Interacting effects of temperature and food on early growth of two plethodontid salamanders

Charlie Holguin

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

In

Fisheries and Wildlife Sciences

Holly Kindsvater, Chair

Nicholas Caruso, Co-Chair

Carola Haas

August 7th, 2023

Blacksburg, VA

Keywords: Body Size, Growth, Temperature, Food Availability, Life history, Climate change

Interacting effects of temperature and food on early growth of two plethodontid salamanders

Charlie Holguin

ABSTRACT (ACADEMIC)

An amphibian's life history includes patterns such as growth, development, and reproduction. These life history traits are essential to the fundamental understanding of population dynamics. Despite the importance of life history traits, key knowledge gaps still exist, hindering key information on birth/death rates, age/size at sexual maturity, size at metamorphosis, and clutch sizes for amphibians. Moreover, these life patterns can vary due to environmental conditions such as temperature and food availability, further emphasizing the importance of knowledge of species demographics. In this study, I aimed to investigate the growth of young-of-the-year and juvenile plethodontid salamanders with different life cycles, *Plethodon cinereus* (direct development) and *Eurycea cirrigera* (metamorphosis). Specifically, I was interested in examining the response of growth trajectories to different temperatures and food availability. I hypothesized that salamanders in the high-temperature treatment will grow faster initially but will be smaller at the conclusion of the experiment, while salamanders in the low-temperature treatment will grow slower initially but will be larger at the end of the experiment. To investigate the interaction between body size and environmental conditions, I exposed both species of salamanders to a factorial design, with two food treatments crossed with two temperature treatments. To analyze the data, I used a generalized additive mixed model (GAMM). The study revealed distinct growth patterns for both species of salamanders. *P. cinereus* experienced linear growth throughout the duration of the study, whereas *E. cirrigera* displayed non-linear growth (Throughout the experiment, salamander experienced a decrease in total length and mass). The results of the GAMM did not support my hypothesis for *P. cinereus*. However, the GAMM results did provide support for my hypothesis in the case of *E. cirrigera* under high food availability treatment. Furthermore, salamanders with limited food availability in both experiments grew at a slower rate. Forecasting the impacts of climate change on salamander populations is complex and will require the understanding of habitat quality and climate factors.

Interacting effects of temperature and food on early growth of two plethodontid salamanders

Charlie Holguin

ABSTRACT (GENERAL AUDIENCE)

With the threats of climate change, habitat fragmentation, and disease looming, it’s crucial we begin to understand amphibian life history traits, which includes the patterns of growth, development, and reproduction. These life history traits can vary due to environmental conditions and are essential to the fundamental understanding of population size and structure. Despite the importance of life history traits, information on amphibian birth/death rates, age/size at sexual maturity, size at metamorphosis, and clutch sizes are unknown, further emphasizing the importance of understanding species demographics. In this study, I investigated the growth of young-of-the-year and juvenile individuals of two plethodontid salamander species: eastern red-backed salamander and the southern two-lined salamander. These species exhibit two different life strategies. The eastern red-backed salamander is a fully terrestrial direct developing salamander without a distinct metamorphic stage, experiencing gradual growth without major morphological changes. However, the southern two-lined salamander semi aquatic salamander that goes through metamorphosis (Undergoes an aquatic larval stage but transitions to a terrestrial salamander), experiencing major morphological and physiological changes. I investigated the growth response of these two species of salamanders when exposed to different temperatures and when food availability varied from high to low. I hypothesized that salamanders in the high-temperature treatment will grow faster but be smaller at the conclusion of the experiment. While salamanders in the low-temperature treatment would grow slower but be larger at the end of the experiment. To understand the interaction between body size and environmental conditions, I exposed both species of salamanders to a 2x2 factorial experiment design that consisted of 2 food treatments and 2 temperature treatments. To analyze the results, I used a generalized additive mixed model. The results of the study indicated that both species of salamander differed in growth. The eastern red-backed salamander demonstrated linear growth throughout the experiment, while the southern two-lined salamander exhibited non-linear growth (During the experiment, salamanders experienced a decrease in total length and mass). However, the results of the eastern red-backed salamander did not support my hypothesis. Conversely, when food availability was high enough, the results supported my hypothesis for the southern two-lined salamander. Moreover, salamanders exposed to limited food availability in both experiments experienced a decrease in growth rates. Forecasting the impacts of climate change on salamander populations is complex and will require comprehension and insight to habitat quality and climate variables.

Table of Contents

Introduction 1

Literature Cited Introduction 3

Chapter I: Interacting effects of temperature and food on the early growth of *Plethodon cinereus* **5**

Abstract 5

Introduction 6

Study Species 9

Methods 9

Results… 12

Discussion 13

Chapter 1 Table 16

Chapter 1 Figures 19

Literature Cited Chapter 1 23

Supplemental Figures Chapter 1 26

Chapter II: Interacting effects of temperature and food on the early growth of *Eurycea cirrigera*..........*27*

Abstract 27

Introduction 28

Study Species 30

Methods 30

Results… 33

Discussion 34

Chapter 2 Table 37

Chapter 2 Figures 40

Literature Cited Chapter 2 46

Supplemental Figures Chapter 2 49

Acknowledgements

The last three years have been nothing but amazing. From beginning to end, my advisors, committee, volunteers, field technicians, and MAOP family have been amazingly helpful and supportive. Moving to Blacksburg, Virginia at the beginning of my research during the pandemic couldn't have gone any smoother due to these individuals. To my family and friends, thank you for being supportive through this process. Without these individuals, I couldn't have accomplished my goals.

I'd like to begin by thanking my co-advisors for the relentless support and guidance I've received over the last three years. Dr. Holly Kindsvater, I'd like to start by thanking you for the continuous support, guidance, and wisdom you've given me. I would not be the scientist I am today if it weren't for you. My experiences in the lab are invaluable, and I look forward to the future. Thank you, Dr. Nicholas Caruso, for teaching me about plethodontid salamanders and for all your support. Thank you for all the R meetings we had and your willingness to mentor me as I embarked on my statistical analysis journey. I will always be grateful and lucky to have had you both as advisors as I navigated my way through graduate school. To my committee member, Dr. Carola Haas, thank you. Thank you for all the support and feedback you've given me over the last three years. Most importantly, thank you for providing me with the opportunity to conduct undergraduate research. Without your guidance and mentorship over the years, I would have never known that graduate school was an option.

To the Multicultural Academic Opportunities Program (MAOP), thank you for the funding and the graduate assistantship. To my MAOP family (Monica Hunter, Amy Ingram, Taylor Swan, Laila Kirkpatrick, and Zoie McMillan), thank you for all the love and support you showed me throughout the last three years. Without MAOP, I'm not sure any of this would be possible. Thanks to programs like MAOP, students like me have the ability to conduct the research they love. To Sam Van Noy, my fellow Blue Ridge Boy, thank you! Your knowledge and enthusiasm for Southwest Virginia are unmatched. Thank you for teaching me how to navigate the field and for being an amazing friend and technician. I appreciate all you have done to help me capture the true beauty of the Appalachian Mountains. Lastly, thank you for all the great times we had over the last three years and our sports debates (Dos a Cero!!!). Thank you, Miriam Hilt, and Julia Coan, for being the best technicians anyone could have asked for and for taking charge when needed. It was truly amazing having you in the lab and being part of the project. To the rest of the Kindsvater lab (Dr. George Brooks, Hailey Conrad, Kaitlyn Theberge, Emma Hultin), thank you all for your help. To the amazing undergraduate volunteers (Joseph Nichols, Tom Fitzpatrick, Corinne Duffy, Alanna Belmont, and Annabelle Muriano), thank you. Thank you for your assistance with fieldwork, data entry, and data management.

To my hometown friends (Samuel and Ivan Gardea, Robert Lazarin, Robert Nieto (and family), thank you for being amazing friends. Thank you for all the love and support you have given me. Thank you for all the times you have reached out to make sure I am okay. To my fellow Beloiters (Adrian Johanningmeier, Victor Pun, Jordan Counihan, and Gopi Patel), thank you for all those times you visited me or had me over while attending graduate school. Most importantly, thank you for all the love and support you have given me the last three years. To my Blacksburg friends Anthony Abe Oliver Gemina, Said and Melisa Abou Draa, Craig Schichtel, Karen Serpa-Perez (and family), Nicolas Dale Burns, Tara Anggita Putri, Hailey Conrad, Babatomiwa Kikiowo, Austin Cantrell), thank you all for the great times and continuous support. To the Biology Department, McNair Program, and Student Excellence and Leadership Program at Beloit College, thank you. Thank you to Dr. Rachel Bergstrom and Dr. Tawnya Cary for being great mentors and advisors and for introducing me to the world of research. To Mrs. Paiz and Coach Avillar, thank you for all the love and support you gave me. Without you both higher education would have never been a thought. To Kristin Frey, Amy Ingram, and Monica Hunter, thank you for always being there for me. My time in Beloit, Wisconsin, and Blacksburg, Virginia, would not have been the same without your guidance, friendships, and support. I truly appreciate it.

Finally, I would like to thank my family. You have all been supportive of my decisions and career choices. Being away from home is never easy, but hearing from you made things easier for me. I love you all. Mom, you instilled in me a sense of adventure and kindness needed to become the person I am today. Dad, thank you for instilling the true meaning of hard work and the necessary skills to navigate the world. Mom and Dad, thank you for always encouraging me to follow my dreams. To my brother and sister, thank you for always being there for me. To my nephew Odin Zachary Holguin and niece Harley Jean Morris, just know anything is possible, and the world is yours. I love you all.

Introduction

Currently, there are a total of 516 described species of salamanders in the Plethodontidae salamander family, accounting for 71% of the global salamander population (Beachy et al. 2017, Frost 2023). Making the Plethodontidae family the largest salamander family in the world (Beachy et al. 2017, Frost 2023). The plethodontid family contains 28 genera, with a wide array of diversity due to its geographic distribution and evolutionary history (Beachy et al. 2017, Rabosky and Adams 2012). Most notability, plethodontid salamanders are known for being lungless, relying on cutaneous respiration for gas exchange (Marshall and Camp 2006). As a result, the salamanders must remain moist to avoid desiccation due to their permeable skin (Feder 1983, Marshall and Camp 2006). In addition to their lungless trait, plethodontid salamanders demonstrate three different life cycles among different species (Direct development, Biphasic, and Paedomorphosis). Due to this, these salamanders can occupy a vast variety of habitats such as streams, forests, and caves. Consequently, it's essential we understand their intricate life cycles for a deeper understanding of plethodontid salamanders.

As a result of anthropogenic influences, climate change has emerged, leading to changes in species ranges and ecological conditions (Velo-Antón et al. 2013). However, not all organisms are facing the same risks of climate change (Devictor et al. 2012). Currently, 41% of amphibians are considered Threatened and approximately 57% of salamanders are listed by the IUCN as: Vulnerable, Endangered, Critically Endangered (IUCN, 2022). Salamanders are experiencing range shifts, disease outbreaks, reduction of water in breeding sites, an increased threat of desiccation, resulting in declines (Wake 1991; Kiesecker et al. 2001; Stuart et al. 2004; Parra-Olea et al. 2005). Due to variety of factors causing the declines, we need to understand life history traits and species demographics to properly manage them.

A plethodontid salamanders' cycle can exhibit one of three different life strategies: direct development, biphasic life cycles (metamorphosis), and paedomorphosis (larval-form) (Beachy et al.

2017). Direct development is the process in which a salamander forgoes the aquatic stage, and their eggs develop directly on land. As a result, the hatchlings are a miniature version of adults (Marks and Collazo 1998). Through this process, the salamander will experience gradual growth without major morphological changes. Direct development is the most common reproductive mode in plethodontid salamanders (Wake and Hanken 1996; Beachy et al. 2017). In a biphasic life cycle, salamanders begin lives in the aquatic larval stage, and then transition into a terrestrial or semi-terrestrial salamander through metamorphosis (Beachy et al. 2017, Bonett et al. 2022). When a salamander undergoes metamorphosis, they experience significant morphological and physiological changes such as limb development and gill resorption. Lastly, paedomorphosis is described as the process of which a salamander forgoes metamorphosis and retains its larval traits as an adult (Johnson and Voss 2013; Beachy et al. 2017). However, no work has examined how differences in early life cycles (a larval period vs. direct development) can affect growth and timing of major milestones, such as metamorphosis for maturation. It is possible plethodontids with different life histories will respond differently to climate change. Given the conflicting results of prior studies, the focus of my study was on body size and early growth. By investigating these factors, I aimed to further understand how early growth and environmental conditions impact a salamander's life history and population dynamics. By studying these relationships, we can gain valuable insights into how growth can directly impact key life history traits such as size at maturity and ecological processes. Understanding these relationships can provide information for conservation efforts and ecological understandings.

Literature Cited Introduction

Beachy, C. K., T. J. Ryan, and R. M. Bonett. 2017. How metamorphosis is different in plethodontids: larval life history perspectives on life-cycle evolution. Herpetologica 73:252–258.

Bonett, R. M., N. M. Ledbetter, A. J. Hess, M. A. Herrboldt, and M. Denoël. 2022. Repeated ecological and life cycle transitions make salamanders an ideal model for evolution and development. Developmental Dynamics 251:957–972.

Devictor, V., C. van Swaay, T. Brereton, L. Brotons, D. Chamberlain, J. Heliölä, S. Herrando, R. Julliard, M. Kuussaari, Å. Lindström, J. Reif, D. B. Roy, O. Schweiger, J. Settele, C. Stefanescu, A. Van Strien, C. Van Turnhout, Z. Vermouzek, M. WallisDeVries, I. Wynhoff, and F. Jiguet. 2012. Differences in the climatic debts of birds and butterflies at a continental scale. Nature Climate Change 2:121–124.

Feder, M. E. 1983. Integrating the ecology and physiology of plethodontid salamanders. Herpetologica 39:291–310.

Frost, Darrel R. 2023. Amphibian Species of the World: an Online Reference. Version 6.2 (*2023*). Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. American Museum of Natural History, New York, USA. doi.org/10.5531/db.vz.0001

IUCN. 2022. The IUCN Red List of Threatened Species. Version 2022-2. https://www.iucnredlist.org. Accessed on [5 August 2023].

Johnson, C. K., and S. R. Voss. 2013. Chapter Eight - Salamander Paedomorphosis: Linking thyroid hormone to life history and life cycle evolution. Pages 229–258 *in* Y.-B. Shi, editor. Current Topics in Developmental Biology. Volume 103. Animal Metamorphosis, Academic Press. Accessed 21 Jul 2023.

Kiesecker, J. M., A. R. Blaustein, and L. K. Belden. 2001. Complex causes of amphibian population declines. Nature 410:681–684.

Marks, S. B., and A. Collazo. 1998. Direct development in Desmognathus aeneus (Caudata: Plethodontidae): A Staging Table. Copeia 1998:637–648.

Marshall, J. L., and C. D. Camp. 2006. Environmental correlates of species and genetic richness in lungless salamanders (family plethodontidae). Acta Oecologica 29:33–44.

Morrison, C., and J.-M. Hero. 2003. Geographic variation in life-history characteristics of amphibians: a review. Journal of Animal Ecology 72:270–279.

Parra-Olea, G., E. Martínez-Meyer, and G. P.-P. De León. 2005. forecasting climate change effects on salamander distribution in the highlands of Central Mexico. Biotropica 37:202–208.

Rabosky, D. L., and D. C. Adams. 2012. rates of morphological evolution are correlated with species richness in salamanders: species diversification and phenotypic evolution. Evolution 66:1807–1818.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Velo-Antón, G., J. L. Parra, G. Parra-Olea, and K. R. Zamudio. 2013. Tracking climate change in a dispersal-limited species: reduced spatial and genetic connectivity in a montane salamander. Molecular Ecology 22:3261–3278.

Wake, D. B. 1991. Declining Amphibian Populations. Science 253:860–860.

Wake, D. B., and J. Hanken. 1996. Direct development in the lungless salamanders: what are the consequences for developmental biology, evolution and phylogenesis? The International Journal of Developmental Biology 40:859–869.

Chapter I: Interacting effects of temperature and food on the early growth of *Plethodon cinereus*

Abstract

For ectotherms, temperature and food availability can lead to plasticity in growth and affect population demography. However, in salamanders, the outcome of this interaction is not clear, hindering knowledge of how species demography will respond to future climate conditions. In this study, I investigated the growth patterns of young-of-the-year and juvenile *Plethodon cinereus* in response to different temperatures (low and high) and food availability. The objective of the study is to understand how environmental conditions influence the growth trajectories of *P. cinereus*. To investigate the relationship between body size and environmental conditions, I compared body growth over time under a factorial design, and the data were analyzed using a generalized additive mixed model. By utilizing this method, I was able to examine the impact of two temperature regimes (high and low) and two food regimes (high and low) on both length (snout-vent length) and mass of the salamanders. The results of the study revealed diet had the largest influence on patterns for both measures of growth. However, length and mass both increased in warmer temperatures. In cooler temperatures, salamander lengths did not change, and mass decreased, especially in the low food treatment. My hypothesis was not supported, as salamanders in the high-temperature treatments outgrew those in the low-treatments. The results suggest that the body size of a directly developing salamander is not limited by temperature.

Introduction

An organism's life history describes its patterns of growth, development, and reproduction (Morrison and Hero 2003). Life history traits are essential to the fundamental understanding of populations and determine how populations respond to environmental changes over time. Specifically, fluctuations in climate can alter life history parameters (growth rate and age at sexual maturity) and alter population demography (Roff 1992; Stearns 1992). This is especially true for ectothermic organisms like amphibians, as both temperature and precipitation alter their life history traits (Trochet et al. 2014). Additionally, the consequences of climate change may also induce changes to body due to changes in food availability (Connette et al. 2015). Both temperature and food availability can lead to variability in growth and lead to differences in age and size at maturity (Rawson and Hilbish 1991, Sorci et al. 1996; Morrison and Hero 2003). Despite the importance of life-history traits to species persistence, knowledge gaps still exist due to missing data, hindering predictions on how species demography will respond to future climate conditions.

As ectotherms, amphibians rely on external sources for the regulation of their body temperatures (Huey and Stevenson 1979; Pough 1980; Huey, 1982; Duellman and Trueb 1994). Consequently, the body temperatures of these organisms fluctuate within the thermal range of their environment (Buckley et al. 2012). Often described as thermal conformers, amphibians such as salamanders are unable to regulate their body temperature (Feder 1983; Farallo et al. 2018). Instead, they adapt their behavior by occupying micro-environments with favorable temperatures and conditions. The body temperature of these ectothermic organisms ultimately impacts their fitness and performance (Huey and Stevenson 1979; Huey and Kingsolver 1989). Nevertheless, both low and high temperatures can significantly impact the organism's behavioral and physiological function by reducing foraging activities and modifying metabolism (Huey and Kingsolver 1989; Angilletta et al. 2004). As a result, ectothermic organisms have variability in growth, which allows them to adapt to varying environmental conditions (Angilletta et al. 2003; Kingsolver et al. 2004; Walters and Hassall 2006). Variability in growth in different temperatures has been suggested to explain the phenomenon known as the Temperature-Size Rule (TSR) for ectothermic organisms (Atkinson 1994; Atkinson et al. 1997; Forster et al. 2012). The TSR describes the pattern that in cool climates, organisms are larger, while individuals in warm climates are smaller (Angilletta et al. 2004; Blackburn et al. 1999).

The TSR has been documented within and between a range of vertebrate taxa, but evidence of TSR within amphibians has so far been mixed, warranting further research. Salamanders in particular have shown an intricate relationship between body size and temperature. In a study conducted by Adams and Church (2008), only three out of 40 species of *Plethodon* salamanders exhibited a negative correlation between body size and temperature. Additionally, seven out of 40 species exhibited a positive relation between body size and temperature, the inverse of the TSR (Adams and Church 2008). In their study, Adams and Church (2008) highlight the complicated relationship between body size and temperature, displaying three different outcomes between species size and temperature. In a separate study, Caruso et al (2014) reported that in the last 5 decades, six species of *Plethodon* displayed a reduction in body size with warming climates, indicating a plastic response to climate). The results of these studies highlight the need for additional research addressing the complex relationship between temperature and size, and the ecological and physiological mechanisms that may be impacting salamander growth. As the climate continues to change, salamanders are experiencing longer durations of dry conditions in addition to elevated temperatures (Liles et al. 2017). These factors can negatively impact a salamander's ability to forage, leading to a reduction in foraging times, and potentially increased metabolic rate and energy expenditure (Jaeger 1972). An increase in metabolic rate, energy expenditure, and temperatures may exacerbate the impact on salamanders, further negatively impacting their ability to adapt to environmental changes.

Studies of amphibian populations that span an elevational gradient, such as plethodontid salamanders in the Appalachian Mountains have demonstrated that elevation is correlated with shifts in growth, development, and reproduction (Morrison and Hero 2003). For example, amphibians at higher altitudes are undergoing extended larval periods and are taking longer to reach minimum size for sexual maturity (Morrison and Hero 2003). These shifts are likely driven by the higher temperatures the salamanders are experiencing at lower elevations compared to higher temperatures (Liles et al. 2017). In a study examining the demographic consequences of climate variation for *Plethodon montanus*, Caruso and Rissler (2019) reported that precipitation had a positive effect on growth and survival rates during the active season. Predicting how changes in climate will affect salamander demography therefore depends on how changes in temperature and precipitation will affect salamander foraging and metabolic demands, and their subsequent effects on salamander growth. In particular, early growth of salamanders before maturation can influence size at maturation and maximum body size, which in turn are likely to affect reproductive output in female, so understanding how these factors effect early growth can inform how population demography and growth rate will change under future conditions (Semlitsch et al. 1988).

To investigate the contributions of temperature and resource availability on early growth, I used controlled lab experiments to test the mechanisms that have been suggested to drive the TSR within *P. cinereus,* the eastern red-backed salamander, which has been studied intensely throughout the eastern United States (Leclair et al. 2006). This allowed me to determine how environmental conditions at early life stages interact to influence the growth of *P. cinereus*. Based on evidence for the TSR from other ectotherms, I hypothesized that at higher levels of food availability, salamanders reared at lower temperatures will grow larger than salamanders reared at higher temperatures. To understand this interaction, I grew young-of-the-year and juvenile individuals, in a fully factorial experimental design, in which two thermal treatments were crossed with two levels of food availability.

Study Species

Plethodon cinereus is a widely distributed salamander ranging from Canada to the southeastern United States (North Carolina/Tennessee) (Nagel 1977; Petranka 1998). This species of salamander is a fully terrestrial woodland salamander in the plethodontid family (Figure 1). Due to its lack of lungs, *P. cinereus* relies on cutaneous respiration for gas exchange (Feder 1983). Consequently, *P. cinereus* must remain moist to avoid desiccation due to their permeable skin (Feder 1983). Development occurs terrestrially via direct development, bypassing the aquatic larval stage (Bergeron et al. 2010). *P. cinereus* reaches sexual maturity after approximately two years (Bausmann and Whitaker 1987).

Methods

Yong-of-the-year and juvenile *P.* were collected from Minie Ball Hill (Salt Pond Mountain; 1128 m elevation; 37.40501, -80.50979) in Giles County, Virginia. A total of 9 nighttime visual encounter surveys were conducted between June and early October in 2021 (Sayler 1966). Captured salamanders were measured and placed in plastic bags with leaf litter for transport back to the Virginia Tech campus in Blacksburg, VA in coolers. To ensure that the salamanders collected were not adults, only salamanders under 29-mm total length were selected for this experiment (Sayler 1966; Petranka 1998). Upon arrival, each salamander was assigned an individual ID, measured (snout-vent length (SVL) (Posterior vent), total length (TL), and mass), and housed by itself in a plastic container (Dart CH48DEF 47 oz Tamper-Resistant Clear Hinged Container). To maintain a moist environment, the plastic containers were filled with moist paper towels.

After an acclimation period (four months), the salamanders were placed in one of two food treatments, and two temperature regimes, which were crossed in a factorial design for a total of four treatments (Figure 2). Animals were randomly assigned to each treatment in September and October 2021 until I had approximately 30 in each treatment for a total 122 animals (Figure 2).

All containers were placed in one of two growth chambers (Conviron Growth Chamber - Model ATC60), which were maintained at each of the experimental temperature regimes. Salamanders were monitored daily at the start, after two months into the experiment, their health was monitored every three days. Containers were cleaned once a week and individuals were fed one of two diets (according to their food treatment) per week on the same day. The salamanders were fed using a wingless strain of *Drosophila melanogaster* obtained initially from Josh's Frogs (online mail order business – Owosso, Michigan) and maintained in the lab where new cultures were made biweekly. The salamanders in the high-food treatment received a total of 30 fruit flies, while the salamanders in the low-food treatment received 20 fruit flies per week from the recommendation from K. Hamed and J. Uyeda, Virginia Tech.

The growth chambers were equipped with built-in thermostats, as well as two backup thermometers (Temp Stick Model TEMP-STICK-TH-W-FBA, and ThermoPro TP50). The lighting was initially set to a 12-12 day-night cycle. Throughout the experiment, the high-temperature treatment temperatures were set up four degrees above ambient (e.g., 10°C vs. 14°C) (ambient air temperature was determined by the temperatures of the month in which the experiment began, which was consistent with published temperatures in similar experiments (Beachy 2018). The treatment groups were Group 1: Temperature at 10°C with low food (20 Wingless Drosophila per week), Group 2: Temperature at 10°C with high food (20 Wingless Drosophila per week), Group 3: Temperature at 14°C with low food (20 Wingless Drosophila per week), Group 4: Temperature at 14°C with 30 Wingless Drosophila per week)

I monitored the growth of individual salamanders from October 2021 to October 2022, taking measurements of mass (g), SVL (mm), and TL (mm) every month. To measure SVL and TL, I used an electric digital caliper (NEIKO 01407: Measurement Accuracy 0.001''/0.02 mm) and a Ziploc bag. Once inside the bag, the salamanders were pressed against the edge and measured in a straightened position.

For mass, using an analytical balance (U.S. Solid Model: USS-DBS15-1: Accuracy 0.001 g/1 mg) salamanders were weighed using a weight boat.

Seasonal variation in experimental conditions

The length of the light cycle and temperature over the 12-month experiment was manipulated to mimic seasonal variation in day length and thermal conditions. At the beginning of the experiment, the growth chambers were set to 10°C and 14°C with the lighting set to a 12:12 hour light cycle Temperatures were decreased incrementally to represent the onset of winter. After an acclimation period to ambient temperatures, on 8 October 2021, the growth chamber temperatures were lowered to 9°C (Low-Temperature Treatment (LT)) and 13°C (High-Temperature Treatment (HT)). On 9 November 2021, the temperatures decreased to 8°C (LT) and 12°C (HT) with lighting was set to 9:15 hour lighting cycle, then on 8 December 2021, I lowered the temperatures to 7°C (LT) and 11°C (HT). Between 26 May 2022 and 4 June 2022, the chambers were incrementally increased to represent the onset of summer. The low-temperature treatment was raised from 7°C to 11°C while the high-temperature treatment was increased from 11°C to 15°C. Between 20 March 2022 and 26 March 2022, the lighting cycle was set to a 12:12 hour lighting cycle. On 3 October 2022, the experiment was concluded. All the salamanders were euthanized humanely in a buffered bath of MS-222. All husbandry and experimental procedures were conducted in accordance with IACUC protocol 20-094.

All data were entered into Excel and checked for quality control. They were analyzed in program R version 4.2.2 (R Core Team 2023). To determine how environmental conditions at early life stages influence the growth of *P. cinereus*, body growth over time in each treatment was compared using a generalized additive mixed model (GAMM). The random effect being individual identity (repeated samples), while our fixed effects encompass food and temperature. Differences in body size (SVL) (mm) and mass (g) at the end of the experiment were analyzed using an ANOVA. To determine significant differences between the group means, a Tukey post-hoc test was used.

Results

Preliminary inspection of the data revealed nonlinear trends in SVL and mass over the experiment (Supplemental Figure 1). Variation in the data on SVL and TL suggested that measurement error from the caliper for some months was greater than incremental growth (Supplemental Figure 1). Therefore, only SVL measurements in October 2021, March 2022, and October 2022 were considered. By using the GAMM, I was able to analyze the smoothed relationship between *P. cinereus* growth (response variable) and temperature and food (explanatory variables) (Figure 3) (Table 3). Differences among treatments in body size after 12 months in each treatment were analyzed using an ANOVA, which revealed a significant interaction between temperature and food on body size ($F_{1,58}$ = 5.606, p = 0.021) (Table 1). A Tukey post-hoc test suggested that the differences in SVL due to food levels were most significant at low temperatures (diff = -3.164, p adj = 0.013) (Table 2).

Figure 4 shows the smooth trends in mass (in g) from the GAMM analysis *over* the duration of the experiment. All mass measurements were included. This GAMM model also supported an interacting effect of temperature and food on body mass (in grams) over the course of the experiment (Table 3). Individuals at high temperatures experienced increases in mass throughout the experiment at both food levels, but the effect was much greater for the high food treatment. Individuals in the low temperature treatment stayed the same or decreased in mass over the study, especially in the low food treatment.

Discussion

The study investigated the influence of temperature and resource availability on the early growth *P. cinereus*. I intended to better understand how environmental conditions during early life stages impact growth patterns and body size. By better understanding the factors that influence growth for a plethodontid salamander such as *P. cinereus* we can gain a better understanding of their population dynamics and ecological interactions. Early growth for salamanders is crucial for size at maturation and maximum body size and can significantly shape the fitness and survival of an organism (Semlitsch et al. 1988). To understand this interaction, young-of-the-year and juvenile individuals were exposed to a fully factorial experimental design, in which two thermal treatments were crossed with two levels of food availability. Based on evidence from other ectotherms that larger animals live in cooler climates, I hypothesized, that salamanders reared at lower temperatures will grow larger than salamanders reared at higher temperatures, especially at higher levels of food availability.

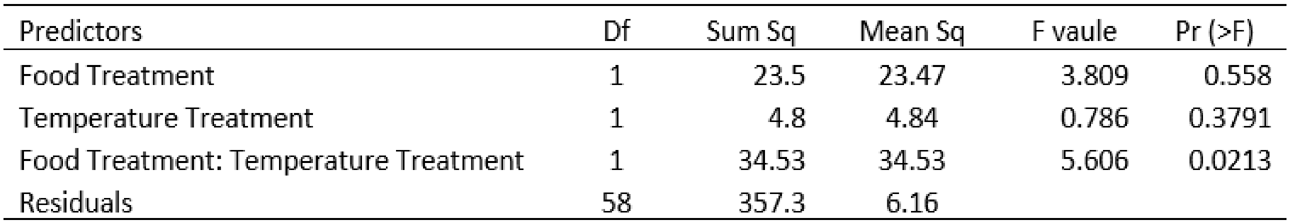
The results of the study indicated a consistent pattern for growth (length) through the duration of the experiment. In the warm temperature treatments, there was gradual increase in both growth measures. Conversely, the individuals subjected to the cool temperature treatments experienced a decrease in mass, especially at low food levels; length appeared to be constant (Figure 3 and Figure 4). The High Temperature: High Food treatment demonstrated the greatest increase in growth for mass, while Low Temperature: Low Food treatment demonstrated lowest decrease in mass (figure 4). Under all the treatments, not all prey was consumed. No treatment exhibited a significantly higher prey consumption. The decline in growth rates in the salamanders exposed to lower temperatures may have been caused by decreases in metabolic activity and/or reduced growth rates (Arendt 2011).

The results of my study did not support my hypothesis, that salamanders reared at lower temperatures will grow larger than salamanders reared at higher temperatures, especially at higher levels of food availability. Regardless of the food availability, the salamanders in the high temperature treatments experienced consistent growth throughout the duration of experiment. Conversely, under the low temperature treatments, variations in growth were due to food levels. The data suggest that the body size of *P. cinereus* was not limited by temperature. Salamanders in high temperature treatments may have experienced heightened metabolic rates and/or enhanced growth rates (Arendt 2011). However, in the field *P. cinereus* has been documented to have experienced temperatures between 18°C and 30°C (Homyack 2011). Furthermore, Homyack (2011), reported a 33% greater basic maintenance cost, indicating a larger number of calories are needed when exposed to higher temperatures. Considering this it’s worth noting that changes in maintenance cost can influence ectotherms growth rate and reproductive output (Sears 2005; Homyack 2011). Unfortunately, due to the experimental conditions, the lab prevented me from exploring higher ranges of temperatures. Therefore, I selected temperatures that matched those used in previous studies (Beachy 1995; Beachy 2018; Hickerson et. al. (2005)).

This study is the first to examine the effects of temperature and food availability on a direct developing plethodontid salamander. Previous similar studies have examined these factors in salamanders with different developmental paths: Allegheny Mountain dusky salamander (*Desmognathus ochrophaeus*) (metamorphosis) (Beachy 1995), Four-toed salamander (*Hemidactylium scutatum*) (postembryonic metamorphosis) (O’Laughlin et. al. 2000), Blackbelly salamander (*D. quadramaculatus*) (metamorphosis) (Hickerson et. al. 2005), and Blue Ridge two-lined salamander (*Eurycea wilderae*) (metamorphosis) (Beachy 2018). The relationship between temperature and food was complex for larvae when examining the Allegheny Mountain dusky salamanders (Beachy 1995). Food regimes appeared to have a greater impact on growth in the high temperature treatment. In contrast to Beachy (1995), the opposite relation between temperature and food was found. In my study, under the low temperature treatments, variations in growth were due to food levels. The results of a significant interaction between food availability and low temperature are consistent with the results reported by Hickerson et. al. (2005). As in Beachy (2018), the salamanders that had limited food availability grew at a slower rate compared to the salamanders with higher food availability. By examining *P. cinereus*, the study contributed to our understanding of the relationship between temperature and food in a direct developing plethodontid salamander. When considering the implications of climate change, the results suggest predicting the effects on salamander populations will be complex. We will have to consider other various factors such as foraging activities, habitat quality/availability, food availability, and phenology to fully understand salamander life history parameters and population demography.

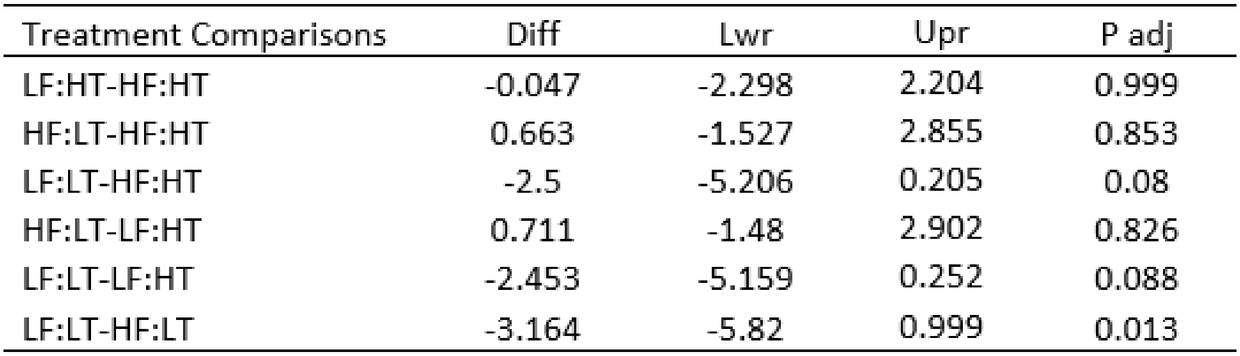
Chapter I Tables

Table 1. ANOVA reveals a significant interaction between temperature and food on body size. In the table, “Df” signifies the degree of freedom, “Sum Sq” represents the sum of squares divided by the degrees of freedom, and “Mean Sq” indicates the mean square.



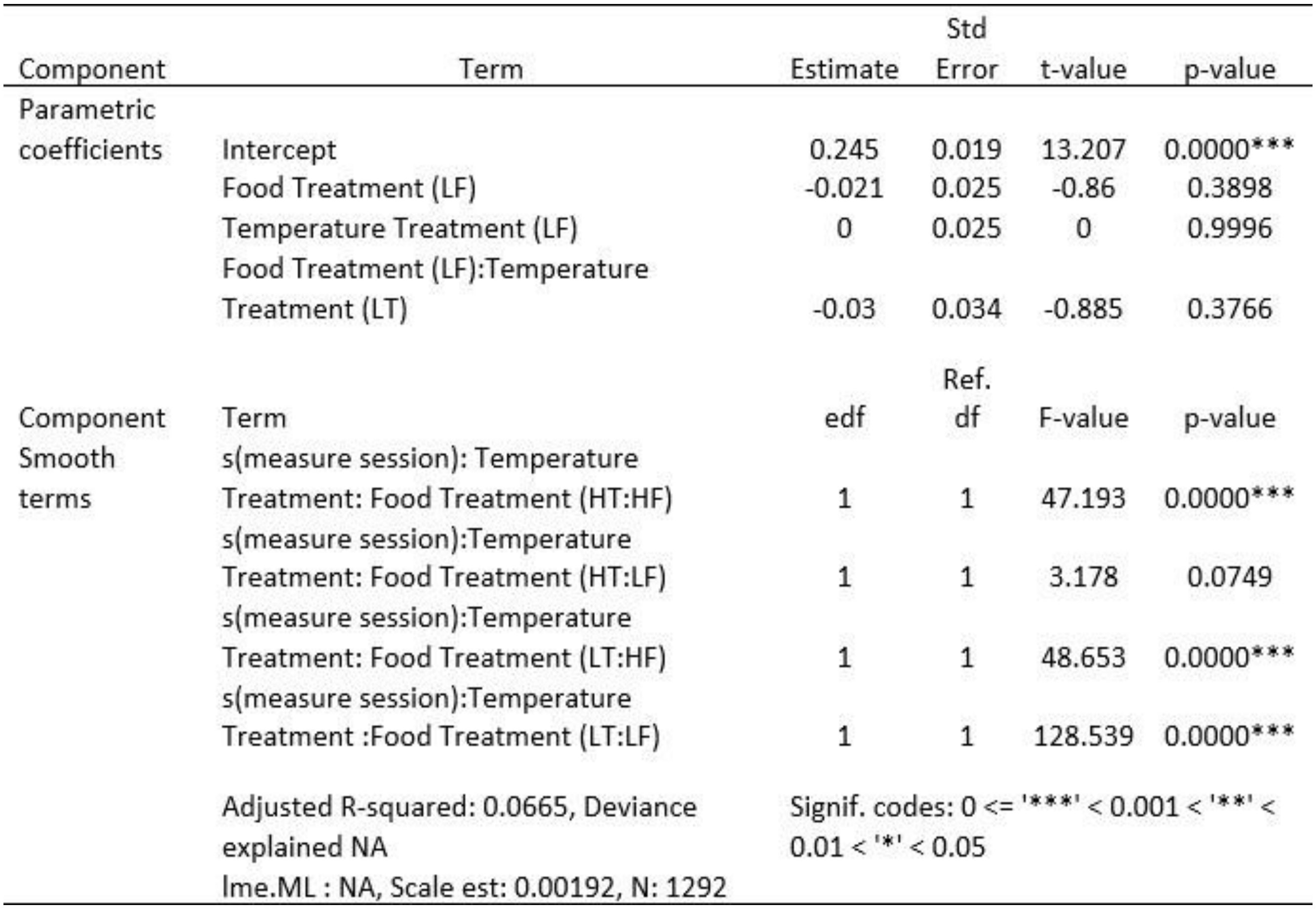
Predictors	Df	Sum Sq	Mean Sq	F vaule	Pr (>F)
Food Treatment	1	23.5	23.47	3.809	0.558
Temperature Treatment	1	4.8	4.84	0.786	0.3791
Food Treatment: Temperature Treatment	1	34.53	34.53	5.606	0.0213
Residuals	58	357.3	6.16		

Table 2. Tukey multiple comparisons of SVL means. The table provides insight to comparison at among the independent variables (Food and Temperature Treatments). The results suggested that differences in SVL were due to food levels. This is most evident in the low temperature treatment. In the table, “Treatment Comparison” denotes the pairwise comparison between treatments, while “Diff” signifies the difference in means between the two treatments, Lwr indicates the lower confidence limit, and Upr represents the upper confidence limit between the treatments. Additionally, the P adj represents the p values that have been adjusted to account for the multiple comparisons.



Treatment Comparisons	Diff	Lwr	Upr	P adj
LF:HT-HF:HT	-0.047	-2.298	2.204	0.999
HF:LT-HF:HT	0.663	-1.527	2.855	0.853
LF:LT-HF:HT	-2.5	-5.206	0.205	0.08
HF:LT-LF:HT	0.711	-1.48	2.902	0.826
LF:LT-LF:HT	-2.453	-5.159	0.252	0.088
LF:LT-HF:LT	-3.164	-5.82	0.999	0.013

Table 3. MASS GAMM summary statistics table. This is an analysis of body mass when exposed to two temperature treatments and two food treatments. This GAMM revealed that there was an interactive effect of temperature and food on body mass (in grams) throughout the duration of the experiment. In the table, “Std error” indicates the standard error, “edf” represents the effective degrees of freedom, and “Ref. df” represents the reference degree of freedom.

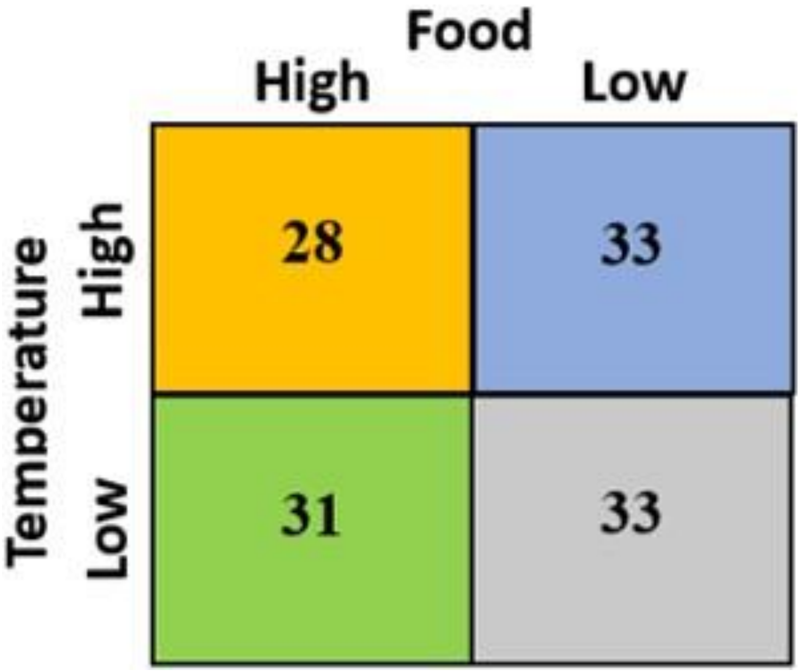


Component	Term	Estimate	Std Error	t-value	p-value
Parametric coefficients	Intercept	0.245	0.019	13.207	0.0000***
	Food Treatment (LF)	-0.021	0.025	-0.86	0.3898
	Temperature Treatment (LF)	0	0.025	0	0.9996
	Food Treatment (LF):Temperature Treatment (LT)	-0.03	0.034	-0.885	0.3766
Component	Term	edf	Ref. df	F-value	p-value
Smooth terms	s(measure session): Temperature Treatment: Food Treatment (HT:HF)	1	1	47.193	0.0000***
	s(measure session):Temperature Treatment: Food Treatment (HT:LF)	1	1	3.178	0.0749
	s(measure session):Temperature Treatment: Food Treatment (LT:HF)	1	1	48.653	0.0000***
	s(measure session):Temperature Treatment :Food Treatment (LT:LF)	1	1	128.539	0.0000***
	Adjusted R-squared: 0.0665, Deviance explained NA lme.ML : NA, Scale est: 0.00192, N: 1292	Signif. codes: 0 <= '***' < 0.001 < '**' < 0.01 < '*' < 0.05			

Chapter I Figures

Figure 1. Photo of an adult *Plethodon cinereus*.

Figure 2. Visual of experimental design. The numbers represent the number of salamanders at the beginning of the experiment.



Temperature	Food: High	Food: Low
High	28	33
Low	31	33

Figure 3. Mean SVL (mm) of *P. cinereus* in two food treatments, crossed with two temperature treatments. treatments (High Temperature: High Food, High Temperature: Low Food, Low Temperature: High Food, Low Temperature: Low Food). The red line represents the High temperature treatment, while the blue line represents the Low Temperature treatment. The left panel represents the High Food treatment, while the right panel represents the Low Food treatment. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.

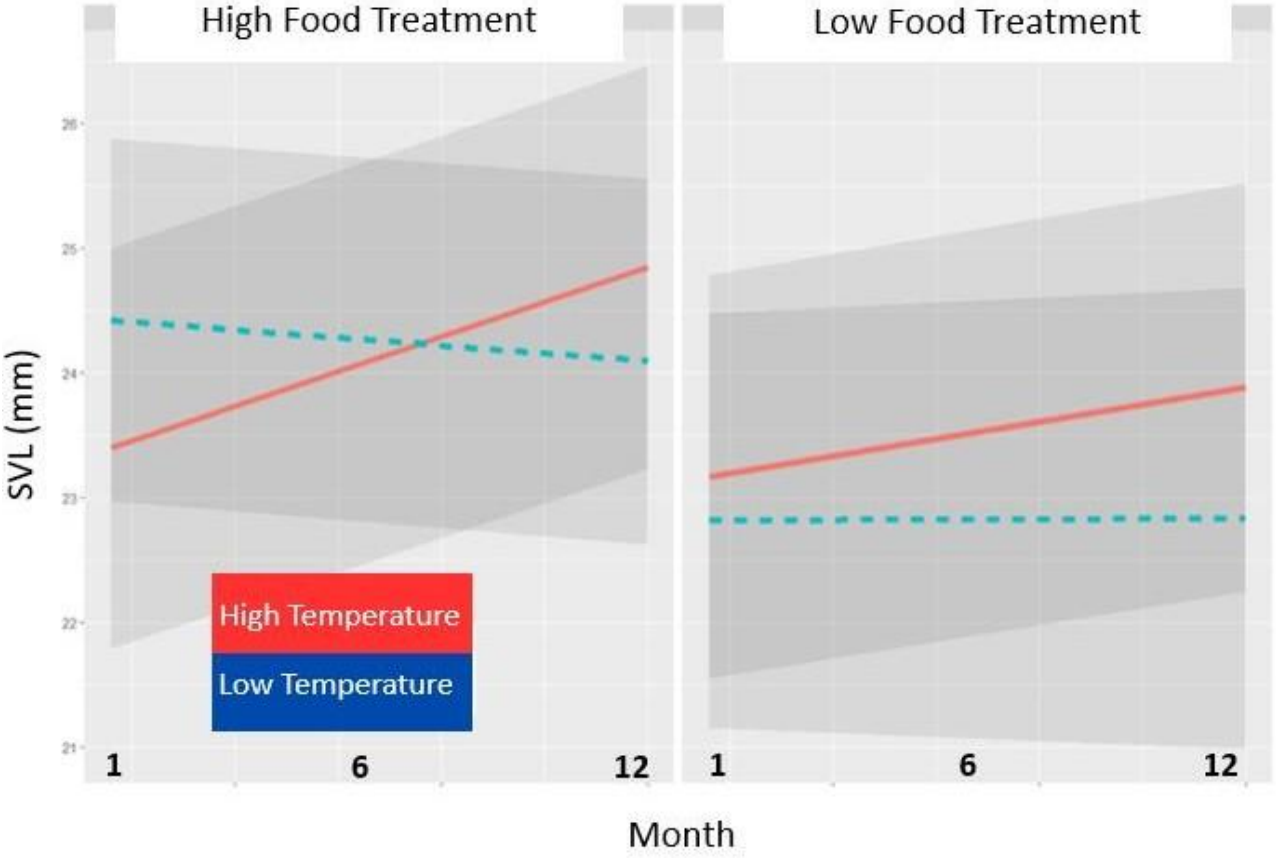
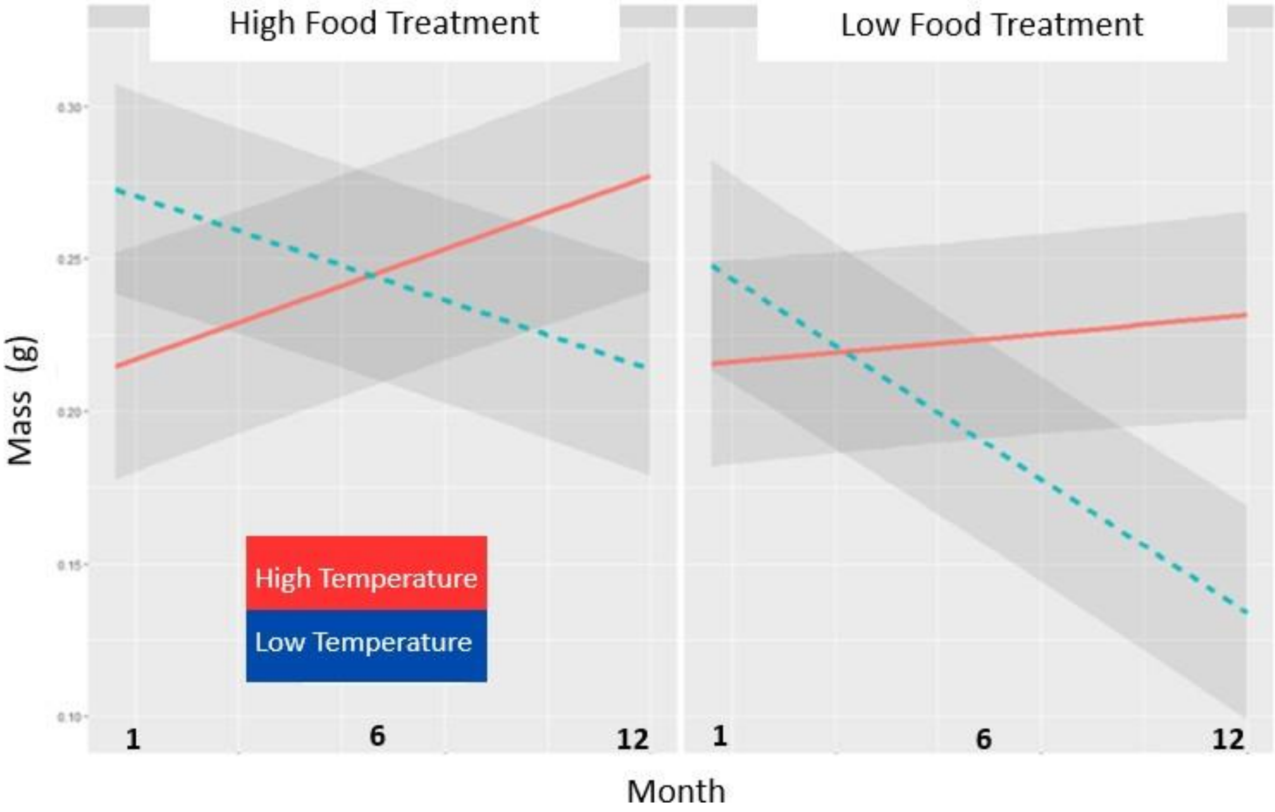


Figure 4. Mean Mass (g) of *P. cinereus* depicted under the four treatments (High Temperature: High Food, High Temperature: Low Food, Low Temperature: High Food, Low Temperature: Low Food). The High Temperature treatment is represented by the red line, while the Low Temperature is represented by the blue line. The left panel how the High Food treatment and right panel represents the Low Food treatment. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.



Literature Cited Chapter I

Adams, D. C., and J. O. Church. 2008. Amphibians do not follow bergmann's rule. Evolution 62:413–420.

Angilletta, M. J., Jr., T. D. Steury, and M. W. Sears. 2004. Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle1. Integrative and Comparative Biology 44:498–509.

Angilletta, M. J., R. S. Wilson, C. A. Navas, and R. S. James. 2003. Tradeoffs and the evolution of thermal reaction norms. Trends in Ecology & Evolution 18:234–240.

Atkinson, D. 1994. Temperature and Organism Size—A Biological Law for Ectotherms? Pages 1–58 in M. Begon and A. H. Fitter, editors. Advances in Ecological Research. Volume 25. Academic Press.

Atkinson, D., and R. M. Sibly. 1997. Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. Trends in Ecology & Evolution 12:235–239.

Bausmann, G. A., and O. Whitaker., Jr 1987. Studies of the Habitat and Food of Sympatric Populations of Plethodon cinereus (Green) and Plethodon dorsalis Cope in South Central Parke County, Indiana. Proceedings of the Indiana Academy of Science 97:513–524.

Beachy, C. K. 1995. Effects of Larval Growth History on Metamorphosis in a Stream-Dwelling Salamander (Desmognathus ochrophaeus). Journal of Herpetology 29:375–382.

Beachy, C. K. 2018. Effects of Growth Rate and Temperature on Metamorphosis in Eurycea wilderae (Caudata, Plethodontidae, Hemidactyliinae, Spelerpini; Blue Ridge Two-lined Salamander). Southeastern Naturalist 17:423–432.

Bergeron, C. M., C. M. Bodinof, J. M. Unrine, and W. A. Hopkins. 2010. Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians. Environmental Toxicology and Chemistry 29:980–988.

Buckley, L. B., A. H. Hurlbert, and W. Jetz. 2012. Broad-scale ecological implications of ectothermy and endothermy in changing environments. Global Ecology and Biogeography 21:873–885.

Connette, G. M., J. A. Crawford, and W. E. Peterman. 2015. Climate change and shrinking salamanders: alternative mechanisms for changes in plethodontid salamander body size. Global Change Biology 21:2834–2843.

Caruso, N. M., and L. J. Rissler. 2019. Demographic consequences of climate variation along an elevational gradient for a montane terrestrial salamander. Population Ecology 61:171–182.

Caruso, N. M., M. W. Sears, D. C. Adams, and K. R. Lips. 2014. Widespread rapid reductions in body size of adult salamanders in response to climate change. Global Change Biology 20:1751–1759.

Duellman, W. E., and L. Trueb. 1994. Biology of Amphibians. JHU Press.

Farallo, V. R., R. Wier, and D. B. Miles. 2018. The Bogert effect revisited: Salamander regulatory behaviors are differently constrained by time and space. Ecology and Evolution 8:11522.

Feder, M. E. 1983. Integrating the Ecology and Physiology of Plethodontid Salamanders. Herpetologica 39:291–310.

Forster, J., A. G. Hirst, and D. Atkinson. 2012. Warming-induced reductions in body size are greater in aquatic than terrestrial species. Proceedings of the National Academy of Sciences 109:19310–19314.

Hickerson, C.-A. M., E. L. Barker, and C. K. Beachy. 2005. Determinants of Metamorphic Timing in the Black-bellied Salamander, Desmognathus quadramaculatus. Southeastern Naturalist 4:33–50.

Highton, R., and T. P. Webster. 1976. Geographic protein variation and divergence in populations of the salamander plethodon cinereus. Evolution 30:33–45.

Homyack, J., C. Haas, and W. Hopkins. 2011. Energetics of Surface-Active Terrestrial Salamanders in Experimentally Harvested Forest. The Journal of Wildlife Management 75:1267–1278.

Huey, R. B., and J. G. Kingsolver. 1989. Evolution of thermal sensitivity of ectotherm performance. Trends in Ecology & Evolution 4:131–135.

Huey, R. B., and R. D. Stevenson. 1979. Integrating Thermal Physiology and Ecology of Ectotherms: A Discussion of Approaches. American Zoologist 19:357–366.

Kingsolver, J. G., R. Izem, and G. J. Ragland. 2004. Plasticity of Size and Growth in Fluctuating Thermal Environments: Comparing Reaction Norms and Performance Curves1. Integrative and Comparative Biology 44:450–460.

Morrison, C., and J.-M. Hero. 2003. Geographic variation in life-history characteristics of amphibians: a review. Journal of Animal Ecology 72:270–279.

Nagel, J. W. 1977. Life History of the Red-Backed Salamander, Plethodon cinereus, in Northeastern Tennessee. Herpetologica 33:13–18.

O'Laughlin, B. E., and R. N. Harris. 2000. Models of metamorphic timing: an experimental evaluation with the pond-dwelling salamander Hemidactylium scutatum (Caudata: Plethodontidae). Oecologia 124:343–350.

Pough, F. H. 1980. The Advantages of Ectothermy for Tetrapods. The American Naturalist 115:92–112.

Rawson, P. D., and T. J. Hilbish. 1991. Genotype-Environment Interaction for Juvenile Growth in the Hard Clam Mercenaria mercenaria (L.). Evolution 45:1924–1935.

Rose, M., and L. Mueller. 2002. Stearns, Stephen C., 1992. The Evolution of Life Histories. Oxford University Press, London xii + 249 pp., £16.95. Journal of Evolutionary Biology 6:304–306.

Sayler, A. 1966. The Reproductive Ecology of the Red-Backed Salamander, Plethodon cinereus, in Maryland. Copeia 1966:183–193.

Sears, M. W. 2005. Resting metabolic expenditure as a potential source of variation in growth rates of the sagebrush lizard. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 140:171–177.

Semlitsch, R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and Size at Metamorphosis Related to Adult Fitness in Ambystoma Talpoideum. Ecology 69:184–192.

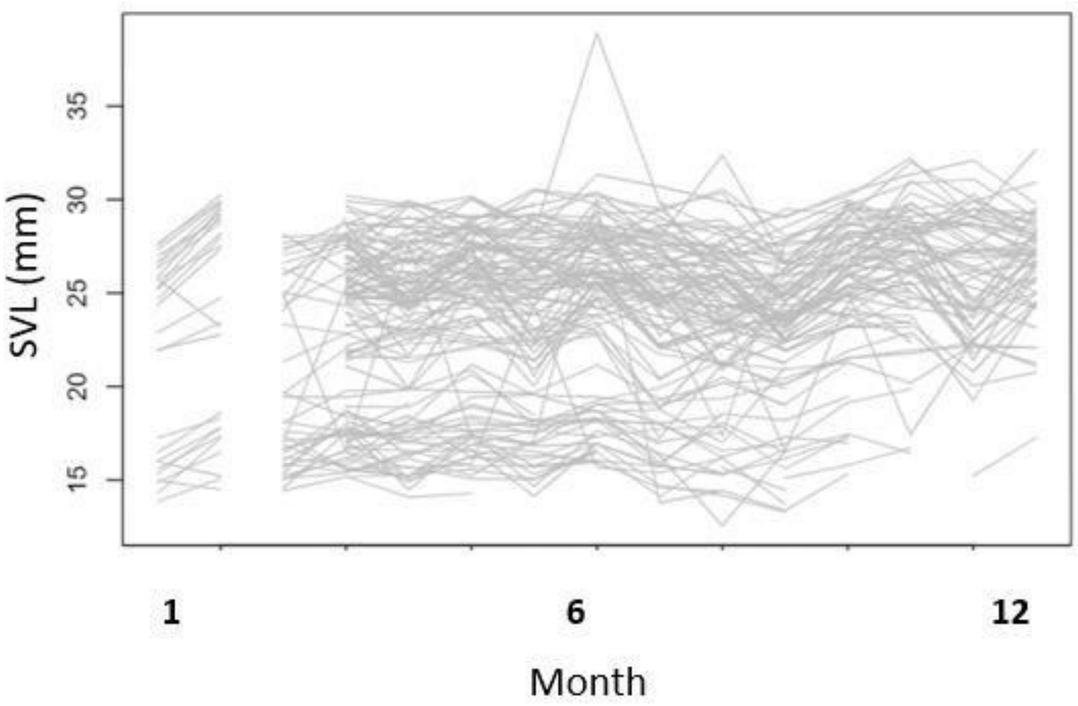
Sorci, G., J. Clobert, and S. Belichon. 1996. Phenotypic Plasticity of Growth and Survival in the Common Lizard Lacerta vivipara. Journal of Animal Ecology 65:781–790.

Trochet, A., S. Moulherat, O. Calvez, V. Stevens, J. Clobert, and D. Schmeller. 2014. A database of life-history traits of European amphibians. Biodiversity Data Journal 2:e4123.

Walters, R. J., and M. Hassall. 2006. The Temperature-Size Rule in Ectotherms: May a General Explanation Exist after All? The American Naturalist 167:510–523.

Supplemental Figures Chapter I

Supplemental Figure 1. Measured variation in SVL for all individuals in all treatments. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.



Chapter II: Interacting effects of temperature and food on the early growth of *Eurycea cirrigera*

Abstract

An amphibian's life history includes patterns such as growth, development, and reproduction, which vary due to different environmental conditions such as temperature and food availability. At high latitudes and altitudes, some amphibians take a longer time to reach a minimum size for sexual maturity and the age of first reproduction. This slower life history can have important effects on population ecology, such as age structure. This pattern can follow the Temperature-Size Rule, which describes the phenotypically plastic response of size at different temperatures. However, resources (food) are another source of variation in body size, and it is unclear how it might interact with temperature. To test the interacting effects of temperature and food resources on growth, I conducted a controlled experiment. In August of 2020, I collected a total of 120 southern two-lined salamanders (*Eurycea cirrigera),* and I exposed the salamanders to a factorial design containing two food treatments (1 and 2 black worms per week) crossed with two temperature treatments (11°C and 16°C). Once a month, the salamanders were measured for total length (TL), and mass (g). The data was analyzed using a generalized additive mixed model (GAMM). The GAMM revealed that growth patterns over the course of the experiment differed according to both food and temperature. When food levels were high enough, my hypothesis was supported, as salamanders in the low-temperature treatments outgrew those in the high-treatments. However, differences in mass among the treatments disappeared by the end of the experiment.

Introduction

Worldwide, organisms are experiencing the negative effects of climate change due to increasing temperatures. Climate change is significantly affecting organisms by interrupting physiological and ecological processes (Liles et al. 2017). The temperature an organism is exposed to can impact their development and growth (van der Have and de Jong 1996; Reading 2007; Sheridan and Bickford 2011; Bickford et al. 2011; Liles et al. 2017). For ectotherms such as amphibians, increased temperatures have been observed to shorten time to metamorphosis and size at metamorphosis (Werner 1986; Blaustein et al. 2010). This trend has been documented in both anurans (frogs) and urodeles (salamanders) (Beachy 1995; Álvarez and Nicieza 2002; Hickerson et al. 2005; Blaustein et al. 2010). Temperature can lead to plasticity in growth, if warmer temperatures accelerate development causing ectotherms to obtain smaller body sizes at maturity. In cooler temperatures ectotherms may experience prolonged development, allowing them to mature with larger body sizes (Morrison and Hero 2003; Angilletta et al. 2004). Despite the importance of growth, further research is needed to evaluate the impact of temperature on early life, particularly in urodeles.

For amphibians such as salamanders, the larval period prior to metamorphosis is a key life stage due to its importance to fitness (Beachy 2018). A salamander's body size can affect their reproductive potential and survivability (Semlitsch et al. 1988). The growth rate of larvae is known to affect both size and age at metamorphosis and sexual maturity (Smith 1987; Semlitsch 1980; Beachy 2018). The growth and development of amphibians are controlled by factors such as food and temperature (extrinsic) and body size (intrinsic) (Morrison and Hero 2003). The two biggest factors influencing metamorphic conditions (growth rates) are both temperature and food (Alford and Harris 1988; Beachy 2018). In the ecological model of metamorphosis, Wilbur and Collins (1973) predicted that larvae experiencing slow growth rates should metamorphose quickly as possible and larvae experiencing rapid growth should delay metamorphosis and continue to grow (Wilbur and Collins 1973; Alford and Harris 1988; Bruce 2005). However, Travis (1984) proposed that early larval experiences set growth rates, and the growth rates can change depending on the available resources (Travis 1984; Alford and Harris 1988). In addition to the ideas of Wilbur and Collins (1973) and Travis (1984), the plasticity in larval growth rates could be attributed to the Temperature-Size Rule (TSR). The TSR describes the patterns to growth rate in response to temperature. In cool climates organisms are larger, while individuals in warm climates are smaller (Angilletta et al. 2004; Blackburn et al. 1999).

Evidence of the TSR within amphibians has received conflicting support from scientists, meriting further investigation. In particular, salamanders have exhibited a complex relationship between body size and temperature. When salamander larvae are exposed to low temperatures, development is delayed resulting in a longer larval period (Beachy 1995, Hickerson et al. 2005; Beachy 2018). In warmer temperatures, larval salamanders experience rapid development and shortened larval stages (Berven and Gill 1983; Voss 1993). Additionally, food availability and density may be contributing to the development of these larval salamanders (Semlitsch et al. 1988, Beachy 2018). However, the relationship between temperature and food on larval growth is complex and is poorly understood (Beachy 2018). Further research is needed to assess the complex relationship between temperature and food on larval growth.

To further investigate the contributions of temperature and resource availability on larval growth, I conducted lab experiments to test the mechanisms that have been suggested to affect early growth and potentially drive the Temperature-Size Rule within *Eurycea cirrigera.* Based on previous research (Wilbur and Collins 1973) (Beachy 2018), I hypothesized that if food availability is abundant, salamanders reared at lower temperatures will grow larger and metamorphose later than salamanders reared at higher temperatures. If food availability is low, salamanders reared at higher temperatures will grow larger than salamanders reared at lower temperatures. To understand this interaction, I grew larval individuals in a fully factorial experimental design, in which two thermal treatments were crossed with two levels of food availability. In contrast to the previous chapter, this experiment will investigate these factors in an aquatic setting in a metamorphic species which spends its early life breathing through external gills.

Study Species

Eurycea cirrigera, the southern two-lined salamander is a common salamander found within southeastern United States (Petranka 1998; Brophy and Pauley 2002; Guy et al. 2004). This species of salamander is a stream salamander that metamorphosis into a terrestrial (Jakubanis et al. 2008). Due to its lack of lungs, as adults *E. cirrigera* relies on cutaneous respiration for gas exchange (Feder 1976; Ruben and Boucot 1989). Adult *E. cirrigera* must remain moist to avoid desiccation due to their permeable skin (Feder 1983). However, juvenile *E. cirrigera* are fully aquatic and breathe through external gills. Prior reports are that *E. cirrigera* reached sexual maturity after approximately two years to four years (Petranka 1998).

Methods

Larval *E.* cirrigera were collected from the Jefferson National Forest (Craig's Creek; 518 melevation; 37.33184, -80.33834) in Montgomery County, Virginia. A total of four nighttime visual encounter surveys were conducted between late June and early August of 2020. Captured salamanders were placed in buckets with air stones (RESUN: DC-160 DC AIR PUMP), then transferred back to the Virginia Tech campus in Blacksburg, VA. Upon arrival, each salamander was assigned an individual ID, measured (total length (TL) (Tip of salamander snout to the end of the tail), and mass), and housed by itself in a plastic container (Dart CH48DEF 47 oz Tamper-Resistant Clear Hinged Container) filled with water purified through reverse osmosis. After an acclimation period in the chambers (4 months), the salamanders were placed in one of two food treatments randomly, and two temperature regimes randomly, which were crossed in a factorial design for a total of four treatments (Figure 8). Animals were added to each treatment in late June and early July 2020 until we had approximately 40 in each treatment for a total of 159 animals (Figure 8).

In the experiment, two growth chambers (Conviron Growth Chamber - Model ATC60) were used to maintain the temperatures regimes. At the start of the experiment, salamander health and water quality were monitored daily, and eventually every three days for health. The containers were cleaned (water changes) every three days, and eventually once a week. Salamanders were fed one of two diets (according to their food treatment) per week. The feeding and cleaning of the salamanders were conducted on the same day. The salamanders were fed using *Lumbriculus variegatus* (California Black Worms) obtained from Eastern Aquatics (Lancaster, Pennsylvania) and maintained in the lab through the duration of the experiment. The salamanders in the high-food treatment received one black worm per week, while the salamanders in the low-food treatment received two black worms per week.

The growth chambers contained built-in thermostats, and two backup thermometers (Temp Stick Model TEMP-STICK-TH-W-FBA, and ThermoPro TP50). At the beginning of the experiment, the lighting was set to a 12-12 day-night cycle. Through the duration of the experiment, the high-temperature treatment remained four degrees above ambient temperatures (e.g., 11°C vs. 15°C). The temperatures of the month in which the experiment began determined the ambient temperature, which was consistent with published temperatures in similar experiments (Beachy 2018). The treatment groups were Group 1: Temperature at 11°C with low food (1 black worm per week), Group 2: Temperature at 11°C with high food (2 black worm per week), Group 3: Temperature at 15°C with low food (one black worm per week), Group 4: Temperature at 15°C with high food (two black worms per week). I monitored the growth of individual salamanders from December 2020 to December 2022, taking measurements of mass (g) and total length (TL) in mm every month (To prevent any harm to the salamanders, I decided to not measure snout-vent-length). To measure TL, I used a microscope (Leica M80 – ACHRO 0.5X Lens) and for mass an analytical balance (U.S. Solid Model: USS-DBS15-1: Accuracy 0.001 g/1 mg) was used. Salamanders were gently straightened using an eraser to measure TL, ensuring no harm was done throughout the experiment.

Seasonal variation in experimental conditions

Over the 24-month experiment, the temperature and light cycle were manipulated to mimic seasonal variation in day length and thermal conditions. At the beginning of the experiment, the growth chambers were set to 11°C and 16°C with the lighting set to a 12:12 hour light cycle. To represent the onset of winter, the temperatures in the chambers were gradually reduced. After an acclimation period to the ambient temperatures, on 22 January 2021, the growth chamber temperatures were lowered to 9°C (Low-Temperature Treatment (LT)) and 13°C (High-Temperature Treatment (HT)). On 7 May 2021, the growth chamber temperatures were lowered to 7°C (Low-Temperature Treatment (LT)) and 11°C (High-Temperature Treatment (HT)). On 20 May 2021, the growth chamber temperatures were increased to 11°C (Low-Temperature Treatment (LT)) and 15°C (High-Temperature Treatment (HT)). Between 1st and 8th of October 2021, the chambers were incrementally lowered to 9°C (LT) and 13°C (HT). On 8 November 2021, the temperatures decreased to 8°C (LT) and 12°C (HT) with lighting being set to 9:15 hour lighting cycle, then 8 December 2021, I lowered the temperatures to 7°C (LT) and 11°C (HT). Between 26 of May and the 4th of June 2022, the chambers were gradually increased to represent the onset of summer. The low-temperature treatment was raised from °7C to 11°C while the high-temperature treatment was increased from 11°C to 15°C. Between 20th and 26th of Match 2022, the lighting cycle was set to a 12:12 hour lighting cycle. In December 2022, the experiment was concluded.

All the salamanders were euthanized humanely in a buffered bath of MS-222. All husbandry and experimental procedures were conducted in accordance with IACUC protocol 20-094.

For quality control, all the data were entered into MS Excel (Microsoft Corporation, 2023) and validated. The data was analyzed in R (Version 4.2.2). To determine how environmental conditions at early life stages influence the growth of *E. cirrigera*, a generalized additive mixed model (GAMM) was used to compare body growth over time in each treatment.). The random effect being individual identity (repeated samples), while our fixed effects encompass food and temperature Differences in body size (TL) and Mass (g) among treatments at the end of the experiment were analyzed using an ANOVA. To determine significant differences between the group means, I utilized a Tukey post-hoc test.

Results

After a preliminary inspection of the data, variable, but nonlinear trends in SVL were found throughout the duration of the experiment (Supplemental Figure. 2). To account for the non-linearity, a generalized additive mixed model (GAMM) was used to describe the trends within each treatment, including a random effect of individual. Through the GAMM, I analyzed the smoothed relationship between *E. cirrigera* growth (response variable) and temperature and food (explanatory variables) (Figure 9). To detect the differences among treatments in body length over 24 months, I used an ANOVA to analyze the data. The ANOVA revealed a non-significant interaction between temperature and food on body length ($F_{1,25} = 1.666$, $p = 0.209$) at the end of the experiment. However, the GAMM revealed that growth patterns over the course of the experiment differed according to both food and temperature. However, similar horseshoe-shaped patterns in both length and mass were observed in all treatments, although the maximum sizes reached were greater in cool treatments. In other words, in the cool temperature treatments, the salamanders displayed a gradual increase in body size followed by a gradual decrease in body size.

Figure 10 shows the smoothed trends of mass over the duration of the experiment from the GAMM analysis. The interacting effects of temperature and food on body mass at the end of the experiment was not significant ($F_{1,106} = 0.037$, $p = 0.848$) (Table 5). However, the timing of growth varied between treatments. The individuals exposed to the high temperatures experienced decreases in mass (g) ahead of the low temperature individuals. The Individuals in the low temperature treatment treatments experienced similar trends in growth, while the high temperature treatments differed in growth according to food levels. With high temperatures, the salamanders experienced rapid growth followed by a gradual decline in both mass and length, although this trend was weaker with low food. At low temperatures and high food, salamanders grew large and then declined in mass; this trend was similar but weaker with low food (Figures 9 and 10).

Discussion

The study explored the impact temperature and resource availability had on the early growth of *E. cirrigera* in a two-year period. By conducting the experiment, I tested how environmental conditions at early life stages influence the body size and growth patterns of larval *E. cirrigera*. By understanding the factors that influence the development and growth of a plethodontid salamander with an aquatic life stage such as *E. cirrigera*, we can gain better insight into their population dynamics and ecological interactions. Early growth plays a pivotal role in determining size at metamorphosis and maximum body size and it can have a profound influence on the salamander's overall fitness and likelihood of survival. To understand the interaction between body size and early growth, larval *E. cirrigera* were exposed to a fully factorial experimental design, crossing two thermal treatments with two levels of food availability. Based on available evidence with other ectotherms in cooler climates, I hypothesized that salamanders reared at higher temperatures would metamorphose at a smaller size than salamanders reared at lower temperatures. When there was an abundance of food, my hypothesis was supported, as salamanders in the low-temperature treatments exhibited greater growth than those in the high-treatments.

Although it may be an artifact of experimental conditions (such as water quality), the clearest effect of the factorial study design was a difference in survival. Larval salamanders in the high temperature, high food treatment had much higher mortality than the other three treatments. However, at least three salamanders in each treatment survived to the experiment's end (Table 6). Salamanders that underwent metamorphosis and survived the entirety of the experiment were classified as survivors. The High Food: High Temperature treatment had two salamanders undergo metamorphosis (21 June 2021 and 15 May 2022). Both Low Food: High Temperature treatment (5 July 2021) and High Food: Low Temperature treatment (29 December 2021) had one salamander undergo metamorphosis.

The decline in body sizes for the larval salamanders may have been caused by pre-metamorphic shrinkage caused by tail resorption (Yaoita 2019). Although I did not observe metamorphs in high enough numbers to relate it to the experimental design, the GAMM shows all the high temperature treatments experienced a decline in mass well before the cool temperature treatments. This could mean that under more natural conditions, salamanders in warmer conditions metamorphose earlier than those in cool conditions.

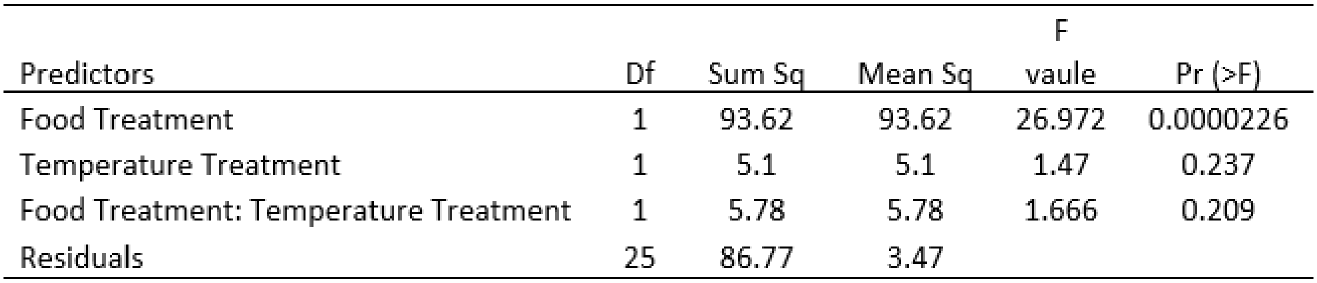
When food levels were high enough, the results of the GAMM were consistent with the hypothesis that salamanders reared at lower temperatures would grow larger than salamanders reared at higher temperatures. However, differences in mass among treatments disappeared by the experiment's conclusion. The salamanders in all treatments were similar in length at the end of the experiment. Overall, the salamanders exhibited similar trends in growth in their temperature treatments, regardless of their food treatment. These results suggest that temperature effects on growth are strongest when food is abundant, and that future studies should consider the effects of resource availability as well as thermal conditions when predicting the effects of climate change on growth.

This study is one of a few studies that have examined the growth of larval plethodontid salamanders in response to temperature and food availability. Previous studies have examined similar factors in different species with similar development: Allegheny Mountain dusky salamander (*Desmognathus ochrophaeus*) (metamorphosis) (Beachy 1995), Four-toed salamander (*Hemidactylium scutatum*) (postembryonic metamorphosis) (O'Laughlin et. al. 2000), Blackbelly salamander (*D. quadramaculatus*) (metamorphosis) (Hickerson et. al. 2005), and Blue Ridge two-lined salamander (*Eurycea wilderae*) (metamorphosis) (Beachy 2018). In the study examining the growth history of the Allegheny Mountain dusky salamander (Beachy 1995), individuals in high temperature treatments appeared to be affected more by the food regimes, growing smaller, suggesting an intricate relationship between food and temperature. Surprisingly, I found the opposite relationship between food and temperature. In my study, high food levels had a greater impact in low temperature treatments, allowing the salamanders to grow larger in mass. My results are more similar to those reported by Hickerson et. al. (2005), high food treatments had bigger impacts on individual growth of *D. quadramaculatus* in lower temperatures. My results are consistent with observations by Beachy (2018), in that salamanders which experienced restricted food grew at a slower rate than those with higher food availability.

By examining *E. cirrigera*, the study contributed to the understanding of growth when examining the relationship between temperature and food availability in a larval plethodontid salamander. When considering the ramifications of climate change, forecasting the effects on salamander populations will be complicated. Factors such as habitat quality, phenology, and climate will need to be considered to understand population demography and life history parameters.

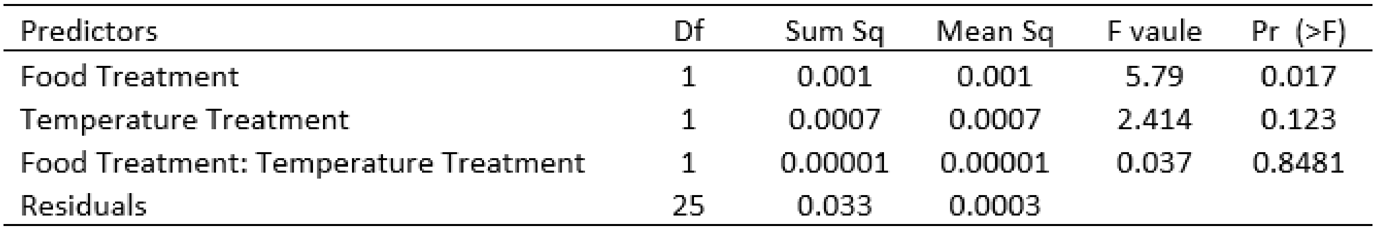
Chapter II Tables

Table 4. ANOVA SVL reveals a non-significant interaction between temperature and food on body length. In the table, “Df” signifies the degree of freedom, “Sum Sq” represents the sum of squares divided by the degrees of freedom, and “Mean Sq” indicates the mean square.



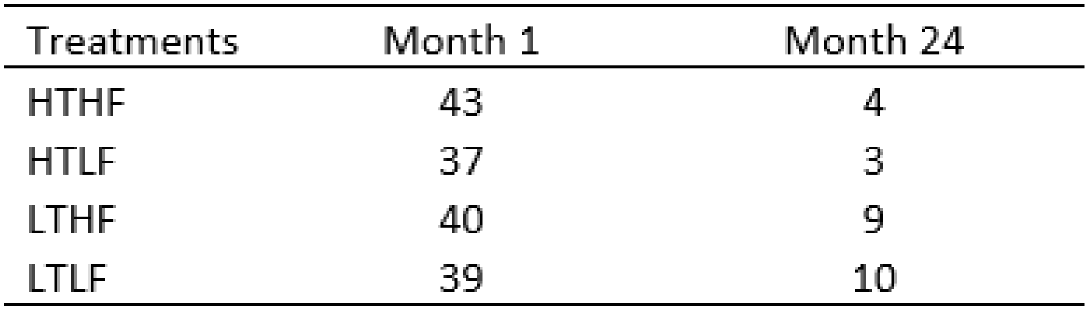
Predictors	Df	Sum Sq	Mean Sq	F vaule	Pr (>F)
Food Treatment	1	93.62	93.62	26.972	0.0000226
Temperature Treatment	1	5.1	5.1	1.47	0.237
Food Treatment: Temperature Treatment	1	5.78	5.78	1.666	0.209
Residuals	25	86.77	3.47		

Table 5. ANOVA Mass reveals the interacting effects of temperature and food on body mass at the end of the experiment were not significant. In the table, “Df” signifies the degree of freedom, “Sum Sq” represents the sum of squares divided by the degrees of freedom, and “Mean Sq” indicates the mean square.



Predictors	Df	Sum Sq	Mean Sq	F vaule	Pr (>F)
Food Treatment	1	0.001	0.001	5.79	0.017
Temperature Treatment	1	0.0007	0.0007	2.414	0.123
Food Treatment: Temperature Treatment	1	0.00001	0.00001	0.037	0.8481
Residuals	25	0.033	0.0003		

Table 6. Represent individual survivorship through the course of the experiment. Individual that underwent metamorphosis are included in the table.



Treatments	Month 1	Month 24
HTHF	43	4
HTLF	37	3
LTHF	40	9
LTLF	39	10

Chapter II Figures

Figure 5. Visual of hypothesis. I hypothesized that if food availability is abundant, salamanders reared at lower temperatures will grow larger and metamorphose later than salamanders reared at higher temperatures.

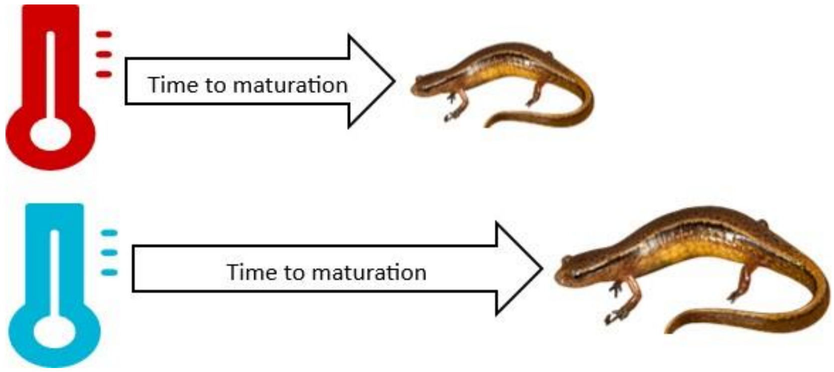
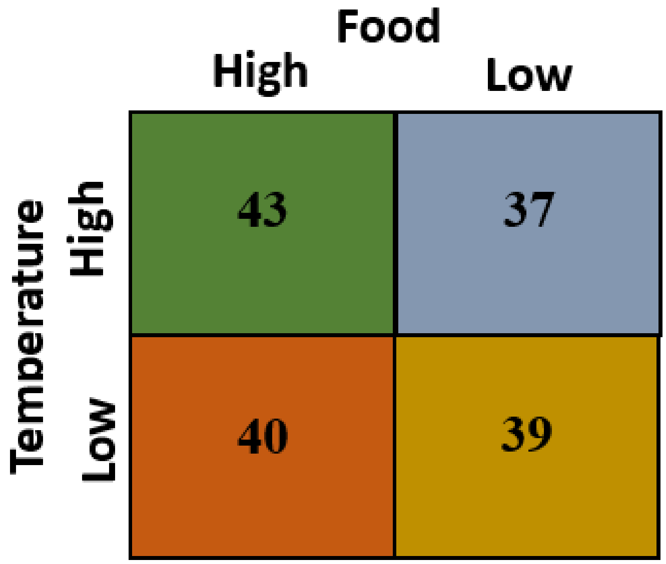


Figure 6. Photo of and adult *E. cirrigera*

Figure 7. Larval *E. cirrigera*

Figure 8. Visual of experimental design. The numbers represent the number of salamanders at the beginning of the experiment.



		Food	
		High	Low
Temperature	High	43	37
	Low	40	39

Figure 9. Mean TL (mm) of *E. cirrigera* in two food treatments, crossed with two temperature treatments (High Temperature: High Food, High Temperature: Low Food, Low Temperature: High Food, Low Temperature: Low Food). The left panel represents the High Food treatment, while the right panel represents the Low Food treatment. In both panels, the red line represents the High Temperature treatment, and the blue line represents the Low Temperature treatment. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.

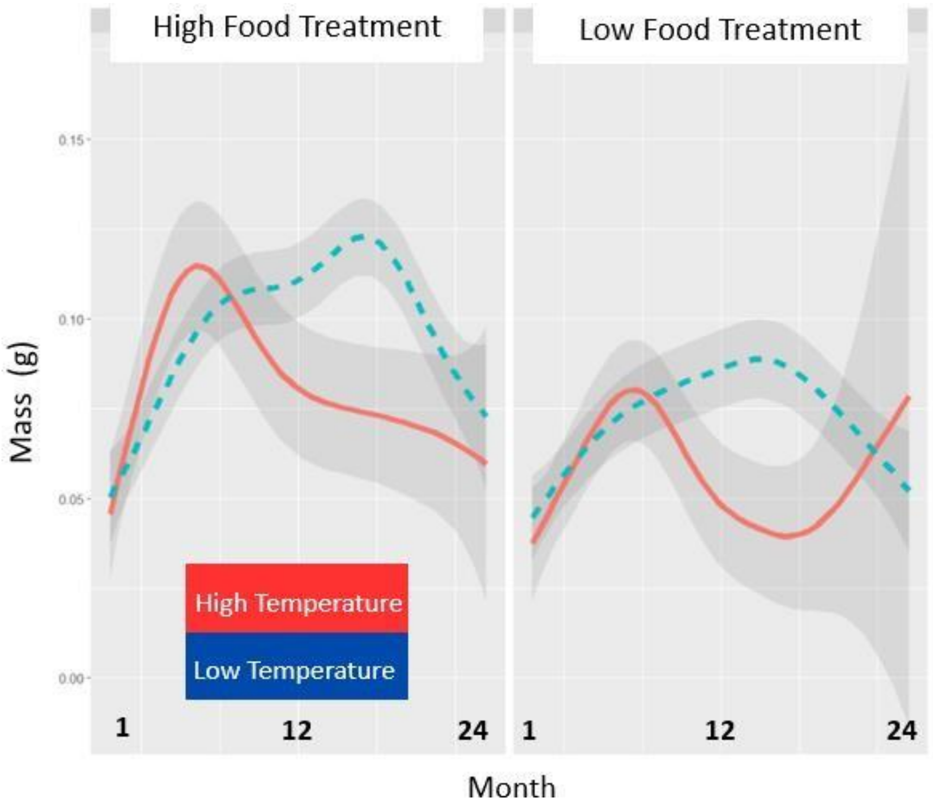
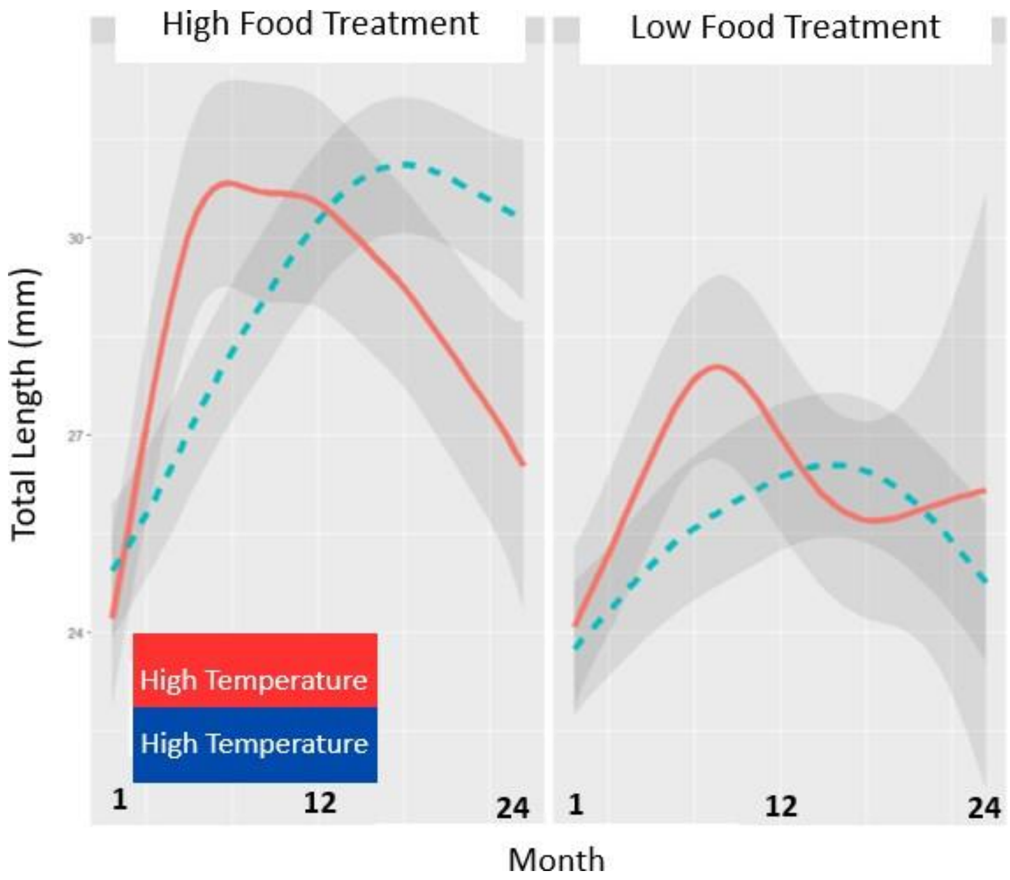


Figure 10. Mass (g) of *E. cirrigera* in two food treatments, crossed with two temperature treatments (High Temperature: High Food, High Temperature: Low Food, Low Temperature: High Food, Low Temperature: Low Food). The left panel represents growth when exposed to the High Food treatment, while the right panel represents growth when exposed to the Low Food treatment. The blue line represents the Low temperature treatment, and the red line represents the high temperature treatment. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.



Literature Cited Chapter II

Alford, R. A., and R. N. Harris. 1988. Effects of Larval Growth History on Anuran Metamorphosis. The American Naturalist 131:91–106.

Álvarez, D., and A. G. Nicieza. 2002. Effects of temperature and food quality on anuran larval growth and metamorphosis. Functional Ecology 16:640–648.

Angilletta, M. J., Jr., T. D. Steury, and M. W. Sears. 2004. Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle1. Integrative and Comparative Biology 44:498–509.

Beachy, C. K. 1995*a*. Effects of Larval Growth History on Metamorphosis in a Stream-Dwelling Salamander (Desmognathus ochrophaeus). Journal of Herpetology 29:375–382.

Beachy, C. K. 1995*b*. Effects of Larval Growth History on Metamorphosis in a Stream-Dwelling Salamander (Desmognathus ochrophaeus). Journal of Herpetology 29:375–382.

Beachy, C. K. 2018. Effects of Growth Rate and Temperature on Metamorphosis in Eurycea wilderae (Caudata, Plethodontidae, Hemidactyliinae, Spelerpini; Blue Ridge Two-lined Salamander). Southeastern Naturalist 17:423–432.

Berven, K. A., and D. E. Gill. 1983. Interpreting Geographic Variation in Life-History Traits. American Zoologist 23:85–97.

Bickford, D. P., J. A. Sheridan, and S. D. Howard. 2011. Climate change responses: forgetting frogs, ferns and flies? Trends in Ecology & Evolution 26:553–554.

Blaustein, A. R., S. C. Walls, B. A. Bancroft, J. J. Lawler, C. L. Searle, and S. S. Gervasi. 2010. Direct and Indirect Effects of Climate Change on Amphibian Populations. Diversity 2:281–313.

Brophy, T., and T. Pauley. 2002. Reproduction in West Virginia Populations of the Southern Two-lined Salamander (Eurycea cirrigera). Faculty Publications and Presentations.

Bruce, R. C. 2005. Theory of Complex Life Cycles: Application in Plethodontid Salamanders. Herpetological Monographs 19:180–207.

Feder, M. E. 1976. Lunglessness, Body Size, and Metabolic Rate in Salamanders. Physiological Zoology 49:398–406.

Guy, C. J., R. E. Ratajczak, and G. D. Grossman. 2004. NEST-SITE SELECTION BY SOUTHERN TWO-LINED SALAMANDERS (EURYCEA CIRRIGERA) IN THE GEORGIA PIEDMONT. Southeastern Naturalist 3:75–88.

van der Have, T. M., and G. de Jong. 1996. Adult Size in Ectotherms: Temperature Effects on Growth and Differentiation. Journal of Theoretical Biology 183:329–340.

Hickerson, C.-A. M., E. L. Barker, and C. K. Beachy. 2005. Determinants of Metamorphic Timing in the Black-bellied Salamander, Desmognathus quadramaculatus. Southeastern Naturalist 4:33–50.

Jakubanis, J., M. J. Dreslik, and C. A. Phillips. 2008. Nest Ecology of the Southern Two-Lined Salamander (Eurycea cirrigera) in Eastern Illinois. Northeastern Naturalist 15:131–140.

Liles, L. A., K. K. Cecala, J. R. Ennen, and J. M. Davenport. 2017. Elevated temperatures alter competitive outcomes and body condition in southern Appalachian salamanders. Animal Conservation 20:502–510.

Morrison, C., and J.-M. Hero. 2003. Geographic variation in life-history characteristics of amphibians: a review. Journal of Animal Ecology 72:270–279.

O’Laughlin, B. E., and R. N. Harris. 2000. Models of Metamorphic Timing: An Experimental Evaluation with the Pond-Dwelling Salamander Hemidactylium scutatum (Caudata: Plethodontidae). Oecologia 124:343–350.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington.

Reading, C. J. 2007. Linking global warming to amphibian declines through its effects on female body condition and survivorship. Oecologia 151:125–131.

Ruben, J. A., and A. J. Boucot. 1989. The Origin of the Lungless Salamanders (Amphibia: Plethodontidae). The American Naturalist 134:161–169.

Semlitsch, R. D. 1980. Growth and Metamorphosis of Larval Dwarf Salamanders (Eurycea quadridigitata). Herpetologica 36:138–140.

Semlitsch, R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and Size at Metamorphosis Related to Adult Fitness in Ambystoma Talpoideum. Ecology 69:184–192.

Sheridan, J. A., and D. Bickford. 2011. Shrinking body size as an ecological response to climate change. Nature Climate Change 1:401–406.

Smith, D. C. 1987. Adult Recruitment in Chorus Frogs: Effects of Size and Date at Metamorphosis. Ecology 68:344–350.

Smith, S., and G. D. Grossman. 2003. Stream Microhabitat Use by Larval Southern Two-Lined Salamanders (Eurycea cirrigera) in the Georgia Piedmont. Copeia 2003:531–543.

Travis, J. 1984. Anuran Size at Metamorphosis: Experimental Test of a Model Based on Intraspecific Competition. Ecology 65:1155–1160.

Voss, S. R. 1993. Relationship between Stream Order and Length of Larval Period in the Salamander Eurycea wilderae. Copeia 1993:736–742.

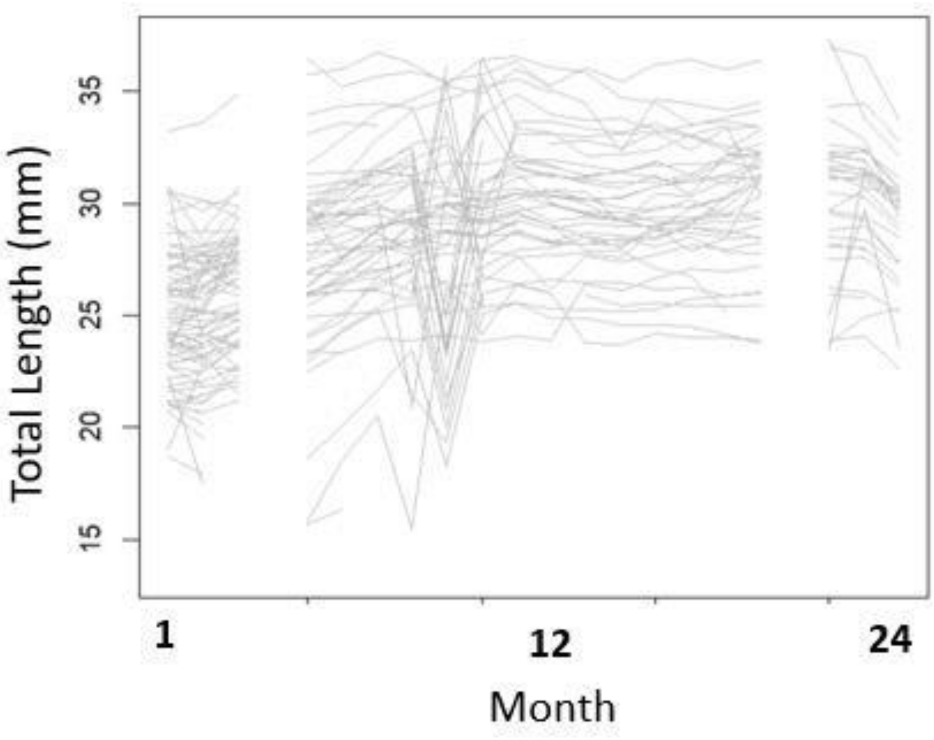
Werner, E. E. 1986. Amphibian Metamorphosis: Growth Rate, Predation Risk, and the Optimal Size at Transformation. The American Naturalist 128:319–341.

Wilbur, H., and J. Collins. 1973. Ecological aspects of amphibian metamorphosis. Science 182:1305–1314.

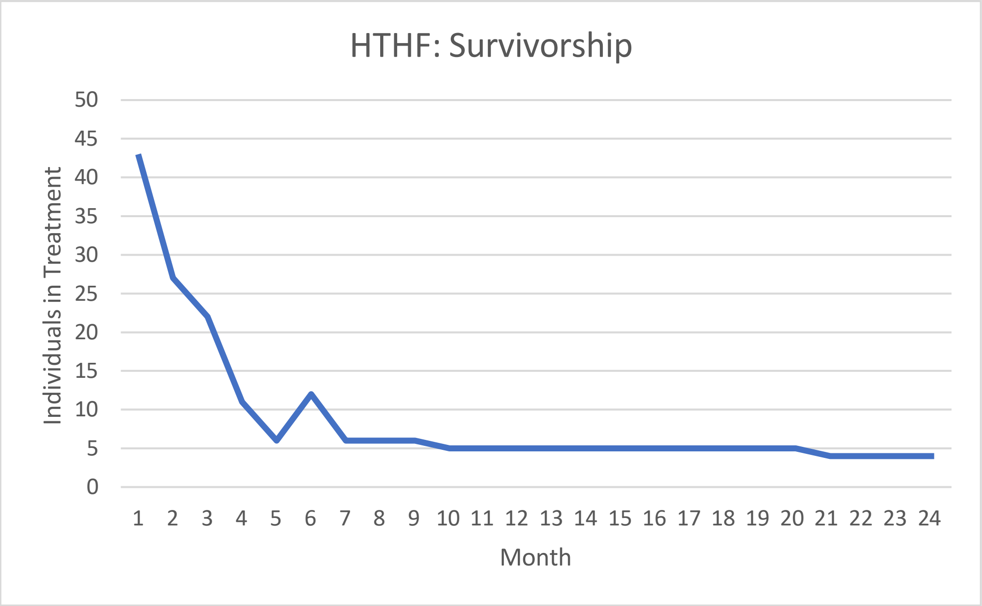
Yaoita, Y. 2019. Tail Resorption During Metamorphosis in Xenopus Tadpoles. Frontiers in Endocrinology 10.

Supplemental Figures Chapter II

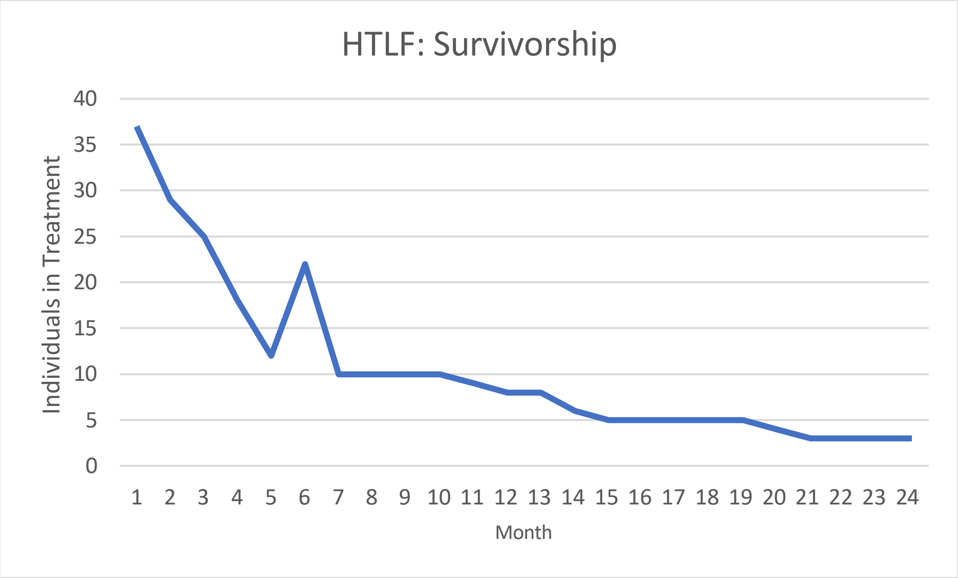
Supplemental Figure 2. Measured variation in TL for all individuals in all treatments. The Y-axis represents SVL (mm), and the X-axis represents the total number of months in the experiment.



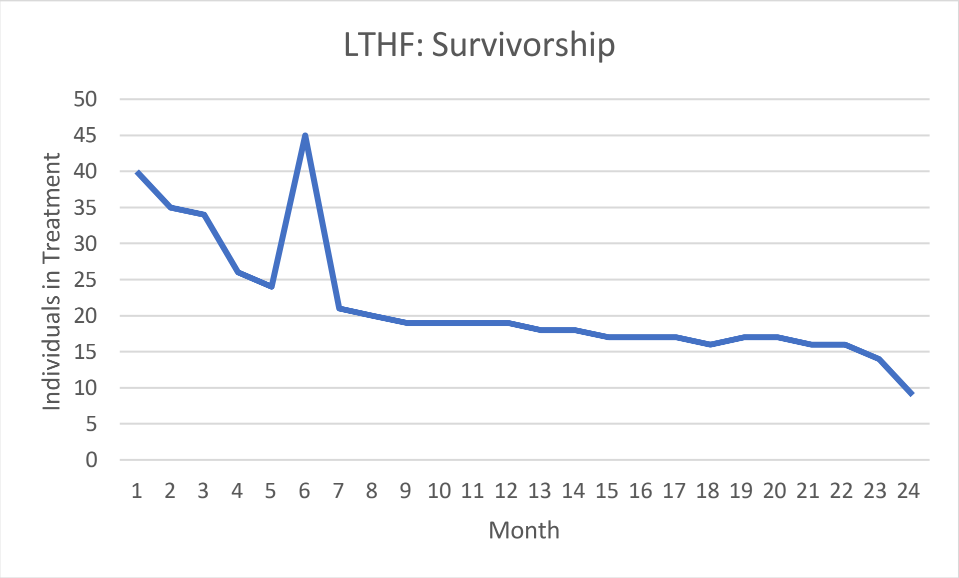
Supplemental Figure 3. Represents High Temperature: High Food (HTHF) survival throughout the experiment. The Y-axis represents the number of individuals in the Treatment, and the X-axis represents the Month. During month 6, salamanders were added to the treatment to increase sample size.



Supplemental Figure 4. Represents High Temperature: Low Food (HTLF) survival throughout the experiment. The Y-axis represents the number of individuals in the Treatment, and the X-axis represents the Month. During month 6, salamanders were added to the treatment to increase sample size.



Supplemental Figure 5. Represents Low Temperature: High Food (LTHF) survival throughout the experiment. The Y-axis represents the number of individuals in the Treatment, and the X-axis represents the Month. During month 6, salamanders were added to the treatment to increase sample size.



Supplemental Figure 6. Represents Low Temperature: Low Food (LTLF) survival throughout the experiment. The Y-axis represents the number of individuals in the Treatment, and the X-axis represents the Month. During month 6, salamanders were added to the treatment to increase sample size.