highly consistent, with IR bulb treatments yielding substantially lower T_{pref} estimates (Fig. 2).

Measurements of field body temperatures (T_b) of the three species yielded similar median values for the two approaches used (cloacal temperature, and surface temperature by infrared laser thermometer): *A. andreanskyi*: $T_{b\text{-}cloaca}$ 28.6°C; $T_{b\text{-}dorsal}$ 28.3°C; *P. vaucheri*: $T_{b\text{-}cloaca}$ 27.1°C; $T_{b\text{-}dorsal}$ 27.0°C; *S. perspicillata*: $T_{b\text{-}cloaca}$ 27.2°C; $T_{b\text{-}dorsal}$ 28.3°C. We tested for possible differences in $T_{b\text{-}cloaca}$ between species and found them to be non-significant (K-W ANOVA, $P = 0.602$).

Comparing field $T_{b\text{-}cloaca}$ with T_{pref} inferred using the four contact thermometer-based methods (pooled for the three species; K-W ANOVA, $H_{4,292} = 141.7121$, $p < 0.001$) revealed significant differences between T_b and T_{pref} inferred with an incandescent bulb (post-hoc tests, $P = 0.0001$ for both photothermal-2h and photothermal-10h) but not between T_b and T_{pref} inferred using an infrared bulb ($P = 1.0$ for thermal-2h, and $P = 0.37$ for thermal-10h) (see Fig. 2 for comparisons for each species).

When scoring lizard cloacal temperatures during the photothermal-10h and thermal-10h treatments, we included values from lizards that were temporarily not active, to obtain a daily average T_{pref} including also resting phases. To test the distribution of these phases without obvious thermoregulatory activity over the day, we analyzed hourly temperatures over the course of the experiment, pooled for all three species (Fig. 3). In the photothermal-10h treatment we found a clear trend of decreasing lizard body temperatures over time, whereas in the thermal-10h treatment, lizard body temperatures increased over time.

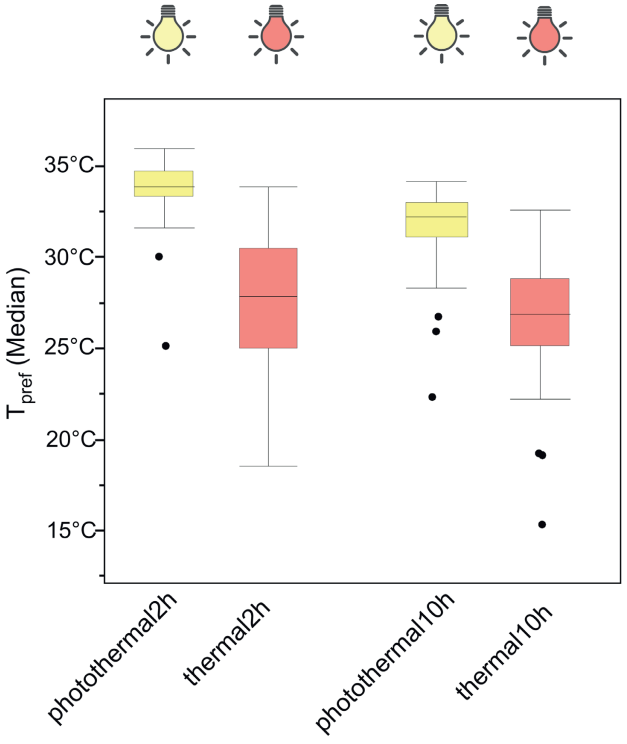


Figure 1. Measured preferred temperatures (T_{pref}), pooled for 66 individuals of three species of lacertids (*Atlantolacerta andreanskyi*, *Podarcis vaucheri, Scelarcis perspicillata*) at Oukaimeden, Morocco, for four different experimental procedures (lizard body temperature measured with contact thermometers, either as thermocouple attached to the venter, every minute for 2 hours; or by cloacal measurements every hour for 10 hours; median body temperature of each lizard in each experiment used as data points for analysis). The boxplots indicate median, quartiles, minimum-maximum values (not considering outliers), and outliers as dots. Yellow colour indicates experiments where thermal gradients were established with incandescent bulbs, red colour indicates an infrared bulb as heat source and uniform cold lighting. Differences between methods are statistically significant (K-W ANOVA, $H_{5,346} = 190.0$, $P < 0.0001$). Differences in all pairwise comparisons are statistically significant in post-hoc tests at $P < 0.0001$, except for the comparison between photothermal-2h and photothermal-10h (significant at $P < 0.002$), and thermal-2h and thermal-10h (not significant).

Treatments where lizard body temperatures in the gradient were measured using an infrared laser thermometer showed a pattern deviant from the overall one (Fig. 4). Here, the higher inferred T_{pref} values corresponded to the thermal-10hL treatment using an IR bulb (32.6°C vs. 25.8°C).

Discussion

Our study focused mainly on comparing experimental approaches that have often been used in lacertid and other lizards, and to describe the amount of difference found among the T_{pref} estimates obtained with these approaches. Because the selected treatments differ at various confounded levels, i.e. heat source, method of temperature measurement, total experimental time, and interval between measurements, we cannot thoroughly discern the physical, behavioural and physiological causes underlying the observed differences, and in the following will develop a series of necessarily speculative but testable hypotheses on these causes. However, as a main strength of our experimental approach, by subjecting a large number of individual lizards each to the various treatments in randomized trials, and by recovering similar among-treatment differences for three species, we provide conclusive evidence for actual differences between often-used approaches of T_{pref} estimation in lacertid and other lizards. We found (i) weak differences between short-term vs. long-term measurements under an incandescent bulb, and important and consistent differences between heat sources, i.e., (ii) between treatments with incandescent vs. IR bulbs. Finally, we also observed strongly different patterns when using (iii) infrared laser thermometers vs. contact thermometers.

The weak differences between the photothermal-2h vs photothermal-10h inferred T_{pref} values are likely explained by inherent differences between the treatments, and by behavioural factors. We hypothesize that the 2h treatments targeted lizards in the initial warming-up phase and during the first phases of activity in the morning and afternoon when thermoregulation is usually a priority, given the known endogenous circadian activity rhythms of lizards (Foà & Bertolucci 2003); our approach in these treatments was to even exclude lizards staying inactive for a long time from further analysis, similar to previous studies (e.g., Kirchhof et al. 2017, Garcia-Porta et al. 2019). In contrast, the 10h treatment attempts to capture the preferred temperature of a lizard over its entire daily cycle, which typically includes phases of inactivity, and phases when thermoregulation is not the main priority for the lizards, resulting in lower body temperatures. The 10h treatments may also exacerbate the hydric costs that can constrain thermoregulation (Sannolo & Carretero 2019), thereby favoring periods when thermoregulation is constrained by other factors. This mechanism might indeed affect some of our target species, in particular *Atlantolacerta*

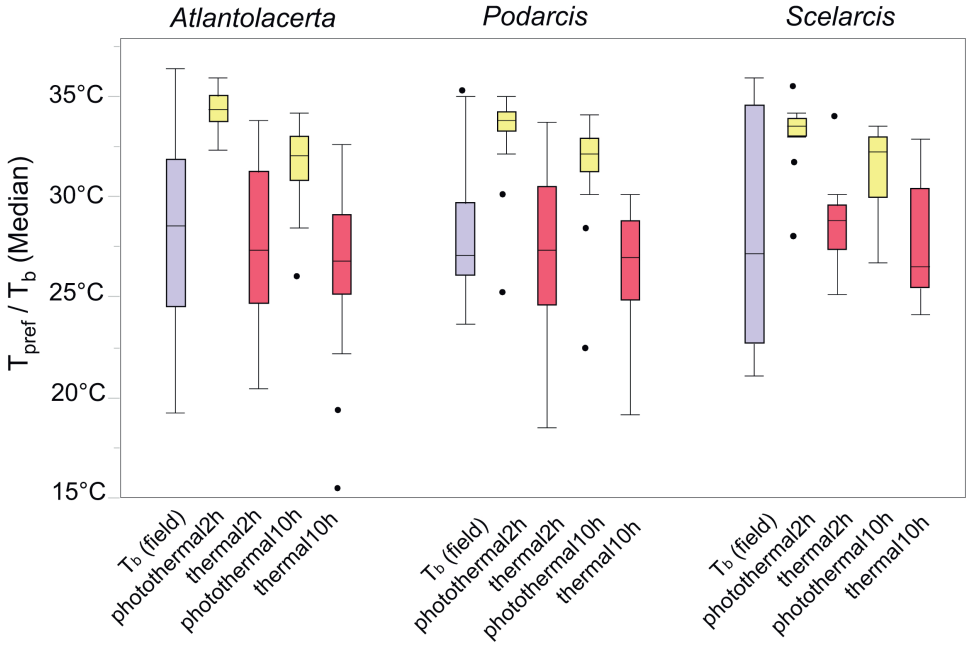


Figure 2. Field body temperatures (T_b) and measured preferred temperatures (T_{pref}), separately for three species of lacertids (*Atlantolacerta andreanskyi*, *Podarcis vaucheri, Scelarcis perspicillata*) at Oukaimeden, Morocco, for four different experimental procedures (lizard body temperature measured with contact thermometers, either as thermocouple attached to the venter, every minute for 2 hours; or by cloacal measurements every hour for 10 hours). The boxplots indicate median, quartiles, minimum-maximum values (not considering outliers), and outliers as dots. Yellow colour indicates experiments where thermal gradients were established with incandescent bulbs, red colour indicates an infrared bulb as heat source and uniform cold lighting.

andreanskyi which requires substantial humidity, as suggested by a previous study (S'KHIFA et al. 2020).

It is also obvious that both the 2h and the 10h treatments involve artificial disturbances that may influence the animal's behaviour in different ways. On one hand, the 2h treatment involves attaching a thermocouple to the animal. According to our observations, animals usually bask very calmly in one particular area of the gradient despite the attached thermocouple, but it cannot be excluded that in some cases this setup leads to increased activity to escape from the thermocouple annoyance. This in turn could lead to a higher variation of measured temperatures, and to higher average temperature values as the animal would not undergo resting activity at lower temperatures. On the other hand, the 10h treatments involve repeated capturing and handling the animals (once per hour) which may lead to a less active behaviour over the entire phase, thus to a higher proportion of time spent in cooler parts of the gradient for resting, and consequently to lower average body temperature measurements. In agreement with the expectations from these differences among the approaches, the photothermal-10h treatment resulted in slightly but significantly lower T_{pref} estimates than the photothermal-2h treatment.

In contrast, the magnitude of differences between heat sources (incandescent vs. IR) observed herein is striking and may in part reflect differences between thermal and

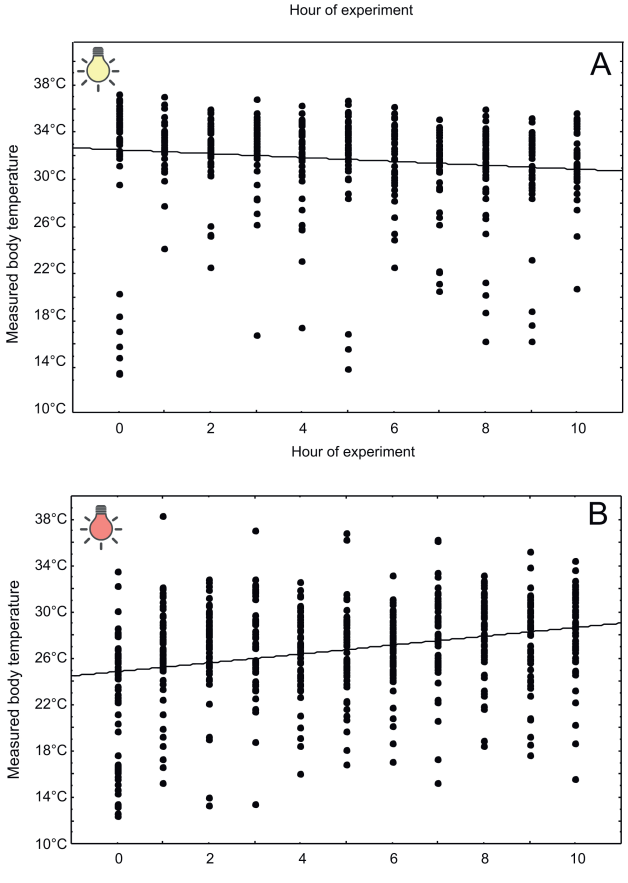


Figure 3. Body temperature measurements of lizards (pooled for all three species, *Atlantolacerta andreanskyi*, *Podarcis vaucheri, Scelarcis perspicillata*) measured per hour over the 10 hours of the (A) thermal-10h and (B) photothermal-10h experiments. Both correlations are statistically significant: (A) r = -0.1304, P = 0.0027; (B) r = 0.2878; P < 0.0001.

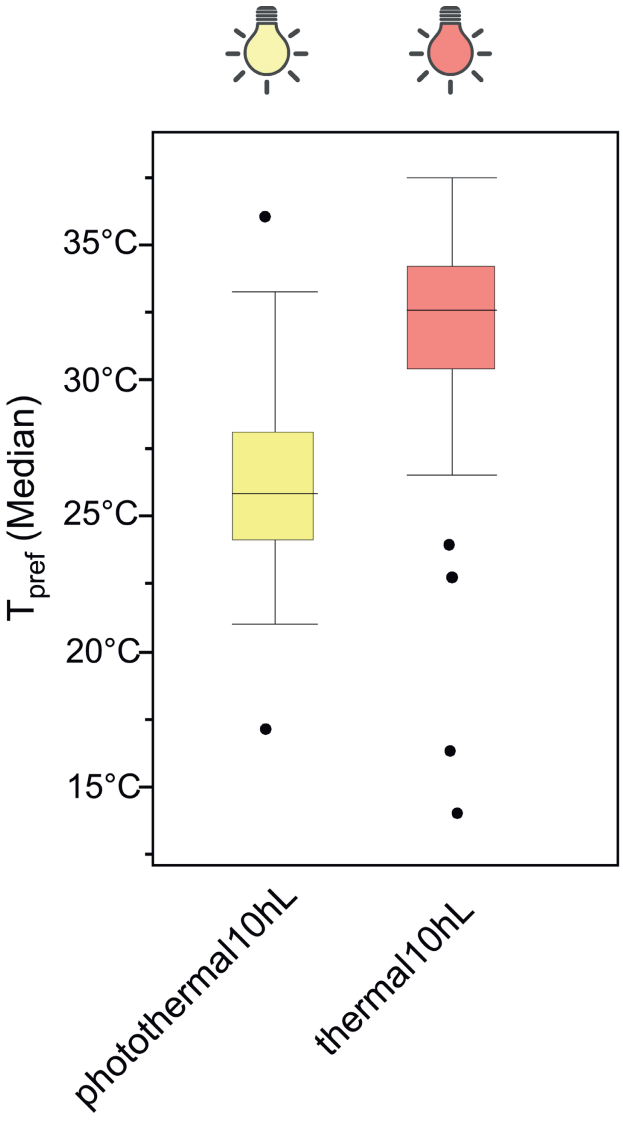


Figure 4. Measured preferred temperatures (T_{pref}), pooled for three species of lacertids (*Atlantolacerta andreanskyi*, *Podarcis vaucheri, Scelarcis perspicillata*) at Oukaimeden, Morocco, for two different experimental procedures (lizard body temperature measured with an infrared laser thermometer every hour for 10 hours). The boxplots indicate median, quartiles, minimum-maximum values (not considering outliers), and outliers as dots. Yellow colour indicates experiments where thermal gradients were established with incandescent bulbs, red colour indicates an infrared bulb as heat source and uniform cold lighting. The differences are statistically significant (U-test; P < 0.001). Note that we here discuss this result as a possible artefact of laser thermometer results under the specific conditions of our indoor treatments, although elucidating the causes of this surprising result will require future testing.

photothermal preferences. Not considering the results obtained with laser thermometers, T_{pref} values obtained in photothermal gradients were consistently higher than those in thermal gradients. We cannot readily explain these differences, but we speculate that this may reflect that heliothermic lizards such as lacertids prefer thermoregulating in bright sunlight, even if this leads to body temperatures above their purely thermal preference. On the other hand, in an IR-thermal gradient with uniform, constant artificial light, lizards may become less active than they would normally be, due to the lacking stimulus of a bright light source emitting at the same time thermal radiation. Therefore, in such an IR-thermal gradient, they would on average attain temperatures below their physiological preference.

Some hints on possible explanations of the observed pattern are provided by the trends of hourly body temperatures measured in the gradient during the 10h experimental treatments (Fig. 3). Under such a photothermal treatment over an entire daily period, according to these observations (data herein and numerous other studies), lizards in the first hours attain comparatively high body temperatures while basking, and then increasingly become active across the gradient, with lower body temperatures, or even become inactive with even lower body temperatures. These results may be further exacerbated by an endogenous circadian activity rhythms of lizards (Foà & Bertolucci 2003) which could trigger initially higher activities in the morning. On the contrary, under a thermal treatment, lizards in the beginning according to our observations (Fig. 3) operate under relatively low temperatures and later attain higher temperatures, which may be due to our use of artificial cold light rather than natural light with natural photoperiod (Tosini & Avery 1994, 1996, Tosini et al. 2001). However, using purely natural light sources in our experimental setup was not feasible (e.g., through a large window) due to constraints in the available rooms at the study site. Perhaps also, in such a thermal treatment (especially in a setup with artificial light source) lizards require some time to learn how to efficiently up-thermoregulate in a purely thermal gradient, without the visual cues provided by an incandescent bulb, or by the sun in their natural environment where also the spatial distribution of temperatures is different.

Further hypotheses for the factors influencing T_{pref} assessments under different experiments of different duration in photothermal setups can be derived from growth experiments in which hours of access to photothermal heat per day were manipulated to 6, 8, 10, and 12 hours, in *Sceloporus* lizards found across altitudinal and latitudinal gradients (Sinervo & Adolph 1989, 1994). The results suggested adaptive differences in thermal "niches", where the cool-adapted species may not have the capacity to use long thermal periods. Additional studies on *S. occidentalis* from a warm desert site and a cool site revealed a similar difference (Sinervo 1990). Lizards from the cooler site did not use the full 6-hour treatment and were in refugia in 9 and 12 hour treatments, while the populations from the warmer site used the entire time in each treatment for thermoregulation. These findings, as well as those of Paranjpe et al. (2013) are germane in the use of short (2h) versus long (10h) experimental duration at revealing evolution in thermoregulatory behaviour. They explain why in the studied lizards, short-term methods for T_{pref} will give high values, while long exposure to a photothermal heat will reveal additional adaptations related to activity period that might impose severe constraints on cool-adapted species under climate change (Sinervo et al. 2018). We hypothesize that similar mechanisms also explain, at least partly, the differences between short- and long-duration photothermal experiments in our study.

Presumably, under field conditions, active lizards may largely operate under their preferred temperatures, and T_b may, therefore, be used as a very approximate yardstick to evaluate the accuracy of experimental T_{pref} measurements. In our study, the temperatures from the thermal treatment (i.e., with IR bulb) were similar to field T_b of the lizards. However, it must be considered that active lizards may have been observed during initial phases, or in-between episodes of basking; and indeed, in these phases they might be more easily spotted and captured, and will have lower body temperatures. Our study did not include a rigorous assessment of operative body temperatures in the field based on fully randomized sampling across the entire range of microhabitats used. Still, our results allow for the preliminary hypothesis that the thermal (IR bulb) T_{pref} estimates may more closely reflect the average body temperatures of active lizards in the field, which alternate between basking and being active in thermally suboptimal microhabitats, e.g. when foraging.

Lastly, the differences between T_{pref} estimates from measurements obtained by an infrared thermometer strongly deviated from the pattern observed with contact thermometers. An obvious and intuitive hypothesis would predict overall higher temperature measurements obtained from laser thermometers, as they measure temperature on the dorsal side of the lizards (exposed to the heating source), compared to thermocouple measurements on their belly or cloaca, thus on their ventral side. This prediction was confirmed since under an IR bulb the laser thermometer measurements (thermal10hL) were consistently higher than the cloacal measurements (thermal10h). However, under an incandescent bulb this trend was inverted and the laser thermometer measurements (photothermal10hL) were consistently lower than the cloacal measurements (photothermal10h). On the contrary, when using the two types of thermometer with natural sunlight in the field, very similar T_b measurements were obtained. This suggests that under the experimental conditions of the thermal gradient, some laser thermometers may suffer from different artefacts. Since we did not perform dedicated experiments on the underlying causes, we can here only speculate on the nature of these artefacts. One of these may be related to the substrate materials that we used to build the gradient: in order to have a lightweight, portable and heat-resistance field setup, we used silver-aluminium coated foamed plastic with a roughened wallpaper as substrate – and the re-

maining exposed aluminium coating at the gradient walls may have caused reflectance influencing the laser thermometer. Other possible biases may have been caused by a too wide measuring angle (Hare et al. 2007; although we took great care to keep this uniform across the experiments), possibly excessive measuring distance, and artefacts we observed from old, partly discharged to fresh batteries. Furthermore, given that our model of infrared laser thermometer is recommended only for altitudes up to 2000 m above sea level (and our study site was located at 2600 m above sea level), we cannot exclude that using different models of laser thermometer these factors and artefacts can be attenuated or avoided. With the data at hand, it seems impossible to clearly understand what has caused the counter-intuitive laser thermometer measurements of lizard body temperature. Given these erratic results, our study calls for caution when using laser thermometers under experimental conditions rather than natural sunlight.

Overall, our comparisons highlight that experimental approaches to determine T_{pref} can yield substantially different results. First and foremost, our experiments confirmed the existence of important differences between laser and contact thermometers (Carretero 2012), and call for extreme caution and need for careful exclusion of possible measuring artefacts when using the former instruments especially in non-natural setups. Second, we obtained surprisingly high differences in median values between setups with incandescent vs. IR bulbs (photothermal vs. thermal), with average differences of about 5°C in inferred T_{pref}. This differs from empirical differences summarized by Garcia-Porta et al. (2019) who found an average difference between these methods of only 1°C, and applied this value as correction factor in order to include in their meta-analysis a few crucial species for which only data from a 10h-thermal setup were available. As discussed above, this may be due to the use of non-natural light in our thermal setups, and highlights the need to consider lighting as important factor when interpreting the results from thermal gradient T_{pref} assessments. Thirdly, it is encouraging that T_{pref} data inferred from different photothermal treatments (2h vs. 10h) under incandescent bulbs, and obtained with contact thermometers, resulted to be roughly comparable, at least in the three sympatric lacertids studied herein, with a suggested correction of +2.2°C to make the photothermal-10h comparable to the photothermal-2h treatment. Yet, given the known inter- and intraspecific differences in thermoregulatory period (see above; Sinervo & Adolph 1989, 1994, Sinervo 1990) it is unlikely that such a correction factor can ever be generally valid for all lacertid species, let alone for other lizards. As previously recommended for laser and contact thermometers (Carretero 2012) and now extended to the other methods, where detailed and thorough comparisons are needed, a rigorous consideration of the optimal experimental setup and its consistent use will remain necessary. Lastly, we envisage that future field and experimental studies on lizard thermoregulation will increasingly make use of thermographic cameras which allow visualizing temperatures even in different lizard body parts, without disturbance, but require careful calibration of the equipment for the target species (Barroso et al. 2016).

Acknowledgments

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Supplementary data

The following data are available online:

Supplementary Table S1. Matrix of Spearman rank correlation coefficients for median T_{pref} values obtained from different measuring methods, and field body temperatures (T_b), for 30 male lizard individuals of *Atlantolacerta andreanskyi* at Oukaimeden.

Supplementary Table S2. Matrix of Spearman rank correlation coefficients for median T_{pref} values obtained from different measuring methods, and field body temperatures (T_b), for 23 male lizard individuals of *Podarcis vaucheri* at Oukaimeden.

Supplementary Table S3. Matrix of Spearman rank correlation coefficients for median T_{pref} values obtained from different measuring methods, and field body temperatures (T_b), for 12 male lizard individuals of *Podarcis vaucheri* at Oukaimeden.