

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) 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 +/-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

552 **Competing interests**

553 The authors declare no competing interests

554

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559 **Data availability:** Full dataset will be available on Dryad after manuscript acceptance.

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

| Block | Experiment time (min) | Terrarium temperature (°C) | Terrarium cold end (°C) | Terrarium warm end (°C) |
|--------------|------------------------------|-----------------------------------|--------------------------------|--------------------------------|
| 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 | 2.076x10⁻¹³ |
| 3-5 | NA | NA | 0 | 0.00055 |
| 1-5 | NA | NA | 78 | 0.00055 |
| 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.

759

| Factors | Estimate | Std Err | t-value | p-value |
|--------------------------|-----------------|----------------|----------------|----------------|
| 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

| Blocks | covariates | Estimate | Std Err | t-value | p-value |
|--|-----------------------------------|-----------------|----------------|----------------|----------------|
| 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 | 0.02 |
| | Temperature*melanistic proportion | 0.0177 | 0.0062 | 2.877 | 0.02 |
| 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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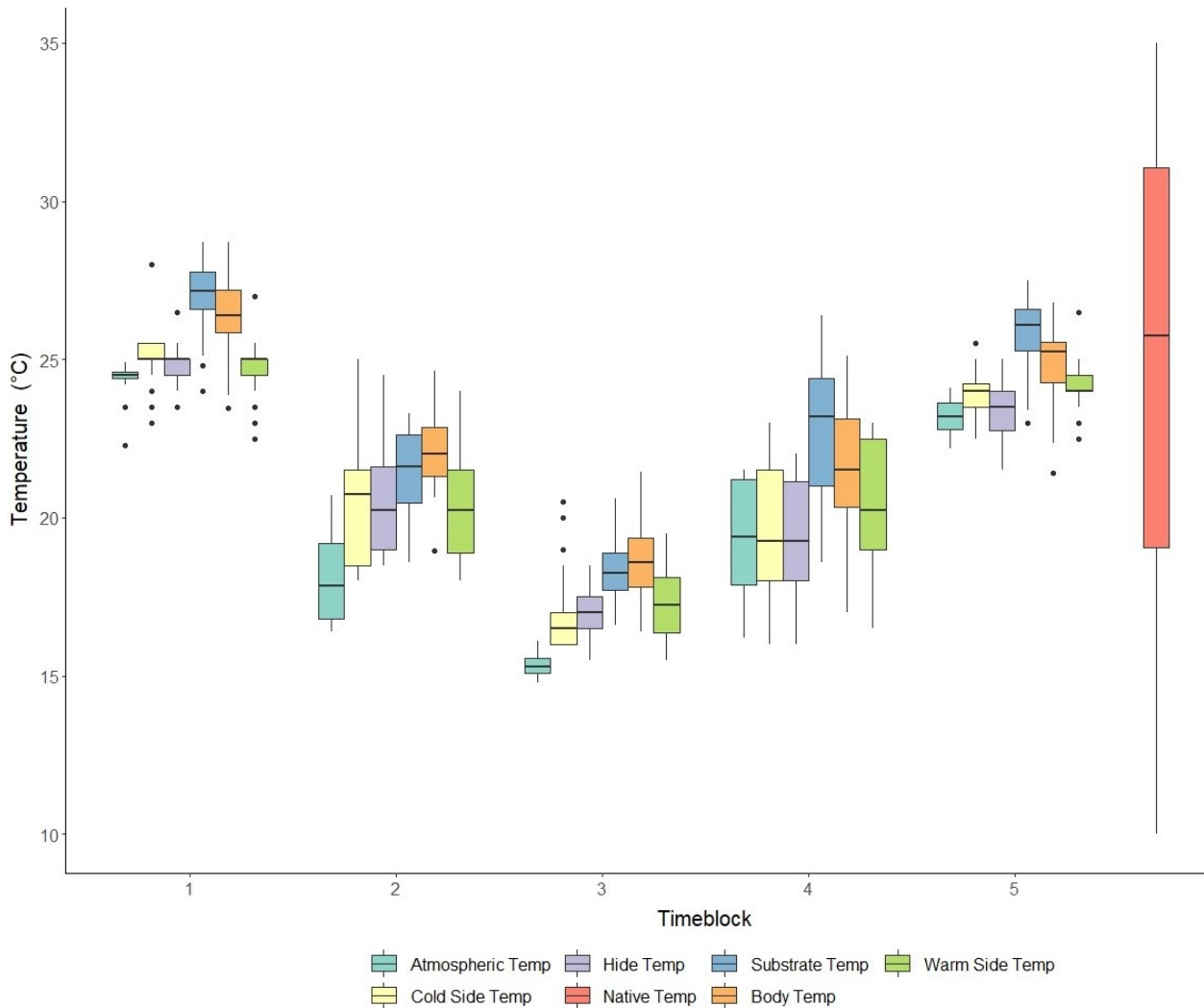
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773 **Figures**

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.

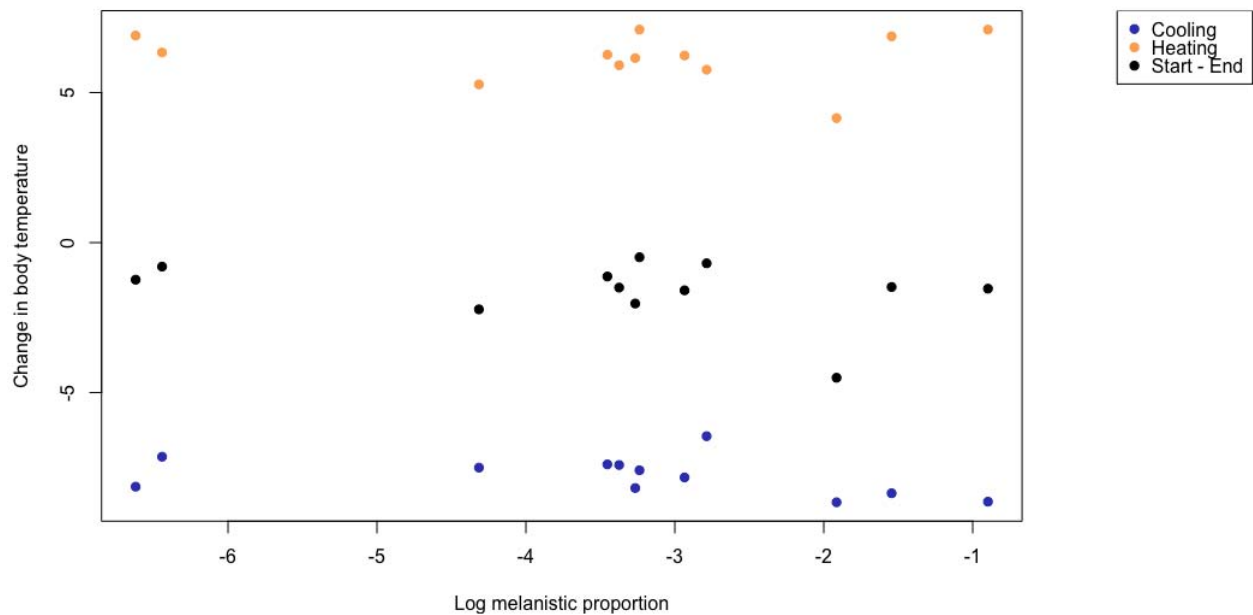
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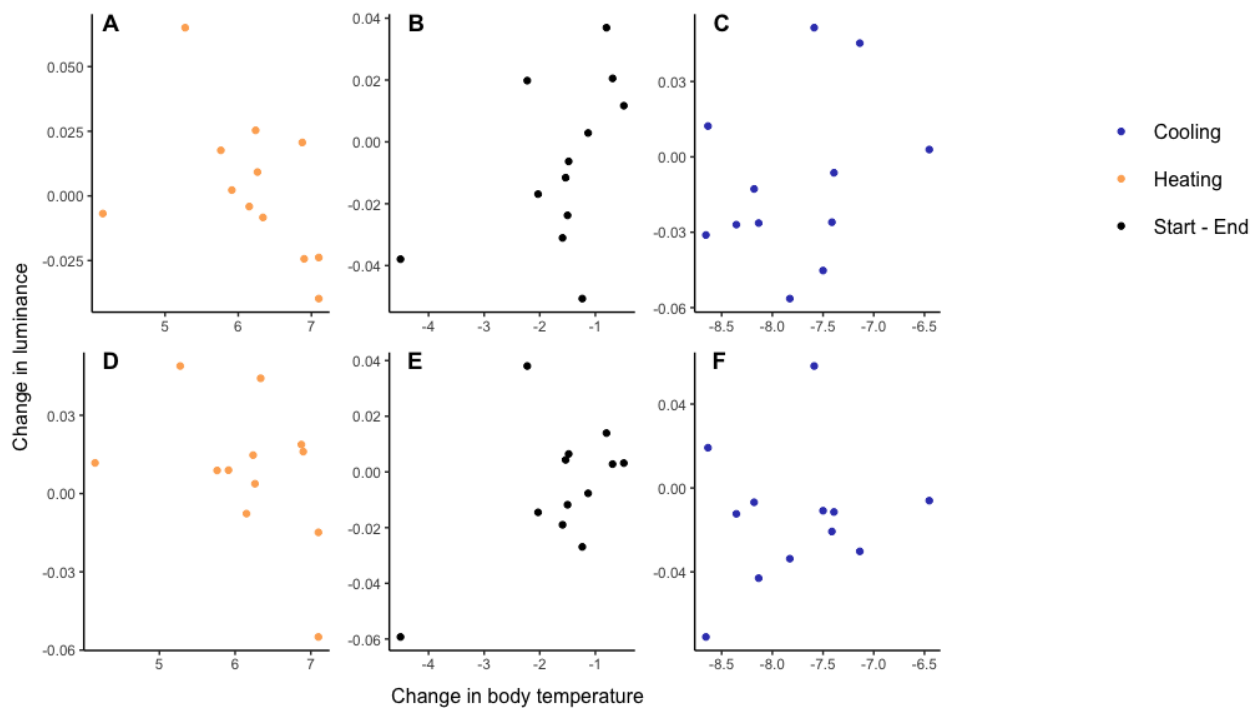
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786 **Figure 2. Influence of melanistic proportion on the change in body temperature.** Logarithm
787 of the average stable melanistic proportion of each individual was used for this plot. Heating
788 rates were calculated by taking the average body 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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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.
814

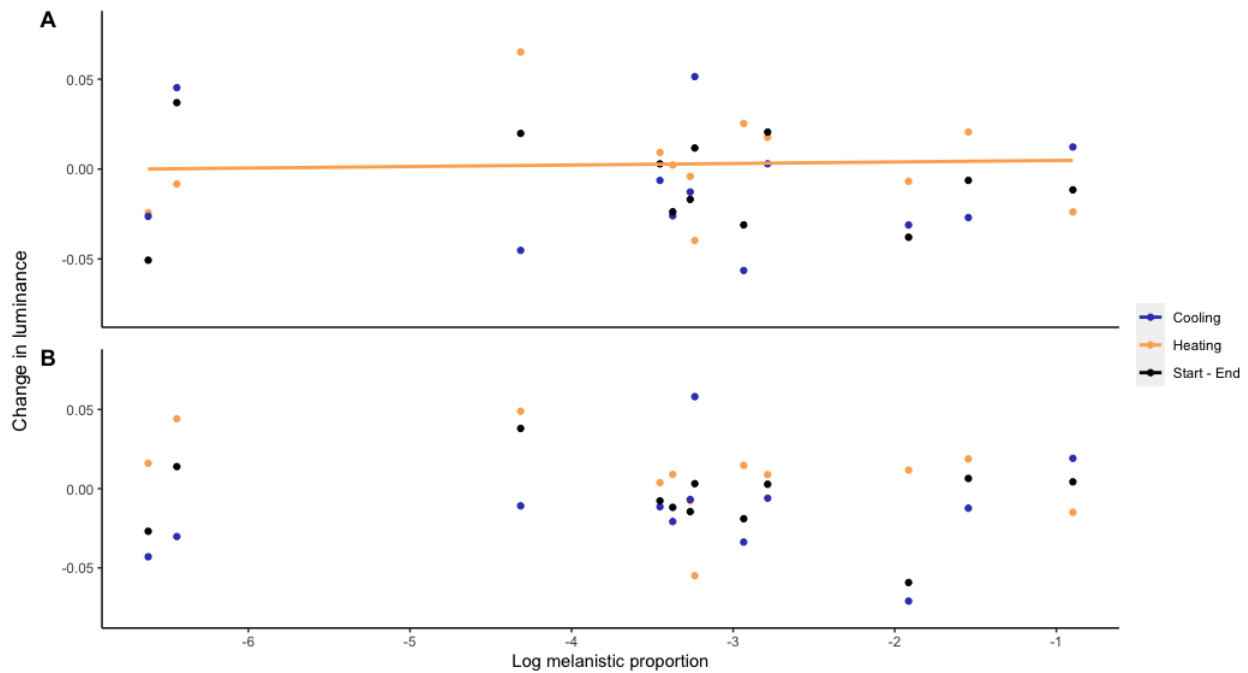


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818 **Figure 4. Change in average luminance for the non-melanistic (A) and melanistic (B) part**
819 **of the body against melanistic proportion plotted on a logarithmic scale.** Average luminance
820 was calculated by weighted RGB values taken from 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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