ABSTRACT

CHAPARRO, RAFAEL. Characterizing Metabolic Responses of *Eleutherodactylus* Frogs in Puerto Rico to Different Thermal Treatments: Implications for Conservation and Management. (Under the direction of Dr. Jaime A. Collazo)

Amphibians are vulnerable to extinction owing, partly, to altered physiological processes induced by projected global warming and drying. Understanding the mechanisms behind their responses is essential to formulate adaptation strategies for their conservation. Puerto Rico harbors 15 endemic *Eleutherodactylus* frogs considered vulnerable to extinction due to poor vagility and sensitivity to environmental variability. Herein I report the effects of four temperature treatments (15, 20, 25, and 30°C) on metabolic rates associated with specific dynamic action (SDA) and standard metabolic rates (SMR) of four representative species of *Eleutherodactylus* employing a respirometer. All species in either experiment increased their excretion of CO_2 with increasing temperature. CO_2 excretion rates were higher immediately post-ingestion, subsiding to low levels by the third day (72 hours). SMR excretion rates of *E. juanariveroi* and *E. antillensis* increased up to 20°C and then curbed. Rates of *E. coqui* increased linearly, whereas rates of *E. wightmanae* increased markedly from 20°C to 25°C, perishing at 30°C. *E. antillensis*, *E. wightmanae* and *E. juanariveroi* exhibited a change in metabolic rates between 20°C and 25°C, the same range where occupancy shifts from lower to higher probability for all species. Climate projections suggest that species will be exposed to 2-3 additional hours during evenings at ≥25°C below 300 m, and about 1 hour at 400-500 m. Species occurring in low elevations (≤400 m) may have to compensate for the additional energy expenditure induced by increased exposure and adjust their evening time budget. A continuing warming trend could begin to infringe on habitats of high elevation specialists like *E. wightmanae*.

Characterizing Metabolic Responses of *Eleutherodactylus* Frogs in Puerto Rico to Different Thermal Treatments: Implications for Conservation and Management.

By

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DEDICATION

I dedicate this work to my parents, Rafael and Ana, who have always supported and pushed me through my academic journey since I was a child. Thank you for fostering my curiosity and giving me the opportunity to explore my interest while supporting my dreams.

To my older brother, André, who I can call for advice at any hour of the day and can count on for support in all aspects of life.

To my Aunt Mildred, who supported my academic endeavors and has mentored me in many aspects of life since I was a child. Thank you so much for our long conversations that led me to jump in headfirst into a world that I knew nothing about.

To my Uncle Ruperto, for always taking me in at your home in Puerto Rico and treating me like a son. You are a true leader that leads by example, Puerto Rico is lucky to have you. Thank you for always being an excellent role model.

To my in-laws, Consuelo and Ferdinand, who have always challenged my thinking and pushed me to excel academically. Ferdinand, you became one of my best friends and I wish you were still here so that I could share this experience with you.

I would like to thank my entire family for the unwavering support that they have given me over the years.

Finally, I want to thank my beautiful wife, Ana Cecilia, for giving me a shoulder to lean on throughout this entire journey. I appreciate the long hours of work in the forest, swamp, laboratory, and office, without you I could not have achieved my goals.

BIOGRAPHY

I was born in Indianapolis, Indiana in 1990 and was raised in the nearby town of Martinsville. During my formative years, I spent much of my time venturing through the woods, cycling, skateboarding, and fishing. As my family is from Puerto Rico, my parents ensured that only Spanish was spoken within the household. This preservation of language and culture created a yearning within me for a place I had never actually lived in. Luckily, throughout my childhood, I frequently traveled to the island and typically spent Christmas and Summer vacations at my uncle's home in Rincon, PR. During these visits, my cousins and I would go the beach, go cycling, and play basketball. I often wished to stay there instead of returning to my Indiana home, which I adored just as much.

Upon graduating high school, I moved to Bloomington, Indiana to attend Indiana University. My free time there was spent playing ultimate frisbee, rock climbing, and exploring the vast forests near the beautiful campus. Since my immediate family had all worked in either pharmaceutical production or medicine, I chose to pursue a degree in Health Administration and Management at what was then known as the School of Public and Environmental Affairs. After completing my Bachelor’s degree in 2014 I decided to move to Puerto Rico to perfect my Spanish and to get to know the island that I loved so much better.

In Puerto Rico I developed an adoration for exploring the mountains and forests of the island. In 2015 I was given the opportunity to work as a field research technician by Sara Prado, a PhD student of my advisor and friend, Dr. Jaime Collazo. Even though I hadn't studied applied ecology, I felt that fieldwork was a perfect fit for my outdoor lifestyle and interests. From that pollination project with bees, I moved on to work with birds and frogs. Through these projects, I

developed a deep appreciation for applied ecology and ecophysiology, which eventually led me to my master's project.

AKNOWLEDGEMENTS

This work was supported by grants from USGS Southeast Climate Adaptation Science Center, USFWS Caribbean Ecological Services Office, and USGS NC Cooperative Research Unit. To begin with, I would like to express my gratitude towards both of my advisors, Dr. Jaime Collazo and Dr. Eloy Martinez. Jaime has been an excellent boss, advisor, and mentor, I consider him family. He pushed me to get my Master's degree after we had been working together for 3 years. I would like to thank him for his willingness to always help me learn and progress, without him I do not know where I would be. Without Eloy's expertise and positive attitude, my ecophysiology project would not have come to fruition. He has been the backbone of the project and has taken me under his wing since 2017, introducing me to the fascinating multidisciplinary field of ecophysiology. Working with him has been a pleasure due to our shared interests and experiences, I consider him family and look forward to working alongside him in the future.

I would like to to express my gratitude to several individuals who have also contributed significantly to the successful completion of my Master's degree. First, I would like to thank Dr. Alberto Puente Rolón for his unwavering support and assistance during field collections. During the sweltering heat and rainy conditions, we worked through in the swamps, his excellent attitude and assistance gave me an invaluable learning experience. I am eagerly anticipating future collaborations with him.

Next, I owe a debt of gratitude to Dr. Adam Terando, who has been an exceptional teacher and role model, always demonstrating an insatiable thirst for knowledge coupled with critical thinking skills. Our discussions during site scouting in the field have been perspective changing.

Furthermore, I am indebted to Mitch Eaton for his invaluable advice and encouragement to continue learning. His support has been instrumental in my development as a professional.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDIX LIST

INTRODUCTION

Amphibians are the taxonomic group most vulnerable to extinction worldwide. The current rate of decline exceeds background rates of extinction by 1000-fold (Pimm et al. 2014). In addition, IUCN listing of amphibians as threatened or endangered is far greater than any other vertebrate taxon (Sn et al. 2004, "The IUCN Red List of Threatened Species" n.d.). As such, amphibians are considered the *canary in the coal mine*, particularly when it comes to predicting how rising global temperatures and changes in the hydrology of tropical systems will affect ectothermic organisms. Global warming is of particular concern in the tropics because almost one half of global amphibian diversity occurs in this region (Heinicke et al. 2007, Hawley Matlaga et al. 2021), and many tropical ectotherms already experience temperatures near their upper thermal limits (Deutsch et al. 2008, Hillman et al. 2009, Scheffers et al. 2012, Bestion et al. 2015, Nowakowski et al. 2016). Current approaches to integrate both ecological effectors and physiological limits of ectotherms through ecophysiological studies are a critical component to assess the 'winners' and losers' of the thermal game of global warming (Somero, 2010)

An important goal in ecophysiology is to anticipate how Earth's biodiversity will respond to changing thermal regimes (Greenberg and Palen 2021). This is essential to understand their influence on various evolutionary, ecological, and behavioral traits (Huey and Stevenson 1979, Homyack et al. 2010). The need for this understanding has gained impetus as climatological models project increasing warming and drying trends for many regions in the world (Neelin et al. 2006, Bowden et al. 2021, Delgado-Suazo and Burrowes 2022). Changes in temperature and water availability represent selective pressures that are driving patterns of amphibian population declines across geographical areas and taxonomic groups (Karmalkar et al. 2013, Burrowes et al.

2021, Delgado-Suazo and Burrowes 2022). Habitat desiccation affects not only the water balance of adult individuals, but largely influence highly vulnerable early life stages, affecting species displaying either direct or indirect development (Townsend and Stewart 1986, Denver et al. 1998, Morey and Reznick 2004). It is expected that species' extinction risks under future climate conditions will be shaped in part by demographic, physiological, and ecological traits (Greenberg and Palen 2021). Therefore, there is a growing interest in comprehending the metabolic and physiological reactions of amphibians to varying temperatures as it is central to conservation because they influence biological processes scaled up to patterns of species abundance, distribution, and key to formulate appropriate adaptation strategies to help amphibians cope with projected changes (McDonald-Madden et al. 2011, Morelli et al. 2016, Burrowes et al. 2021).

Temperature has a significant impact on nearly all aspects of amphibian physiology, including processes involved in sensory, locomotor, and biosynthetic pathways. All these pathways are reflected in the energy demands (i.e. metabolism) and supply (i.e assimilated energy after digestion), thus comprising critical processes for their energy budgets (Huey and Stevenson 1979, Secor 2001, Hillman et al. 2009, Timpone et al. 2020). Generally, amphibians experience an acute increase in metabolic rate with rising temperatures, which is the net result of increased substrate utilization and energy expenditure in the absence of short-term compensatory strategies such as behavioral thermoregulation (Hillman et al. 2009, Schulte 2015, Andrade 2016). Despite this generalization, the response of amphibians to changes in temperature over time is rather complex, and based on the available literature, taxon and species-specific (Andrade, 2016). When species are faced with increasing temperature over longer time spans, biochemical pathways are restructured to reduce metabolic demands, with impacts into substrate

type (e.g. carbohydrates, lipids, proteins) utilization and altering energy balance of amphibians, where most of the assimilated energy is often utilized to fuel the biochemical 'maintenance' of the individual (Schulte 2015). Consequently, this trade-off could influence growth or other energy budget components that contribute to an individual's fitness (Baškiera and Gvoždík 2020). Drying conditions also impinge on amphibian's life history traits, and water availability has been shown to be more influential to individual survival than temperature (Greenberg and Palen 2021). When rainfall patterns shift to a drier regime, the chance of amphibian eggs, larvae, and adults impacted by dehydration increases, with a myriad of ecological and physiological implications. For example, water deficit can impact their interactions with predators, as well as their ability to regulate water levels, potentially leading to a decrease in their population over time. When a frog's body tissues become dehydrated, its resting metabolic rate increases, and its maximum rate of aerobic metabolism decreases (Pough et al. 1983), thus strongly affecting its metabolic scope. Recent research has found that exposure to desiccation in the more arid *Xenopus laevis* influences the regulatory pathways for critical genes involved in neuroprotection (Luu and Storey 2015) and bioenergetic pathways (Hawkins and Storey 2020), indicating a complex molecular response to anuran desiccation. Meanwhile, in tropical Eleutherodactylids, Burrowes et al. (2009) observed that droughts influence the behavior and activity patterns of *E. coqui*, leading to juvenile mortality after three days without rain, and adult males ceasing to call and remaining in their retreat sites after five days without water. These studies highlight the significant water-dependent changes in the biochemical and behavioral response of anurans, although the combined effect of environmental temperature and water availability on metabolic demands remains unresolved for this genus.

In addition to water availability, amphibians are highly influenced by the spatial and temporal heterogeneity of the thermal profile within their habitats. As other ectotherms, they primarily rely on external heat sources and behavioral adjustments to regulate their body temperature (Hillman et al. 2009, Timpone et al. 2020). The physiology of tropical anurans is reflective of their high biodiversity, differentiated by cold-adapted specialists commonly found in cloud forests while generalists are distributed across thermally heterogeneous forests along elevation gradients and vegetative grounds (Navas et al. 2013). Despite the adaptability of some species, most tropical anurans have highly specialized thermal niches, making them vulnerable to gradual warming trends (Navas 1996, Barker and Ríos-Franceschi 2014). For this reason, relatively small changes to their thermal environment could lead to a supraoptimal environment, which may negatively affect the survival of individuals or the persistence of the species in the habitat (Clusella-Trullas et al. 2021, Anderson et al. 2022). Previous studies have demonstrated that some tropical anurans operate with a reduced warming tolerance (i.e. difference between habitat temperature and the critical thermal threshold for the species), and may not withstand temperatures above the mean values during the wet season (Delgado-Suazo and Burrowes 2022). Moreover, long-term sub-lethal effects including metabolic demands under suboptimal and supraoptimal temperatures, remain understudied in amphibians (Somero et al. 1996, Gouveia et al. 2014, Hawley Matlaga et al. 2021).

While most studies on anuran metabolism have focused on temperate species, tropical anurans and ectotherms, in general, have been underrepresented in the scientific literature (Alves-Ferreira et al. 2022). The underrepresentation is alarming because the tropics support nearly half of amphibian species globally (Heinicke et al. 2007). This includes the Caribbean archipelago, which harbors ~200 species of amphibians, of which the *Eleutherodactylus* genus

comprise 164 species characterized by high endemism (Hedges et al. 2019). Puerto Rico has 25 species of amphibians, 17 of those belonging to the genus *Eleutherodactylus* (Joglar et al. 2011). Of these, 3 are listed as endangered (*E. jasperi, E. juanariveroi, E. cooki*), and the remaining 12 are considered at risk of becoming endangered ("The IUCN Red List of Threatened Species" n.d.). Their conservation is justified because they represent a diverse suite of endemic species, are bioindicators of environmental change, and because their total number and biomass affect ecosystem function through complex trophic interactions (Dodd and Seigel 1991, Rohman et al. 2020). 2020). For example, it is estimated that an average of 20,000 individuals ha^{-1} of *E. coqui* occur in El Yunque National Forest, and they can consume up to 114,000 prey ha^{-1} $evening^{-1}$ (Beard 2007, Harmon 2018). Their presence in forest ecosystems thus represents a significant contribution to nutrient cycling for lower trophic levels (Beard 2002). It is also noteworthy that the common coqui (*E. coqui*) and all *Eleutherodactylus* frogs hold tremendous cultural value for the inhabitants of Puerto Rico (Joglar 1998).

The vulnerability of *Eleutherodactylus* species in Puerto Rico to environmental change is rooted in their low vagility, high philopatry, and sensitivity to environmental variability, particularly to extremes in temperature and humidity (Joglar and Burrowes, 1996; Ríos-López et al. 2023). Available climatological models forecast a warming, drying trend for Puerto Rico (Khalyani et al. 2016, Bowden et al. 2021), underscoring the importance of understanding the effects of environmental climate change on the physiological performance of vulnerable species. Previous work on the effects of temperature on *Eleutherodactylus* frogs has focused on thermal limits (Christian et al. 1988, Rivera-Burgos 2019, Delgado-Suazo and Burrowes 2022), and these limits define the ambient conditions where frogs will fail to meet self-maintenance functions (Vieira de Andrade 2016). However, less apparent, but equally important are the sub-lethal

effects of changing environmental conditions on metabolic rates and energy budgets (Blaustein 2010). For example, in a recent work with *E. coqui* at El Yunque National Forest, Hawley-Matlaga et al. (2021) reported that common coquis increased their locomotor activity in response to a 4°C increase in environmental temperature, linked to behavioral thermoregulation as individual searches for thermal refugia. One tradeoff of this response is that individuals might be at a greater risk of predation. Greater mobility also carries with it an additional expenditure of energy and a question of interest is whether individuals reallocate (i.e. redirect) energy to address the increased mobility at the expense of other functions and processes such as reproduction (Secor 2009).

Arguably, the vulnerability of *Eleutherodactylus* frogs species depend on the thermal plasticity of biochemical adjustment, which is ultimately observed in their physiology. The relationship between an ectotherm's metabolic demands and body temperature can be assessed through the standard metabolic rate (SMR), which can be assessed directly via calorimetry or indirectly using respiratory gases. In similar way, the energetic costs associated with the food handling, digestion and nutrient absorption termed the specific dynamic action (SDA), can also be measured using direct or indirect methods. These metrics are essential for determining an organism's fitness under different thermal conditions, and are therefore considered a valuable tool for this purpose (Ríos-Franceschi et al. 2019). SDA is described as the increase in metabolism after a meal, including the combined costs of eating, digesting, absorbing, and assimilating the food (Secor 2009, Hillman et al. 2009). SMR is described as the energy budget or mandatory energy cost of maintaining basic life functions, measured in a post-absorptive state (Hillman et al. 2009).

In this study, I broaden the understanding of how *Eleutherodactylus* frogs perform physiologically at sub-lethal temperatures in Puerto Rico by addressing two objectives. First, characterize the effects of temperature on the metabolic rate associated with SDA for *E. coqui* and *E. antillensis*, and second, characterize the thermal performance curve (TPC) of SMR for *E. coqui, E. antillensis, E. wightmanae,* and *E. juanariveroi*. To address these objectives, I employed high-resolution respirometry (i.e. indirect calorimetry) to measure ppm-level emission of CO_2, corresponding to the metabolic rate of frogs exposed to one of four treatment temperatures (i.e., 15, 20, 25 and 30 °C). High resolution (ppm-level sensitivity), high throughput (repeated measurements and multiplexed chamber capabilities) respirometry settings are considered the next generation of field metabolic measurements, as the equipment allows continuous measurements of respiratory gases from small (less than 1g) individuals, and is sensitive enough to configure a flow through measurement configuration. Flow through respirometry is considered ideal (compared to closed system respirometry), as the partial pressure of respiratory gases are kept constant during non-sampling periods. My study expands the body of knowledge about *Eleutherodactylus* ecophysiology in two important ways. First, it quantifies metabolic responses of *Eleutherodactylus* species to varying temperatures, thereby; providing a basis to a create thermal performance curves (Angilletta 2009, Tattersall et al. 2012, Andrade 2016, Greenberg and Palen 2021, Seebacher and Little 2021, Kefford et al. 2022) for each species, to make inferences about potential triggers and impacts on their energy budgets under future conditions. Second, it will help guide efforts to identify climate refuges and inform decisions about managed translocations to supplement extant populations or reintroduce species to new, suitable locations (Morelli et al. 2016, Burrowes et al. 2020; McDonald-Madden et al. 2011). In these two contexts, I discuss the ecophysiological insights gained from my work, and I

identify next steps to strengthen the application of physiological work to adaptation science. I also discuss conservation implications of findings using downscaled climate projections contrasting two time periods (1985-2005 and 2040-2060; Bowden et al. 2021), combined with findings from field studies quantifying the relationships between occupancy and environmental factors (Rivera-Burgos et al. 2021).

HYPOTHESES

Observation: Digestive, absorptive and biosynthetic processes are complex biochemical pathways, which are temperature dependent. Based on this observation, I hypothesize that

- Metabolic demands associated with SDA will increase with temperature.
- Both species will display a shorter time in return to baseline metabolic rates with increasing temperatures.

Observation: High elevation specialists (e.g., *E. wightmanae*) occur in a narrower range of environmental conditions, compared to widely distributed species (e.g., *E. coqui*, *E. antillensis*; (e.g., *E. coqui*, *E. antillensis*; Joglar 1998, Ríos López and Heatwole 2023, Rivera-Burgos et al. 2021). Based on this observation, I hypothesize that

- Metabolic demands will increase with temperature for all species,
- TPC for *E. wightmanae* will be more sensitive, compared to the more generalists *E. coqui*, *E. antillensis*.

STUDY AREA AND METHODS

Focal species

For this study, I selected *E. coqui*, *E. antillensis*, *E. wightmanae*, and E. *juanariveroi* to yield valuable insights about metabolic performance. Their varied occurrence patterns across

elevations, contrasting habitat associations, and body sizes (e.g., body mass) make them ideal for this purpose. *E. wightmanae* represents eight other species (*E. portoricensis*, *E. richmondi*, *E. gryllus*, *E. hedriki*, *E. eneidae*, *E. jasperi*, *E. locustus*, and *E. unicolor*) known as high elevation specialists that inhabit areas characterized by low temperatures and high humidity (Rivero 1976; Joglar 1998). *E. coqui* is a widespread species but is most commonly found in higher, cooler environments (Beuchat et al. 1984, Rivera-Burgos 2019). In contrast, *E. antillensis* is typically associated with lower elevation areas that have higher air temperatures and lower relative humidity. Lastly, *E. juanariveroi*, an endangered species, represents lowland species associated with freshwater swamps (Ríos-López et al. 2014).

Specimen Collection

I maintained consistent collection sites throughout the study (Table 1). I collected *E. antillensis* and *E. coqui* specimens at 547m in Las Marias, *E. wightmanae* specimens at 890m in Maricao, and *E. juanariveroi* specimens at 0-5m in Arecibo (location not shared, contact USFWS; Figure 1). Using a pocket weather meter (Kestrel® 3500 Pocket Weather Meter; accuracy = ±1 °C, ±3%, respectively), I recorded the relative humidity and ambient temperature at collection sites. I also recorded the in-situ temperature immediately after collection using a Fluke 62 Max Infrared Thermometer, -30-500; ± 1.5°C. *In-situ* temperature is defined as the temperature at the structure or substrate where frogs were located at capture, and given that focal species have a high surface to volume ratio, in-situ temperature is viewed as a better approximation of the conditions frogs are exposed to in the field. I collected individuals by hand, placed them in 16oz polypropylene food-grade cups with sterile napkins and dechlorinated water, and transported all specimens to the NCSU field lab in Las Marias (18.21049, -67.00166) following IACUC approved protocol # 19-028-O.

Laboratory protocols

I placed the specimens in 15L containers and held them at ambient temperatures for a minimum of 2 days to reduce handling stress and tend to specimens showing signs of illness or abnormal behavior. I lined each container with sterile napkins misted with dechlorinated water, and cleaned them daily to avoid waste buildup. I kept the frogs in the laboratory for 2 to 4 days to adapt to their housing before experiments, adjusting the acclimation period based on whether they accepted food (commercially-available small crickets) or not. For both SMR and SDA experiments, I subjected all specimens to a total 48 hour fasting period prior to the treatment protocols.

Treatment protocols

Standard Metabolic Rate Experiments (SMR)

I divided twenty-eight (28) specimens for each of the four focal *Eleutherodactylus* species into four groups of seven individuals each. Then, I exposed each experimental group to four temperature treatments, namely, 15, 20, 25, and 30°C and monitored metabolic gases under saturated water vapor conditions for 24 hours for each treatment. The temperature treatments represented a range that started with temperatures close to what I recorded in *E. coqui* daytime refuges (i.e., lowest 17.5°C in bromeliads) up to 30°C, which is the closest to CT_{Max} temperatures allowed by the IACUC. After the 48 hour fasting period, I weighed all specimens of each species to the nearest milligram using a top loading balance (VWR-123P). Next, I placed individual specimens into individual 30mL modified syringes (serving as respirometry chambers) and acclimated them for an hour before starting the experiment. Experiments were initiated between 1800-2100 hours.

Specific Dynamic Action Experiments (SDA)

I exposed frogs to the same four temperature treatments and measured the metabolic rate associated with the SDA for a period of 72 hours. To ensure accuracy, I only used species with high CT_{Max} values, specifically *E. coqui* and *E. antillensis* (Rivera-Burgos et al. 2019). To prepare for the experiments, I acclimated the frogs to laboratory conditions and weighed them after a 48-hour fasting period. I then placed each specimen into an individual 15L container with live crickets (following Secor 2009 guidelines) and allowed them to feed for an hour. After measuring the ingested ration's fresh weight by re-weighing the individuals, I transferred them to 30mL modified syringes (used as respirometry chambers) and allowed them to acclimate for another hour before beginning the trials. Experiments were initiated between 1800-2100 hours. Throughout the entire 72-hour period, I monitored the respiratory gases as previously described. As the specimens were not physically constrained within the chamber, all metabolic rate measurements are regarded as routine metabolic rates.

Respirometry equipment setup and data acquisition

As noted above, each experimental group was exposed to four temperature treatments. Every experimental run, included an empty chamber for single baselining, along with the 7 specimens per treatment, held individually in 30mL sealed chambers, (i.e. one chamber per channel; Sable Systems MF-8). Multiplexers were controlled by the FMS using a universal interface (Sable Systems; UI), and the commands to the multiplexers corresponded to control codes that I prepared to sample selected chambers at specific time intervals, using Sable System's ExpeData data acquisition software.

Sub-sampling was controlled by the FM-8 unit, upon the command of the ExpeData software. Basically, the air circuit of a selected chamber shifted from the MFS to the FMS air

circuit for gas analysis (Figure 2). The FMS air circuit is produced by the action of a built-in centrifugal pump in the FMS. Air supplied to the FMS was CO_2 and H_2O free, as it moved towards the air pump, the flow control needle valve and the flow meter. The metered air stream was then re-humidified, using a customized dew point generator, saturating the incoming air of water vapor 2-3°C below the assay temperature. Moisturized air was directed to the selected chamber, and respiratory gases from this chamber were routed to the gas analyzers.

Gas analysis involved the sequential measurement of water vapor pressure, CO_2 and O_2, via independent sensors connected in series. First, the excurrent bolus of air was routed to the WVP sensor, then water vapor was removed from the airstream after measurement, using a column of magnesium perchlorate. The water-free air stream is then routed to a CO_2 sensor, then CO_2 is removed from the airstream with a column of ascarite, followed by a column of magnesium perchlorate. Dry, CO_2-free air is then routed to the O_2 sensor, which quantifies the percent composition of O_2 in the sample, and the excurrent air leaves the FMS and is released to the atmosphere. This process continues, as ExpeData selects each of the chambers in an automated fashion. All air lines are furnished with 1/8" ID inert tubing with polyethylene liner (Cole Palmer Bev-a-Line), to route the air to and from the specimen chambers and sensors. Each specimen chamber sampled for a total 10 min for SDA every 1.5 hours and 15 minutes for SMR every 2.25 hours. The baseline chamber was sampled twice for 10 and 15 min respectively; at the beginning and end of each 1.5 and 2.25-hour interval, to allow compensation for baseline drift. Each complete sampling bout was automatically recorded and stored in ExpeData. Upon completion, the automated sampling system repeated the process for a period of 24 hours for SMR and 72 hours for SDA.

Unselected chambers were provided of an incoming bolus of air at a rate of 30mL min^{-1}, by connecting a mass flow generator (Sable Systems, MFS) to the purge inlet of each MF-8. This flow circuit ensured that unselected chambers were not CO_2 enriched or O_2 depleted during the waiting period of the experiment. The complete equipment setup described above was enclosed to create a temperature-controlled environment by means of an independent air conditioning unit (Martinez and Agosta 2016), allowing the calibration and experimental trials to take place at a stable, but programmable temperature regime.

Data processing

The SDA and SMR experiments utilized an ExpeData Macro Utility to convert sampled gases from percent composition to molar concentration, as previously described (Lighton 2008). This was accomplished using the following equations:

Water vapor $VH_2O = FR*(FeH_2O-FiH_2O)/ (1-FeH_2O)$

To calculate the rate of water vapor exchange, where VH_2O represents the rate of water vapor exchange, FR is the flow rate of gas, FeH_2O is the fraction of water vapor in the exhaled gas (Fe is fractional excurrent), and FiH_2O is the fraction of water vapor in the inhaled gas (Fi is fractional incurrent).

Carbon dioxide $VCO_2 = FR*(FeCO_2-FiCO_2)/(1-FeCO_2)$

To calculate the rate of carbon dioxide exchange, where VCO_2 represents the rate of carbon dioxide exchange, FR is the flow rate of gas, $FeCO_2$ is the fraction of carbon dioxide in the exhaled gas, and $FiCO_2$ is the fraction of carbon dioxide in the inhaled gas.

Oxygen $VO_2 = FR^* (FiO_2-FeO_2)/(1-FeO_2)$

To calculate the rate of oxygen exchange, where VO_2 represents the rate of oxygen exchange, FR is the flow rate of gas, FiO_2 is the fraction of oxygen in the inhaled gas, and FeO_2 is the fraction of oxygen in the exhaled gas.

The equations account for fluctuations in the flow rate (FR) and the fractional composition of gases (incurrent or excurrent, in relation to the chamber) into the baseline chamber and each specimen chamber. The FiH_2O, $FiCO_2$, and FiO_2 values were obtained from the baseline chamber, while the FeH_2O, $FeCO_2$, and FeO_2 values correspond to the flow of gases from the specimen's chamber. As the specimens required incoming air to be saturated with water vapor, WVP measurements were not further processed for analysis. Instead, CO_2 values were used as the proxy for metabolic activity, as the sensor type (laser beam) is significantly more thermally stable than the O_2 sensor (paramagnetic O_2 cell) in the FMS. To express the most accurate metabolic rates, all the data presented is based on CO_2 emissions.

Data extraction

To process the data using the macro utility, there are several steps that need to be taken when using Expedata, the proprietary software that accompanies Sable Systems respirometry equipment. The O_2 channel needs to be made active, then the starting O_2 levels need to be spanned to 0.2095, which is the fraction of O_2 in the dry CO_2 free air. After these steps the O_2 channel can be renamed to FeO_2. The next step is to create the delta O_2 channel by copying the O_2 channel to a new channel and changing the name. After making the delta O_2 channel active, use the drift correction function with a zero baseline to make this channel (FeO_2-FiO_2). Then, multiply by -1 to make the deltaO_2 channel (FiO_2-FeO_2). Next, activate the CO_2 channel and

align the CO_2 levels with the markers. After this, divide by 100 to convert $\%CO_2$ to $FeCO_2$. Again, copy the CO_2 channel to a new channel and name it delta CO_2. Next make deltaCO_2 the active channel and use a drift correction with a zero baseline to make the values in this channel ($FeCO_2$-$FiCO_2$). Proximate make the barometric pressure the active channel, use transform, corrections smooth to reduce the noise in the BP signal by averaging signal every 9 seconds, and repeat this process 2 consecutive times in the trace. Then, make the WVP the active channel and use transform, corrections, lag to make the WVP signal line up with the markers. Use the General Transform to create a new channel called FeH_2O, which is simply WVP/BP. Copy the FeH_2O channel to a new channel and name it deltaH_2O. Use transform corrections, drift correction, with a zero baseline to make the deltaH_2O channel (FeH_2O-FiH_2O). The flow rate (FR) signal was smoothed in the same fashion as described previously for BP. At this point, everything is in place to calculate VO_2, VCO_2, and VH_2O. To do this, use the General Transform to create three new channels:

- VO_2 = FR*deltaO_2/(1-FeO_2)
- VCO_2 = FR*deltaCO_2/(1-$FeCO_2$)
- VH_2O = FR*deltaH_2O/(1-FeH_2O)

Once these steps are completed, save a modified file with the calculations.

DATA ANALYSIS

I analyzed data from the SMR and SDA experiments using a split-plot ANOVA, a univariate analytical framework suitable for repeated measures data. The main plot are the temperature treatments assigned to 7 individual frogs within each treatment (sub-plots). For SMR analyses, I pooled data for *E. antillensis, E. coqui,* and *E. juanariveroi* because I wanted to determine if there were interspecific differences in responses. The response variable in the

model was mass specific VCO_2 (uL/min). We used a mass-adjusted response variable because there was a positive relationship between excretion of VCO_2 and body mass (Repeated measures ANCOVA, $P < 0.05$). Model terms were: species, assay temperature (hereafter referred to as treatment), and individuals within species and treatment chamber was a random effects term. The model included a "time" term that accounted for the fact that data (i.e., excretion of CO_2) represented a repeated measure (i.e., measurements were taken from 2 to 24 hours). Models included relevant interactions (e.g., species*assay temperature, species*time, species*assay temperature*time). I analyzed *E. wightmanae* data separately because specimens perished at 30C, causing an unbalanced design if kept together with the others species in a model. The model for *E. wightmanae* was similar to the one described above, but without the species term, but only for 15, 20 and 25°C temperature treatments. For SDA analyses, I pooled data for *E. antillensis* and *E. coqui*, with repeated measurements taken from 0 to 72 hours. I ran F tests (contrasts) to compare levels for nominal and ordinal effects and their interactions. For SDA experiments, I compared VCO_2 (mass adjusted) from the first 24 hours (0-24) to the last 24 hours (48-72) using t-tests. Data on mass specific VCO_2 per treatments met homogeneity of variance assumption (O'Brien tests, $p > 0.05$). All results are reported as means (±SE).

I conducted an additional analysis to determine the point at which rates of CO_2 excretion changed as a function of treatment temperature for *E. antillensis* and *E. juanariveroi*. I used the LSmean values of CO_2 emission per treatment and fitted a quadratic regression model. To find the inflection point (Arrhenius breakpoint), I employed a piecewise regression as described by Nickerson et al. (1989). The response variables for the piecewise regression were the LSmean values of CO_2 excretion (single species) and predicted values of CO_2 excretion from the quadratic model. The independent variable was the treatment temperatures (e.g., 15, 20, 25, and

30°C) converted to absolute temperature (Kelvin = °C+273), expressed as 1000/K. In Arrhenius breakpoint analysis, respiration rates are measured at various temperatures and then plotted against the reciprocal of the absolute temperature (1/T) (Farrell 2016). This generates a curve that can be used to determine the breakpoint temperature, which is the temperature at which the rate of respiration suddenly increases or decreases rapidly (Tattersall et al. 2012, Farrell 2016).

Finally, I integrated previous field data on occupancy from Rivera-Burgos et al. (2021) and climate projections to gain insights on the possible conservation implications of my work. First, I plotted occupancy probability values along two environmental/physical factors, that is, *in-situ* temperature (°C) and elevation (m). Then I overlaid the SMR results to relate to metabolic performance of *E. coqui*, *E. wightmanae*, and *E. antillensis*, and linear/non-linear fit through the SMR points. For climate projections, I used Bowden et al. (2021), who developed high resolution, dynamically downscaled simulations of output from two Global Climate Models (GCMs) for the US Caribbean. The projection period is 2040-2060 for a high greenhouse gas emissions scenario (RCP 8.5). The resulting projections of daily maximum and minimum daily temperature, developed at a 2km resolution, were then bias-corrected using a quantile regression model fit to long-term observations for each month and GCM. Although the dynamically downscaled output is available on an hourly time step, observed hourly data are sparse (spatially and temporally) on the island and not available for the bias-correction procedure. Therefore, the average diurnal temperature cycle was estimated from the available hourly weather station data from the US Department of Agriculture SCAN network (USDA Natural Resource Conservation Service, 2022). The average diurnal cycle at the closest station to each grid cell was then applied to the bias-corrected modeled temperature data to estimate the hourly projected temperature.

RESULTS

Environmental parameters (e.g., relative humidity, air temperatures at 1 m) measured at all collection sites (>500 m) are listed in Tables 2 and 3. Air temperatures ranged from 22.10°C to 25.80°C. *In-situ* temperatures ranged from an average of 18.31°C (0.17) to 23.79° (0.25). The average body mass for *E. wightmanae* was 0.67 ± 0.005g, and 0.29 ± 0.003g for *E. juanariveroi*, both used for SMR experiments. Body mass of *E. coqui* and *E. antillesis* used in SDA experiments was 2.15 ± 0.04g and 1.32 ± 0.02g, respectively. Body mass of frogs used in SMR experiments was 2.16 ± 0.05g (*E. coqui*) and 1.38 ± 0.02g (*E. antillensis*).

Contrary to my prediction, the rates of CO_2 excretion, reflecting the metabolic rate for each individual, differed between *E. coqui* and *E. antillesis* during SDA experiments ($F = 1.89$; $df = 72, 72$; $P < 0.001$; $R^2 = 0.61$; Appendix 1). However, as predicted, metabolism increased with temperature treatments ($F = 10.15$; $df = 3, 3$; $P < 0.001$; $P < 0.001$, Figure 3). The thermal sensitivity *E. coqui* was found to be lower than *E. antillensis* for 15, 20 and 25°C as performance curves of the former exhibited less spread relative to each other (Figure 3, bottom panel). In all but two cases (*E. antillensis* at 20°C and 30°C), metabolic rates were significantly higher during the first 24 hours as compared to the last 24 (48-72 hrs) as predicted (Table 4). Higher metabolic demands consistently corresponded with evening hours (Figure 3).

Rates of CO_2 excretion during SMR experiments differed among *E. coqui*, *E. antillensis*, and *E. juanariveroi* ($R^2 = 0.60$; $F = 3.56$; $df = 54, 54$; $P < 0.001$; Appendix 2). Nevertheless, metabolic rates generally increased as assay temperatures increased towards 25°C (Figure 4). Interestingly, as temperatures rose to 25°C and beyond, interspecific variability in metabolic rate was significant. In the case of *E. coqui*, metabolic demands increased with temperature, although the rates found at 20-25°C were statistically similar ($F = 11.47$, $df = 1, 70$; $P = 0.73$).

In the case of *E. juanariveroi*, CO_2 excretion increased up to 25°C, but decreased at 30°C ($F = 5.04$, $df = 1, 70$; $P = 0.03$). The opposite was recorded for *E. antillesis* at 25°C. CO_2 excretion for the high-elevation, cold-adapted *E. wightmanae* varied by assay temperature as predicted ($F = 3.48$; $df = 10, 10$; $P = 0.001$; Appendix 3). Metabolic rates exhibited a significant increase at 25°C relative to 20°C ($R^2 = 0.98$; $F = 258.74$; $df = 1, 16$; $P < 0.001$, Figure 4). At 30°C, metabolic rates were not reliably measured as none of the experimental frogs survived the treatment. The sensitivity to temperature by *E. wightmanae*, as predicted, is illustrated by depicting all SMR results, dwarfing responses by all other species (Figure 5).

Piecewise regressions used to calculate the Arrhenius Breakpoint in Temperature (ABT) for *E. antillensis* and *E. juanariveroi* indicate an inflection point in metabolism between 21.23°C and 22.45°C, respectively (Figure 6), indicating a reduction in metabolism with increasing assay temperatures. This is in sharp contrast to *E. coqui*, which did not exhibit a similar response, increasing linearly throughout the range of assay temperatures. Based on the similarity in responses between *E. juanariveroi* and *E. antillensis*, I created an interspecific model that describes a decreasing trend in emissions ($R^2 = 0.69$; $F = 5.68$; $df = 2, 5$; $F = 0.05$). The combined data on metabolic rates yields an ABT of 22.78°C (Appendix 4; Figure 7).

Based on climate projections, it is anticipated that frogs will experience higher temperatures for longer periods as they move to higher elevations (Figure 8). For example, at 250-300 m, frog will, on average, be exposed an additional 2.5 and 3 hours at ≥25°C. Not surprisingly, species distributed throughout lower elevation habitats (e.g., *E. antillensis*, *E. juanariveroi*) will be exposed most strongly to warming trends during mid-century. The greater exposure of *E. antillensis* is evident by plotting occupancy data (Figure 9, left, top panel). Interestingly, metabolic rates curb along the transitional range of occupancy probabilities below

and above 25°C, that is, there is a corresponding change in metabolism. In contrast, *E. coqui* exhibited higher occupancy at higher elevations and lower temperatures, which coincided primarily within the range of suboptimal SMR measured (e.g., 20-25°C; Figure 9, right, top panel). At ~600 m, *E. coqui* will be exposed to 30-45 additional minutes at 25°C (Figure 8). Highest occupancy of *E. wightmanae* is strongly associated with elevations between ≥500 and temperatures between 20-25°C (Figure 9, bottom panel). That range points at a transition zone between lower or higher occupancy probabilities, aligning with drastic changes in the metabolic response obtained in this study for *E. wightmanae*.

DISCUSSION

This study was aimed at determining the role of environmental temperature in the energy demands of four representative species of the genus *Eleutherodactylus* in Puerto Rico, namely, *E. coqui, E. antillensis, E. wightmanae* and *E. juanariveroi*. Additionally, I assessed the thermal dependence of the heat increment associated with digestion and absorption processes (i.e. specific dynamic action; SDA) after a meal in *E. coqui* and *E. antillensis*. The study employed an indirect calorimetry approach to evaluate the metabolic performance of these species under different thermal environments. This study was primarily motivated by the need to understand how future conditions induced by climate change could affect the energy balance of these endemic frogs, thus affecting organismal physiology, abundance and distribution (Nowakowski et al. 2017, Amphibian and Reptile Adaptations to the Environment: Interplay Between Physiology and Behavior" n.d.). This study becomes part of a growing body of work that could inform decision makers regarding the implementation of adaptations strategies such as managed translocations and identifying climate resilient habitats in Puerto Rico or elsewhere where

Eleutherodactylus frogs occur (McDonald-Madden et al. 2011, Morelli et al. 2016, Burrowes et al. 2021).

In this study, I show the postprandial effect of meal ingestion, reflected in CO2 excretion, for both E. coqui and E. antillensis increasing with temperature. Similar to previous studies evaluating SDA in anurans (Powell et al. 1999, Andrade et al. 2005, Secor and Boehm 2006, Secor 2009) CO2 excretion for both species were higher during the first 24 hours after ingestion. By the third day (48-72 hours), CO2 excretion rates declined for both species and all treatment temperatures, but only significant for E. coqui (all treatment temperatures), and for 15°C and 25°C for E. antillensis. These findings are in agreement with the expected pattern of SDA response, which typically peaks between 7-48 hours after ingestion and returns to baseline values within 3 to 7 days (Secor 2009, Hillman et al. 2009). Interestingly, results from my study do not portrait the classical SDA curve describing SDA in anurans (Secor and Boehm 2006, Secor 2009). This lack of alignment with a conventional view of SDA can be attributed to the body mass of the species evaluated and the sampling method; my experimental species are likely one of the smallest anuran species used for SDA measurements using flow-through respirometry. Most SDA measurements in anurans rely on closed-systems and the subsampling of respiratory gasses (Secor and Boehm 2006, Secor 2009). Most SDA measurements in anurans rely on closed-systems and the subsampling of respiratory gasses (Secor and Boehm 2006, Secor 2009). Although widely used because of its convenience, closed-system respirometry introduces variability to the respiratory gasses within the chamber over time, which can lead to artifactual physiological responses of the individual to O2 -depleted and CO2 -enriched environments (Lighton 2008). Regardless of methodological approaches, my findings show a significant, temperature-dependent, postprandial effect for both species studied. When both SDA and SMR

rates are compared, it is clear that meal consumption, digestion and absorption has an important toll over the metabolic demands of these species. Indeed, all else being equal, warming trends will increasingly undercut the net energy balance of individuals derived from foraging. In other words, such increases in energy demand influence the biochemical energy available for other ecologically relevant processes including locomotion, mating and growth, all important elements of individual survival and species persistence in the field.

Applying my results to estimate field metabolic rate is possible, but only after incorporating the role of circadian rhythms, meal ingestion and temperature into the analysis for each species. This is because it is likely that frogs may ingest food every evening, and thus, their metabolic demands might seem elevated at first glance. Paradoxically, energy assimilation comes with increasing energy expenditure, likely during evening hours. Thus, it is important to account for the digestive state of individuals to avoid spurious inferences about metabolic responses to air temperature or alternatives (e.g., operative temperatures). It should be kept in mind that metabolic rates for *E. coqui* and *E. antillensis* also adhered to the local diel cycle, indicating a strong biological control of metabolism during inactive, diurnal periods. Circadian rhythmicity in metabolism is a widely documented trait in many vertebrates, including amphibians ("Amphibian and Reptile Adaptations to the Environment: Interplay Between Physiology and Behavior" n.d.). Metabolic control has several benefits, particularly to those species showing marked feeding periods during the day. With a larger mass specific energetic demands, metabolic control ensures that assimilated energy use is optimized during periods of inactivity, potentially fueling biosynthetic pathways. As thermal regimes are forecasted to rise, and as the data suggest, metabolic control over increasing treatments shows a progressive loss or

lack of control in metabolism; as temperature rise, diurnal metabolic demands approach those found during nocturnal periods.

Experimental trials monitoring the thermal performance curve of standard metabolism showed an overall increased metabolic rate with increasing temperature for each species (*E. wightmanae*, *E. antillensis*, *E. coqui*, and *E. juanariveroi*), but patterns were species-specific. *E. coqui* stood out as having a linear relationship between metabolic rate and temperature. Interestingly, this species is the only Eleutherodactylid from Puerto Rico who have been attributed to multiple known invasions in Central America, Florida and Hawai'i (Austin and Schwartz 1975, Wilson and Porras 1983, Stewart and Woolbright 1996, Beard and Pitt 2012, Westrick et al. 2022), thus it is a plausible interpretation of this relationship that *E. coqui* thermotolerant physiology reflects its ability to withstand a wider range of temperatures. Standard metabolism of *E. antillensis* and *E. juanariveroi* also increased linearly with temperature, but both species, individually or combined, began to plateau beyond 20°C. Indeed, ABT in standard metabolism of both species changed rates around 21-22°C, suggesting a shift in the processes controlling metabolic pathways around these temperatures. Although instructive, due caution must be exercised when interpreting inflection points such as the ABT (Schulte 2015). It is certainly unwarranted to assume that inflection points reflect the thermal optimum of a species performance curve or that only metabolic pathways govern the response of individuals with rising temperatures.

There is agreement, however, that results from applying the Arrhenius equation, as in this study, indicate that a change in metabolic rates occurs with rising temperatures. This is interesting because both species have strong affinities for low elevation habitats characterized by warmer temperatures (Ríos-López et al. 2014, Rivera-Burgos et al. 2021). Indeed, *E.*

juanariveroi occurs only in lowland, freshwater wetlands. Because *E. coqui* is also widely distributed, it was not unreasonable to ask why *E. coqui* did not respond in a similar fashion. One possible explanation is that *E. antillensis* has a low probability of occurring at high elevations (Campos-Cerqueira et al. 2021, Rivera-Burgos et al. 2021), and thus, may have a unique metabolic response. Likewise, *E. juanariveroi* may employ unique metabolic control pathways to deal with increasing temperatures. Control of metabolism in response to temperature can occur at multiple levels (Schulte 2015), including allosteric regulation of enzymes, membrane restructuring (Hazel 1995), enzyme-membrane interactions (Wodtke 1981) and micro RNA-mediated regulation of metabolic pathways (Hawkins and Storey 2020).

Thermal performance curve of metabolism for *E. antillensis* and *E. juanariveroi* may indicate that warm-dwelling species undergo further shifts in their metabolic pathways to persist in these habitats. It is noteworthy that Rohr et al. (2010), Narayan et al. (2012), Navas et al. (2013), Schulte (2015) also argued that such adaptations to high temperature might come at the cost. That is, warm-tolerant species may not be able to efficiently harness the assimilated energy into useful cellular energy when faced with supraoptimal temperatures, thus compromising the energy available for whole-organism processes such as growth and reproduction (Somero 1996, Wintrode and Arnold 2001). Some of the many tradeoffs for warm tolerant or warm adapted species are reduced metabolic efficiency due to the temperature dependent nature of the biochemical reactions (Cossins and Bowler 1987, Somero et al. 1996, Tattersall et al. 2012, Schulte 2015). This means that they may require more energy to maintain their physiological processes, impacting overall fitness. Another metabolic trade-off from being adapted to warm environments is the increased rate of water loss through evaporation. Amphibians that are adapted to warm habitats such as *E. antillensis* may face challenges in maintaining proper

hydration levels. Prior research showed that when compared to *E. coqui*, *E. antillensis* could rehydrate much faster than both lowland and highland *E. coqui* (Berkum et al. 1982). Some studies suggest that warm adapted amphibians may be more susceptible to diseases such as chytridiomycosis, a fungal disease caused by a chytrid fungus (*Batrachochrytium dendrobatidis*) that can be fatal for amphibians, because higher temperatures can increase the growth and transmission rates of such pathogens (Longo et al. 2009, Joglar et al. 2011). Amphibians that are adapted to warm habitats can more efficiently regulate their body temperature, allowing them to maintain optimal body temperatures for various physiological functions such as digestion, movement, and reproduction (Freed 1980, Preest and Pough 1989, Anderson and Andrade 2017, Forget-Klein and Green 2021). Warm-adapted amphibians may have access to more resources, such as food and breeding sites, than those living in cooler environments (Lyu and Alexander 2022). Studies have also shown that direct development of eggs hatching days vary according to temperature, with higher temperatures shortening the time to maturity (Townsend et al. 1984, Townsend and Stewart 1986).

As predicted, standard metabolic rate experiments confirmed that *Eleutherodactylus wightmanae*, a high-elevation specialist, had markedly higher metabolism at high temperatures compared to widely distributed species. *E. wightmanae* exhibited a nearly 10-fold increase in metabolism between treatments at 20°C and 25°C. The response is likely the result of metabolic control loss, induced by the stress of the exposure to supraoptimal temperatures. Supporting this view, all specimens exposed to 30°C assay temperature did not survive the treatment. Similar results were reported by Beuchat et al. (1984) and Delgado-Suazo and Burrowes (2022) for another high elevation specialist, *E. portoricensis*. The response from *E. wightmanae* demonstrates how sub-lethal temperatures can impact survival when constant exposure for

multiple hours occurs. Exposure to shorter, but incremental periods of time during evening hours, could undermine species abundance and distribution through its impact on the all aspects dependent on a positive energy balance including growth, reproduction, and locomotion (Delgado-Suazo and Burrowes 2021, Hawley-Matlaga et al. 2021).

Insights on the potential impacts of climate change and conservation implications can be gained by integrating available field and laboratory studies. Projections suggest that frogs will be exposed to an increasingly amount of time to warmer temperatures (Bowden et al. 2021). For example, the highest increase in thermal exposure will occur at ≤300m (Figure 8). Under this projection, the majority of *E. antillensis* populations, particularly on the southern slopes of Puerto Rico, will face nearly 2-3 extra hours of exposure to 25°C compared to the current conditions. Our SMR and CT_{Max} experiments (39.33 ± 0.41; Rivera-Burgos et al. in prep) suggest that they could withstand such exposure, albeit perhaps following alternative physiological pathways and energetic trade-offs suggested in this study. Species may also avoid making such adjustments by reducing their nocturnal period of activity. In contrast, *E. coqui* is strongly associated with higher elevation and cooler temperatures, but as shown in this study, the species shows increasing metabolic demands through the entire assay temperature range. Assuming temperature is the only factor influencing their metabolism, *E. coqui* has the potential to sustain increased metabolism with warming temperatures, a potential backed by its comparable CT_{Max} to *E. antillensis* (E.c. = 38.27 ± 0.21 vs E. a. = 39.33 ± 0.41; Rivera-Burgos et al. in prep.). This apparent capability, however, is belied by the fact that occupancy levels exhibit a decreasing trend past 25°C. It follows that other factors (e.g., moisture) may be lacking despite their potential to deal with temperature levels as discussed below. Additionally, the effectiveness of energy transduction (i.e. nutrients and carbon molecules turned into cellular

energy in the form of ATP) at the subcellular level might be reduced with increasing temperatures, hindering the efficiency of a higher metabolic rate at warmer temperatures. The latter statement is yet unresolved for any *Eleutherodactylus* species, and it constitutes the next step of our ongoing research efforts.

Clearly, *E. wightmanae* was the most thermosensitive of the species studied in this work. Their responses during SMR experiments suggests that temperatures between 20°C and 25°C are a transitional zone between suboptimal and supraoptimal temperatures for the species. That same range seems to signal the onset of metabolic adjustments by other species as suggested by ABT values (e.g., *E. antillensis*, *E. juanariveroi*), and arguably appear to partition increasing or decreasing occupancy probabilities for all species in this study. Further work with *E. wightmanae* should focus on increasing the resolution of the metabolic rate curve developed in this study, with particular emphasis in additional measurements in the transition zone described above.

As noted previously with *E. coqui*, the interplay between temperature and water availability on metabolic rates adds to the complexity of predicting the effects of climate change on amphibians. While rising temperatures are expected to harm amphibian populations, the impacts of water availability changes must also be considered, particularly given amphibians' high sensitivity to such changes. For example, studies have shown that dehydration of a frog's body tissues can increase its RMR and reduce its maximum rate of aerobic metabolism (Pough et al. 1983). *Eleutherodactylus* experiencing high metabolic rates under dry conditions are likely to face greater energy demands and may have less energy available for crucial life history traits such as growth and reproduction (Berkum et al. 1982, Pough et al. 1983, Beuchat et al. 1984). These frogs rely on calling behavior for reproductive success, which can stop during extended

dry spells (Berkum et al. 1982). Frogs can deal with these conditions through thermal/humidity refuges. For example, *E. coqui* and *E. antillensis* can endure prolonged dry periods if they have access to moist retreats (Burrowes 2009). Retreats can be a major contributor of patchy distributions in amphibians (e.g., Burrowes et al. 2022), but such distribution patterns can be correlated with higher risks of being exposed to chytrid fungus and parasites (Longo et al. 2009, Joglar et al. 2011). To increase the complexity of the responses on these species to changes in temperature and water, infection rates are shown to reduce thermal tolerance in frogs (Greenberg and Palen 2021),and the immune response relies vastly on cellular energy, tapping on the already compromised energy budget of individuals (Grogan et al. 2018). Thus, understanding the thermal physiology of these species relies on complex, multilevel analysis of all the factors mentioned above, and the synergistic effects found amongst them.

My work expanded our foundational knowledge of how *Eleutherodactylus* species respond metabolically to increasing temperatures, conditions forecasted for Puerto Rico (Bowden et al. 2021). It is an example a physiological frame of work designed to explore a critical component in the energy budget of these threatened species, while including an assessment of how changes in thermal conditions might influence decisions concerning the implementation of climate adaptation strategies in Puerto Rico (Barrows et al. 2021). My work, however, might also provide insights on potential impacts of climate change for other *Eleutherodactylus* species in the Caribbean. Certainly, there is a need for additional physiological studies addressing thermal tolerance across a broader geographic (e.g. altitudinal gradients, seasons, habitat types) to increase the capacity to group thermal generalists, while allowing the study of those thermal specialists whose persistence might be dependent of active management in the face of climate change. Specifically, I recommend that new studies be

conducted to ascertain, more precisely, the pathways and potential consequences incurred under different stressors (e.g., temperature, water vapor, diseases). Examples of those studies include 1) expand the resolution and the range of temperatures for metabolic rate measurements in the current species 2) expand the number of specialists included in the study to gauge the diversity of physiologies within vulnerable species, 3) evaluate whether energy transduction efficiency at the cellular level is thermos dependent, 4) analyze the expression response of genes linked to metabolic pathways, by evaluating the transcriptomic signature, of the individuals exposed to supraoptimal temperatures and finally, 5) Explore the adaptive plasticity (i.e. rapid adaptation) of vulnerable species to stressors under controlled, mesocosm and laboratory experiments.

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Figure 1. Map of Puerto Rico depict the locations (inset) were samples of four species of *Eleutherodactylus* species were collected. *E. juanariveroi* was collected on north-central wetlands, and the remaining (*E. coqui*, *E. wightmanae*, and *E. antillensis*) were collected ≥ 500 m in the municipalities of Maricao and Las Marias.

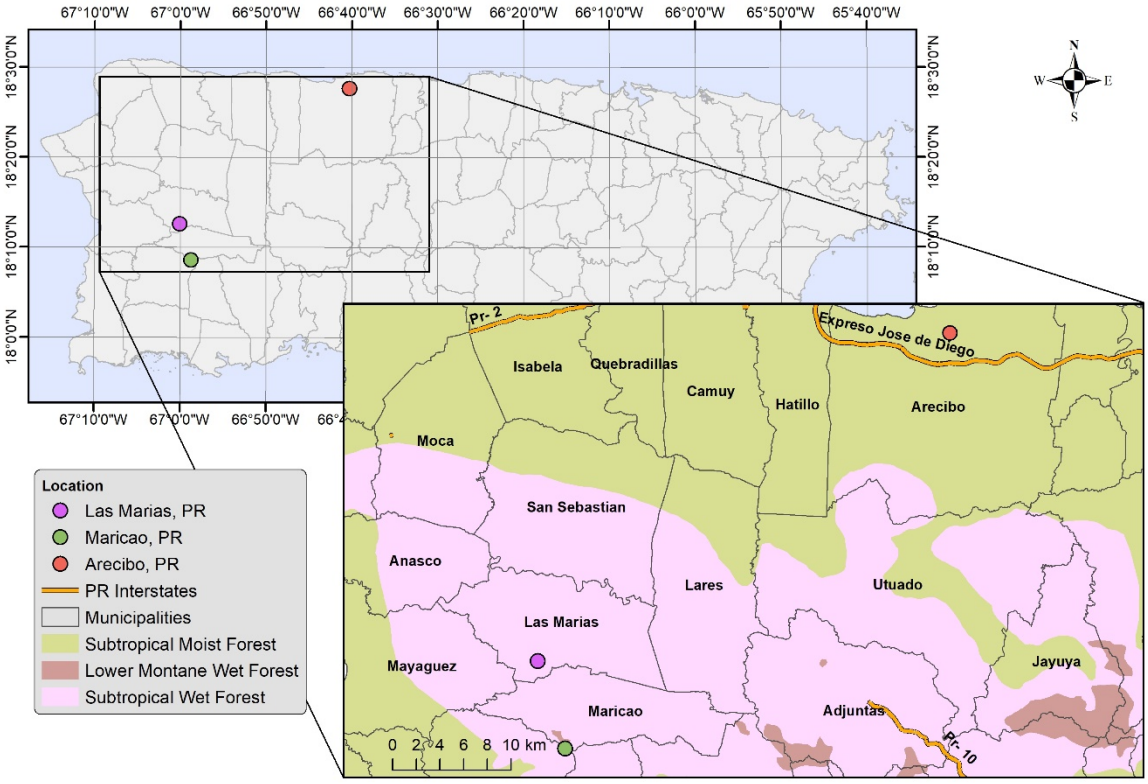


Figure 2. Diagram of a Sable Systems Field Metabolic System, configured in flow-through, push model, used to measure rates of CO_2 production with four species of *Eleutherodactylus* frogs in Puerto Rico.

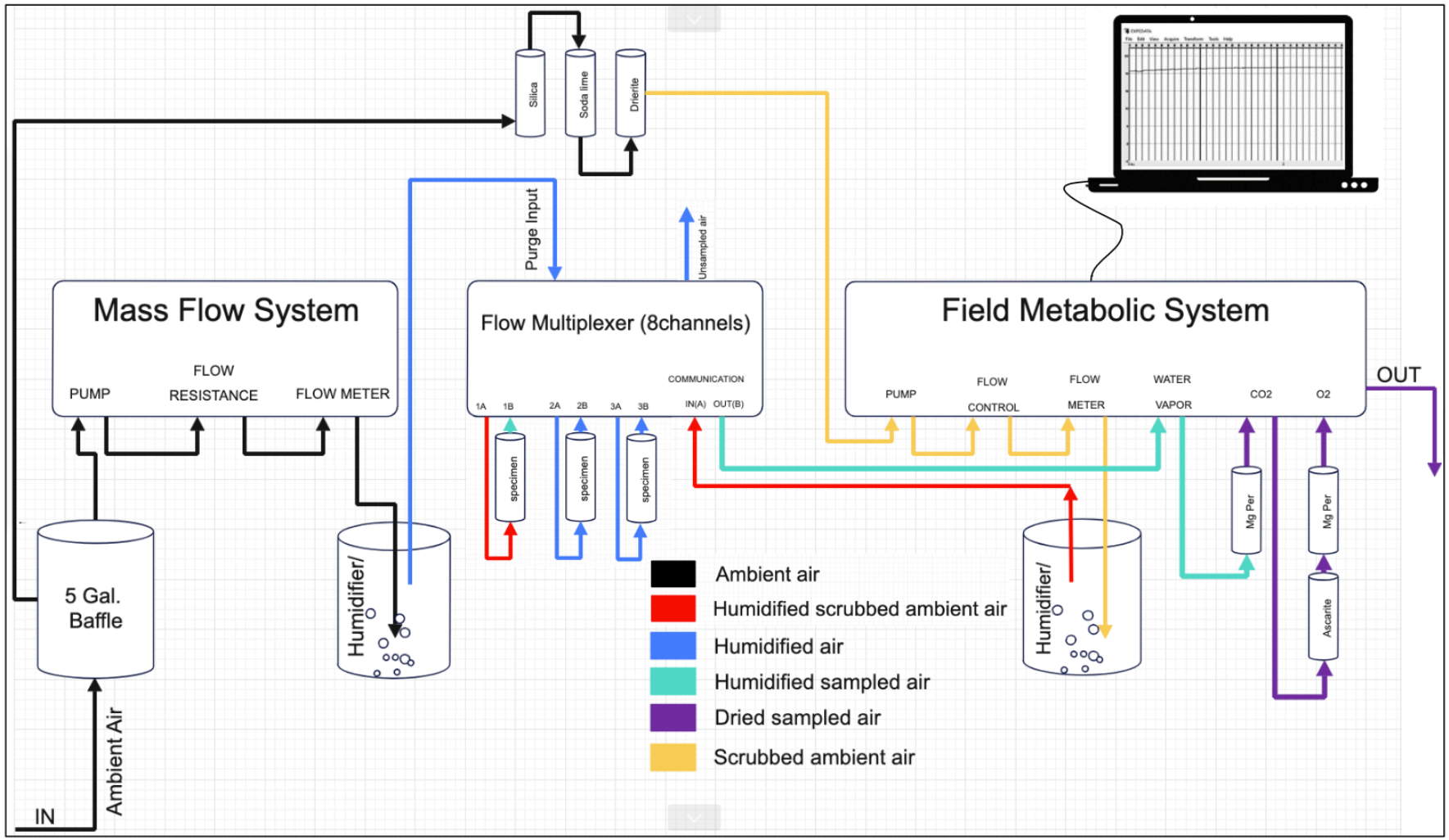


Figure 3. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis* and *E. coqui* during specific dynamic action experiments (SDA) run for 72 hours in west-central, Puerto Rico. Bars indicate 24 hour cycles (diel) during the elapsed time of the experiments.

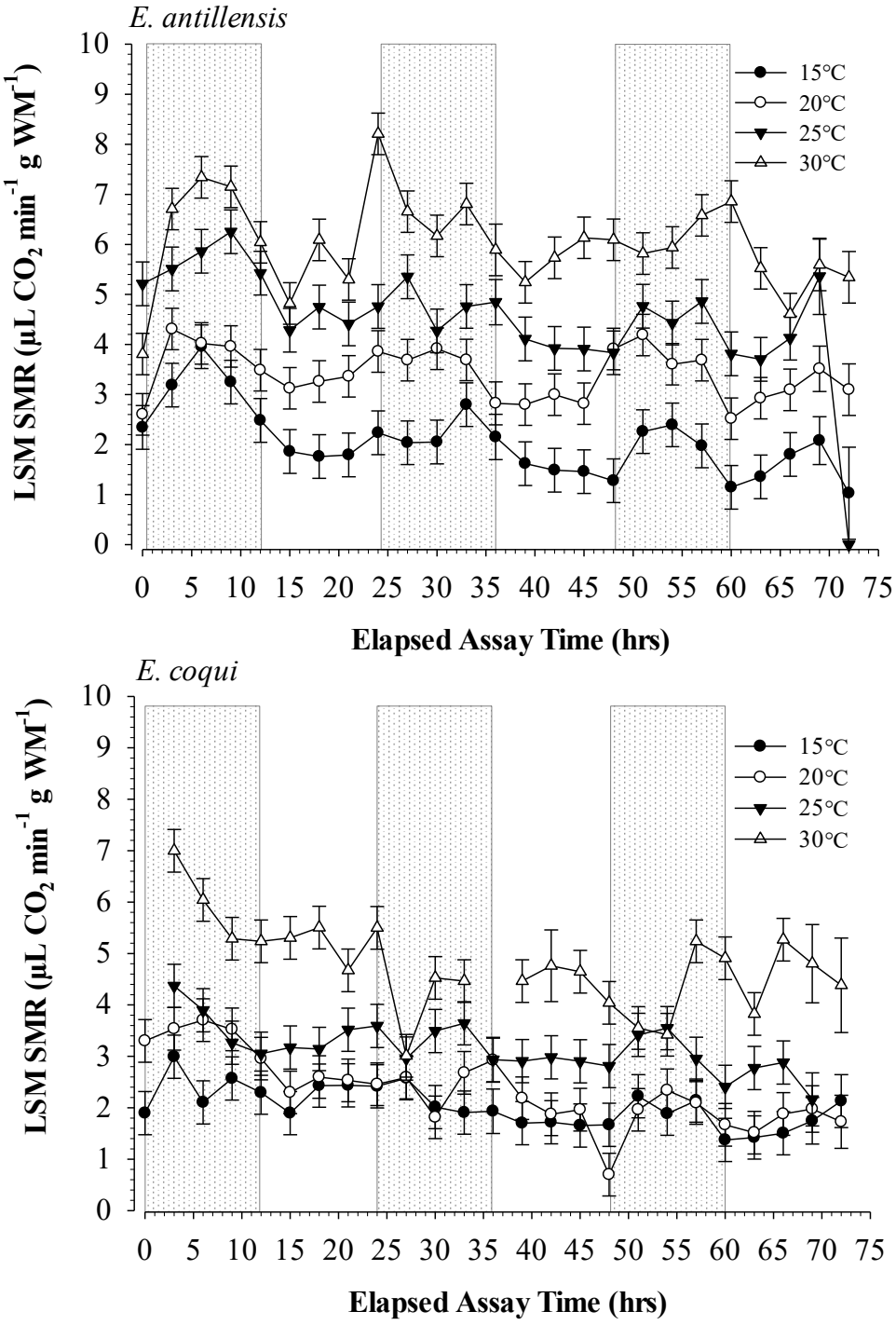


Figure 4. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis*, *E. coqui*, *E. juanariveroi* (top-panel) and *E. wightmanae* (bottom panel) during standard metabolic rate (SMR) experiments run for 24 hours in west-central, Puerto Rico.

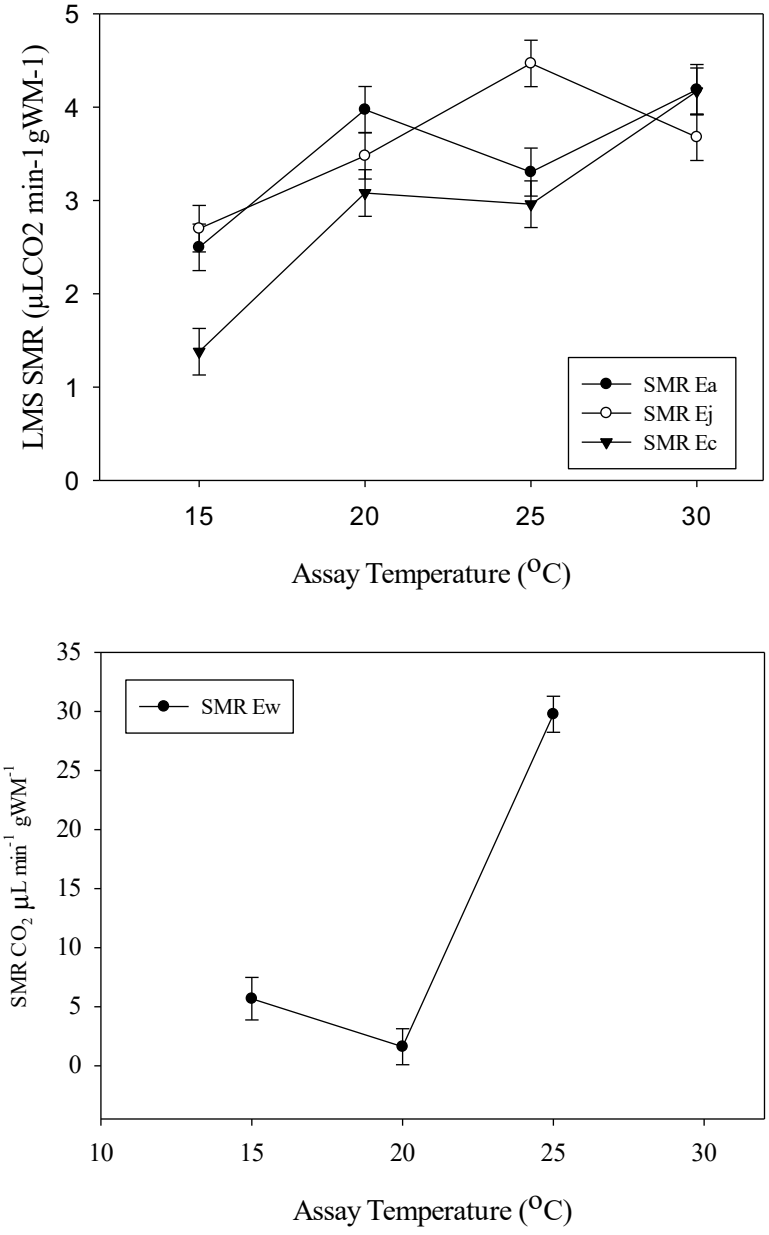


Figure 5. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis*, *E. coqui*, *E. juanariveroi* and *E. wightmanae* during standard metabolic rate (SMR) experiments run for 24 hours in west-central, Puerto Rico.

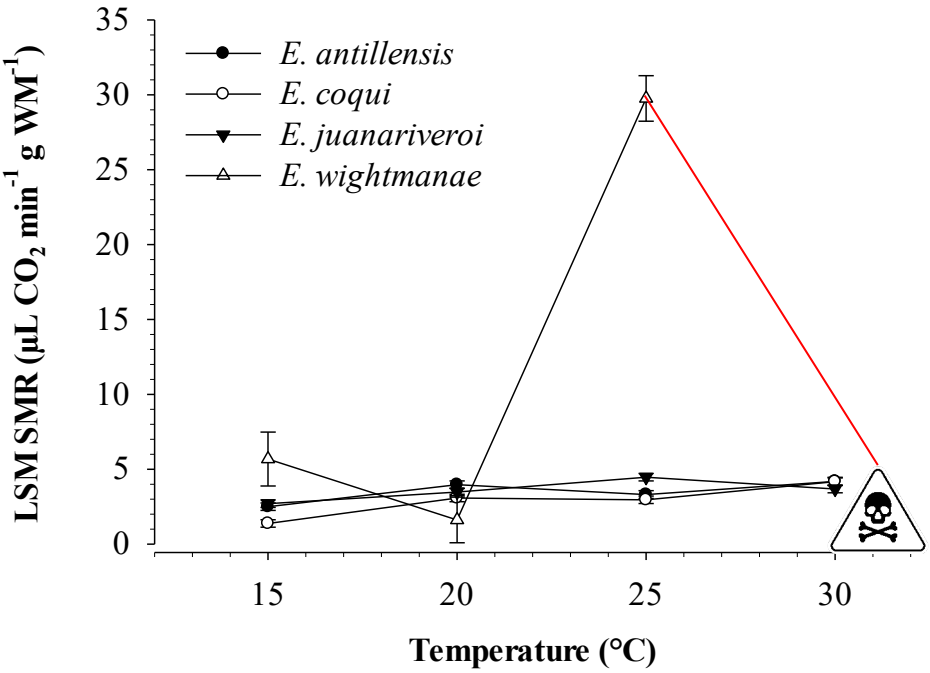


Figure 6. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis*, *E. coqui*, and *E. juanariveroi* during standard metabolic rate (SMR) experiments run for 24 hours in west-central, Puerto Rico. Overlaid is the predicted SMR based on piecewise regression to estimate the Arrhenius Breakpoint in Temperature (ABT; blue line).

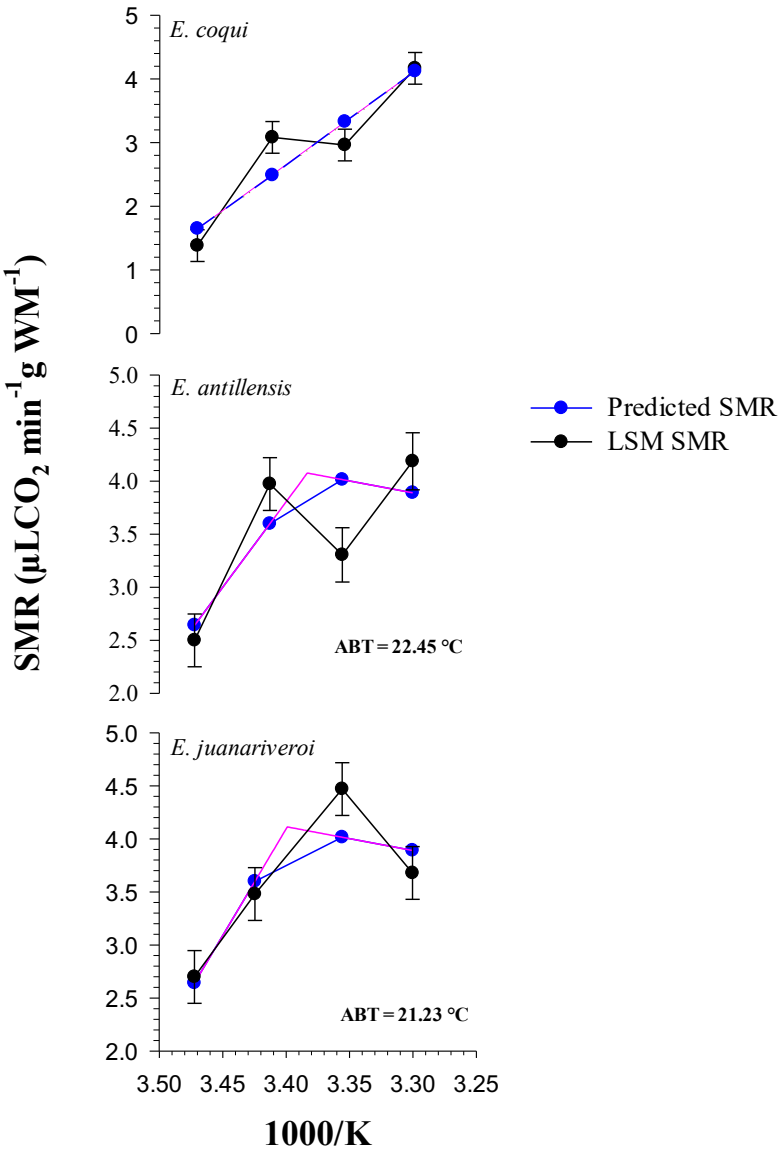


Figure 7. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis* and *E. juanariveroi* during standard metabolic rate (SMR) experiments run for 24 hours in west-central, Puerto Rico. Overlaid are the predicted SMRs based on based on piecewise regression to estimate the Arrhenius Breakpoint in Temperature (ABT; blue line), and a quadratic regression model (red dotted line).

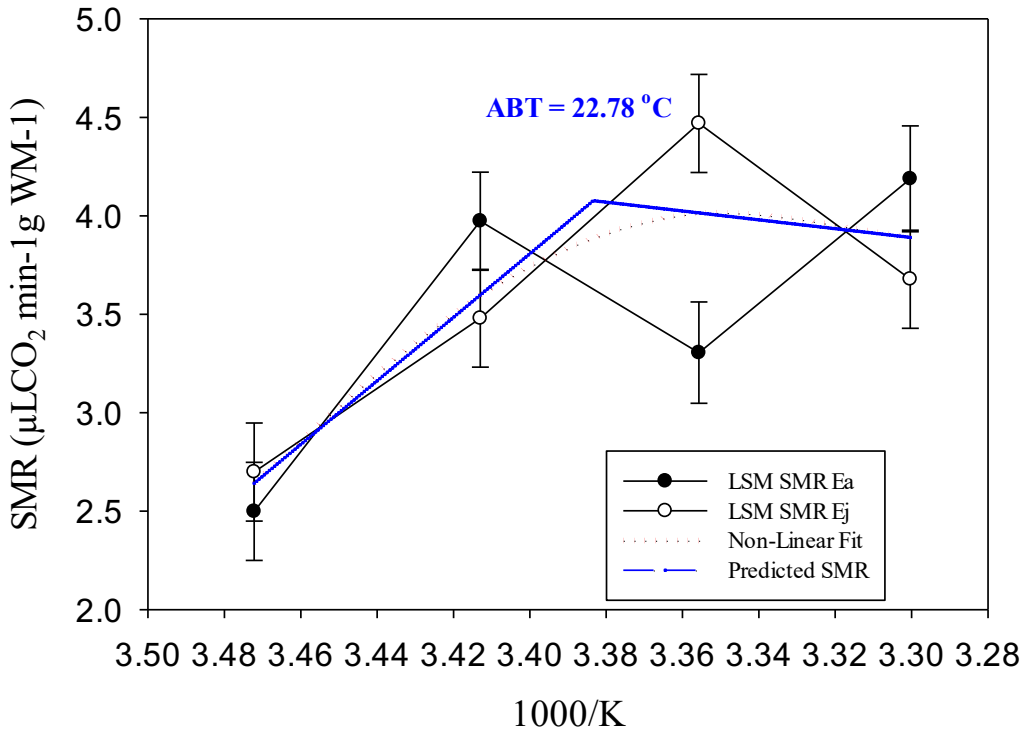


Figure 8. Average exposure time (in hours) for temperature thresholds at and above 25-30ºC for evening hours (1800-2300 LST) across Puerto Rico for the period 2040-2060 under a higher greenhouse gas emissions scenario. The curves represent individual loess models fit to the projected exposure time for each 2km grid cell (filled circles) from the dynamically downscaled climate model output. 95% confidence intervals are depicted by gray shading for each loess curve.

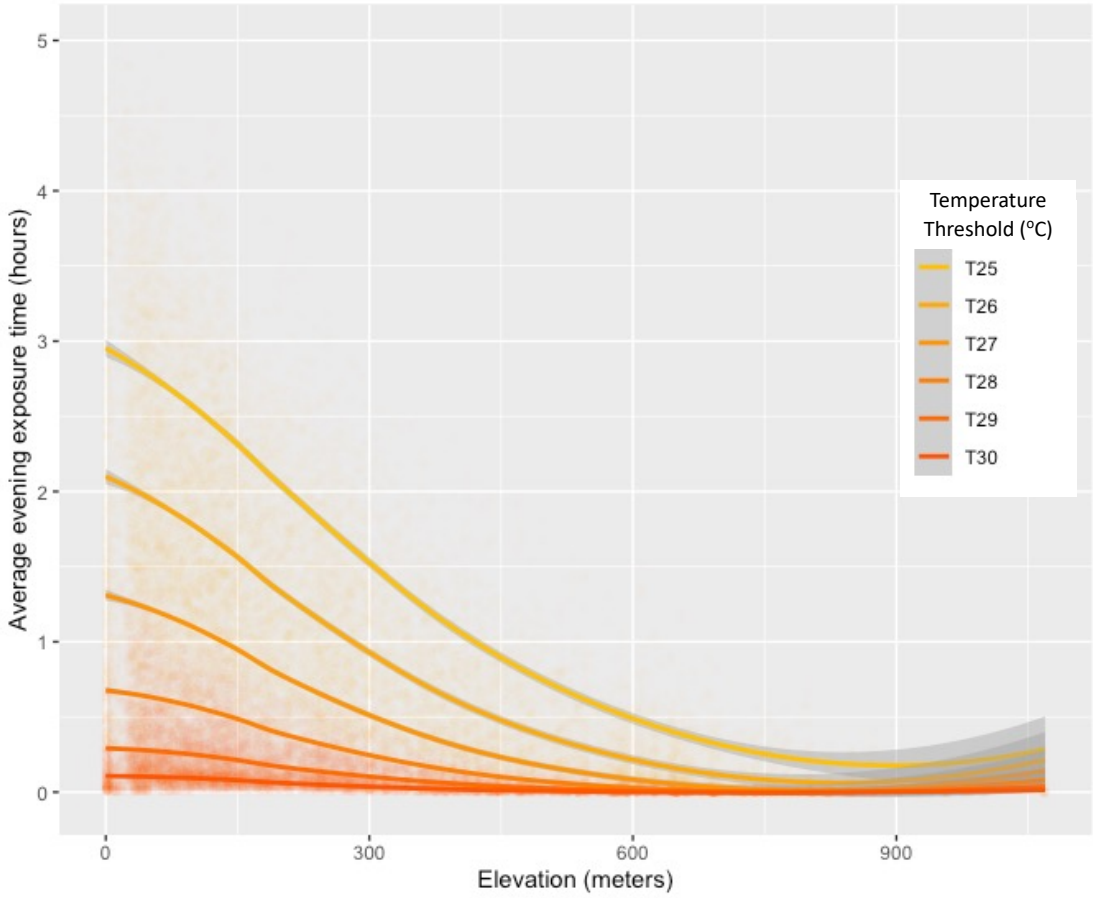
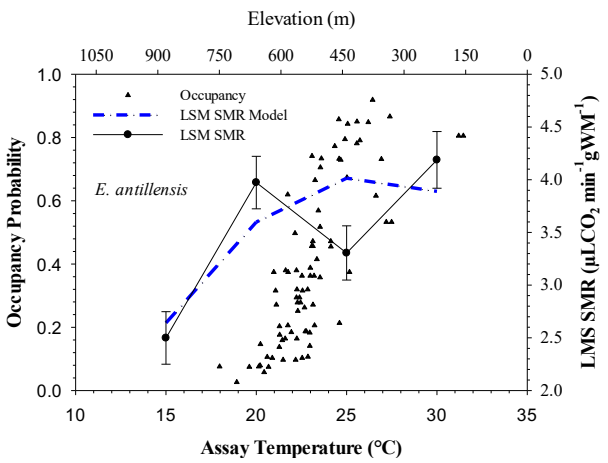
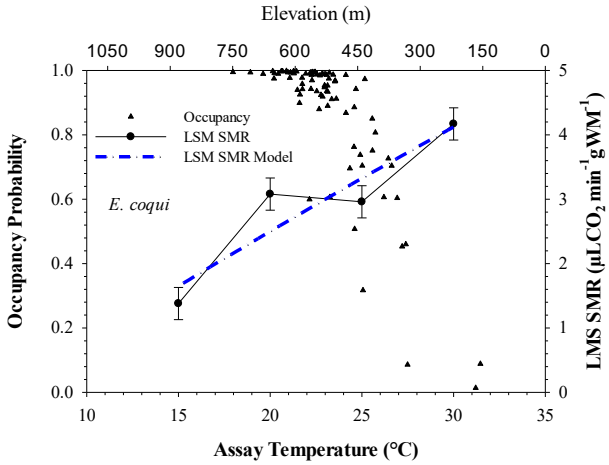


Figure 9. Least-square means (±SE) of CO_2 (µL/min gWM) excreted by *E. antillensis*, *E. coqui*, and *E. wightmanae* during standard metabolic rate (SMR) experiments ran for 24 hours in west-central, Puerto Rico. Overlaid is the predicted SMR based on piecewise regression to estimate the Arrhenius Breakpoint in Temperature (ABT; blue line), and occupancy probabilities (small triangles) based on in-situ temperature (°C) and elevation (m) from Rivera-Burgos et al. (2021).





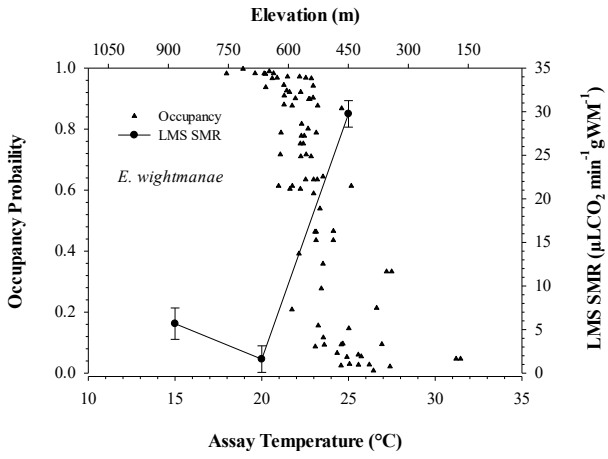


Table 1. Name and location of sites where four species of *Eleutherodactylus* frogs were collected in west-central Puerto Rico. Specimens were used to conduct specific dynamic action (SDA) and standard metabolic rates (SMR) experiments.

Species	Location	Coordinates	Elevation (m)
E. antillensis	Hacienda Ana Luisa	18.2103, -67.00087	526
E. coqui	Las Marias		
E. wightmanae	Parque Ecologico	18.14353, -66.97875	879
	Monte del Estado		
	Maricao		
E. juanariveroi	Wetland near Caño Tiburones	18.46105, -66.67182	3
	Arecibo		

Table 2. Environmental conditions at field sites at the time when four *Eleutherodactylus* frogs were collected for specific dynamic action (SDA) experiments in west-central Puerto Rico. Collections for SDA experiments were conducted during winter months, 2022. All values are means (±SE), N= 7. Difference is ambient minus in-situ temperatures. -- measurement not taken.

Species	Ambient Temperature (°C)	Relative Humidity (%)	In-Situ Temperature (°C)	Difference Amb – In-situ
E. antillensis				
15	22.20	84.60	18.31 (0.17)	3.88 (0.17)
20	24.00	87.40	20.58 (0.25)	3.41 (0.25)
25	23.50	89.00	20.86 (0.69)	5.61 (3.03)
30	--	--	20.43 (0.25)	--
E. coqui				
15	22.70	78.30	20.82 (0.47)	1.87 (0.47)
20	20.50	91.8	18.68 (0.24)	1.81 (0.25)
25	22.50	78.20	20.65 (0.19)	1.84 (0.19)
30	--	--	21.14 (0.30)	

Table 3. Environmental conditions at field sites at the time when four *Eleutherodactylus* frog species were collected for SMR experiments in west-central Puerto Rico. Collections were conducted during late-summer and fall months 2022. All values are means (±SE), N= 7. Difference is ambient minus in-situ temperatures. -- measurement not taken.

Species	Ambient Temperature (°C)	Relative Humidity (%)	In-Situ Temperature (°C)	Difference Amb – In-situ
E. antillensis				
15	22.50	88.70	19.77 (0.39)	2.73 (0.39)
20	25.70	82.60	20.52 (0.24)	5.18 (0.24)
25	22.40	80.10	21.96 (0.38)	0.44 (0.38)
30	24.70	78.90	20.95 (0.48)	6.74 (3.02)
E. coqui				
15	25.80	90.20	23.79 (0.25)	2.01 (0.25)
20	23.20	82.70	21.68 (0.48)	1.52 (0.48)
25	24.70	78.90	22.04 (0.45)	2.66 (0.45)
30	23.40	84.30	21.06 (0.41)	2.34 (0.41)
E. wightmanae				
15	23.80	85.40	19.50 (0.50)	4.30 (0.50)
20	22.10	95.80	20.48 (0.19)	1.62 (0.19)
25	22.00	89.10	19.60 (0.14)	2.40 (0.14)
30	20.50	91.90	18.83 (0.39)	1.67 (0.39)
E. juanariveroi				
15	26.10	100.00	24.25 (0.25)	1.85 (0.25)
20	26.10	100.00	24.15 (0.33)	1.95 (0.33)
25	27.00	100.00	25.17 (0.22)	1.83 (0.22)
30	27.00	100.00	24.60 (0.28)	2.40 (0.28)

Table 4. Contrasts between mass specific VCO_2 (uL/min gWM) recorded at 15, 20, 25 and 30°C (treatments) during the first 24 and last 24 hours during specific dynamic action (SDA) experiments for the *Eleutherodactylus coqui* and *E. antillensis* in west-central Puerto Rico. Means (±SE) are reported. N = 9 for all contrasts. *= $P < 0.05$

Species	TRT	0-24 hours	48-72 hours	
E. antillensis	15*	2.54 ± 0.25	1.70 ± 0.17	0.84
	20	3.55 ± 0.18	3.39 ± 0.17	0.16
	25*	5.16 ± 0.22	3.88 ± 0.52	1.28
	30	6.16 ± 0.45	5.82 ± 0.22	0.34
E. coqui	15*	2.34 ± 0.11	1.79 ± 0.11	0.55
	20*	2.99 ± 0.18	1.76 ± 0.15	1.23
	25*	3.41 ± 0.16	2.55 ± 0.35	0.86
	30*	5.28 ± 0.36	4.24 ± 0.33	1.04

Appendix 1. Split-plot Anova summary tables for *E. coqui* and *E. antillensis* specific dynamic action (SDA) experiments in west-central Puerto Rico, 2022. The experimental set up, sample sizes, data extraction are detailed in the methods section.

Summary of Fit

RSquare	0.61
RSquare Adj	0.56
Root Mean Square Error	1.36
Mean of Response	3.54
Observations	1304

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F Ratio**
Model	150.00	3417.18	22.78	12.25
Error	1153.00	2144.82	1.86	**Prob > F**
C. Total	1303.00	5562.00		<.0001*

Effect Tests

Source	**Nparm**	**DF**	**SS**	**F Ratio**	**Prob > F**
Species	1.00	1.00	0.76	0.41	0.52
Assay Temp	3.00	3.00	1771.15	317.38	<.0001*
chamber[Assay Temp]	24.00	24.00	425.05	9.52	<.0001*
Time	24.00	24.00	291.71	6.53	<.0001*
Species*Time	24.00	24.00	80.67	1.89	0.0070*
Species*Assay Temp	3.00	3.00	56.65	10.15	<.0001*
Species*Assay Temp*Time	72.00	72.00	250.04	1.89	<.0001*

Appendix 2. Split-plot Anova summary tables for *E. coqui*, *E. juanariveroi*, and *E. antillensis* standard metabolic rate (SMR) experiments in west-central Puerto Rico, 2022. The experimental set up, sample sizes, data extraction are detailed in the methods section.

Summary of Fit

RSquare	0.60
RSquare Adj	0.55
Root Mean Square Error	1.08
Mean of Response	3.32
Observations	823.00

Fixed Effects Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Species	2.00	2.00	72.86	8.94	0.0003*
Assay Temp	3.00	3.00	72.87	29.54	<.0001*
Time	9.00	9.00	650.10	2.08	0.0294*
Species*Assay Temp	6.00	6.00	72.76	4.40	0.0008*
Assay Temp*Time	27.00	27.00	650.10	2.55	<.0001*
Assay Temp*Time*Species	54.00	54.00	650.10	3.56	<.0001*

Appendix 3. Split-plot Anova summary tables for *E. wightmanae* SMR experiments in west-central Puerto Rico, 2022. The experimental set up, sample sizes, data extraction are detailed in the methods section.

Summary of Fit

RSquare	0.98
RSquare Adj	0.97
Root Mean Square Error	2.56
Mean of Response	12.99
Observations	114.00

Fixed Effects Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Assay Temp	2.00	2.00	16.00	149.97	<.0001*
Time	5.00	5.00	80.00	12.72	<.0001*
Time*Assay Temp	10.00	10.00	80.00	3.48	0.0008*

Appendix 4. Non-linear regression (quadratic) summary tables for the combined data of LSMeans CO_2 excretion values ($\mu L/min^{-1}$ gWM^{-1}) for *E. juanariveroi* and *E. antillensis* based on standard metabolic rate (SMR) experiments in west-central Puerto Rico, 2022. The experimental set up, sample sizes, data extraction are detailed in the methods section.

Summary of Fit

RSquare	0.6942
RSquare Adj	0.571881
Root Mean Square Error	0.452071
Mean of Response	3.536574
Observations	8

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F Ratio**
Model	2.00	2.32	1.16	5.68
Error	5.00	1.02	0.20	**Prob > F**
C. Total	7.00	3.34		0.05

Parameter Estimates

Term	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	2.00	0.69	2.89	0.03*
TRT	0.08	0.03	2.91	0.03*
(TRT)^2	-0.01	0.01	-1.69	0.15