

Color and Temperature Are Not Related in the Crepuscular Reptile, *Eublepharis macularius*

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Brandon Hastings  
Bachelor of Science  
Longwood University, 2020

Director: Ylenia Chiari, Assistant Professor  
Department of Biology

Fall 2022  
George Mason University  
Fairfax, VA

Copyright 2022 Brandon Hastings  
All Rights Reserved

## **DEDICATION**

I dedicate this work to my parents, Kim and Brian Hastings, who strived to ensure that I would have access to an education and supported my choice to pursue graduate school.

## **ACKNOWLEDGEMENTS**

I wish to thank my advisor, Dr. Ylenia Chiari, for her guidance and support throughout this process, sticking with me through a changing thesis project, and constant revisions of this manuscript. I want to thank the members of my committee for their input and patience: Dr. Patrick Gillevet, Dr. Daniel Hanley, and Dr. Andrea Weeks. I would also like to thank Anastasiya Melnyk and Emma White for their help with running the experiment and extracting data, Dr. David Lattanzi and Mehrdad Ghyabi for their assistance in devising the luminance extraction methods. Finally, this work would not have been possible without a generous grant by the GMU OSCAR program.

## TABLE OF CONTENTS

	Page
List of Tables .....	vi
List of Figures .....	vii
Abstract .....	viii
Introduction .....	1
Tables .....	19
Figures .....	191
References .....	27

## LIST OF TABLES

Table	Page
Table 1: Proposed terrarium temperatures at different time points in the experiment .....	19
Table 2: Spearman correlation coefficients between body temperatures .....	20

## LIST OF FIGURES

Figure	Page
Figure 1: Experimental setup.....	21
Figure 2: Box plots of the individual body temperatures, temperatures within terrarium and environmental temperatures for each time block.....	22
Figure 3: Change in body temperature by melanistic proportion .....	23
Figure 4: Influence of melanistic proportion on heating and cooling rates .....	24
Figure 5: Change in average luminance by body temperature between time blocks.....	25
Figure 6: Change in melanistic and non-melanistic luminance values by melanistic proportion across the experiment.....	26

## **ABSTRACT**

### **COLOR AND TEMPERATURE ARE NOT RELATED IN THE CREPUSCULAR REPTILE, EUBLEPHARIS MACULARIUS**

Brandon Hastings

George Mason University, 2022

Thesis Director: Dr. Ylenia Chiari

Ectothermic organisms depend on external sources for heating and cooling to regulate their internal body temperature. Some ectotherms rely on physiological color change by adjusting melanistic body coloration to increase or decrease heat absorption. Furthermore, ectotherms with a greater proportion of stable melanistic body coloration seem to have better thermoregulatory performance in cold climates due to better absorption of solar energy to buffer body temperature in relation to environmental temperatures. Together with its influence on thermoregulation, body coloration is also used in ectotherms for other ecological functions, from escaping predators to communicating with conspecific individuals. As fluctuations in extreme temperatures due to global warming are becoming more frequent, understanding the relationship between coloration and suboptimal temperature is essential to predict the influence that this may have on other functions. In this study, we tested if the exposure to suboptimal low temperatures produces a

physiological color in the crepuscular reptile *Eublepharis macularius*. Furthermore, we tested if the proportion of melanism on the body of these animals is related to differences in heating and cooling rates of the internal body temperature during suboptimal temperature exposure. Temperature measurements were taken using infrared photography and temperature loggers, and coloration was obtained using objective photography and analyzed using a newly developed custom software package. As the dorsal part of the body is the one mostly involved in physiological color changes in ectotherms, we tested if dorsal coloration would change as a response to lowering environmental temperatures, analyzing melanistic and non-melanistic areas of the body separately for twelve geckos. We expected a higher degree of color change in non-melanistic versus melanistic coloration and a relative higher average body temperature in geckos with a higher proportion of melanistic coloration. We found that body temperature reflects substrate temperatures, but that the proportion of melanistic coloration has a moderate negative correlation with cooling rates of body temperature. We also did not observe a relationship between body temperature and physiological color change for melanistic and non-melanistic color. These findings suggest that in *E. macularius* melanistic coloration may be used for other purposes such as predator avoidance and that physiological color change may not be used for thermoregulation to increase heat absorption, but may occur for background matching, similarly to what described for other nocturnal/crepuscular reptiles. Future research should further test these hypotheses to elucidate the function of melanistic coloration in crepuscular and nocturnal geckos and to understand the evolution of pattern development in organisms active in low light conditions.

## INTRODUCTION

Optimal body temperature is essential for animals for mating, foraging, predator escape, and other biological functions (Dunham et al., 1989; Seebacher & Franklin, 2005). In the face of global climate change and recurrent temperature fluctuations caused by climate change (Rummukainen, 2012; Vasseur et al., 2014), organisms will be subjected to recurrent suboptimal temperatures. Thus, understanding the influence of these suboptimal temperatures on the organismal physiology and ecology, is essential to predict how their biology will be affected (Deutsch et al., 2008; Kearney et al., 2009). Ectotherms, such as non-avian reptiles, generally do not have an internal mechanism for maintaining a relatively constant body temperature; thus, their basic life functions depend on external heat absorption and loss.

Ectothermic vertebrates have thus evolved a wide range of behavioral, physiological, and morphological characteristics that aid in maintain optimal body temperatures. Although microhabitat changes and posturing are the most common thermoregulatory behaviors observed in ectotherms (Aubret & Shine, 2010 and references within; Bauwens et al., 1996; Kearney & Predavec, 2000), skin coloration may also influence thermoregulation. In areas with low mean annual solar radiation, ectotherm vertebrates may for example have higher concentrations of dermal melanin to increase heat absorption (the so called thermal melanism hypothesis, TMH, Clusella Trullas et al., 2007;

Clusella-Trullas et al., 2008). The darker skin coloration in this case is dependent on melanistic pigmentation of the dermis caused by the concentration of melanophores, specifically seen in reptiles (Forsman, 1995; Gibson & Falls, 1979; Walton & Bennett, 1993). Vertebrate ectotherms living in areas with low levels of solar radiation – i.e., areas further from the equator – have melanophores distributed relatively equally throughout the dermis to create an overall lower skin reflectance and higher rate of heat absorption. Ectotherms occurring in areas of higher solar radiation levels, however, are less constrained and can exhibit clusters within their dermis that have a higher concentration of melanophores relative to other areas (Martínez-Freiría et al., 2020; Moreno Azócar et al., 2015; Szydlowski et al., 2020), suggesting that these organisms have other methods of heat absorption aside from melanin. Furthermore, dermal melanophores also facilitate a phenomenon known as rapid physiological color change. This is caused by the movement of melanosomes within dermal melanophores, with melanosomes moving toward the surface during skin darkening (Sherbrooke & Frost, 1989). Rapid physiological color change may be used for thermoregulation by darkening or lightening the skin surface of ectotherms and increasing or decreasing the level of solar radiation absorption, respectively, which then alters the internal temperature of the organism (Lowe & Norris, 1956; Sherbrooke et al., 1994; Sherbrooke & Frost, 1989; Smith, Cadena, Endler, Kearney, et al., 2016; Smith, Cadena, Endler, Porter, et al., 2016). Although thermal melanism has been studied across and within species in ectotherms, thermal melanism has been measured as overall darker or lighter skin colors, without inferring how the proportion of melanistic patterns may influence thermoregulation (though see Forsman, 1995) and if melanistic

patterns may also be influenced by physiological color change (i.e., melanistic and non-melanistic coloration becoming lighter or darker at different rates).

The leopard gecko, *Eublepharis macularius*, a crepuscular species primarily active in low light conditions, is an ideal organism on which to test the thermal melanism hypothesis and the influence of physiological color change on body temperature. *E. macularius* naturally occurs in Pakistan, India, Iran, and surrounding regions (Agarwal et al., 2022), with an estimated preferred temperature range of 28-29°C (Angilletta et al., 1999). This species in nature is characterized by melanistic spots (at the adult stage) or stripes (at the juvenile stage) on a lighter, often yellow, background of base dermal coloration. It is also commonly bred for the pet-trade resulting in a variety of melanistic patterns of different types and proportion (Glimm et al., 2021; Kiskowski et al., 2019; Szydłowski et al., 2020). The melanistic pattern diversity allows investigating how the proportion of melanism influences body temperature and physiological color change during exposure to suboptimal lower temperatures, when the animals would be expected to use darker coloration to increase heat absorption. Previous studies investigating rapid physiological color change in other crepuscular/nocturnal lizards have found a greater influence of background matching over thermoregulation on physiological color change (Vroonen et al., 2012; Zaidan III & Wiebusch, 2007), suggesting that in nocturnal species melanistic body color and patterns may primarily be used for different functions than thermoregulation.

In this work, we used multispectral objective photography (Troscianko & Stevens, 2015) and a newly developed data extraction pipeline to extract the melanistic coloration

from the background animal coloration on live and free to move *E. macularius*. Although other algorithms and pipelines have been used to extract color data, including segmenting color patterns from background colors (see Glimm et al., 2021, Abramjan et al., 2020, and Troscianko & Stevens, 2015), our approach allowed for extracting the same type of color data over time – i.e., every 30 min at which the images of the gecko were taken - on the same free to move individual while also standardizing the color information through this time series. In this study we tested if: 1. individuals with a greater proportion of melanistic pattern across the body experience a slower change in body temperature as a consequence of lower suboptimal temperatures; 2. exposing individuals to lower suboptimal temperatures elicits a physiological color change in the skin of the geckos; 3. individuals with a greater proportion of melanistic spots experience a lower amount of overall color change (background color and melanistic color). Conclusions from this study will help to uncover the function that the proportion of melanistic coloration serves in nocturnal ectotherms as a basis for understanding drivers of pattern development in organisms active in low light conditions.

## **MATERIALS AND METHODS**

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

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

All geckos were housed in the same room at George Mason University, with one gecko per cage, and exposed to a 12hr day-night cycle. The housing containers for *E. macularius* were plastic boxes with newspaper bedding. The room temperature was kept stable between 25-28°C and each enclosure contained moist and dry hides and a heat pad for thermoregulation. Humidity level and temperature in the room were checked daily using a digital thermometer/hygrometer. The health condition of each individual was checked daily by visual inspection, and no gecko was tested during the shedding process. Geckos were fed three times a week with a combination of crickets and mealworms dusted with calcium and vitamin powder. Feeding was withheld 3 days prior to testing in order to avoid digestive problems for the animals due to exposure to low temperature and to also avoid confounding effects due to potential food digestion and resumed after testing. Water was always available except during testing. Testing was carried out on 12 adult geckos (n=12, 7 males, 5 females).

### **Experimental Setup**

Experiments were conducted in a temperature controlled room of 4x2 m at George Mason University. This room has a 1x3 m open area in the middle of the room, where the 51x25x30 cm glass terrarium used as a testing terrarium was placed on a rubber mat on the floor. The walls and floor of the terrarium were covered with white Teflon (SS Shovan) to remove any potential effect of background coloration, and the lid was removed for the entirety of the experiment to facilitate data collection. The Teflon on the bottom of the terrarium consisted of three layers, while the one on the side was a single layer. Black electrical tape (3M) was used to adhere the bottom sheet of Teflon to the side sheets. A

clean cardboard egg carton of 15 cm X 15 cm was placed in the middle of the terrarium as a hiding spot for the gecko, because reptiles are known to thermoregulate behaviorally by seeking shelter in suboptimal temperatures (Aubret & Shine, 2010; Kearney et al., 2009; Woods et al., 2015). Except for the cardboard egg carton and four iButtons (DS1921G Thermochron, Maxim Integrated Products), nothing else was placed in the terrarium and the gecko was free to move and use its entire space in the terrarium. A heating pad was placed underneath one side 15 cm from the end of the terrarium (to have a warm spot throughout the experiments). A broad-spectrum UV-VIS light (Zoo Med PowerSun H.I.D Metal Halide UVB Lamp, 6500 K, 70 W, 95 CRI) was placed 160 cm above the center of the testing terrarium to ensure photographs were taken under proper lighting (Troscianko & Stevens, 2015), as melanin has strong absorption in the UV-Vis spectrum (McNamara et al., 2021). This light was turned on 45 minutes prior to the start of each experiment and remained on for the length of the experiment. Fig. 1 shows the set up for the experiment.

A thermometer (ThermoPro) was placed on the floor adjacent to the terrarium to measure the temperature and humidity of the room before and during the experiments. To measure the temperature throughout the experiments and to ensure that room and terrarium temperatures were similar across experiments, we also placed a total of four iButtons in the terrarium and in temperature controlled room. Specifically, one iButton was placed on the floor outside of the middle section of the terrarium 20 cm away. Of the three iButtons placed in the terrarium, two were placed on the opposite ends (one on top of the heating pad and the second on the opposite end of the terrarium) and one under the cardboard egg carton. The iButtons were programmed to start collecting temperatures data 30 minutes

prior to the start of the experiment and continued to collect temperature data every five minutes for the entire duration of the experiment. Following each experiment, iButtons were sanitized with isopropyl alcohol and the terrarium was cleaned with soap and hot water to remove any potential scent or residue left from the previously tested individual. The top layer of the Teflon at the bottom of the terrarium was replaced after each experiment, while the Teflon sheets on the sides were sanitized with isopropyl alcohol after each experiment. A new cardboard egg carton was used for each tested individual.

### **Experimental temperature ranges**

The range of temperatures used in this experiment was from 15–25°C (Table 1). The high temperature was chosen to be at 25°C, as this is the overall most frequent temperature at which these geckos are exposed in their housing environment. 15°C was used as the lower temperature to resemble natural low temperature experienced by this species in its natural habitat (Fig. 2), without eliciting hibernation (Khan, 2009).

To set up the maximum and minimum temperatures for this experiment, the temperature control for the room was set at 25°C and then when it needed to be lowered the temperature control was set to 15°C; temperature lowering between 25 and 15°C (or vice versa) took one hour. To bring the temperature back to 25°C from 15°C a space heater was placed 1 m from the warm end of the terrarium and turned on after the temperature control of the room was adjusted to 25°C (stage 4, Table 1). At the beginning of each experiment, we used the room thermometer (ThermoPro) to confirm that the room was at 25°C. The temperature of the terrarium was confirmed for each end of it by pointing an infrared thermometer (Etekcity Corporation) held 30 cm from the surface pointing

perpendicular towards the bottom of the terrarium. Temperature checks of the room and terrarium were repeated every 30 minutes during the experiment using the in-room thermometer and the infrared thermometer, respectively. Temperature readings from iButtons were used to confirm temperature readings after each experiment.

### **Native Environmental Temperatures for *E. macularius***

To determine native environmental temperatures for *E. macularius*, occurrences for this species were downloaded from the Global Biodiversity Information Facility (GBIF, October 2022; [www.gbif.org](http://www.gbif.org)) and imported into Rstudio (V4.1.2, R Core Team 2021) using the “occ\_download\_get” function from the *rgbif* package (Chamberlain et al., 2022). Species occurrences for *E. macularius* were filtered for species’ scientific name mismatches as well as NA values for latitude, longitude, species’ scientific names, and country codes. Species occurrences were also cleaned and cross-checked for coordinate validity using the “clean\_coordinates” function from the package, *Coordinate Cleaner* (Zizka et al., 2019). Species occurrences which resulted in at least one flagged test labeled as, “FALSE,” were removed from the dataset. From a total of 37 occurrences for *E. macularius*, maximum and minimum monthly temperatures were extracted from raster files containing climate data downloaded from the WorldClim v2.0 database (Fick & Hijmans, 2017) using the “extract” function from the *raster* package in R (Hijmans et al., 2022).

### **Data collection**

Geckos were tested in a random order. Individuals were weighed using a digital scale before the start of the experiment and snout vent length (SVL) was obtained for all

tested geckos. Only one gecko per day was tested and geckos were always tested within the same time frame for 7 hours, starting at 11:00am each day. At the end of the experiment, the gecko was returned to its housing terrarium. Geckos were visually monitored after the experiments to check for any health concern. No geckos had any issues after the experiments.

To extract temperature data from multiple body parts of the gecko, as different body parts may have different temperatures, a CAT S62 smartphone Pro camera (Caterpillar Inc.) was used to take an infrared (IR) image (Barroso et al., 2016). The camera was held approximately 30 cm directly above the individual in order to maintain the same resolution across IR images, regardless of the body size of the animal or its position in the terrarium. To standardize IR images and determine reflective temperatures, an 8x8 cm square piece of aluminum foil was placed next to the gecko in the terrarium when capturing the image of a gecko each time an IR image was taken, following Barroso et al. (2016). The square piece of aluminum foil in each IR image was used to extract average reflective temperature from IR images only and was not used in color analysis. Average reflective temperature is required in each IR image to standardize temperatures for the gecko and the terrarium. After taking the IR image of the gecko, the relative humidity and temperature of the temperature-controlled room were recorded.

After taking the IR image of the gecko, to obtain the color data for each gecko, visible images were taken using a full spectrum converted Canon 1300D with a Kolari Vision UV/IR cut filter (410–700 nm transmission). Images were taken approximately 40cm directly above the gecko to ensure a good resolution across images and with a

greyscale standard built from Teflon following the methods of Abramjan et al. (2020) in the frame of the image. Visible images were obtained only for the dorsal part of the geckos, as melanistic patterns are generally absent from the ventral side of the animals and as such the ventral side was not relevant to the study questions (Glimm et al., 2021). Two people (Anastasiya Melnick, Emma White) took the IR images making sure to standardize the method between them and another person (Brandon Hastings) always took the visible images. Due to possible movement of the gecko, multiple images of the animal were taken every time. If the gecko was under the cardboard hide, the hide was lifted prior to taking the images and replaced afterwards. The time at which the temperature of the room was changed, the thermostat/thermometer readings, as well as the times at which visible and thermal photos were taken were manually recorded.

### **Data extraction**

Temperature measurements from the IR images were extracted in FLIR Tools (Teledyne FLIR 2022) from five body parts of each gecko (head – base of parietal scales, left knee, left foot, central dorsum, eyes – right and left, snout, and tail - above the cloaca), as body temperature is known to vary across the body (Barroso et al., 2016). Temperature data averaged from both eyes were used as the internal body temperature for the analyses, as suggested for reptiles by Barroso et al. (2016). The average temperature from both eyes was found to be highly correlated with the temperature of the snout ( $r_s = 0.96$ ) which has also been suggested to be a good proxy for internal body temperature (Tabh et al., 2021). For each IR image, relative humidity, environmental temperature, distance (0 m), and emissivity (1) were first entered into FLIR Thermal Studio following Barroso et al. (2016)

and Barroso (pers. comm.) to calibrate the temperature readings of the thermal camera. Relative humidity and environmental temperature values were obtained from the data recorded at the same time as the gecko IR images. Reflective temperature was obtained as the average reflective temperature of the aluminum foil standard. This value was extracted from the IR image of the aluminum foil by overlaying a box entirely over the aluminum foil using the *Rectangle* function in FLIR Tools. After calibrating and entering the average reflective temperature in FLIR, distance and emissivity were re-entered as 0.3 m and 0.96, respectively (Barroso et al., 2016). The substrate temperature of the terrarium was also measured by using the *Rectangle* function to overlay a small box over the electrical tape on the bottom of the terrarium for each image taken, as the electrical tape more accurately reflects the temperature of the terrarium (Barroso et al., 2016).

Digital images obtained with the Canon camera were processed using a custom software suite in Python. First, images were corrected for potential changes in lighting condition across images of the same individual for the different time blocks by converting each image to Hue -Saturation -Value (HSV) color space and using the greyscale reference in the first image of that individual as the baseline. The Value parameter of following images were then adjusted so that the greyscale reference matched that of the first image. All images were then converted back to RGB color space. Next, because the limbs of the geckos were sometimes obscured from the camera view due to movement, images were cropped to only include the head, trunk, and tail of each individual. For image color segmentation, hierarchical k-means clustering was run on each image (the specific parameters used can be found in the available codes), as manual object segmentation can

be inaccurate and time consuming, especially when dealing with a large number of images. In this process every pixel is assumed to be a datapoint in the RGB color space, then pixels are grouped into a predefined number of clusters based on their distances to each other in the three-dimensional RGB color space. All color space conversions were done using OpenCV (Bradski, 2008). Because the result of this method is sensitive to its initialization, cluster centers were initiated following a K-means++ algorithm to account for this. While the K-means algorithm is computationally inexpensive and fast, there were some limitations caused by lighting conditions with a high incidence of shadows. It was common for coloration in areas of discoloration due to shadowing to be incorrectly assigned to a cluster, resulting in a misrepresentation of the pattern for that image. Because of the unsupervised nature of k-means, there is no way to correct for this error once the segmentation step is started. To account for this, a visual confirmation step by the user was implemented before segmentation to ensure that the color clusters would accurately represent the pattern to be segmented.

The results of K-means clustering for visible images was a Boolean mask representing melanistic and non-melanistic coloration of the entire body. The Boolean mask was applied to the color corrected image to extract the mean luminance value for melanistic and non-melanistic coloration and the proportion of the coloration that was either melanistic or non-melanistic relative to the total coloration captured of the gecko. Luminance value – derived from a weighted calculation of RGB color channels (Bradski, 2008) - was used in this approach because luminance has been identified as the most important color component affecting the absorption of solar radiation (Smith, Cadena,

Endler, Kearney, et al., 2016). All Python codes used to extract color data are available at <https://github.com/mghyabi/Gecko.Repo>. The full dataset will be publicly available on Dryad after manuscript acceptance.

### **Statistical analyses**

Spearman's correlation coefficients were used to evaluate the relationship between the gecko body temperature and the substrate temperature, both calculated from the IR image, or the average of the three datalogger temperatures. To confirm the accuracy of IR substrate readings, a Spearman's correlation test was also run on the average data logger temperatures from within the terrarium and the substrate temperature taken with IR imaging. To ensure there was no correlation between the proportion of melanism and snout-vent length (SVL) or sex, a Pearson's correlation test was performed between melanistic proportion and SVL while a t-test was performed to detect any relationship between melanistic proportion and sex. Additionally, as heating and cooling rates may be influenced by body weight, a Pearson's correlation test was conducted between body weight and heating and cooling rates separately. Heating and cooling rates were calculated taking the average change in body temperature between time blocks 1-2, 2-3, and 1-3 (cooling) and time blocks 3-4, 4-5, and 3-5 (heating).

To investigate the influence of melanistic proportion on heating and cooling rates, we calculated the Pearson's correlation coefficients between the log average melanistic proportion taken for each gecko and heating/cooling rates.

To assess the influence that changes in the gecko body temperature - following changes in the environmental temperature - may have on physiological color changes

(luminance) on the entire dorsal area (head, trunk, and tail) of the gecko, we ran a one way ANOVA using the time block as a nested factor of body temperature to account for potential differences in body temperature between the time blocks. This analysis was run using luminance data for melanistic and non-melanistic coloration separately.

Finally, to see if the proportion of melanin on the body influences the amount of physiological color change (luminance) for melanistic and non-melanistic coloration separately, we ran a one way ANOVAs with melanistic and non-melanistic color change as the dependent variable, respectively, with the log proportion of melanin as the independent variable and time blocks as a factor to account for potential differences in luminance change between time blocks. ANOVAs that were run with factors were followed by post-hoc tests (Tukey test) to further investigate which relationships may be significant. All statistical analyses were run in R (V4.1.2, R Core Team 2021).

## **RESULTS**

For each gecko, 15 visible and 15 IR images were used for the analyses, giving 180 data point (15 x 12 individuals) for each image type in total. Similarly, 180 temperature measurements were used for each temperature type (IR images or average of the three dataloggers inside the terrarium) for the analyses as well. All temperatures taken from the experiments were visualized against the geckos' native temperature range taken from worldclim data (Fig. 2). Experimental temperatures stayed within the quartiles of native temperature as planned by the experimental design. Internal body temperature was strongly correlated with the terrarium substrate temperature ( $r_s = 0.96$ ; both temperatures are

estimated on the same IR image for each individual and time block). Correlation between the average temperature of all the three dataloggers within the terrarium and the substrate temperature estimated from the IR images were also highly correlated ( $r_s = 0.91$ ), although the temperature estimated by the IR images was relatively higher for each time block than the one based on the datalogger (Fig. 2). Correlation tests between internal body temperature corresponding to the average of the two eyes and other body regions showed strong correlation, all showing a Spearman correlation coefficient above 0.98 (Table 2).

Of the 12 geckos tested, nine exhibited a melanistic proportion between 0 and 11% of the dorsal coloration across the entire body, while the three others had 16%, 22%, and 41% of melanistic proportion. We found no correlation between the proportion of melanin and SVL ( $r^2 = -0.13$ ), weight ( $r^2 = 0.03$ ), or sex (T Stat=0.97,  $p = 0.19$ ). When examining the effect of body weight on heating and cooling rates, no correlation was found with heating rates ( $r^2 = 0.32$ ), while a moderate positive correlation was found for cooling rates ( $r^2 = 0.50$ ). The proportion of melanism – taken as a logarithmic value – had no influence on an individual's change in body temperature across the time blocks throughout the experiment ( $p = 0.68$ , Fig. 3) or the heating rate ( $r^2 = 0.18$ ), but had a moderate negative correlation with cooling rates ( $r = -0.56$ ) (Fig. 4). Finally, we found no influence of body temperature variation among any of the time blocks on variation in luminance for each gecko for either the melanistic or non-melanistic areas of the body ( $p = 0.20$  and  $p = 0.52$ , respectively; Fig. 5). Post-hoc comparisons further indicated that none of the comparisons among time blocks was significant (all  $p > 0.05$ ). Finally, we did not observe any significant difference between

melanistic proportion and the amount of melanistic or non-melanistic luminance change ( $p=0.86$ , Fig. 6).

## DISCUSSION

In ectotherms, melanistic coloration – both as a stable coloration and as a physiological darkening of the skin has been proposed among other potential functions to be used for thermoregulation. Melanin can be used to absorb UV radiation and convert it in heat by photon-phonon transformation (McNamara et al., 2021). Our results indicate that the proportion of melanin on the entire dorsal side of *E. macularius* is independent of the size (as a snout vent length) and the sex of the animal. We found that the proportion of melanin does not affect the heating rate, but moderately influences the cooling rate, with geckos with a higher proportion of melanin cooling faster than the ones with less. This result is contrary to our prediction, for which we expected an increased proportion of melanin to increase radiation absorbance and heat conversion. Previous studies investigating the effect of melanin on heating and cooling rates in insects have found a positive relationship between the proportion of melanin and heating rates (Hegna et al., 2013; Kingsolver, 1987) and this has been observed experimentally in reptiles, but not in the wild (Forsman, 1995). The moderate correlation that we found between the melanin proportion and cooling rates may be influenced by several of the individuals in our sample having low proportions and only three having higher proportions of melanin. Further studies involving individuals with a greater diversity of melanistic proportion are needed to further confirm our findings.

The role of proportion of melanin and melanism in crepuscular/nocturnal ectotherms can be complex, as most research into melanism has been conducted on diurnal ectotherms. Previous studies have suggested that crepuscular/nocturnal reptiles may be thigmothermic – absorbing heat from the surrounding environment, rather than directly from solar radiation - due to the low exposure to solar radiation during their active times (Garrick, 2008; Kearney & Predavec, 2000). Our study is the first to directly compare substrate temperatures – instead of just environmental temperatures - with the internal body temperature in *E. macularius*. We found a strong correlation between body temperatures and terrarium substrate temperatures, further supporting that *E. macularius* – a crepuscular species - may in fact rely on absorbing heat from the ground and be thigmothermic. This confirms previous findings (Garrick 2008) based on measures of body surface temperature and ambient temperature differentials in relation to body length. Although *E. macularius* may rely on the substrate temperature for thermoregulation, exposure to prolonged suboptimal low temperatures may trigger a physiological response to darken the skin color to increase heat absorbance, though this has only been observed in heliothermic reptiles to date. We found that a decrease in body temperature from 26°C to 18/15°C and permanence at 18/15°C for 120 minutes does not trigger a change in the luminance of the dorsal skin for either the melanistic or non-melanistic coloration. Taken together, our results suggest that in *E. macularius* coloration may not be related to body temperature regulation. Instead, melanistic pattern may serve a different function in this species, such as camouflage. A previous study found that in geckos spotted patterns may represent a more specialized type of camouflage (Allen et al., 2020). In this context, previous studies on

crepuscular/nocturnal geckos have indicated that physiological color change may be used more for background matching and camouflage than for thermoregulation (Vroonen et al., 2012; Zaidan III & Wiebusch, 2007).

The few studies that investigated physiological color change for thermoregulation and camouflage in other nocturnal geckos propose that these functions are mutually exclusive phenomena (Vroonen et al., 2012; Zaidan III & Wiebusch, 2007), suggesting that physiological color change in *E. macularius* could be used for background matching only. Furthermore, in *E. macularius* the UV coloration limited to the tail has also been suggested to be used for predator avoidance as a multipurpose signaling mechanism, either to deflect a predator attack to a less vulnerable part of the body or to deter larger conspecifics, especially in young individuals (Abramjan et al., 2020). Future studies should therefore further investigate the extent to which melanistic coloration and physiological color changes for thermoregulation versus camouflage or signaling are mutually exclusive or not in crepuscular and nocturnal reptiles and how these coloration interplay with UV coloration for predator escape. The conclusions of those studies could help to understand the function of coloration and color pattern development in nocturnal/crepuscular reptiles, and how their function may potentially differ from the functions in diurnal reptiles.

## TABLES

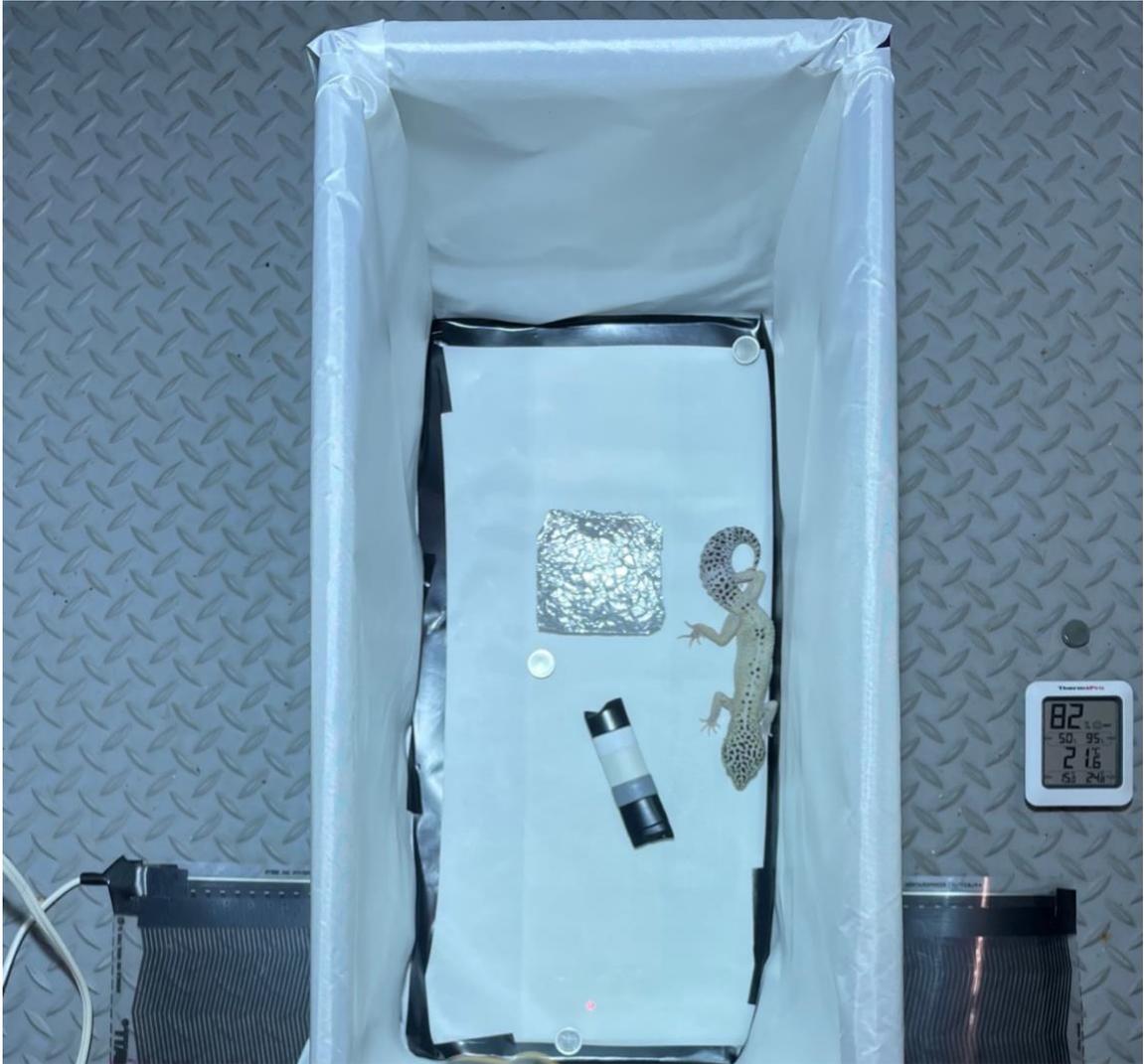
**Table 1. Experimental terrarium temperatures at different time points in the experiment.** Time blocks were used for the statistical analyses. Time blocks were selected to minimize temperature variance within blocks and maximize variance across them. For minimum and maximum for each time block, see also Figure 2.

<b>Time 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

**Table 2. Spearman correlation coefficients between body temperatures.** Body temperature was taken from the average of the two eyes and tested for correlation of temperatures of other body regions.

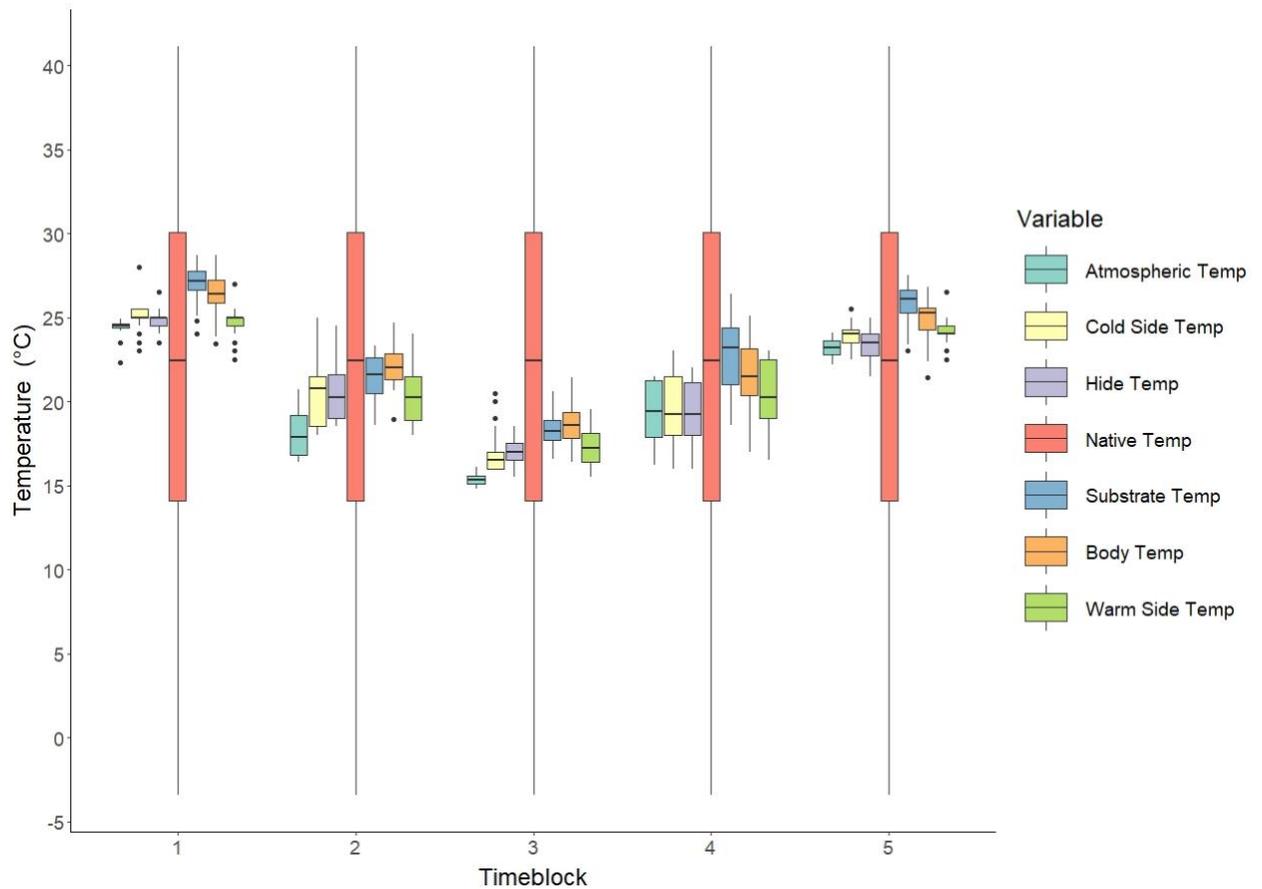
<b>Body region where the temperature was obtained from</b>	<b>Spearman correlation</b>
Snout	0.991
Head	0.989
Dorsum	0.985
Back Leg	0.988
Front Foot	0.985
Tail	0.984

## FIGURES

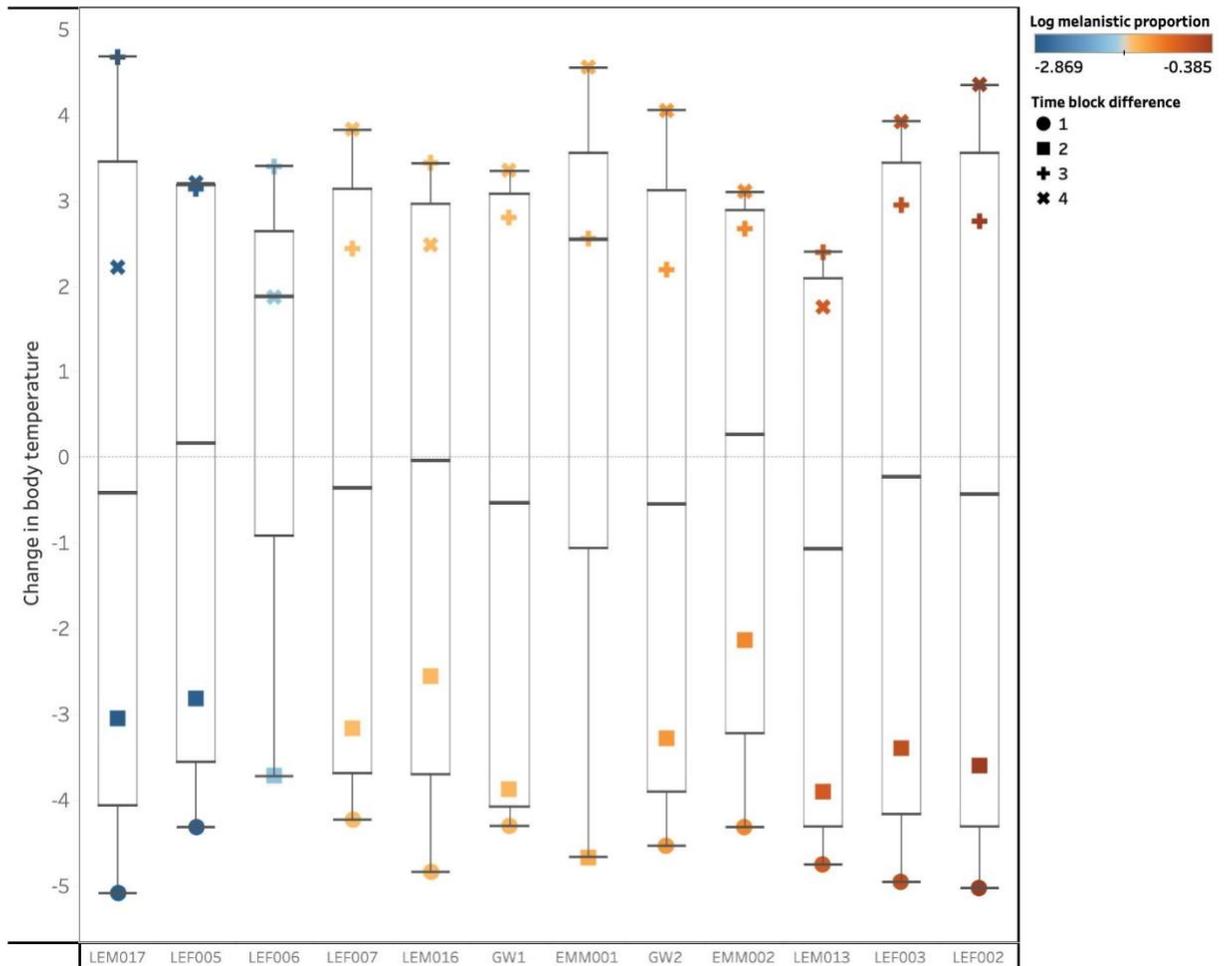


**Figure 1. Experimental setup.** Testing terrarium covered in white sheets of Teflon bordered by black electrical tape. A heating pad is set up at the warm end of the terrarium. Three iButton dataloggers are inside on the opposite ends and in the middle (where the cardboard hide would be while not capturing images), while one is outside with a digital

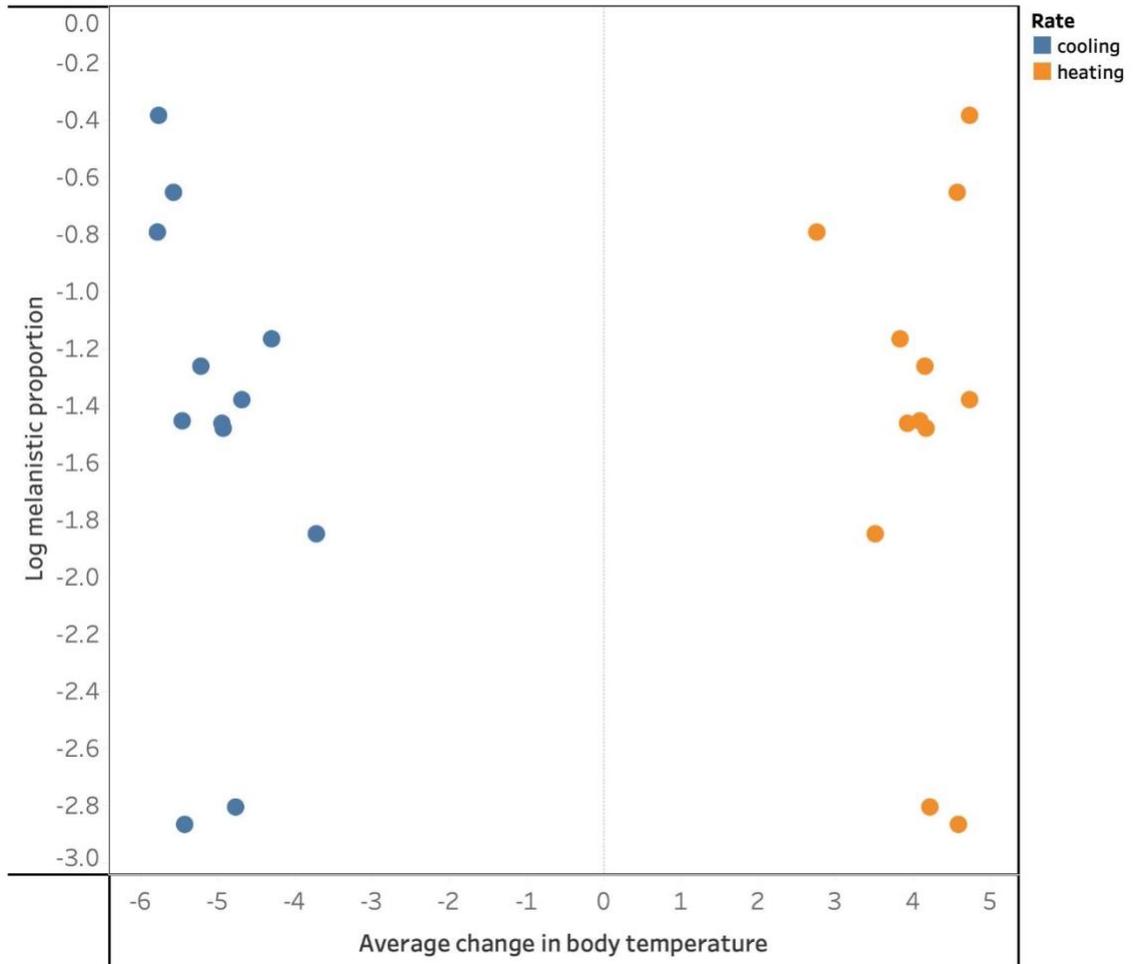
thermometer/hygrometer. The standards used for image capture are temporarily positioned in the terrarium in range of the gecko.



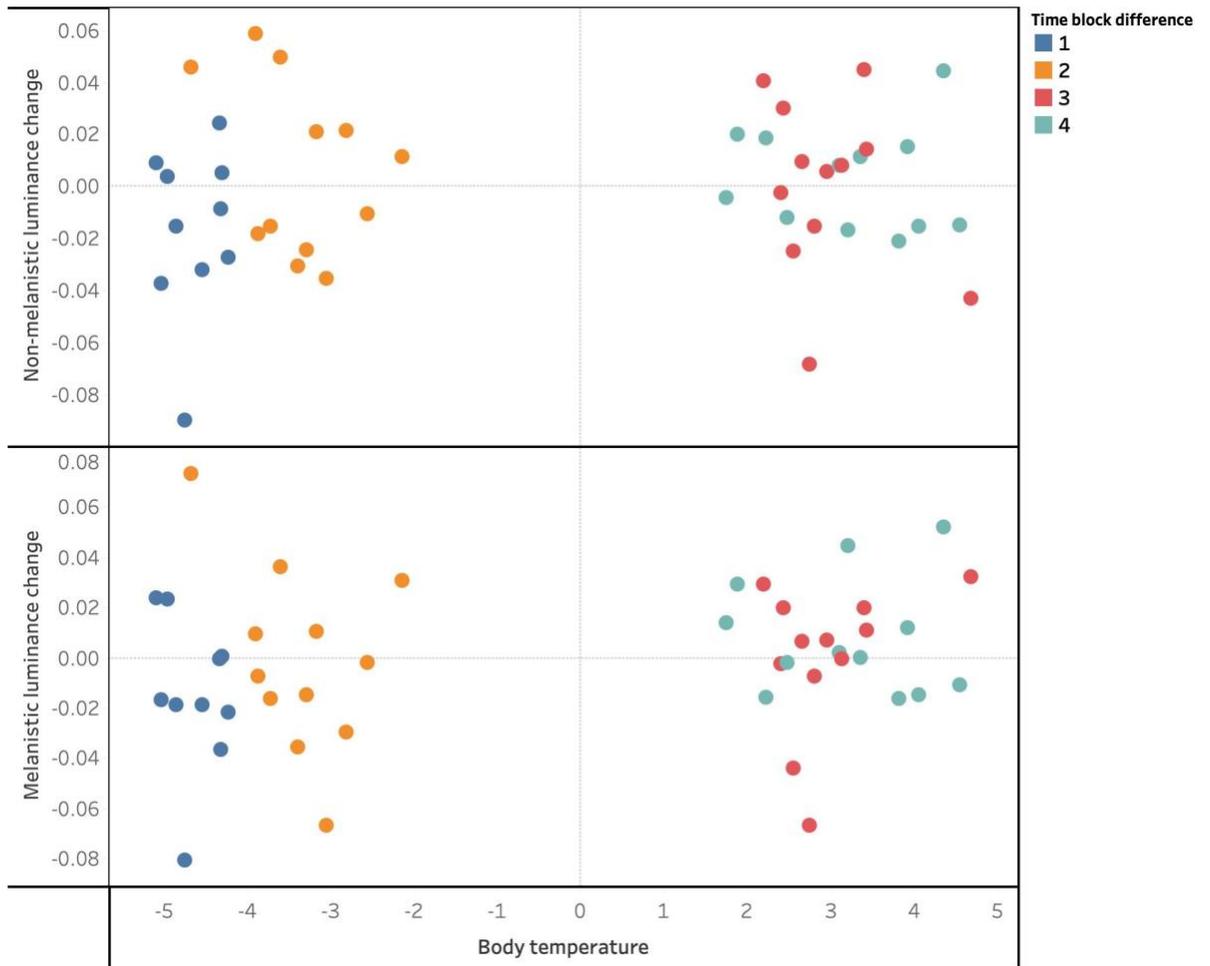
**Figure 2. Box plots of the individual body temperatures, temperatures within terrarium and environmental temperatures for each time block.** Temperature values for each individual were averaged and plotted to visualize temperature variations between environmental terrarium, and body temperatures in comparison to the native temperature range of *E. macularius* for each time block. Each box plot of different color corresponds to a different temperature measurement as indicated in the Figure legend.



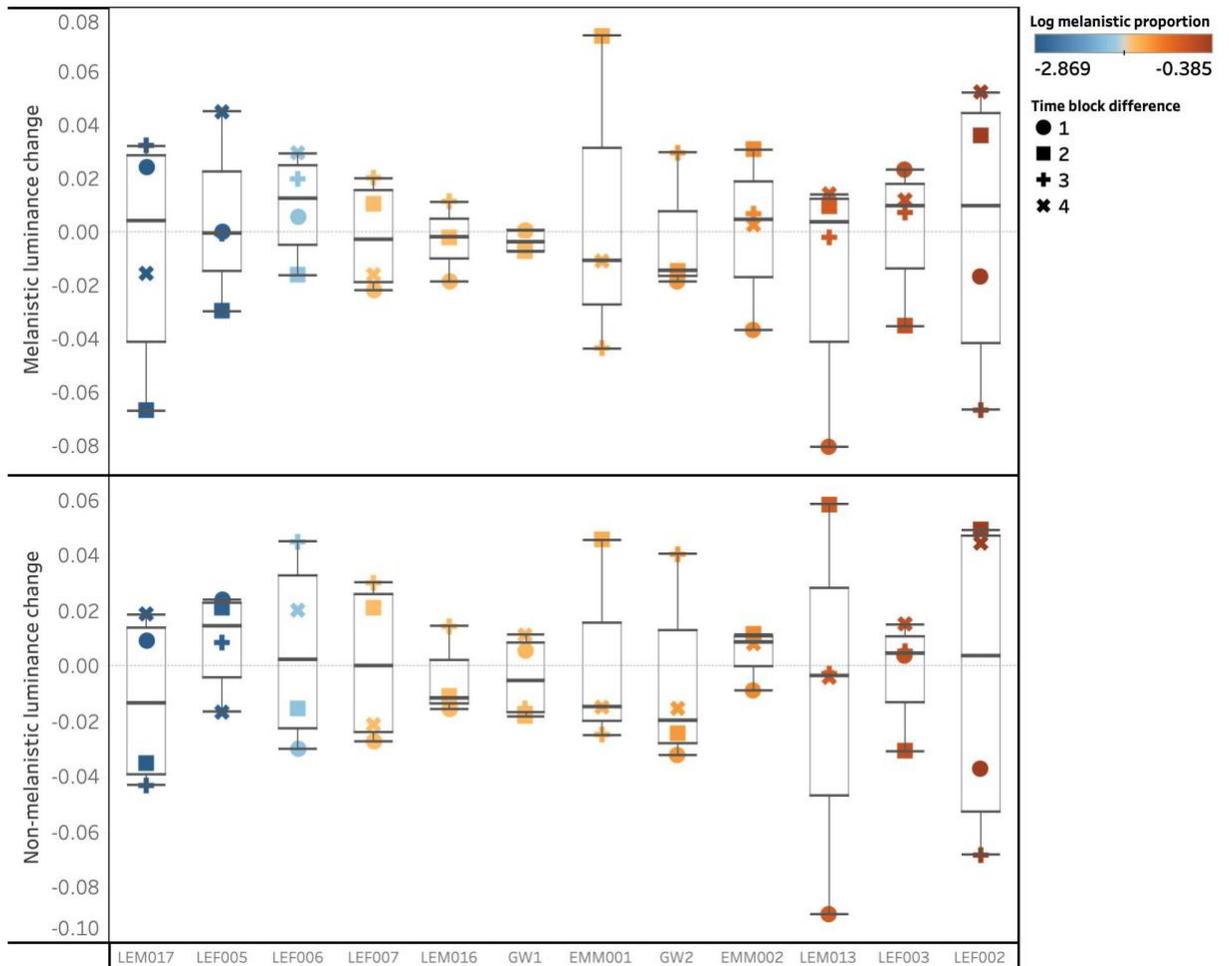
**Figure 3. Change in body temperature by melanistic proportion.** Average body temperatures across the experiment were taken for each individual. Melanistic proportion values were determined by the proportion of melanistic luminance relative to the entire dorsal area of the gecko, and are log transformed to visualize the values normally distributed. The range of melanistic proportion is color coordinated and increasing from left to right, with the lowest value having the lowest proportion of melanin. Each value within the histogram area represents the change in body temperature between time blocks, represented by shapes. If the proportion of melanistic coloration was to affect temperature changes, we would expect to see average temperature changes decreasing from left to right. Time block differences in the legend 1 corresponds to time block 2 – time block 1, etc. The codes on the x-axes represent the gecko IDs.



**Figure 4. Influence of melanistic proportion on heating and cooling rates.** Heating rates were calculated by taking the average body temperature difference between each time block for blocks 3, 4, and 5 per individual. Cooling rates were calculated using the same methods for time blocks 1, 2, and 3. Each dot represents an individual. Blue dots refer to the cooling phase, while orange dots to the heating phase.



**Figure 5. Change in average luminance by body temperature between time blocks.** Average luminance - calculated by weighted RGB values taken from images – and body temperature were calculated for each time block per individual. The difference of those averages was then taken between consecutive time blocks. Each color corresponds to the difference between time blocks. Time block difference 1 is time block 2- time block 1, etc. If results were consistent with our hypothesis regarding thermoregulatory color change, we would expect to see luminance values increase during low temperature changes and decrease during high temperature changes.



**Figure 6. Change in melanistic and non-melanistic luminance values by melanistic proportion across the experiment.** Luminance values were extracted from visible images and calculated by weighted RGB values. Melanistic proportion values were determined by the proportion of melanistic luminance relative to the entire dorsal area of the gecko, and are log transformed to visualize the values normally distributed. The range of melanistic proportion is color coordinated and increasing from left to right, with the lowest value having the lowest proportion of melanin. Each value within the histogram area represents the change in body temperature between time blocks, represented by shapes. Time block difference 1 corresponds to time block 2 – time block 1, etc. The codes on the x-axes represent the gecko IDs.

## REFERENCES

- Agarwal, I., Bauer, A. M., Gamble, T., Giri, V. B., Jablonski, D., Khandekar, A., Mohapatra, P. P., Masroor, R., Mishra, A., & Ramakrishnan, U. (2022). The evolutionary history of an accidental model organism, the leopard gecko *Eublepharis macularius* (Squamata: Eublepharidae). *Molecular Phylogenetics and Evolution*, *168*, 107414. <https://doi.org/10.1016/j.ympev.2022.107414>
- Allen, W. L., Moreno, N., Gamble, T., & Chiari, Y. (2020). Ecological, behavioral, and phylogenetic influences on the evolution of dorsal color pattern in geckos\*. *Evolution*, *74*(6), 1033–1047. <https://doi.org/10.1111/evo.13915>
- Angilletta, M. J., Montgomery, L. G., & Werner, Y. L. (1999). Temperature Preference in Geckos: Diel Variation in Juveniles and Adults. *Herpetologica*, *55*(2), 212–222.
- Aubret, F., & Shine, R. (2010). Thermal plasticity in young snakes: How will climate change affect the thermoregulatory tactics of ectotherms? *Journal of Experimental Biology*, *213*(2), 242–248. <https://doi.org/10.1242/jeb.035931>
- Barroso, F. M., Carretero, M. A., Silva, F., & Sannolo, M. (2016). Assessing the reliability of thermography to infer internal body temperatures of lizards. *Journal of Thermal Biology*, *62*, 90–96. <https://doi.org/10.1016/j.jtherbio.2016.10.004>
- Bauwens, D., Hertz, P. E., & Castilla, A. M. (1996). Thermoregulation in a Lacertid Lizard: The Relative Contributions of Distinct Behavioral Mechanisms. *Ecology*, *77*(6), 1818–1830. <https://doi.org/10.2307/2265786>

- Bradski, G. (2008). The OpenCV Library. *Dr. Dobb's Journal of Software Tools*.
- Chamberlain, S., Oldoni, D., Barve, V., Desmet, P., Geffert, L., Mcglinn, D., Ram, K.,  
 rOpenSci (<https://ropensci.org/>), Waller [aut, J., & cre. (2022). *rgbif: Interface to  
 the Global Biodiversity Information Facility API (3.7.3)*. [https://CRAN.R-  
 project.org/package=rgbif](https://CRAN.R-project.org/package=rgbif)
- Clusella Trullas, S., van Wyk, J. H., & Spotila, J. R. (2007). Thermal melanism in  
 ectotherms. *Journal of Thermal Biology*, 32(5), 235–245.  
<https://doi.org/10.1016/j.jtherbio.2007.01.013>
- Clusella-Trullas, S., Terblanche, J. S., Blackburn, T. M., & Chown, S. L. (2008). Testing  
 the thermal melanism hypothesis: A macrophysiological approach. *Functional  
 Ecology*, 22(2), 232–238. <https://doi.org/10.1111/j.1365-2435.2007.01377.x>
- Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak,  
 D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial  
 ectotherms across latitude. *Proceedings of the National Academy of Sciences*,  
 105(18), 6668–6672. <https://doi.org/10.1073/pnas.0709472105>
- Dunham, A. E., Grant, B. W., & Overall, K. L. (1989). Interfaces between Biophysical  
 and Physiological Ecology and the Population Ecology of Terrestrial Vertebrate  
 Ectotherms. *Physiological Zoology*, 62(2), 335–355.
- Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: New 1-km spatial resolution climate  
 surfaces for global land areas. *International Journal of Climatology*, 37(12),  
 4302–4315. <https://doi.org/10.1002/joc.5086>

- Forsman, A. (1995). Heating rates and body temperature variation in melanistic and zigzag *Vipera berus*: Does colour make a difference? *Annales Zoologici Fennici*, 32(4), 365–374.
- Garrick, D. (2008). Body surface temperature and length in relation to the thermal biology of lizards. *Bioscience Horizons: The International Journal of Student Research*, 1(2), 136–142. <https://doi.org/10.1093/biohorizons/hzn014>
- Gibson, R. A., & Falls, B. J. (1979). Thermal biology of the common garter snake *Thamnophis sirtalis* (L.): II. The effects of melanism. *Oecologia*, 43(1), 99–109. <https://doi.org/10.1007/BF00346675>
- Glimm, T., Kiskowski, M., Moreno, N., & Chiari, Y. (2021). Capturing and analyzing pattern diversity: An example using the melanistic spotted patterns of leopard geckos. *PeerJ*, 9, e11829. <https://doi.org/10.7717/peerj.11829>
- Hegna, R. H., Nokelainen, O., Hegna, J. R., & Mappes, J. (2013). To quiver or to shiver: Increased melanization benefits thermoregulation, but reduces warning signal efficacy in the wood tiger moth. *Proceedings of the Royal Society B: Biological Sciences*, 280(1755), 20122812. <https://doi.org/10.1098/rspb.2012.2812>
- Hijmans, R. J., Eten, J. van, Sumner, M., Cheng, J., Baston, D., Bevan, A., Bivand, R., Busetto, L., Canty, M., Fasoli, B., Forrest, D., Ghosh, A., Golicher, D., Gray, J., Greenberg, J. A., Hiemstra, P., Hingee, K., Ilich, A., Geosciences, I. for M. A., ... Wueest, R. (2022). *raster: Geographic Data Analysis and Modeling* (3.6-3). <https://CRAN.R-project.org/package=raster>

- Kearney, M., & Predavec, M. (2000). Do Nocturnal Ectotherms Thermoregulate? A Study of the Temperate Gecko *Christinus Marmoratus*. *Ecology*, *81*(11), 2984–2996. [https://doi.org/10.1890/0012-9658\(2000\)081\[2984:DNETAS\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2000)081[2984:DNETAS]2.0.CO;2)
- Kearney, M., Shine, R., & Porter, W. P. (2009). The potential for behavioral thermoregulation to buffer “cold-blooded” animals against climate warming. *Proceedings of the National Academy of Sciences*, *106*(10), 3835–3840. <https://doi.org/10.1073/pnas.0808913106>
- Khan, M. (2009). Leopard gecko *Eublepharis macularious* from Pakistan. *Reptilia*.
- Kingsolver, J. G. (1987). Evolution and Coadaptation of Thermoregulatory Behavior and Wing Pigmentation Pattern in Pierid Butterflies. *Evolution*, *41*(3), 472–490. <https://doi.org/10.1111/j.1558-5646.1987.tb05819.x>
- Kiskowski, M., Glimm, T., Moreno, N., Gamble, T., & Chiari, Y. (2019). Isolating and quantifying the role of developmental noise in generating phenotypic variation. *PLOS Computational Biology*, *15*(4), e1006943. <https://doi.org/10.1371/journal.pcbi.1006943>
- Lowe, C. H., & Norris, K. S. (1956). A Subspecies of the Lizard *Sceloporus undulatus* from the White Sands of New Mexico. *Herpetologica*, *12*(2), 125–127.
- Martínez-Freiría, F., Toyama, K. S., Freitas, I., & Kaliontzopoulou, A. (2020). Thermal melanism explains macroevolutionary variation of dorsal pigmentation in Eurasian vipers. *Scientific Reports*, *10*(1), Article 1. <https://doi.org/10.1038/s41598-020-72871-1>

- McNamara, M. E., Rossi, V., Slater, T. S., Rogers, C. S., Ducrest, A.-L., Dubey, S., & Roulin, A. (2021). Decoding the Evolution of Melanin in Vertebrates. *Trends in Ecology & Evolution*, *36*(5), 430–443. <https://doi.org/10.1016/j.tree.2020.12.012>
- Moreno Azócar, D. L., Perotti, M. G., Bonino, M. F., Schulte II, J. A., Abdala, C. S., & Cruz, F. B. (2015). Variation in body size and degree of melanism within a lizards clade: Is it driven by latitudinal and climatic gradients? *Journal of Zoology*, *295*(4), 243–253. <https://doi.org/10.1111/jzo.12193>
- Rummukainen, M. (2012). Changes in climate and weather extremes in the 21st century. *WIREs Climate Change*, *3*(2), 115–129. <https://doi.org/10.1002/wcc.160>
- Seebacher, F., & Franklin, C. E. (2005). Physiological mechanisms of thermoregulation in reptiles: A review. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, *175*(8), 533–541. <https://doi.org/10.1007/s00360-005-0007-1>
- Sherbrooke, W., Castrucci, A., & Hadley, M. (1994). Temperature Effects on in vitro Skin Darkening in the Mountain Spiny Lizard, *Sceloporus jarrovi*: A Thermoregulatory Adaptation? *Physiological Zoology*, *67*, 659–672. <https://doi.org/10.2307/30163763>
- Sherbrooke, W., & Frost, S. K. (1989). Integumental chromatophores of a color-change, thermoregulating lizard, *Phrynosoma modestum* (Iguanidae; Reptilia). *Am. Mus. Novit.*, *2943*, 1–14.
- Smith, K. R., Cadena, V., Endler, J. A., Kearney, M. R., Porter, W. P., & Stuart-Fox, D. (2016). Color Change for Thermoregulation versus Camouflage in Free-Ranging

Lizards. *The American Naturalist*, 188(6), 668–678.

<https://doi.org/10.1086/688765>

Smith, K. R., Cadena, V., Endler, J. A., Porter, W. P., Kearney, M. R., & Stuart-Fox, D. (2016). Colour change on different body regions provides thermal and signalling advantages in bearded dragon lizards. *Proceedings of the Royal Society B: Biological Sciences*, 283(1832), 20160626.

<https://doi.org/10.1098/rspb.2016.0626>

Szydłowski, P., Madej, J. P., Duda, M., Madej, J. A., Sikorska-Kopyłowicz, A., Chełmońska-Soyta, A., Ilnicka, L., & Duda, P. (2020). Iridophoroma associated with the Lemon Frost colour morph of the leopard gecko (*Eublepharis macularius*). *Scientific Reports*, 10(1), Article 1. <https://doi.org/10.1038/s41598-020-62828-9>

Tabh, J. K. R., Burness, G., Wearing, O. H., Tattersall, G. J., & Mastro Monaco, G. F. (2021). Infrared thermography as a technique to measure physiological stress in birds: Body region and image angle matter. *Physiological Reports*, 9(11), e14865. <https://doi.org/10.14814/phy2.14865>

Troscianko, J., & Stevens, M. (2015). Image calibration and analysis toolbox – a free software suite for objectively measuring reflectance, colour and pattern. *Methods in Ecology and Evolution*, 6(11), 1320–1331. <https://doi.org/10.1111/2041-210X.12439>

Vasseur, D. A., DeLong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., McCann, K. S., Savage, V., Tunney, T. D., & O'Connor, M. I. (2014). Increased temperature

variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society B: Biological Sciences*, 281(1779), 20132612.

<https://doi.org/10.1098/rspb.2013.2612>

Vroonen, J., Vervust, B., Fulgione, D., Maselli, V., & Van Damme, R. (2012).

Physiological colour change in the Moorish gecko, *Tarentola mauritanica* (Squamata: Gekkonidae): effects of background, light, and temperature.

*Biological Journal of the Linnean Society*, 107(1), 182–191.

<https://doi.org/10.1111/j.1095-8312.2012.01915.x>

Walton, B. M., & Bennett, A. F. (1993). Temperature-Dependent Color Change in

Kenyan Chameleons. *Physiological Zoology*, 66(2), 270–287.

<https://doi.org/10.1086/physzool.66.2.30163690>

Woods, H. A., Dillon, M. E., & Pincebourde, S. (2015). The roles of microclimatic

diversity and of behavior in mediating the responses of ectotherms to climate change. *Journal of Thermal Biology*, 54, 86–97.

<https://doi.org/10.1016/j.jtherbio.2014.10.002>

Zaidan III, F., & Wiebusch, P. L. (2007). Effects of temperature and illumination on

background matching in Mediterranean geckos (*Hemidactylus turcicus*). *Texas Journal of Science*, 59, 127–136.

Zizka, A., Silvestro, D., Andermann, T., Azevedo, J., Duarte Ritter, C., Edler, D., Farooq,

H., Herdean, A., Ariza, M., Scharn, R., Svantesson, S., Wengström, N., Zizka, V., & Antonelli, A. (2019). CoordinateCleaner: Standardized cleaning of occurrence

records from biological collection databases. *Methods in Ecology and Evolution*,  
10(5), 744–751. <https://doi.org/10.1111/2041-210X.13152>

## **BIOGRAPHY**

Bandon Hastings received his Bachelor of Science from Longwood University in the Spring of 2020, where he conducted research on avian and insect flight biomechanics and behavior. He then went on for a Master of Science at George Mason University, with a broad interest in animal behavior, coloration, and technologies to improve scientific research. Brandon currently works as a data analyst at Redhorse Corporation as part of a team assisting the US Air Force.