

1 **On the role of melanistic coloration on thermoregulation in the crepuscular gecko**  
2 ***Eublepharis macularius***  
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4 **Running title:** Melanism and thermoregulation in geckos

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38 **ABSTRACT**

39

40 Body coloration in ectotherms serves multiple biological functions, including avoiding predators,  
41 communicating with conspecific individuals, and involvement in thermoregulation. As  
42 ectotherms rely on environmental sources of heat to regulate their internal body temperature,  
43 stable melanistic body coloration or color change can be used to increase or decrease heat  
44 absorption and heat exchange with the environment. While the function of melanistic coloration  
45 for thermoregulation has been found to increase solar radiation absorption for heating in many  
46 diurnal ectotherms, research on crepuscular and nocturnal ectotherms is lacking. Since  
47 crepuscular and nocturnal ectotherms generally absorb heat from the substrate, coloration is  
48 likely under different selective pressures than in diurnal ectotherms. We tested if the proportion  
49 of dorsal melanistic body coloration is related to differences in body temperature heating and  
50 cooling rates in the crepuscular gecko *Eublepharis macularius* and whether changes in  
51 environmental temperature trigger color changes in this species. Temperature measurements of  
52 the geckos and of the environment were taken using infrared thermography and temperature  
53 loggers. Color data were obtained using objective photography and a newly developed custom  
54 software package. We found that body temperature reflected substrate temperatures, and that the  
55 proportion of melanistic coloration has no influence on heating or cooling rates or on color  
56 changes. These findings suggest that, in *E. macularius*, melanistic coloration may not be used for  
57 thermoregulation. Future research should further test the function of melanistic coloration in  
58 other crepuscular and nocturnal vertebrates to understand the evolution of melanistic pattern in  
59 animals active in low light conditions.

60

61 **Keywords:** color data extraction, color pattern, melanism, reptiles, thermal physiology

62

## 63 INTRODUCTION

64 Optimal body temperature is essential for animals for mating, foraging, predator escape,  
65 and other biological functions (Dunham et al., 1989; Seebacher & Franklin, 2005). In the face of  
66 global climate change and frequent temperature fluctuations caused by climate change  
67 (Rummukainen, 2012; Vasseur et al., 2014), organisms will be exposed to recurrent suboptimal  
68 temperatures. Thus, examining the performance of organisms under suboptimal temperatures is  
69 essential to predict how their biology will be affected (Angilletta, 2009; Deutsch et al., 2008; M.  
70 Kearney et al., 2009; Mader et al., 2022; Pérez i de Lanuza et al., 2018). Ectotherms, such as  
71 non-avian reptiles, generally do not have a built-in physiological mechanism for internal heat  
72 production; hence, the maintenance of their body temperature, and thus of the temperature-  
73 dependent basic life functions, relies on a thermal exchange with their environment (M. Kearney  
74 et al., 2009; Kiefer et al., 2007).

75 Many ectothermic vertebrates have evolved a wide range of behavioral, physiological,  
76 and morphological characteristics that aid in maintaining close to functionally optimal body  
77 temperatures. Although microhabitat changes and posturing are the most common  
78 thermoregulatory behaviors observed in ectotherm vertebrates (Aubret & Shine, 2010 and  
79 references within; Bauwens et al., 1996; Kearney & Predavec, 2000), skin coloration may also  
80 influence thermoregulation. In areas with low mean annual solar radiation, ectotherm vertebrates  
81 may have higher concentrations of dermal melanin caused by the concentration of melanophores  
82 to increase heat absorption, the so-called Thermal Melanism Hypothesis (Clusella Trullas et al.,  
83 2007; Clusella-Trullas et al., 2008; Forsman, 1995; Gibson & Falls, 1979; Walton & Bennett,  
84 1993). Vertebrate ectotherms living in areas with low levels of solar radiation – i.e., areas further  
85 from the equator – are more uniformly dark in color due to the melanophores distributed  
86 relatively equally throughout the dermis - to create an overall lower skin reflectance and higher  
87 rate of heat absorption. Ectotherms occurring in areas of higher solar radiation, however, are less  
88 constrained in increasing heat absorption from solar radiation and can exhibit clusters within  
89 their dermis that have a higher concentration of melanophores relative to other areas, causing for  
90 example a spotted pattern (Martínez-Freiría et al., 2020; Moreno Azócar et al., 2015; Szydlowski  
91 et al., 2020). Similarly, ectotherms that are mostly active during hours of low solar radiation –  
92 i.e., crepuscular and nocturnal species may rely on other strategies to absorb heat (Garrick, 2008)  
93 and melanism in these species may be used for other functions, including for cooling rather than

94 heating (Geen & Johnston, 2014). Together with stable melanistic coloration, melanosomes may  
95 move within the dermal melanophores, producing a skin darkening when moving towards the  
96 surface of the skin, or skin lightening when moving away from it, a phenomenon called rapid  
97 physiological color change (Sherbrooke & Frost, 1989). Rapid physiological color change may  
98 be used for thermoregulation by darkening or lightening the skin surface of ectotherms and  
99 increasing or decreasing the level of solar radiation absorption and/or body heat exchange with  
100 the environment, thus altering the internal temperature of the organism (Lowe & Norris, 1956;  
101 Sherbrooke et al., 1994; Sherbrooke & Frost, 1989; Smith, Cadena, Endler, Kearney, et al., 2016;  
102 Smith, Cadena, Endler, Porter, et al., 2016). Although thermal melanism has been studied within  
103 and among species in ectothermic vertebrates, thermal melanism has been measured as overall  
104 darker or lighter skin colors, without inferring how the proportion of melanistic patterns may  
105 influence the rates of heating and cooling and the internal body temperature of the organism  
106 (though see Forsman, 1995 for snakes). Furthermore, to our knowledge, physiological color  
107 change in response to temperature changes influenced by the proportion of melanistic pattern  
108 (i.e., melanistic and non-melanistic coloration becoming lighter or darker at different rates) has  
109 not been examined.

110         The leopard gecko, *Eublepharis macularius*, a species with widespread melanistic pattern  
111 (Glimm et al., 2021), is characterized by melanistic spots (at the adult stage) or stripes (at the  
112 juvenile stage) on a lighter, often yellow, background of base dermal coloration. It is also  
113 commonly bred for pet-trade resulting in a variety of melanistic patterns of different types and  
114 quantity (Glimm et al., 2021; Kiskowski et al., 2019; Szydlowski et al., 2020) that is ideal for  
115 testing the influence of melanistic proportion on biological processes. To our knowledge, no  
116 study has assessed and quantified rapid physiological color change in this species. *Eublepharis*  
117 *macularius*, a species that naturally occurs in Pakistan, India, Iran, and surrounding regions  
118 (Agarwal et al., 2022), is a crepuscular lizard primarily active in low light conditions. As such,  
119 we expect that heat absorption in this organism does not strongly depend on solar radiation – as  
120 it is active during times of low solar radiation – and that dorsal melanistic proportion would not  
121 affect heating rates in this species. On the other hand, melanistic proportion may have an  
122 influence on increasing cooling rates during especially hot days, while the organism is hiding.  
123 We expect *E. macularius* to be thigmothermic – absorbing heat from the direct contact with

124 warm substrates while hiding during the day or moving on surfaces that have absorbed heat  
125 during the day at night.

126 In this work, we used multispectral objective photography (Troscianko & Stevens, 2015)  
127 and a newly developed data extraction pipeline to extract the melanistic coloration from the  
128 background animal coloration on live and free to move *E. macularius*. Although other algorithms  
129 and pipelines are available to extract color data, including segmenting color patterns from  
130 background colors (see Glimm et al., 2021, Abramjan et al., 2020, and Troscianko & Stevens,  
131 2015), our approach allowed for extracting the same type of color data over time – i.e., every 30  
132 min at which the images of the gecko were taken - on the same freely moving individual  
133 allowing coloration and color pattern to be compared over time. In this study, we tested if: 1.  
134 individuals with a greater proportion of stable dorsal melanistic pattern across the body  
135 experience increase heating or cooling rates than individuals with lower proportion; 2. exposing  
136 individuals to lower suboptimal temperatures for this species elicits a physiological color change  
137 in the skin of the geckos; 3. individuals with a greater baseline proportion of melanistic  
138 coloration experience a lower amount of overall physiological color change (background light  
139 color and melanistic color). Experimental results on this model are expected to contribute to  
140 elucidate the function of melanistic coloration and physiological color change in this species, and  
141 further our understanding on the role of melanistic coloration on thermoregulation vs. crypsis in  
142 crepuscular and nocturnal species.

143

## 144 **MATERIALS AND METHODS**

145 All capture, handling, and experimental protocols were approved by George Mason  
146 University IACUC committee (Permit number 1718778). Experiments were carried out to  
147 minimize stress and disturbance to the animals and in accordance with relevant guidelines and  
148 regulations.

### 149 **Study subject and captivity conditions**

150 All geckos were housed in the same room at George Mason University, with one gecko  
151 per terrarium (61x30x20 cm) and exposed to a 12-12hr day-night cycle. The housing containers  
152 for *E. macularius* were plastic boxes with newspaper bedding. The room temperature was kept  
153 stable between 25-28°C, with the average temperature generally being close to 25.3°C +/-0.2°C,  
154 and each terrarium contained moist and dry hides and a heat pad for thermoregulation. Humidity

155 (41+/-10%) and temperature in the room were checked daily using a digital  
156 thermometer/hygrometer. The health condition of each individual was checked daily by visual  
157 inspection, and no gecko was tested during the shedding process. Geckos were fed three times a  
158 week with a combination of crickets and mealworms dusted with calcium and vitamin powder.  
159 Feeding was withheld 3 days prior to testing in order to avoid interference in digestion due to  
160 exposure of animals to low temperature and to also avoid confounding effects due to potential  
161 food digestion. Feeding was resumed after testing. Drinking water was always available except  
162 during testing. Testing was carried out on 12 adult geckos (n=12, 7 males, 5 females). None of  
163 the females used in this experiment were gravid, and neither any of the tested individuals had  
164 been engaging in mating or any social interaction as each individual was housed and tested in  
165 isolation from the others.

## 166 **Experimental Setup**

167 Experiments were conducted in a temperature controlled room of 4x2 m at George  
168 Mason University. This room has a 1x3 m open area in the middle, where the 51x25x30 cm glass  
169 terrarium used as a testing enclosure was placed on a rubber mat on the floor. The walls and  
170 floor of the terrarium were covered with white Teflon (SS Shovan) to remove any potential  
171 effect of background coloration, and the lid was removed for the entirety of the experiment to  
172 facilitate obtaining the data. The Teflon on the bottom of the terrarium consisted of three layers,  
173 while the one on the side was a single layer. Black electrical tape (3M) was used to adhere the  
174 bottom sheet of Teflon to the side sheets to prevent geckos from hiding underneath of the Teflon.  
175 The use of black electrical tape was chosen following guidelines on thermal camera calibration  
176 (F. Barroso, pers. comm.). A clean cardboard egg carton of 15x15 cm was placed in the middle  
177 of the terrarium as a hiding spot for the gecko, because reptiles are known to thermoregulate  
178 behaviorally by seeking shelter in suboptimal temperatures (Aubret & Shine, 2010; M. Kearney  
179 et al., 2009; Woods et al., 2015). Except for the cardboard egg and three iButtons (DS1921G  
180 ThermoChron, Maxim Integrated Products, precision = 0.5°C) to record the temperature (see  
181 below), nothing else was placed in the terrarium and the gecko was free to move and use the  
182 entire space available in the terrarium. A heating pad was placed underneath one side 15cm from  
183 the end of the terrarium (to have a warm spot throughout the experiments). A broad-spectrum  
184 UV-VIS light (Zoo Med PowerSun H.I.D Metal Halide UVB Lamp, 6500K, 70W, 95CRI) was  
185 placed 160cm above the center of the testing terrarium to ensure photographs were taken under

186 proper lighting (Troscianko & Stevens, 2015), as melanin has strong absorption in the UV-Vis  
187 spectrum (McNamara et al., 2021). This light was turned on 45 minutes prior to the start of each  
188 experiment and remained on for the length of the experiment (Supplementary Materials Fig. S1).

189 A thermometer/hygrometer (ThermoPro TP50, precision=0.1°C, 1% humidity) was  
190 placed on the floor adjacent to the terrarium to measure the temperature and humidity of the  
191 room before and during the experiments. To monitor the temperature throughout the experiments  
192 and to ensure that room and terrarium temperatures were similar across experiments, a total of  
193 four iButtons were placed in the terrarium and in the temperature controlled room. Specifically,  
194 one iButton was placed on the floor outside of the middle section of the terrarium 20 cm away.  
195 Of the three iButton placed in the terrarium, two were placed on the opposite ends (one on top of  
196 the heating pad and the second on the opposite end of the terrarium) and one under the cardboard  
197 egg carton. The iButtons were programmed to start collecting temperatures data 30 minutes prior  
198 to the start of the experiment and continued to collect temperature data every five minutes for the  
199 entire duration of the experiment. Following each experiment, iButtons were sanitized with  
200 isopropyl alcohol and the terrarium was cleaned with soap and hot water to remove any potential  
201 scent or residue left from the previously tested individual. The top layer of the Teflon at the  
202 bottom of the terrarium was replaced after each experiment, while the Teflon sheets on the sides  
203 were sanitized with isopropyl alcohol after each experiment. A new cardboard egg carton was  
204 used for each tested individual.

#### 205 **Native environmental temperatures for *E. macularius***

206 To estimate the native environmental temperatures for *E. macularius* and compare them  
207 with the temperatures tested in this study, occurrences for this species were downloaded from the  
208 Global Biodiversity Information Facility (GBIF, October 2022; [www.gbif.org](http://www.gbif.org)) and imported into  
209 Rstudio (V4.1.2, R Core Team 2021) using the “occ\_download\_get” function from the *rgbif*  
210 package (Chamberlain et al., 2022). Species occurrences for *E. macularius* were filtered for  
211 species’ scientific name mismatches as well as NA values for latitude, longitude, species’  
212 scientific names, and country codes. Species occurrences were also cleaned and cross-checked  
213 for coordinate validity using the “clean\_coordinates” function from the package, *Coordinate*  
214 *Cleaner* (Zizka et al., 2019). Species occurrences which resulted in at least one flagged test  
215 labeled as, “FALSE,” were removed from the dataset. Microclimate temperatures were extracted  
216 using the global model from the *NicheMapR* package (M. R. Kearney & Porter, 2017).

217 Microclimate temperatures represent temperatures at 3 cm above ground with full sun (no shade)  
218 using 23 native coordinates for leopard geckos from GBIF. The model was run over 365 days for  
219 10 years. Microclimatic temperatures for each day were taken as the average of each 60-min time  
220 interval across the day.

### 221 **Experimental temperature ranges**

222         The range of experimental temperatures used in this experiment was 15-25°C (Table 1).  
223 For the purposes of analysis, time periods in the experiment were separated into five “blocks” to  
224 measure differences in variables between different phases of the experiment (Table 1). Briefly,  
225 blocks 1 and 5 correspond to the beginning and end of the temperature experiment, block 2  
226 corresponds to the temperature going down, block 4 corresponds to the temperature going up  
227 back to 25°C, and block 3 corresponds to the lower temperature used in this study (Table 1). The  
228 highest temperature was chosen as 25°C because this is the overall most frequent temperature at  
229 which these geckos are exposed in their housing environment. 15°C was used as the lower  
230 temperature to resemble natural low temperature experienced by this species in its natural habitat  
231 (Fig. 1), without eliciting hibernation (Khan, 2009). Experiments focused on studying the effects  
232 of lower temperatures as *E. macularius* is a crepuscular species that is less active during the  
233 warmer parts of the day.

234         To set up the maximum and minimum temperatures for this experiment, the temperature  
235 control for the experimental room was set at 25°C and then lowered by setting the room  
236 temperature control at 15°C when needed for the experiments (Table 1); temperature lowering  
237 between 25 and 15°C (or vice versa) took one hour. To bring the temperature back to 25°C from  
238 15°C a space heater was placed 1 m from the warm end of the terrarium and turned on after the  
239 temperature control of the room was adjusted to 25°C (block 4, Table 1). At the beginning of  
240 each experiment, we used the room thermometer (ThermoPro) to confirm that the room  
241 temperature was at 25°C. The temperature of the terrarium was confirmed for each end of it by  
242 pointing an infrared thermometer (Etekcity Corporation) held 30 cm from the surface pointing  
243 perpendicular towards the bottom of the terrarium. Temperature checks of the room and  
244 terrarium were repeated every 30 minutes during the experiment using the in-room thermometer  
245 and the infrared thermometer, respectively. Temperature readings from iButtons were used to  
246 confirm atmospheric temperature readings after each experiment.

### 247 **Data collection**



248           Geckos were tested in a random order. Individuals were weighed to the nearest 0.01g.  
249 using a digital scale before the start of the experiment and snout vent length (SVL) was measured  
250 to the nearest 1mm. Only one gecko per day was tested within the same time frame for 7 hours,  
251 starting at 11:00am each day. Although this species is crepuscular, experiments were carried out  
252 during the day to replicate the conditions of the housing room, where geckos are exposed to light  
253 conditions during the day. At the end of the experiment, each gecko was returned to its housing  
254 terrarium. Geckos were visually monitored after the experiments to check for any health concern.  
255 No geckos had any issues during or after the experiments.

256           To extract temperature data from multiple body parts of the gecko, as different body parts  
257 may have different temperatures, a CAT S62 smartphone Pro camera (Caterpillar Inc.,  
258 resolution=12MP, emissivity=0.95) was used to take an infrared (IR) image (Barroso et al.,  
259 2016). The camera was held approximately 30 cm directly above the individual in order to  
260 maintain the same effective pixel size (i.e., the actual area each pixel represents in the  
261 photographed subject) across IR images, regardless of the body size of the animal or its position  
262 in the terrarium. To standardize IR images and determine reflective temperatures, an 8x8 cm  
263 square piece of wrinkled aluminum foil was placed next to the gecko in the terrarium when  
264 capturing the image of a gecko each time an IR image was taken, following Barroso et al. (2016).  
265 The square piece of aluminum foil in each IR image was used to extract average reflective  
266 temperature from IR images only and was not used in color analysis. Average reflective  
267 temperature is required in each IR image to standardize temperatures for the gecko and the  
268 terrarium. After taking the IR image of the gecko, the relative humidity and temperature of the  
269 temperature-controlled room were recorded.

270           After taking the IR image of the gecko, to obtain the color data for each gecko, visible  
271 images were taken using a full spectrum converted Canon 1300D with a Kolari Vision UV/IR  
272 cut filter (410-700nm transmission). Images were taken approximately 40cm directly above the  
273 gecko to ensure a good resolution across images and with a grayscale standard built from Teflon  
274 following the methods of Abramjan et al. (2020) in the frame of the image. Visible images were  
275 obtained only for the dorsal part of the geckos, as melanistic patterns are generally absent from  
276 the ventral side of the animals and as such the ventral side was not relevant to the study questions  
277 (Glimm et al., 2021). Two people (AM and EW) took the IR images making sure to standardize  
278 the method between them and another person (BH) always took the visible images. If the gecko

279 was under the cardboard hide, the hide was lifted prior to taking the images and replaced  
280 afterwards. The time at which the temperature of the room was changed, the  
281 thermostat/thermometer readings, as well as the times at which visible and thermal photos were  
282 taken were manually recorded.

### 283 **Data extraction**

284 Temperature measurements from the IR images were extracted in FLIR Tools (Teledyne  
285 FLIR 2022) from five body parts of each gecko (head – base of parietal scales, left knee, left  
286 foot, central dorsum, eyes – right and left, snout, and tail-- above the cloaca), as body  
287 temperature is known to vary across the body (Barroso et al., 2016). Temperature data averaged  
288 from both eyes were used as the internal body temperature for the analyses, as suggested for  
289 lizards by Barroso et al. (2016). Overall, the average of the temperature between two eyes and  
290 other body regions showed strong correlation, with a Spearman correlation coefficient above  
291 0.98 ( $p < 0.001$ , Supplementary Material Table S1). Furthermore, the average temperature from  
292 both eyes was found to be highly correlated with the temperature of the snout ( $r_s = 0.96$ ) which  
293 has also been suggested to be a good proxy for internal body temperature (Tabh et al., 2021). For  
294 each IR image, relative humidity, atmospheric temperature, distance (0 m), and emissivity (1)  
295 were first entered into FLIR Thermal Studio following Barroso et al. (2016) and Barroso (pers.  
296 comm.) to calibrate the temperature readings of the thermal camera. Reflective temperature was  
297 obtained as the average reflective temperature of the aluminum foil standard. This value was  
298 extracted from the IR image of the aluminum foil by overlaying a box entirely over the  
299 aluminum foil using the *Rectangle* function in FLIR Tools. After calibrating and entering the  
300 average reflective temperature in FLIR, distance and emissivity were re-entered as 0.3 m and  
301 0.96, respectively (Barroso et al., 2016). The substrate temperature of the terrarium was also  
302 measured by using the *Rectangle* function to overlay a small box over the black electrical tape at  
303 the bottom of the terrarium for each image taken, as the electrical tape more accurately reflects  
304 the temperature of the terrarium (F. Barroso, pers. comm.).

305 Digital images obtained with the Canon camera were processed using custom image  
306 processing software written in the Python programming language; color space conversions and  
307 luminance calculations were done using the OpenCV package in Python (Bradski, 2008).  
308 Luminance was used as a measure color change as this is the most important color component  
309 that affects solar radiation absorption (Smith, Cadena, Endler, Kearney, et al., 2016). First,

310 images were normalized for potential changes in lighting conditions across images of the same  
311 individual for the different blocks by converting each image to Hue -Lightness -Saturation (HLS)  
312 color space and using the grayscale reference in the first image of that individual as the baseline.  
313 The Lightness parameter (HLS) of following images were then standardized so that the greyscale  
314 reference matched that of the first image of that individual. This standardization step therefore  
315 allows comparisons among images taken for the same individual, but not across individual. All  
316 images were then converted back to the RGB color space. Next, because the limbs were  
317 sometimes obscured from the camera view due to the posture of the gecko in the image, images  
318 were cropped to only include the head, trunk, and tail of each individual. For image color  
319 segmentation – to segment the studied areas into color regions-, hierarchical k-means clustering  
320 was run on each image (the specific parameters used can be found in the available codes hosted  
321 on GitHub), as manual object segmentation can be inaccurate and time consuming, especially  
322 when dealing with a large number of images. In this process every pixel is assumed to be a  
323 datapoint in the RGB color space, then pixels are grouped into a predefined number of clusters  
324 based on their distances to each other in the three-dimensional RGB color space. Because the  
325 result of this method is sensitive to its initialization, cluster centers were initiated following a K-  
326 means++ algorithm to account for this (Arthur & Vassilvitskii, 2007). While the K-means  
327 algorithm is computationally inexpensive and fast, there were some limitations caused by  
328 lighting conditions with a high incidence of shadows. It was common for coloration in areas of  
329 discoloration due to shadowing to be incorrectly assigned to a cluster, resulting in a  
330 misrepresentation of the pattern for that image. Because of the unsupervised nature of k-means,  
331 there is no way to correct for this error once the segmentation step is started. To account for this  
332 effect, a visual confirmation step by the user was implemented before segmentation to ensure  
333 that the color clusters would accurately represent the pattern to be segmented. For the present  
334 study, k=2 was used for clustering. While this is conservative, it avoided overestimation of the  
335 amount of melanistic coloration (Supplementary Materials Fig. S2).

336         The results of K-means clustering for visible images was a Boolean mask representing  
337 stable melanistic and non-melanistic coloration of the entire body for each image. Melanistic  
338 proportion was calculated as the area of stable melanistic coloration relative to the entire dorsal  
339 area (head, trunk, and tail), averaged across all images (15) of a single individual. The average  
340 standard deviation of melanistic proportion across all 12 geckos - calculated as average of the

341 standard deviation for each gecko based on the 15 images taken for each individual's melanistic  
342 proportion - was  $\pm 3.5\%$ . Variation in melanistic proportion could be attributed to variation in  
343 lighting conditions, different positioning of the animal across the 15 images, imaging cropping,  
344 and the fact that K-means is an iterative, unsupervised algorithm (Lloyd, 1982). The Boolean  
345 mask was applied to the color corrected image to extract the mean luminance value for  
346 melanistic and non-melanistic coloration and the proportion of the coloration that was either  
347 melanistic or non-melanistic relative to the total coloration of the gecko. Luminance values were  
348 derived from a weighted calculation of RGB color channels (specific information are included in  
349 the data extraction pipeline on GitHub). All Python codes used to extract color data are available  
350 on GitHub. The full dataset will be publicly available on Dryad *after manuscript acceptance*.

### 351 **Statistical analyses**

352 Pearson's correlation coefficients were used to evaluate the relationship between the  
353 gecko body temperature and the terrarium substrate temperature, both calculated from the same  
354 IR image, or between the gecko body temperature and the average of the three datalogger  
355 temperatures placed inside the terrarium, or between the gecko body temperature and the  
356 atmospheric temperature as estimated on the data logger outside the terrarium. To confirm the  
357 accuracy of IR substrate readings, a Pearson's correlation test was also run on the average data  
358 logger temperatures from within the terrarium and the substrate temperature taken with IR  
359 imaging. Because one temperature value from the data loggers had a reading of zero, that value  
360 was removed from the analyses. Previous studies have found that humidity may influence body  
361 temperature (Galliard et al., 2021); as such, we also tested the potential correlation between  
362 humidity and body temperature using a Pearson's correlation test.

363 To test for overall differences in body temperature throughout the experiment, we  
364 performed paired t-tests or Wilcoxon-tests (based on normality of the data) between the average  
365 body temperatures of each gecko in each block. The rate at which geckos heat and cool down  
366 may be influenced by body weight or by the animal size; as such, a linear model was fit between  
367 body weight and heating and cooling rates separately and the analyses were then repeated for  
368 SVL. Furthermore, as different sexes may respond differently to heating and cooling, we also  
369 used a linear model to test the influence of sex effects on heating and cooling rates separately.  
370 Heating and cooling rates were calculated taking the change in body temperature between blocks  
371 1 and 3 (cooling) and blocks 3 and 5 (heating).

372 To ensure that there was no correlation between the proportion of dorsal melanism (taken  
373 for each gecko as the average log of melanistic proportion over the 15 images) and snout-vent  
374 length (SVL) or sex, a linear model was fit between melanistic proportion and SVL with sex as a  
375 factor. Melanistic proportion was transformed to log of melanistic proportion to obtain normality  
376 of the data. To investigate the influence of melanistic proportion on heating and cooling rates, we  
377 ran a linear model based on data obtained on all 12 geckos between the log of the average  
378 melanistic proportion taken for each gecko and heating/cooling rates as defined above.

379 Paired T-tests or Wilcoxon-tests (depending on normality of the data) were used to  
380 evaluate any changes in luminance values (physiological color change) between blocks. To  
381 assess the influence of body temperature variation and log of the average melanistic proportion  
382 on physiological color changes (luminance) on the entire dorsal area (head, trunk, and tail) of the  
383 gecko, we used linear models with the luminance change between two blocks as the dependent  
384 variable with body temperature change between two blocks or log of averaged melanistic  
385 proportion of each individual as the independent variable. We also ran more general models  
386 where these two independent variables (melanistic proportion and body temperature) were  
387 jointly tested with their interaction. As the results do not vary between the simpler and more  
388 complex model, we only report the results of the complex model to also assess the influence of  
389 the interaction between melanistic proportion and body temperature. Analyses were run  
390 independently for changes between blocks 1 and 3 (cooling), 3 and 5 (heating), and 1 and 5  
391 (initial and final body temperatures). Luminance for melanistic and non-melanistic parts of the  
392 body of each gecko was tested in separate models. Any statistically significant linear model  
393 results were investigated further with diagnostic tests, specifically the Cook's distance to  
394 evaluate how much leverage each individual exerted on the model. All statistical analyses were  
395 run in R (V4.1.2, R Core Team 2021).

396

## 397 **RESULTS**

398 For each of the 12 tested geckos, 15 IR and 15 visible images were used for the analyses,  
399 with the exception of one IR image each for two geckos being of too low quality for data  
400 extraction, giving 178 IR data points and 180 visible data points (15 x 12 individuals). Similarly,  
401 179 temperature measurements were used for each temperature type (IR images or average of the  
402 three dataloggers inside the terrarium) for the analyses as well. All temperatures taken from the

403 experiments were evaluated against the geckos' native temperature range based on WorldClim  
404 data (Fig. 1). Experimental temperatures stayed within the quartiles of native temperature as  
405 planned by the experimental design, with the starting and ending temperature of 25°C  
406 corresponding to the median of native temperatures (Fig. 1). As a consequence, blocks 1 and 5  
407 are closest in temperature to the interquartile range of the native temperature (Fig. 1), while  
408 block 3 had the greatest temperature difference from the median native temperature. Specifically,  
409 in block 3 all the experimental temperatures have a median temperature lower than the lower  
410 quartile of the native temperatures (warm side of the terrarium=17.25°C, cold side=16.5°C, hide  
411 spot=17°C, native lowest quartile=19.04°C;  $p < 0.001$  based on Wilcox tests for all data logger  
412 temperatures compared to native low quartile of native temperature), supporting that the  
413 temperatures we selected as suboptimal in this study are in fact suboptimal for this species in its  
414 native environment.

415 The geckos' body temperature was strongly correlated with the terrarium substrate  
416 temperature ( $r^2 = 0.97$ ,  $p = 0.33$ ), both obtained from IR imaging. Correlation between the geckos'  
417 body temperature and the atmospheric or terrarium temperatures based on data loggers were also  
418 strong ( $r^2 = 0.92$  or  $0.93$  depending on the comparison,  $p < 0.001$  for all correlations). Although,  
419 correlation between body temperature and each of the three dataloggers placed in the terrarium  
420 (cold side, warm, and hide) and between body temperature and atmospheric have identical  $r^2$ , the  
421 median atmospheric temperature is generally lower than the body temperature and the terrarium  
422 temperature based on data loggers (Fig. 1). Finally, correlation between the average temperature  
423 of all the three dataloggers within the terrarium and the substrate temperature estimated from the  
424 IR images were also high ( $r^2 = 0.85$ ,  $p = 0.40$ ), although the temperature estimated by the IR  
425 images was relatively higher for each block than the one based on the datalogger (Fig. 1). We  
426 also found that relative humidity was not correlated with body temperature ( $r^2 = -0.23$ ,  $p = 0.82$ ).  
427 To notice that while the median gecko temperature at the beginning and end of the experiment is  
428 lower than the median substrate temperature estimated by IR imaging, as the temperature of the  
429 experiment decreases (block 2-3), the median body temperature is higher than the median  
430 substrate temperature and closer to the lower quartile of the native temperatures (Fig. 1).

431 Significant differences in body temperature were detected during cooling (blocks 1 and 3,  
432 t-test  $t = 41.2$ ,  $p = 2.08 \times 10^{-13}$ ), heating (blocks 3 and 5, Wilcox test  $V = 0$ ,  $p = 0.0005$ ), and between  
433 beginning and end of the experiment (blocks 1 and 5, Wilcox test  $V = 78$ ,  $p = 0.0005$ ) (Table 2).

434 The heating and cooling rates were found to be independent of body weight ( $r^2=-0.02$   $p=0.39$  for  
435 heating and  $r^2=0.19$   $p=0.09$  for cooling), SVL ( $r^2=-0.09$   $p=0.76$  for heating and  $r^2=0.06$   $p=0.22$   
436 for cooling), or sex ( $r^2=-0.05$   $p=0.50$  for heating and  $r^2=-0.07$   $p=0.60$  for cooling) (Table 3).

437 Of the 12 geckos tested, nine had a melanistic proportion between 0% and 11% across the  
438 entire dorsal part of the body, while the other three had a melanistic proportion of 16%, 22%, and  
439 41%. We found no influence of SVL ( $r^2=-0.27$ ,  $t=-0.70$ ,  $p=0.50$ ), sex ( $r^2=-0.27$ ,  $t=-0.73$ ,  $p=0.49$ ),  
440 or their interaction ( $t=0.71$ ,  $p=0.49$ ) on melanistic proportion (Table 3). Based on linear models,  
441 the melanistic proportion had no influence on heating rates (blocks 3 and 5,  $r^2=-0.09$ ,  $t=-0.292$ ,  
442  $p=0.78$ ) or cooling rates (blocks 1 and 3,  $r^2=0.06$ ,  $t=-1.327$ ,  $p=0.21$ ) (Table 3, Fig. 2).

443 Using t-tests to evaluate differences in luminance between blocks, we found no  
444 significant differences between blocks 1 and 3 (cooling), blocks 3 and 5 (heating), and beginning  
445 and end of the experiment (blocks 1 and 5) for the melanistic and non-melanistic areas of the  
446 body (Table 2b and c for p-values). Linear models ran to test the influence of the proportion of  
447 stable melanistic coloration and body temperature changes on changes in luminance for the  
448 melanistic and non-melanistic dorsal areas of the body indicate that during heating (blocks 3-5),  
449 the melanistic proportion has an influence on change in luminance for the non-melanistic area of  
450 the body ( $r^2=0.45$ ,  $t=-2.861$ ,  $p=0.02$ , Table 4). Specifically, individuals with a greater melanistic  
451 proportion had higher luminance (luminance increases, thus the animal becomes lighter in color)  
452 in the non-melanistic areas of the body, while the melanistic part of the body did not experience  
453 any change in luminance (Figs 3A and 4A). Furthermore, we found an interaction between  
454 melanistic proportion and body temperature on changes in luminance during heating (blocks 3  
455 and 5) for the non-melanistic areas of the body ( $r^2=0.45$ ,  $t=2.877$ ,  $p=0.02$ ) (Table 4, Figs 3A and  
456 4A). Diagnostic tests revealed that one individual exerted significant leverage on the results of  
457 the model and influenced the significant results (Cook's distance > 1). Melanistic proportion and  
458 body temperature had no influence on changes in luminance during cooling (blocks 1-3) or  
459 beginning and end of the experiment (blocks 1-5) for the non-melanistic area or the melanistic  
460 area of the body and for heating and the melanistic part of the body (Table 4).

461

## 462 **DISCUSSION**

463 Crepuscular and nocturnal reptiles experience low exposure to solar radiation during their  
464 active times and have been suggested to primarily rely on thigmothermy. Thigmothermy refers to

465 the absorption of heat from the surrounding environment, rather than directly from solar  
466 radiation (Garrick, 2008; M. Kearney & Predavec, 2000). Despite relying mostly on substrate  
467 and surrounding temperatures, crepuscular and nocturnal reptiles can also bask for  
468 thermoregulation when needed (Angilletta et al., 1999).

469 In this study, we directly compare how substrate and atmospheric temperatures correlate  
470 with the internal body temperature in *E. macularius* and the role of melanistic pattern on  
471 physiological color change and thermoregulation in these animals. In our study, the main sources  
472 of heat during the cooling down of our experiments (blocks 1-3) were provided by the heat pad  
473 placed at one side of the terrarium and the lamp placed above the terrarium. We found a stronger  
474 correlation between body temperatures and terrarium substrate temperatures than for atmospheric  
475 temperatures, suggesting that *E. macularius* relies on absorbing heat from the ground and is  
476 thigmothermic. To notice that while the substrate temperature of the spot at which the gecko was  
477 located and the body temperatures were both estimated from IR photographs, the median  
478 atmospheric temperature - as estimated from data loggers - was generally lower than the gecko  
479 body temperature and the substrate temperature estimated from data loggers, suggesting that the  
480 higher correlation observed between substrate temperatures and body temperatures is not due to  
481 how the temperatures were measured. Our findings also support what previously suggested  
482 (Craioveanu et al., 2017; Garrick, 2008) based on measures of body surface temperature and  
483 ambient temperature differentials.

484 Although *E. macularius* mostly rely on the substrate temperature for thermoregulation,  
485 melanistic coloration – both as a stable coloration and as a physiological darkening of the skin –  
486 can be used for thermoregulation to increase or decrease the animal’s body temperature during  
487 basking (Angilletta et al., 1999; Clusella Trullas et al., 2007; Smith, Cadena, Endler, Kearney, et  
488 al., 2016; Smith, Cadena, Endler, Porter, et al., 2016). Specifically, melanin can be used to  
489 absorb UV radiation and convert it into heat by photon-phonon transformation (McNamara et al.,  
490 2021) thus contributing to increased heating rates, but can also increase the rate of heat transfer  
491 for cooling in reptiles (Geen & Johnston, 2014). as most research into melanism has been  
492 conducted Compared to what we know on the role of melanistic coloration on diurnal ectotherms  
493 (Belluere & Carrascal, 2002; Garrick, 2008), the function(s) of melanistic coloration and  
494 especially of melanistic pattern in crepuscular and nocturnal reptiles are largely unknown. Our  
495 results based on 12 individuals of *E. macularius* with different proportions of melanistic pattern,



496 indicate that melanistic proportion is independent from body size (SVL), body weight, or sex,  
497 and that body size (SVL), body weight, or sex do not influence the heating or cooling rates. We  
498 found that the proportion of melanistic pattern does not influences cooling or heating rates in *E.*  
499 *macularius*. As *E. macularius* is crepuscular and only active for a few hours of daylight and  
500 mostly relies on thigmothermy, the characteristic spotted melanistic pattern of *E. macularius* in  
501 nature may be used for purposes different from thermoregulation.

502         However, exposure to prolonged suboptimal low temperatures, as tested here, may trigger  
503 a physiological response to darken the skin color to increase heat absorbance. This phenomenon  
504 to date has only been observed in heliothermic reptiles – reptiles that regulate body temperature  
505 through solar radiation (Cowles, 1940). As *E. macularius* is crepuscular and shelters during  
506 daylight hours (Angilletta et al., 1999), physiological color change may be used more for cooling  
507 purposes, for example to increase heat transfer to the environment if shelters exceed optimal  
508 temperatures during daylight. In our study, we found that luminance (changes in luminance  
509 corresponds to physiological color change) does not change significantly between blocks during  
510 cooling temperatures, but it does during the heating phases of the experiment for the non-  
511 melanistic part of the body and depending on the melanistic proportion of the animals. We found  
512 that for the non-melanistic areas of the body, individuals with higher proportions of melanistic  
513 pattern experienced less darkening of the skin, and vice-versa. Physiological color changes  
514 during heating – along with other mechanisms such as changes in peripheral blood flow  
515 (Bartholomew et al., 1965; Rice & Bradshaw, 1980) - may provide protection against  
516 overheating, as the rate of heat gain has been observed to increase at higher environmental  
517 temperatures (Belluire & Carrascal, 2002). A caveat of these results is however that the  
518 significant relationship between melanistic proportion, heating, and physiological color change  
519 are strongly influenced by one individual with high melanistic proportion.

520         Taken together, our results suggest that in *E. macularius* melanistic pattern may not be  
521 used for thermoregulation, while physiological color change may occur to prevent overheating.  
522 Further research is needed to understand the role of melanistic coloration and melanistic pattern  
523 in geckos and the extent to which physiological color change occurs, especially in crepuscular  
524 and nocturnal species. In geckos, spotted patterns – such as the one observed in *E. macularius* –  
525 have been suggested to represent a more specialized type of camouflage, although bands and not  
526 spots have been proposed to be associated with nocturnal activity (Allen et al., 2020). Previous

527 studies on crepuscular and nocturnal geckos have also indicated that physiological color change  
528 may be used more for background matching and camouflage than for thermoregulation (Vroonen  
529 et al., 2012; Zaidan III & Wiebusch, 2007). The few studies investigating physiological color  
530 change for thermoregulation and camouflage in other nocturnal geckos propose that these  
531 functions are mutually exclusive phenomena (Vroonen et al., 2012; Zaidan III & Wiebusch,  
532 2007), but based on our results, this may depend on the occurrence and proportion of  
533 melanistic pattern. Future studies should therefore investigate the functional trade-off between  
534 melanistic coloration, including melanistic pattern and physiological color change, for  
535 thermoregulation versus its use in camouflage or signaling in crepuscular and nocturnal reptiles  
536 in captivity and in the wild. Finally, although our study is based on captive bred animals and  
537 wild *E. macularius* do not show the same extent of variation in melanistic proportion as for the  
538 captive bred animals, our study is noteworthy mostly from a methodological and theoretical  
539 point of view. First, we developed a freely available software package that can be used to extract  
540 color patterns information from digital images of freely moving organisms with soft bodies, as in  
541 the case of geckos. Secondly, our results highlight the importance of melanistic pattern for study  
542 on thermoregulation and coloration and suggest that melanistic pattern and melanistic coloration  
543 may be used for multiple non-exclusive functions. The conclusions of our study could help with  
544 further understanding the function of coloration and color pattern development in nocturnal and  
545 crepuscular reptiles, and how their function may differ from those of diurnal reptiles.

546

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551

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553 The authors declare no competing interests

554

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558

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560 **Code availability:** <https://github.com/brandon-hastings/Lumeleon>

561

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733 **Tables**

734 **Table 1: Selected experimental temperatures at different time points in the experiment.**

735 Blocks were used for the statistical analyses. Blocks were selected to minimize temperature  
736 variance within blocks and maximize variance across them. For actual experimental minimum  
737 and maximum temperatures for each block, see Fig. 1.

738

<b>Block</b>	<b>Experiment time (min)</b>	<b>Terrarium temperature (°C)</b>	<b>Terrarium cold end (°C)</b>	<b>Terrarium warm end (°C)</b>
1	0-120 min	25	25	25
2	121- 180 min	25 decreasing to 15	25 decreasing to 15	25 decreasing to 18
3	181-300 min	15	15	18
4	301-360 min	15 increasing to 25	15 increasing to 25	18 increasing to 25
5	361-420 min	25	25	25

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741 **Table 2. Differences in body temperature (a), and luminance for the melanistic (b) and non-**  
742 **melanistic (c) part of the body during cooling, heating, and between the beginning and end**  
743 **of the experiments.** Cooling occurs between blocks 1-3, heating during blocks 3-5, and  
744 beginning and end of the experiment between blocks 1-5. More information about blocks  
745 specific temperatures can be found in Table 1. Depending on the data distribution, paired t-tests  
746 or Wilcox test were run; as such we report corresponding t-value for t-tests and V-value for  
747 Wilcox test. Significant p-values (<0.05) are indicated in bold.

748

Block comparisons	df	t-value	V	p-value
a) Body temperature				
1-3	11	41.232	NA	<b>2.076x10<sup>-13</sup></b>
3-5	NA	NA	0	<b>0.00055</b>
1-5	NA	NA	78	<b>0.00055</b>
b) Luminance for melanistic body areas				
1-3	11	1,5257	NA	0.16
3-5	11	-1.0559	NA	0.31
1-5	11	0.84798	NA	0.41
c) Luminance for non-melanistic body areas				
1-3	11	1.0359	NA	0.32
3-5	11	-0.34277	NA	0.74
1-5	11	0.94218	NA	0.37

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752 **Table 3. Influence of snout-vent length (SVL), sex, and body weight on melanistic**  
753 **proportion (a), heating rates (b), and cooling rates (c).** In (b) and (c), we used the simplest  
754 models testing one variable at the time. Melanistic proportion was calculated as the area of stable  
755 melanistic coloration relative to the entire dorsal area (head, trunk, and tail) across all the 15  
756 images of a single individual. Melanistic proportion was transformed as log of melanistic  
757 proportion for the analyses. Cooling rates correspond to the difference in body temperature  
758 between blocks 1 and 3, while heating rates correspond to blocks 3 and 5.

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<b>Factors</b>	<b>Estimate</b>	<b>Std Err</b>	<b>t-value</b>	<b>p-value</b>
a) Melanistic proportion				
SVL	-0.9906	1.4138	-0.701	0.50
Sex	-16.584	22.826	-0.727	0.49
Sex* SVL	1.3674	1.9087	0.716	0.49
b) Heating rates				
Weight	-0.0670	0.0753	0.890	0.39
SVL	-0.1315	0.4189	-0.314	0.76
Sex	-0.3546	0.5087	-0.697	0.50
Melanistic proportion	-0.0451	0.1542	-0.292	0.78
c) Cooling rates				
Weight	0.0980	0.0517	1.898	0.09
SVL	0.3897	0.2997	1.300	0.22
Sex	0.2126	0.3954	0.538	0.60
Melanistic proportion	-0.1458	0.1099	-1.327	0.21

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762 **Table 4. Influence of melanistic proportion and body temperature on changes in dorsal**  
763 **luminance during cooling (blocks 1-3), heating (blocks 3-5), and beginning and end of the**  
764 **experiment (blocks 1-5) for the melanistic (a) and non-melanistic (b) dorsal areas of the**  
765 **body.** Melanistic proportion was calculated as the area of stable melanistic color relative to the  
766 entire dorsal area, averaged across all images of a single individual and transformed to a log  
767 scale for analyses. Significant p-values (<0.05) are indicated in bold. \* indicates interaction of  
768 variables tested in the models.

769

<b>Blocks</b>	<b>covariates</b>	<b>Estimate</b>	<b>Std Err</b>	<b>t-value</b>	<b>p-value</b>
a) Change in luminance for the melanistic area of the body					
1-3	Temperature	0.0215	0.0293	0.733	0.49
	Melanistic proportion	0.1826	3.1282	0.058	0.96
	Temperature*melanistic proportion	0.0077	0.3685	0.021	0.98
3-5	Temperature	-0.0348	0.0180	-1.934	0.09
	Melanistic proportion	-1.1748	0.7829	-1.501	0.17
	Temperature*melanistic proportion	0.1683	0.1157	1.455	0.18
1-5	Temperature	-0.0108	0.01429	-0.755	0.47
	Melanistic proportion	0.3099	0.1655	1.872	0.10
	Temperature*melanistic proportion	0.1936	0.1046	1.851	0.10
b) Change in luminance for the non-melanistic area of the body					
1-3	Temperature	-0.0348	0.0353	-0.984	0.35
	Melanistic proportion	-0.1337	0.0860	-1.554	0.16
	Temperature*melanistic proportion	-0.0168	0.0109	-1.547	0.16

3-5	Temperature	0.0293	0.0168	1.740	0.12
	Melanistic proportion	-0.1164	0.0407	-2.861	<b>0.02</b>
	Temperature*melanistic proportion	0.0177	0.0062	2.877	<b>0.02</b>
1-5	Temperature	0.0077	0.0216	0.357	0.73
	Melanistic proportion	-0.0020	0.0117	-0.176	0.87
	Temperature*melanistic proportion	-0.0019	0.0086	-0.221	0.83

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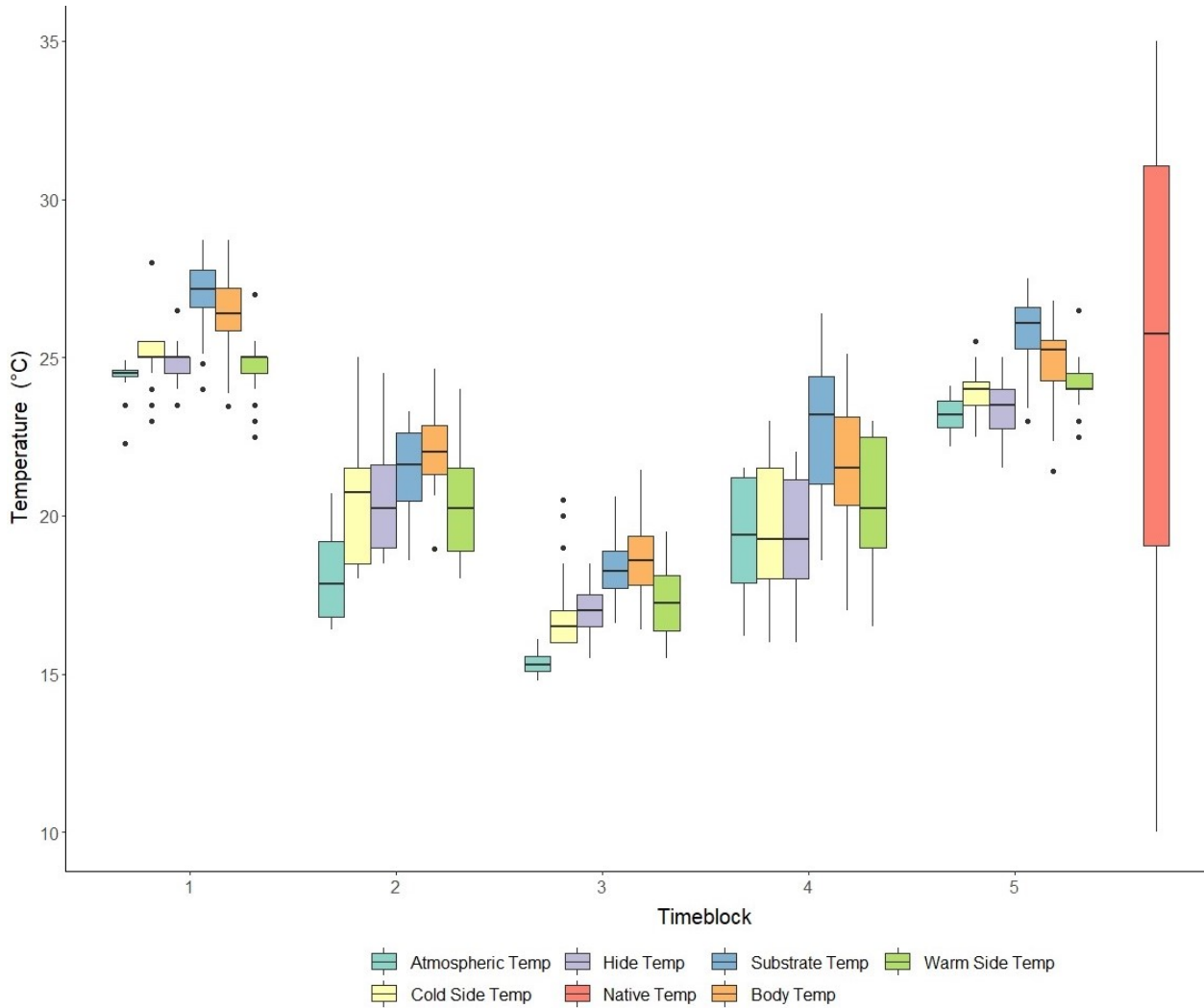
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773 **Figures**

774 **Figure 1. Box plots of the individual body temperatures, temperatures within terrarium**  
775 **and atmospheric temperatures for each block.** Temperature values for each individual were  
776 plotted to visualize temperature variations for within the terrarium, atmospheric temperatures,  
777 and body temperatures in comparison to the native temperature range of *E. macularius* for each  
778 block. Blocks are as in Table 1. Body and substrate temperatures are based on IR (infrared)  
779 images. Cold side, hide, and warm side of the terrarium and atmospheric temperatures are based  
780 on temperatures recorded by dataloggers. Native temperatures are based on Global Biodiversity  
781 Information Facility. The colors used for each block correspond to the different temperatures are  
782 indicated in the figure legend.

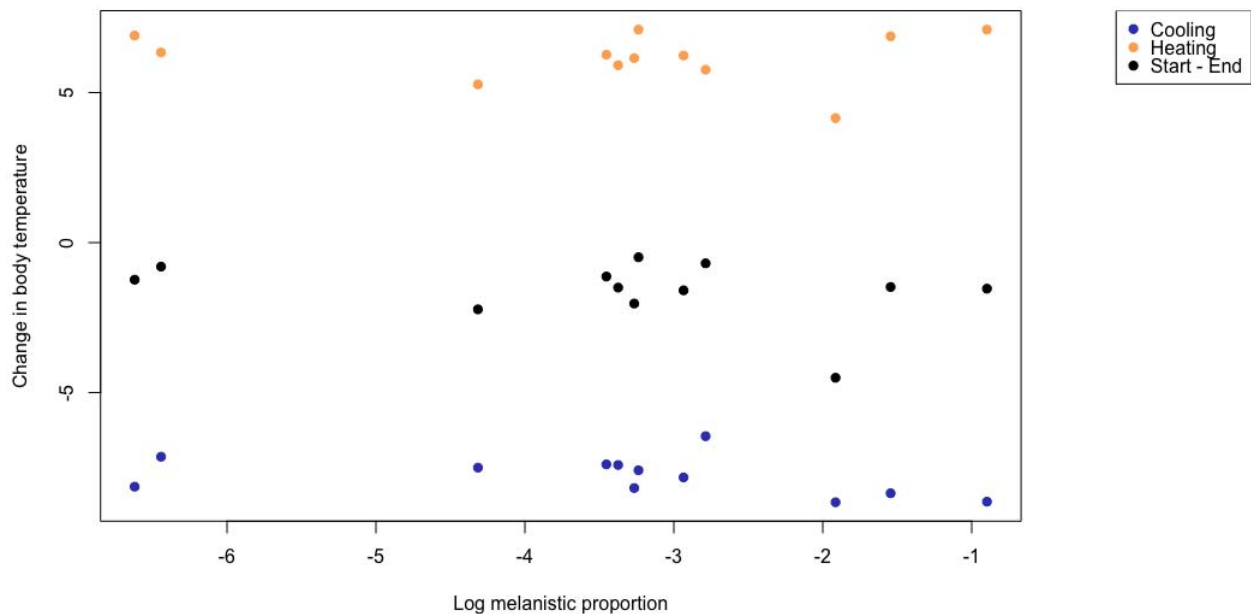
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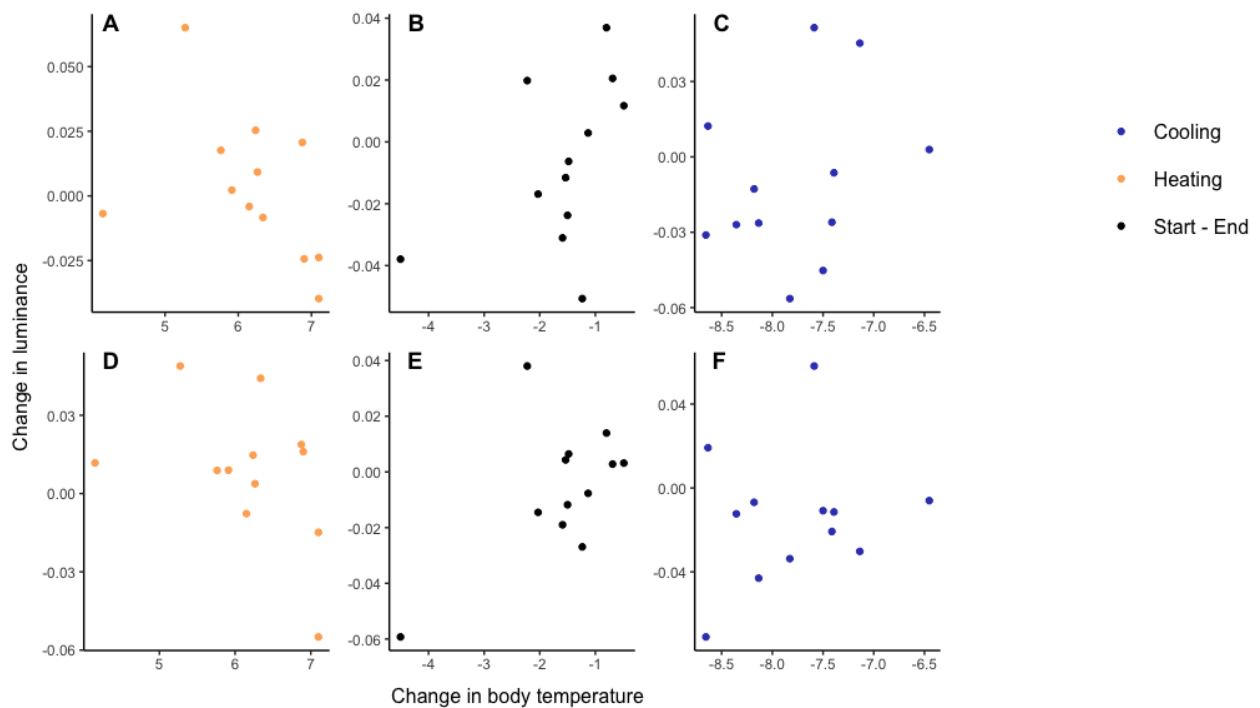
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786 **Figure 2. Influence of melanistic proportion on the change in body temperature.** Logarithm  
787 of the average stable melanistic proportion of each individual was used for this plot. Heating  
788 rates were calculated by taking the average body temperature difference between each block for  
789 blocks 5 and 3 per individual. Cooling rates were calculated using the same methods for blocks 1  
790 and 3. Each dot represents an individual. Blue dots refer to the cooling phase (blocks 1-3),  
791 orange dots to the heating phase (blocks 3-5), and black dots refer to the start and end of the  
792 experiment (blocks 1-5). Changes in body temperature are similar within each group (cooling,  
793 heating, start-end) independently of the melanistic proportion of each individual.  
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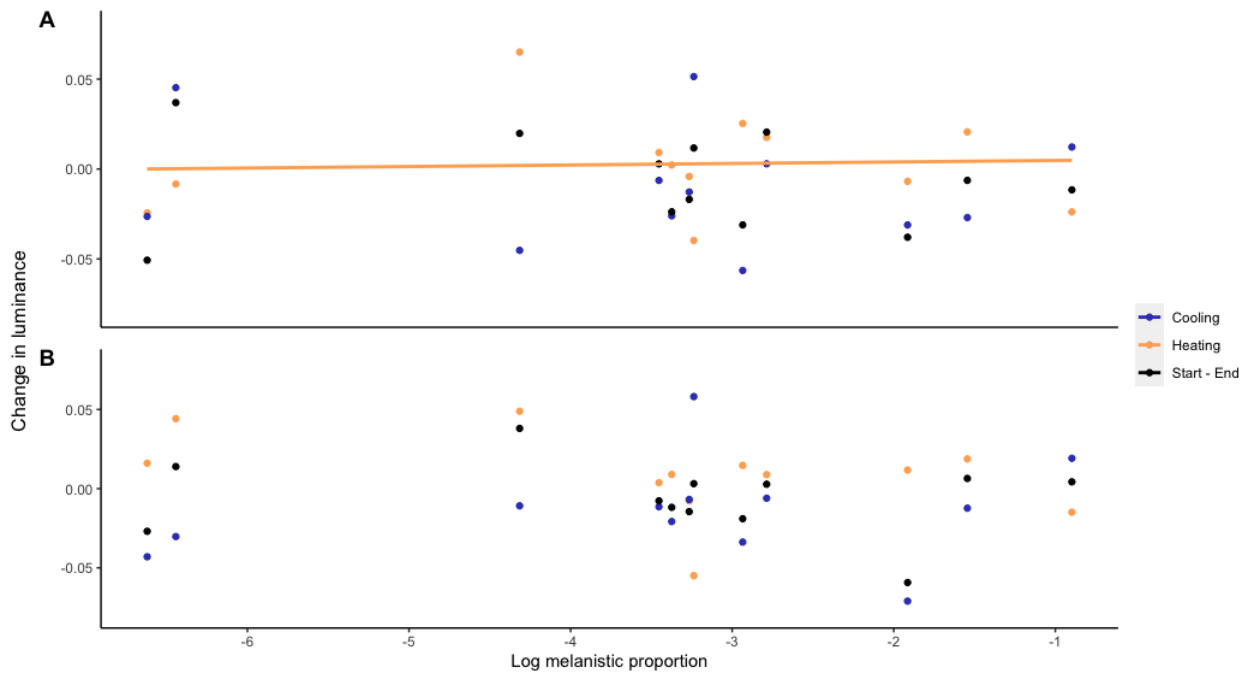
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806 **Figure 3. Change in average luminance for non-melanistic (A, B, C) and melanistic (D, E,**  
 807 **F) areas of the body against change in body temperature.** Average luminance was calculated  
 808 by weighted RGB values taken from images. Heating rates were calculated by taking the average  
 809 body temperature difference between each block for blocks 5 and 3 per individual. Cooling rates  
 810 were calculated using the same methods for blocks 1 and 3. Each dot represents an individual.  
 811 Blue dots refer to the cooling phase (blocks 1-3, plots C and F), orange dots to the heating phase  
 812 (blocks 3-5, plots A and D), and black dots refer to the start and end of the experiment (blocks 1-  
 813 5, plots B and E). Solid line indicates a significant p-value via linear models.  
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818 **Figure 4. Change in average luminance for the non-melanistic (A) and melanistic (B) part**  
819 **of the body against melanistic proportion plotted on a logarithmic scale.** Average luminance  
820 was calculated by weighted RGB values taken from images. Heating rates were calculated by  
821 taking the average body temperature difference between each block for blocks 5 and 3 per  
822 individual. Cooling rates were calculated using the same methods for blocks 1 and 3. Each dot  
823 represents an individual. Blue dots refer to the cooling phase (blocks 1-3), orange dots to the  
824 heating phase (blocks 3-5), and black dots refer to the start and end of the experiment (blocks 1-  
825 5). Solid line indicates a significant p-value via a linear model.  
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