THERMAL TOLERANCE OF THE LARVAL STADIA OF TWO FORENSICALLY IMPORTANT BLOW FLY SPECIES, *CHRYSOMYA RUFIFACIES* (MACQUART) AND *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE)

A Thesis

by

LAUREN ELIZABETH JOANN BEEBE

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Aaron M. Tarone
Committee Members,	Jeffery K. Tomberlin
	Hsiao-Hsuan Wang
Head of Department,	Pete D. Teel

December 2019

Major Subject: Entomology

ABSTRACT

The growth and development of insects are heavily temperature dependent. Generally, development time decreases as temperature increases, up to an optimal temperature. Beyond the optimal temperature, development and performance slow until a knockdown temperature or critical thermal maximum (CT_{max}) is reached, or enough time is spent at stressful temperatures, resulting in death. Blow fly dependent ecological processes and forensic entomology rely on this temperature-development relationship and are impacted by knockdown and lethality at critical thermal limits. This thesis focuses on two forensically important blow flies, *Chrysomya rufifacies* and *Cochliomyia macellaria* (Diptera: Calliphoridae). Both are currently found in Texas; *C. macellaria* is native, while *C. rufifacies* is an invasive species from the Eastern Hemisphere. Temperatures in Texas can exceed 40˚C, which often results in maggot deaths. Knowledge of a species' thermal tolerance is important to forensics as a heatwave could easily disrupt estimates of the time of colonization. The purpose of this research was to observe the thermal tolerance using the ramping and the static method, for all larval instars of *C. rufifacies* and *C. macellaria*.

Using the ramping method to determine CT_{max} and rates of survival, larvae were heated on a metal plate until knockdown was observed. Larvae were kept at room temperature with food for a 24-hour recovery period. It was found that *C. rufifacies* had slightly higher CT_{max} than *C. macellaria.* Average knockdown temperatures increased with instar for both species. *Cochliomyia macellaria* tended to have slightly higher rates

of survival than *C. rufifacies.* Using the static method to determine likelihood of knockdown and rates of survival, larvae with and without food were placed in incubators set at 25, 35, 45 and 50˚C for half an hour, one or two hours. After knockdown was recorded, all larvae were placed with food into an incubator set at 25˚C for a 24-hour recovery period. Older larvae were able to withstand warmer temperatures for longer durations of time and tended to have higher rates of survival. Access to food greatly improved performance at higher temperatures for longer periods. *Cochliomyia macellaria* tended to perform better than *C. rufifacies* after longer exposure periods.

DEDICATION

This thesis is dedicated to my family. To my dad, who has always inspired me to do greater things. Life's not fair, but you have certainly given me a head start and provided me with the skills necessary to be successful throughout my career. To my mom, who has been my greatest supporter and provider of encouragement (as well as the occasional reminder of deadlines). I will always appreciate you being a mom. To my sister and best friend, who has been my role model and kept me motivated and mindful of what is in my best interest. Who would Adam be without Madame? To my Mormor, Morfar and Grandma Beebe, whose backyards sparked my early curiosity of all living things. You always joined in for outdoor adventures and never discouraged me from getting a little scuffed up and dirty. To Andrew, my other best friend, who accompanied me across the country to pursue this degree (hopefully this thesis was worth it). You have made everywhere we've ended up feel like home. To Tora (page 23), Mitano and Piper, whose unconditional love and excitement upon my arrival home could brighten any day. You all have shaped me into the person I am today, and I thank you from the bottom of my heart.

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my committee chair, Dr. Tarone. You allowed me the independence to structure my path of study and set my own goals. You were also always available to provide the guidance needed when I reached my limits, and necessary for me to grow as a researcher. I always left your office motivated and ready to face the next task.

I would like to thank my committee member Dr. Tomberlin, who provided advice for my writing and future aspirations. Your positive attitude and encouragement were greatly appreciated. I would also like to thank my committee member Dr. Wang, who introduced me to new concepts and supported me through several projects. Our meetings never failed to cheer me up.

Thanks also go to Dr. Rusch, who assisted in nearly every aspect of my research. You set an excellent example for me to follow in the lab and with my work. I am extremely grateful for all of your help, and everything I have learned from you.

Finally, thanks to my close friends and colleagues Jeffrey Yung and Dr. Lesne for their contribution to my projects. As well as Alex Payne, Constance Lin, Iris Tsing, James Grindell, Ming-Ray Liao and Pierre Lau. You all have made my time at Texas A&M University an amazing experience.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Doctors Aaron Tarone and Jeffery Tomberlin, faculty of the Department of Entomology and Doctor Hsiao-Hsuan Wang, faculty of the Department of Wildlife and Fisheries Sciences.

The data analyses depicted in Chapter II was performed in collaboration with post-doctoral researchers Travis Rusch and Pierre Lesne of the Department of Entomology. Data collection for Chapter III was assisted by Travis Rusch and graduate student Jeffrey Yung of the Department of Entomology, and data analyses depicted was performed in collaboration with Pierre Lesne.

All other work conducted for this thesis was completed by the student independently.

Funding Sources

The first year of graduate study was supported by the Excellence Fellowship from Texas A&M University. Subsequent graduate study and research was supported by the National Institute of Justice under Grant Number 2016-DN-BX-0204. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Justice.

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Chapter I serves as a literature review over pertinent topics to this thesis. The purpose of this thesis is to dissect the thermal biology of blow flies, which can affect a variety of applications associated with this family of Dipterans. To do this, a full understanding of thermal biology in general is required. Therefore, I have included a comprehensive review of thermal and blow fly biology before transitioning into more specific topics of this thesis. The review of thermal biology begins on page 1, blow fly biology on page 7 and their applications to forensic entomology on page 14.

Thermal Biology

Temperature affects all living organisms on earth. It has been linked with numerous aspects of survival, growth and reproduction (Bogert 1949, Hutchison 1961, Byrd & Butler 1996, Angilletta 2009, Boatright & Tomberlin 2010, Zinn et al. 2010, Bala & Singh 2015, Rusch & Angilletta 2017) – therefore, studying what kind of impacts temperature can have various life-history traits of an organism is important. For chemical reactions within the body of an organism, the amount of reactants with the free energy necessary for a reaction are determined by temperature (Angilletta 2009). For example, a higher proportion of reactants surpass the energy threshold for activation when temperature is increased. Enzymes speed up reactions by lowering the energy

needed for activation. However, at extreme temperatures both high and low, the conformation of enzymes required to bind to substrate is disrupted, denaturing the enzyme. When an organism approaches its upper or lower temperature thresholds, enzymatic function slows, inhibiting any number of biochemical processes (Hochachka & Somero 2002). Because all life is dependent on these chemical reactions, the performance curves for certain enzymes are strikingly similar to those of the organisms themselves (Licht 1967).

Another vital cellular component determined by temperature is membrane structure. Low temperatures can inhibit movement of the phospholipid bilayer, which produces a membrane that is gel-like. High temperatures can accelerate movement to the point of destroying structure altogether. There is a place between temperature extremes where structure is semifluid, and membrane function stabilizes (Angilletta 2009, Hazel & Williams 1990). This range of stable temperatures varies between organisms due to phospholipid composition. Saturated and unsaturated fatty acids have different optimal temperatures – they function better at higher and lower temperatures respectively (Hazel 1995). It is possible for an organism to change its membrane makeup, adjusting the ratio of fatty acids to adapt to warmer or cooler environments. However, once the time and energy have been expended adapting to a higher or lower extreme temperature, the organism is then maladapted to the other (Angilletta 2009).

Since temperature does not affect every organism equally, numerous studies have documented different thermal responses based on species, sex, size, age and food availability among others (Bakken 1976, Huey & Stevenson 1979, Joern & Chapman

1990, Chen et al. 2005, Falk & Dotan 2008, Angilletta 2009, Rusch & Angilletta 2017, Rusch et al. 2019). For example, in Chen et al. (2005) the female phorid fly *Pseudacteon tricuspis* (Borgmeier) (Diptera: Phoridae) duration of survival was longer when kept at lower temperature than higher temperatures. Sugar deprived individuals lived on average 7 days at 20°C and 2 days at 33°C. Longevity also increased with access to sugar. Sugar-fed individuals lived on average 15 days at 20°C and 4 days at 33°C. The authors found similar results for male phorids. Another example can be found in Rusch et al. 2019, where the blow fly *Cochliomyia macellaria* (Fabricius) responds differently to temperature based on sex. In experiment 3, adult blow flies were exposed to three stressful temperature treatments (42, 44 and 45°C) for different durations of time (1, 2, 4 or 6 hours). It was determined that males had a higher probability of losing motor control after the treatments, 10% higher than females. Males also had lower rates of survival, 12% lower than females. It is apparent that variations in temperature or individual will result in different thermal responses. Outside of a controlled lab setting, it is very unlikely an organism will experience constant, ideal temperatures. In the wild, individuals must take preventative measures to avoid a reduction of fitness or even death.

To adapt to inconsistent environmental temperatures, an organism needs to thermoregulate. Thermoregulation is the ability to maintain body temperature with physiological or behavioral adaptations independent of environmental temperature (Cowles & Bogert 1944, Bogert 1949, Bakken 1979, Joern & Chapman 1990). For example, mammals such as humans are thermal regulators. They sweat in an effort to

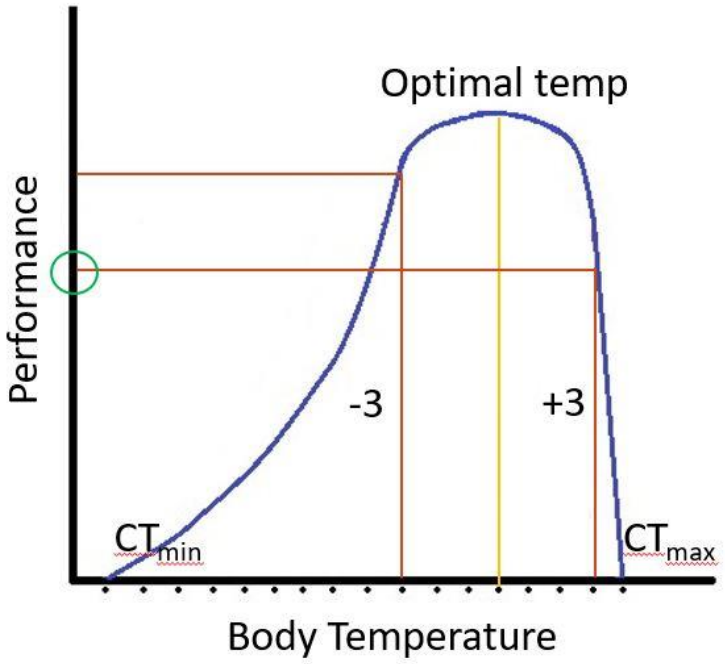
prevent overheating and shiver when they are exposed to lower temperatures to produce heat (Osilla & Sharma 2019). Ectotherms, or thermal conformers, are more susceptible to variations in temperature because they have little to no inherent physiological adaptations and thus rely more on environmental conditions for behavioral thermoregulation (Bakken 1976). For example, a reptile like a snake or lizard will bask in the sun to raise its body temperature (Cowles & Bogert 1944, Seebacher & Franklin 2005) and seek out shade or an underground burrow to lower it (Rusch & Angilletta 2017, Cowles & Bogert 1944). Furthermore, every living thing has upper and lower thermal limits, outside of which survival is not possible. There is an even smaller range in which fitness for an organism is optimized (Angilletta 2009, Lutterschmidt & Hutchison 1997, Hazell & Bale 2011), also termed thermal performance breadth, or the range of temperatures in which an organism performs well (Huey & Stevenson 1979).

Performance is defined as the measure of an organism's ability to function. Common assessments include growth, development, locomotion and survivorship (Angilletta 2009). Previous studies (Moore 1939, Fry & Hart 1948, Brett 1971) established the theory that one could measure an organism's performance over a large range of temperatures, fit a curve to these data and then predict thermal performance breadth, optimal temperature, or performance at any temperature within the tested range (Huey & Stevenson 1979). Measuring an organism's thermal tolerance can help estimate the range of temperatures within which survival is possible (Beers & Sidell 2011, Terblanche et al. 2011). As mentioned before, temperature does not affect every organism equally. These same variables (i.e. size, sex, age) will also affect an

individual's thermal tolerance. Thermoregulation is only possible within this window of survivability, temperatures outside the range can be lethal (Angilletta 2009).

Development is also restricted within this range of survivable temperatures and is regulated by temperature (Angilletta 2009, Hanks & Ritchie 1991, Byrd & Butler 1996). For ectotherms like insects (Insecta), the rate of development changes according to temperature (Byrd & Butler 1996, Lactin et al. 1995). As temperatures reach the edge of the window of survivability (as slope nears x axis in Figure 1), development is slowed and eventually halted. These thermal limits are known as an organism's critical thermal minimum (CT_{min}) and critical thermal maximum (CT_{max}), the temperatures at which coordination is lost (Hazell & Bale 2011, Becker & Genoway 1979). When looking at a performance curve (Figure 1) a much steeper slope down is seen after reaching optimal temperatures. This research will be focusing on upper thermal limits because the consequences of a change in temperature are much more severe on the right side of the curve (Martin & Huey 2008). For example, in Figure 1 when we move three degrees higher than optimal temperature, the performance (circled in green) is significantly lower than when we move three degrees lower than optimal temperature.

Figure 1: Performance curve across a range of body temperatures. Critical thermal minimum, maximum and an optimal temperature are noted.



One method for determining the CT_{max} is by observing knockdown (Berrigan & Hoffmann 1998), which can be defined several ways: onset of spasms, loss of righting response, or loss of motor function (Lighton & Turner 2004, Angilletta 2009). There are two standard techniques used to measure CT_{max}, the 'static' (Lutterschmidt & Hutchison 1997) or 'total immersion method' (Lighton & Turner 2004), and the 'dynamic' (Lutterschmidt & Hutchison 1997) or 'temperature ramp method' (Lighton & Turner 2004). The former method involves exposing organisms to constant temperatures, while the latter exposes the organism to steadily increasing (or decreasing) temperatures.

When employing the total immersion method, the time at which knockdown (i.e., loss of motor function or righting response) is observed is factored into that organism's thermotolerance but is not considered the organisms CT_{max} (Lighton & Turner 2004). An exact CT_{max} cannot be reached with this method, because the reaction of the organism at the temperatures leading up to and following the chosen temperature are unknown, but a probability of knockdown at exact temperatures for certain durations can be determined. Knowing the duration of time spent at high temperatures before knockdown is important because it is possible to be exposed to stressful high temperatures, or even CT_{max} and survive (Angilletta 2009, Hutchison 1961). When employing the other method, temperature ramping, the temperature at which knockdown is observed is considered that organism's CT_{max} (Lighton & Turner 2004). Care must be taken that the temperature does not start too low, so that the tested organism does not spend too much time under stress and without resources which could lead to death before an actual CT_{max}. The rate of increase in temperature cannot be too fast either, this would risk a lagging body temperature of the tested organism (Lighton & Turner 2004). Since there are arguments for and against both methods throughout the literature, both will be utilized in this study.

Blow Fly Biology

Blow flies (Diptera: Calliphoridae) are typically metallic green or blue flies that in general are best recognized for their ability to colonize decomposing material

(Triplehorn & Johnson 2005, Byrd and Castner 2010). Blow flies are easily identifiable by a row of bristles on the meron (also called the hypopleuron, which is a sclerite on the thorax resting between the second and third pair of legs) (Figure 2), undeveloped postscutellum (the area of thorax resting below the mesoscutellum/scutellum) (Figure 3), and plumose arista (a large bristle located on the distal end of the antennae) (Figure 4) (Triplehorn & Johnson 2005, Whitworth 2006).

Figure 2: Characteristic meron of the blow fly with bristles on *Chrysomya megacephala*. Photo by S. Freitas, University of California, Riverside.

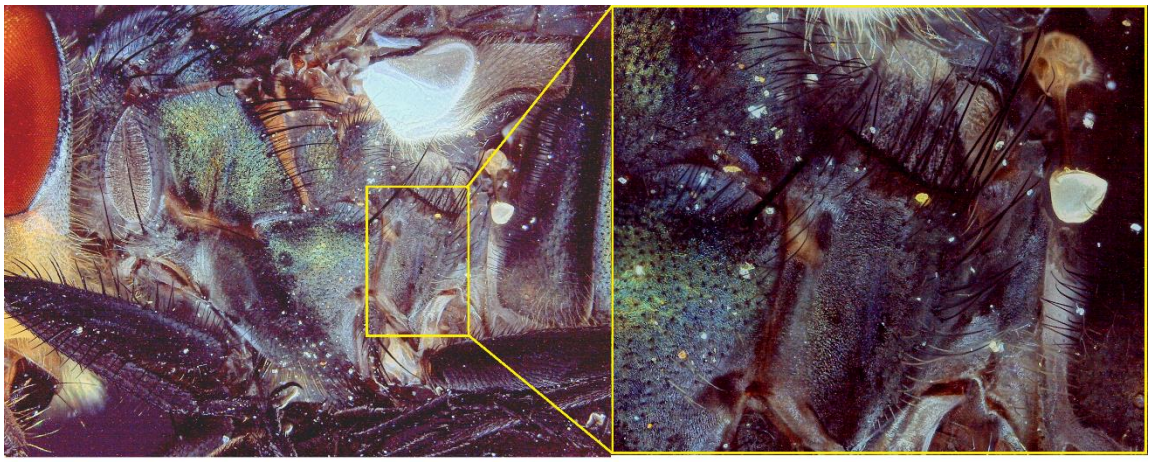


Figure 3: Characteristic undeveloped subscutellum of the blow fly on *Phormia regina.* Photo by S. Freitas, University of California, Riverside.

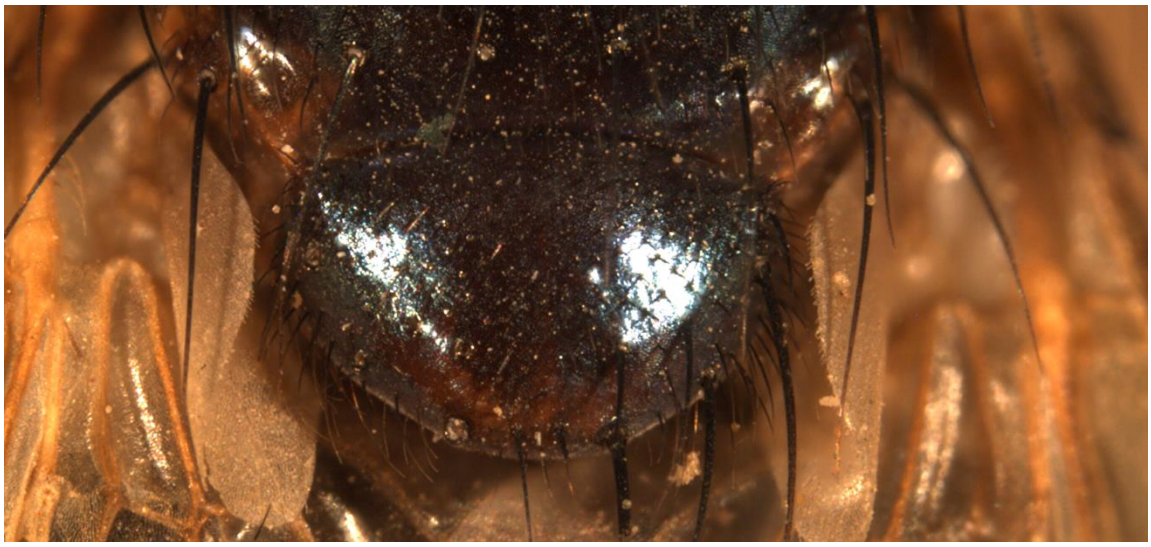
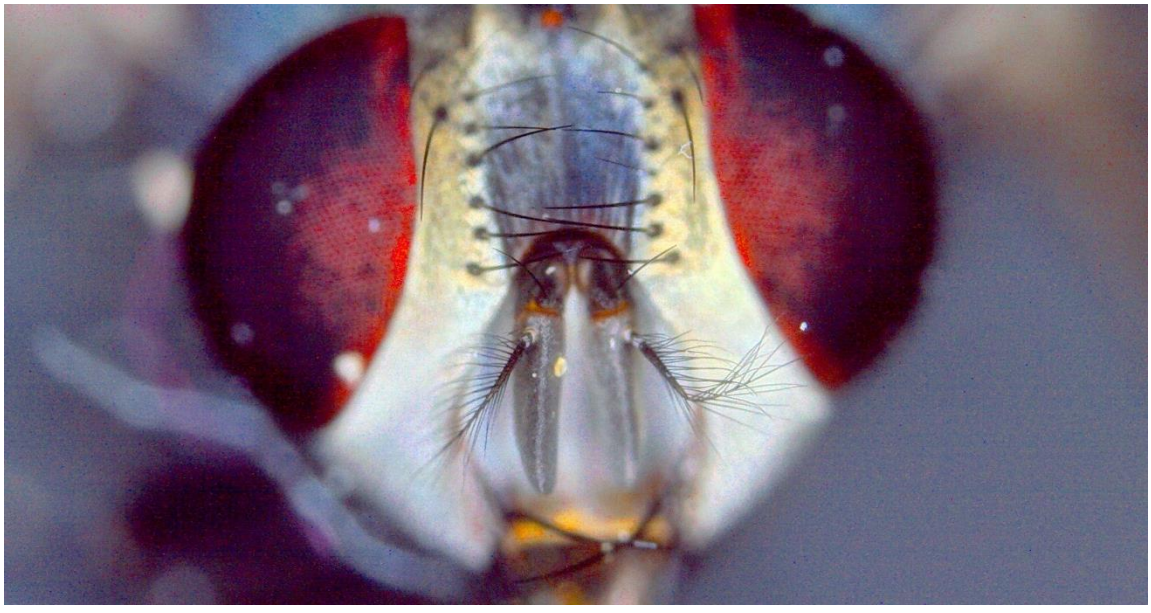


Figure 4: Characteristic plumose arista of the blow fly on *Lucilia sericata* (Meigen). Photo by S. Freitas, University of California, Riverside.



The calliphorid family contains dozens of genera and over 1000 species worldwide (Byrd & Castner 2010). Blow flies can tolerate an immense range of temperatures. *Protophormia terraenovae* (Robineau-Desvoidy), also known as the Holarctic blow fly, is one of the more cold tolerant species (Byrd & Castner 2010) has been identified within 550 miles of the North Pole (Smith 1986). In North America *P. terraenovae* is most often found in Canada and Alaska but is present in the lower 48 states during colder months, and in higher altitudes (Byrd and Castner 2010). Another species that has been documented north of the Arctic Circle, but less frequently found, is *Boreellus atriceps* (Zetterstedt) (Smith 1986, Erzinçlioğlu 1988). On the other end of the spectrum, there are also species that thrive in hot tropical and even desert regions. The species *C. macellaria* has been documented as the most abundant carrion frequenting species during a summer study in the Chihuahuan desert (Schoenly & Reid 1983). Several species within the genus *Chrysomya* originated from and frequent warmer regions. *Chrysomya albiceps* (Wiedemann) is a tropical species native to Africa (Hall & Wall 1995, Byrd & Castner 2010), and is found in almost every country and coastal island south of the Sahara Desert. It is also common in the Mediterranean and Oriental regions and has expanded into South America as well as farther north into Europe (Hall & Wall 1995, Riback & Godoy 2008, Szpila et al. 2008, Makovetskaya & Verves 2018). It can often be found during warmer times of the year and has even been documented in Poland, but is only able to complete development during hot summers due to its temperature requirements (Szpila et al. 2008). The species *Chrysomya rufifacies* (Macquart) originated from the Australasian regions and is still abundant

within its indigenous range and throughout the Old-World tropics (Baumgartner 1993, Byrd & Castner 2010). In the early 20th century, *C. rufifacies* was identified in Hawaii (Van Dine 1908) and after being discovered in Central America in 1978 (Jirón 1989) has since spread throughout the Western hemisphere (Baumgartner 1993). The species is not well adapted to the cold and in the United States is found primarily in the south (Byrd & Castner 2010). A study in Australia concluded that the high temperatures produced by the metabolic energy of heat tolerant calliphorids like *C. rufifacies* produced unfavorable environments for another blow fly, *Lucilia cuprina* (Wiedemann) (Waterhouse 1947). However, in 2004, *C. rufifacies* was found in southwestern Ontario, Canada during the fall. It is thought that the species was able to make its way up through range expansion once reproducing slightly south in the United States during the spring and summer. *C. rufifacies* is also expected to establish itself in southern Canada in the future due to global warming (Rosati & VanLaerhoven 2007).

Blow flies play significant roles in many ecological processes. Though many people immediately think of insects like bees or butterflies (Lepidoptera), flies are actually very important pollinators. For example, *Chrysomya megacephala* (Fabricius) plays a vital role in the production of mangos in Australia and Taiwan, where populations are even increased to improve pollination (Anderson et al. 1982, Sung et al. 2006). Flower visitation is not just coincidence, as consumption of pollen could aid in ovary development in female flies, and it has been shown that specific colors and odors are more attractive to adult blow flies (Brodie et al. 2015). There are also plants that have evolved specifically to mimic traits of carrion, like smell, to attract calliphorids and

other carrion frequenting insects as pollinators (Vereeken & McNeil 2010, Jürgens & Shuttleworth 2015).

Blow flies also are known to spread disease by vectoring various parasites and pathogens. In Australia, rabbit hemorrhagic disease virus (RHDV) is a very lethal disease that was considered by authorities for the biological control of wild rabbits. The escape of RHDV from a quarantined compound prompted concern, and it was shown that the virus was present in calliphorid flies collected from an area with a recent outbreak. RHDV was also detectable in 'flyspots' (oral/anal excretions) exuded from the species *Calliphora stygia* (Fabricius) and *C. rufifacies* up to nine days after exposure to contaminated material. These fly spots also contained enough of the virus to transmit the disease to susceptible rabbits (Asgari et al. 1998). It has also been shown that the blow fly *Phormia regina* (Meigen) can pick up *Salmonella enterica* (ex Kauffman & Edwards) (Le Minor & Popoff) and *Escherichia coli* (Migula) (Castellani & Chalmers) from manure and transmit the bacterium to lettuce plants (Pace et al. 2017). In Malaysia, *C. megacephala* is the most common vector of eggs from parasitic helminths and *C. rufifacies* has been documented as a vector as well (Gabre et al. 2005, Sulaiman et al. 1988, Sulaiman et al. 1989).

Fly strike is another serious problem posed by calliphorids. This is when blow fly larvae infest and feed on the living flesh of animals. In Australia alone, sheep fly strike costs $280 million annually (Smith & Curnow 2017). This not only causes direct damage to the animals, but a loss of animal productivity, cost of treatment, and even unethical alterations of the animals to prevent infestation (Morris 2000). Death

immediately after strike is not uncommon, and in a study conducted by Horton et al. in 2018 it was shown that in severe cases of infestation, mortality can reach upwards of 15% within 10 days. It was also discovered that strike closely following mating reduced the number of lambs born and weaned.

Blow flies are arguably most well-known for the role they play in nutrient recycling. They are commonly associated with dead or decaying organic matter, because this is where their larvae feed and develop. An excerpt from Went and Stark 1968 reads, "the bulk of minerals available in the tropical rain forest ecosystem is tied up in dead and living organic systems." Once an organism dies it is essential that the nutrients be processed and reintroduced into the system, otherwise our ecosystems would fail (Parmenter & MacMahon 2009). Due to the ephemeral nature of this type of resource, there is extreme competition among organisms which utilize carrion. Scavengers, both vertebrate and invertebrate, and microbes are the driving forces of the breakdown and decomposition of organic matter (Tomberlin et al. 2017). A change in the species diversity can even vary the decomposition processes of that system (Hättenschwiler et al. 2005, Gessner et al. 2010). With up to 90 gigatons of plant biomass (Gessner et al. 2010) and as much as 5,000 kg/km^2 of mammalian biomass (Carter et al. 2007) alone entering the dead organic matter pool each year, it is apparent our need for decomposers is constant and critical.

Forensic Application

An excellent field for the utilization of insect thermal tolerance is in forensic entomology. Forensic entomology is the application of the study of insects to a legal investigation (Greenberg 1991, Byrd & Castner 2010). The field is split into three categories: urban, stored product, and medicolegal (Lord & Stevenson 1986). The medicolegal category involves the identification and examination of insects, necrophagous or other, near a body (Byrd & Castner 2010, Boatright & Tomberlin 2010). A corpse is an attractive source of food and mating site for insects. Because, insects are ectotherms, they are extremely sensitive to changes in temperature. Temperature limits their activity, ability to reproduce, species distributions, and survival (Byrd & Butler 1996, Ames & Turner 2003, Boatright & Tomberlin 2010, Bala & Singh 2015). Their thermal tolerance can give us valuable information for forensically important timelines. The calculation of a minimum post-mortem (mPMI), period of insect activity (PIA), time of colonization (TOC), etc. (Amendt et al. 2007, Tomberlin et al. 2011, Tarone & Sanford 2017) can help a medical examiner determine the time of death. Knowledge of insect succession and development on a corpse is essential for these calculations.

Calliphorids are widely associated with decomposing remains and are typically among the first insect colonizers (Payne 1965, Byrd & Castner 2010). Aggregations of blow fly larvae (maggot masses) on cadavers are frequently observed and create heat in their micro-environment by constantly consuming and quickly metabolizing (Charabidze

et al. 2011). Blow flies are a unique group of insects because larvae are able to generate a limited amount of heat (Cianci & Sheldon 1990, Slone & Gruner 2007). Larvae in a maggot mass are observed to thermoregulate by cycling through positions in the mass (Johnson et al. 2014). However, it is thought that smaller maggot masses lack the metabolic output necessary to regulate temperatures, and larger masses may generate more heat than their cooling mechanisms can combat (Slone & Gruner 2007). These factors compounded by very high external temperatures have the potential to exceed the upper thermal tolerances of larvae.

It is vital in a forensic investigation to consider the effect of high temperatures on developing maggots. Blow fly larvae progress through three larval stages (Byrd & Castner 2010), or instars, each instar progressing at different rates (Boatright & Tomberlin 2010, Grassberger & Reiter 2001). Development of blow fly larvae is temperature dependent, each instar duration changing at varying temperatures as shown in Byrd & Butler (1996), where *C. macellaria* larval stage durations increased with drops in temperature. In Bala and Singh (2015) it was found that larvae of *C. megacephala* and *C. rufifacies* of the same age decreased in weight and length across increasing temperature treatments. Larvae then enter a post-feeding stage, where they search for a place to pupariate (Byrd & Butler 1996, Byrd & Castner 2010). Pupariation can occur on or near a cadaver, duration of which is also temperature dependent (Byrd & Butler 1996, Grassberger & Reiter 2001). A forensic entomologist would use environmental temperatures and larval stadia to determine insect or larval age, which is

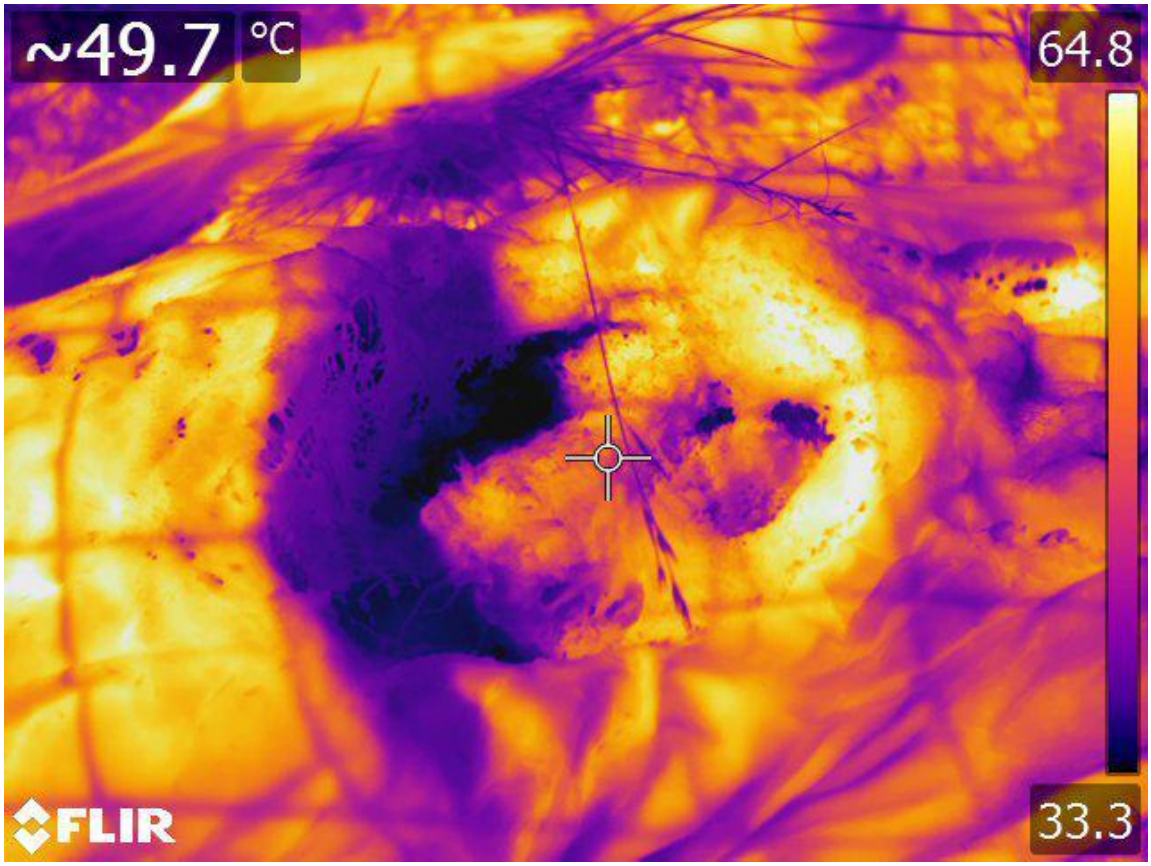
interpreted as a mPMI (Tarone & Sanford 2017). This can assist a medical examiner in determining the postmortem interval (PMI) or time of death.

For this thesis, I have selected two blow fly species commonly found in Texas. The species *C. macellaria* is native to North America, ranges from South America to southern Canada (Byrd & Castner 2010) and is frequently found in Texas (Owings et al. 2014). I would predict this species to be a thermal generalist – it is capable of withstanding and reproducing in a wide variety of environmental temperatures. The species *C. rufifacies* is not native to North America, originating in the Asian tropics and Australia (Byrd & Castner 2010). *C. rufifacies* over the past 30 years has become established in the United States (Baumgartner & Greenberg 1984). The larvae of *C. rufifacies* are predatory and cannibalistic, they will eat other larvae present on a food source (Byrd & Castner 2010). I predict this species to be a thermal specialist – native to an environment where there is not much fluctuation in temperature, its window of survivable temperatures is narrower than *C. macellaria*. These two species are frequently observed together on carcasses. They were selected for the study because of their potentially different thermal tolerances, as well as their similar preference in habitat and food source when the seasons allow for interspecies interaction.

In a place like the state of Texas, where temperatures can easily reach 40˚C, members of my lab have observed die-off of maggot masses on a cadaver in extreme heat (Figures 5 & 6). Insects and their developing larvae are often exposed to high temperatures and can be at risk of death.

Figure 5: Image of a dead maggot mass on a cadaver. Located at the Forensic Anthropology Research Facility (FARF) on Texas State University's Freeman Ranch, San Marcos, Texas. Photo by T. Rusch, Texas A&M University.

Figure 6: Thermal image of a dead maggot mass on a cadaver. Located at the FARF, taken May 29th, 2018 at 10:09 am. Photo by T. Rusch, Texas A&M University. Area recorded ambient and soil temperatures 28.9 and 26.1°C respectively at 10:00 am "MesoWest, University of Utah" May 2018.



However, duration of time spent at these high temperatures is just as important as the temperature readings themselves. During hot days, the temperature does not quickly climb, reach a maximum and descend. Hours can be spent at various temperatures throughout the day, and it is important to know what effect this could have on larvae. It is possible to be exposed to lethal temperatures for a brief amount of time and survive

(Angilletta 2009, Hutchison 1961). Without knowledge of knockdown temperatures and thermal tolerance, extremely high temperatures could disrupt calculations of the forensically important timelines listed previously (Gennard 2012). This information could also help determine a species distribution or predicted expansion range for an invasive species like *C. rufifacies*. If *C. macellaria* is a true generalist and able to withstand a wider range of temperatures, and *C. rufifacies* a specialist and more thermally restricted, this could also explain why the predatory *C. rufifacies* has not yet displaced the native *C. macellaria* where they interact.

Objectives and Hypotheses

The objectives for the proposed research are as follows:

I. To determine critical thermal maxima and proportion of survival for maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria*

- Record knockdown temperatures for individual maggots and proportion of survival post-24 hours for all instars of both species during a constant ramp in temperature

H_0: There is no significant difference in critical thermal maxima or survival between species and stadia

H_a: There is a significant difference in critical thermal maxima and survival between species and stadia

II. To observe the likelihood of knockdown and survival of maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria*

- Record proportion of knockdown and survival post-24 hours for maggots of all instars of both species at different temperatures for varying amounts of time with and without food

H_0: Knockdown and survival of larvae at various temperatures between species, stadia, duration of exposure and access to food will be the same

H_a: Knockdown and survival of larvae at various temperatures between species, stadia, duration of exposure and access to food will differ

The above hypotheses address several questions about the processes about this biological system. The questions I am most interested in are: At what upper thermal limit do larvae of various stadia knockdown (CT_{max}), potentially disrupting a timeline estimate? What are the rates of survival after knockdown? Do the thermal tolerances of larvae change between stadia? What is the probability of knockdown and survival after exposure to other stressful temperatures for different durations of time? Does access to food change the response? Do the responses to extreme temperatures differ between species? By testing these hypotheses in the following chapters, I hope to answer these questions.

CHAPTER II

CRITICAL THERMAL MAXIMA OF THE HAIRY MAGGOT BLOW FLY, *CHRYSOMYA RUFIFACIES* (MACQUART), AND THE SECONDARY SCREWWORM, *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE)

Introduction

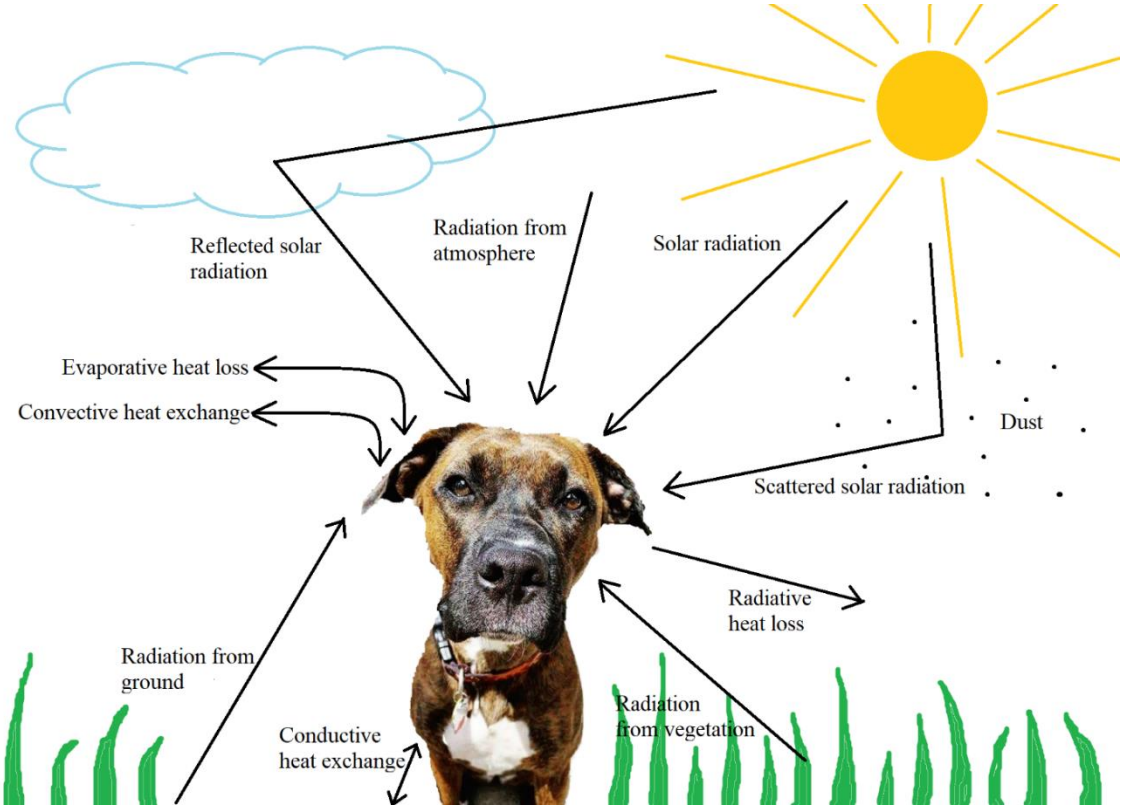
As discussed in Chapter I, temperature is a very important factor in the biology and life history of an ectotherm. In this Chapter, I am interested in exploring how temperature can affect the carrion-feeding larvae of the blow fly species *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) and *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae). In locations, such as Texas, USA where temperatures can easily reach 40˚C (104˚ F) (Table 1) ("Texas Climate Report: July 2017," n.d.), insects and their developing larvae are often exposed to high temperatures and can be at risk of death. College Station, Texas (where this objective took place) had 20 combined days in which the maximum daily temperature reached 37.78°C (100°F) or higher during the months of July and August in 2017 ("College Station Summary: July 2017," n.d., "College Station Summary: August 2017," n.d.). In recent history, the record high temperature was 48.89°C (120°F) in Monahans, Texas in 1994 ("Texas Extremes," n.d.). In addition to this, heat produced within a maggot mass can compound the ambient air

Table 1: Texas July 2017 temperature report from selected stations. Adapted from "Texas Climate Report: July 2017" n.d., recorded ambient temperatures.

	MAX TEMP °C		MIN TEMP °C		AVG TEMP °C
Station	**Mean**	**Max.**	**Mean**	**Min.**	**Mean**
Abilene	35.5	39.4	22.0	17.2	28.8
Amarillo	34.6	37.8	18.8	13.9	26.7
Austin	38.6	41.7	24.2	20.0	31.6
Brownsville	33.5	36.7	24.4	21.7	28.9
College Station	36.4	40.0	24.4	22.2	30.4
Corpus Christi	34.4	36.7	23.7	21.7	29.1
Dallas-Fort Worth	35.6	38.9	25.1	21.1	30.3
Del Rio	37.4	41.7	24.6	21.7	31.0
El Paso	35.0	40.0	22.4	20.0	28.7
Galveston	32.2	33.9	26.8	23.3	29.5
Houston	34.7	37.8	24.6	22.2	29.6
Lubbock	33.8	36.7	20.7	17.2	27.3
Midland	35.7	38.9	22.4	19.4	29.0
San Angelo	36.8	40.6	21.4	18.3	29.1
San Antonio	37.1	40.6	24.7	22.2	30.9
Victoria	35.1	40.0	23.9	22.2	30.0
Waco	36.3	41.1	23.8	20.6	30.1
Wichita Falls	35.8	39.4	22.1	17.8	28.9

temperature and result in stressful temperatures even without high environmental heat. Upper internal carcass temperatures have been measured above 50°C while lower ambient temperatures remained near 10°C (Anderson & VanLaerhoven 1996). This indicates that in some instances while ambient temperatures read within a tolerable range, larvae present on a body could be experiencing temperatures as much as 40 degrees higher than ambient. In addition to the maggot mass producing heat, there are other factors at play in the environment affecting heat transmission (Figure 7).

Figure 7: Heat exchange between an organism and its surroundings. Adapted from Angilletta 2009 (Fig. 2.1).



Knowledge of larval critical thermal maxima (CT_{max}) is important to determine at what environmental temperatures to expect maggot and maggot mass die off. These limits are also of importance to explain larval behavior, interactions on a food source and project species ranges. Chapter I detailed the role blow flies play in other ecological applications like pollination and nutrient recycling. This concept is important because if temperatures reach critical limits, flies cannot participate in their various ecological roles. As discussed previously, Dipterans can serve as pollinators to various types of plants, from orchids (Sugiura 1996) to mango trees (Sung et al. 2006). It has even been suggested in some studies that flies may be the primary pollinators of some plants (Kumar et al. 2016).

It is widely accepted that flies in the family Calliphoridae are associated with decomposition. Due to the nearly incomprehensible amount of plant (Gessner et al. 2010) and vertebrate biomass (Carter et al. 2007) entering the dead organic matter pool each year, the presence of insects involved in decomposition is imperative. An extreme example of this involves mass mortality events (MMEs). A MME is a swiftly occurring, disastrous die-off of organisms, and in recent years these events have had an increase in reported frequency (Fey et al. 2015). MMEs are often correlated with extreme weather perturbations (like heat waves/thermal stress), which are expected to increase because of climate change. The global average of the warm spell duration index has increased by about eight days since the mid 1900's (Donat et al. 2013). MMEs attributed to fluctuations in climate account for about 24.7% of known reports (Fey et al. 2015). Early works like that of Payne 1965, detailed the importance of insects

in nutrient recycling, especially when it comes to vertebrate carcasses. Invertebrates and microbes are responsible for the bulk of the decomposition when large or extremely large amounts of carrion are present (Tomberlin et al. 2017). If instances of MMEs increase due to a warming climate, it is vital that carrion frequenting insects be present for the decomposition process. Even in the presence of invertebrates, MMEs have the potential to devastate an ecosystem – killing herbaceous plants and even trees (Tomberlin et al. 2017). However, if the high environmental temperatures causing certain MMEs also nears upper thermal limits for blow flies, adults would be unable to forage for protein meals or mating sites, and larvae would be unable to develop. Even if higher temperatures allow for invasive species to establish (Rosati & VanLaerhoven 2007), it could impact the native fauna and have serious consequences for the recycling of organic matter and the survival of the environment (Baumgartner 1993). The absence of insects has been shown to greatly lengthen the decomposition process. Simmons et al. 2010 found that the exclusion of insects played the most significant role in affecting the rate of decomposition, regardless of species, environment or season. In a regular death event, vertebrate scavengers would likely pick up the slack when given access. However, in the event of a MME the extreme amount of carrion will over saturate the environment, proving too much for the vertebrate and invertebrate communities to handle (Tomberlin et al. 2017).

For ectotherms like insects (Insecta), the rate of development changes according to temperature (Byrd & Butler 1996, Lactin et al. 1995). Estimates of their development rely on accumulated degree day (ADD) and accumulated degree hour (ADH) models,

and the units added together to be used in determining the time of development are referred to degree days and hours (Gennard 2012). As temperatures reach an insects upper and lower thermal limits, growth is slowed and eventually halted, but up until these points the rate of development is considered linear as temperature increases or decreases. Some have suggested that these upper thermal limits or thresholds are not often encountered, and that accounting for them is 'infrequently important' (Gennard 2012). While some states or countries may not often experience stressful high environmental temperatures, it is obvious from the data collected in Anderson & VanLaerhoven 1996 that it is possible for internal temperatures of a cadaver to reach 40°C above ambient. Many of these degree day models account for base temperatures required for development (Gennard 2012), but not upper temperatures capping development. Since these models are implemented worldwide, it is important that we consider upper thermal limits when calculating growth rate.

It is also vital in a forensic investigation to consider the effect of temperature on feeding and developing maggots. Development data are based solely on temperature and life stage of the larvae. Critical temperatures have the potential to disrupt forensically important timelines (Gennard 2012) (mPMI/PIA/TOC) (Amendt et al. 2007, Tomberlin et al. 2011, Tarone & Sanford 2017), for example - thermal stress or knockdown can slow or halt development and delay pupariation or result in death (Donovan et al. 2006). It is worth noting that upper thermal limits are mentioned in some works, but only temperatures resulting in death. Knockdown temperatures are rarely/never mentioned but are equally as important in the field. There have been various unreported

observations of dead maggot masses and larvae experiencing thermal stress in the field. The thermal variability of a cadaver results in stressful areas and thermal refuges (Figure. 1, Chp. I). Under exposure to sun and high heat in Texas, maggots trapped on the exposed surface of a body have been observed convulsing and experiencing knockdown if they are not able to retreat to cooler parts of the cadaver (Beebe, Rusch, Tarone, Tomberlin; personal observations). In some cases, these maggots have rolled off the body and have the potential to recover on the cooler ground. In this Chapter, we will test the larval CT_{max} and survival post-knockdown employing the temperature ramping method.

The objective of this chapter is as follows:

I. To determine critical thermal maxima and proportion of survival for maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria*

- Record knockdown temperatures for individual maggots and proportion of survival post-24 hours for all instars of both species during a constant ramp in temperature

H_0: There is no significant difference in critical thermal maxima or survival between species and stadia

H_a: There is a significant difference in critical thermal maxima and survival between species and stadia

Materials and Methods

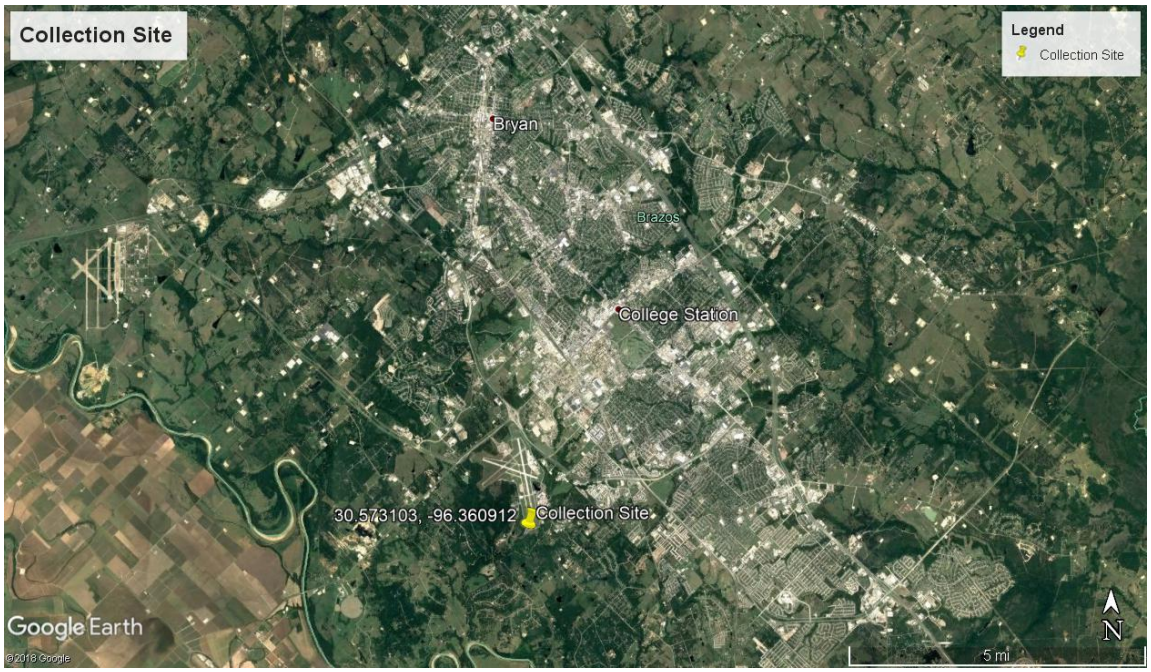
This experiment took place in the F.L.I.E.S. facility between February 10th and May 24th 2018, and is of a novel experimental design. Voucher specimens (739) were submitted to the Texas A&M University Insect Collection (Whitworth 2006).

Source of Blow Fly Larvae

The Forensic Laboratory for Investigative Entomological Sciences (FLIES) facility at Texas A&M University maintains a colony of both *Chrysomya rufifacies* and *Cochliomyia macellaria* for research purposes. Larvae for this experiment came from the FLIES facility colonies. Induction of oviposition for both colonies is kept on a rigorous schedule, and each newly eclosed group of adult flies is labeled as one generation above their precursors. Generations of interbreeding flies within the laboratory do not exceed ten for experimentation. Addition of wild type flies or members from a separate colony can reduce the generation number.

Collection of wild type flies occurred within the College Station area near the TAMU Ecology & Natural Resources Teaching Pavilion (located at 1852 Observatory Road, College Station, TX, approximately 30.573103, -96.360912) (Figure 8).

Figure 8: Google Earth satellite image of the fly colony collection site in College Station, Texas.



Adults were collected with aerial sweep nets and transported in BugDorms (Bioquip Products, Rancho Dominguez, CA) back to the FLIES facility for identification with Whitworth (2006). Immatures were also collected in the field; larvae were taken from carcasses and transported in jars, egg masses were carefully collected in moist tissue and transported in jars. Both were reared to adulthood for identification.

Adult colonies kept at the FLIES facility were housed at room temperature (23.89°C/75°F) in BugDorms and maintained on a diet of powdered milk and sugar (approximate ratio of 50:50). Bovine blood dripped onto a single Kimwipe (Kimberly-Clark Worldwide Inc., Roswell, GA) was offered as an extra protein source for egg

development. Blood was set out around the fourth day post-eclosion, and fresh blood continued to be offered every other day for a duration of one week. After the last day on which blood was offered, two egg cups (3 oz Great Value ™ Bath Plastic Cups, Wal-Mart Stores, Inc, Bentonville, AR) containing a single Kimwipe and a small amount of beef liver were placed in each cage and checked daily. During routine egg collection, around sixteen 946 ml mason jars (Ball® Corporation, Broomfield, CO) filled approximately halfway with vermiculite (Sun Gro® Agawam, MA) were collected, each containing several hundred eggs. For this experiment, 20 – 25 mason jars were collected to ensure a sufficient number of eggs for colony maintenance and experimentation. The mason jars were kept at room temperature within the FLIES facility and were checked daily during experimentation. Beef liver was added as needed to guarantee the larvae for trials were well fed.

Source of Animal Diet

Blow fly larvae for this experiment were fed on beef liver for the entirety of their immature stadia. Beef liver and blood were collected and purchased from the Rosenthal Meat Science and Technology Center. Beef liver was also occasionally purchased from Readfield Meats and Deli (Bryan, TX) and HEB (San Antonio, TX). No vertebrate animals were killed for the purpose of this experiment, and appropriate forms have been completed and approved by Texas A&M for the purchase and use of vertebrates in this study.

Experimental Design

A heat conductive stage approximately 25.5cm L x 20cm W x 14cm H was constructed out of a satin aluminum 8in x 34in kick plate (Everbilt™, Home Depot Product Authority, LLC, Atlanta, GA). Four walls of the same material were added, two 20cm L x 4cm H and two 14cm L x 4cm H. Three thermocouples and an A0188598 4 channel K thermometer SD card data logger (AZ® Instrument, Tantzu, Taichung, Taiwan) were used for this experiment. Two of the couples were taped on both ends of the metal arena, cardboard was then placed between the walls to prevent contact with the tape/couples (Figure 9). The third thermocouple was taped onto an empty petri dish as a control temperature measurement (Figure 10). Two 10L water baths (PolyScience, Niles, IL) were used for this experiment, the stage was rotated between baths after each group of larvae to allow for complete cooling between treatments. Water was added to the basin at approximately 2 mm above the base of the metal stage walls, to ensure the entire underside of the stage was uniformly heated.

Figure 9: Water bath used in Chapter II containing aluminum stage, cardboard dividers and thermocouples. Used to measure larval knockdown with the ramping method.

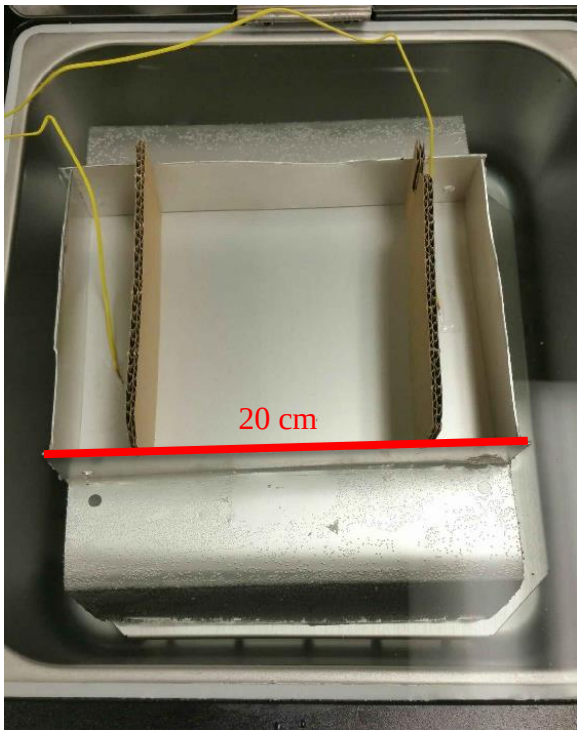
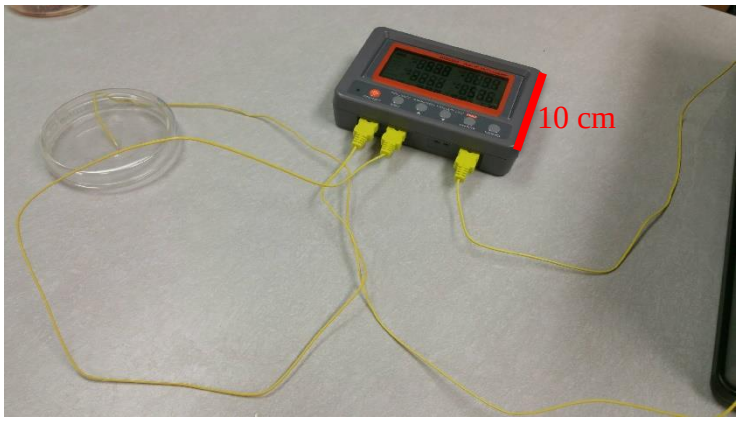


Figure 10: Data logger connected to three thermocouples. Used to measure surface temperature of both ends of the stage, and control petri dish.

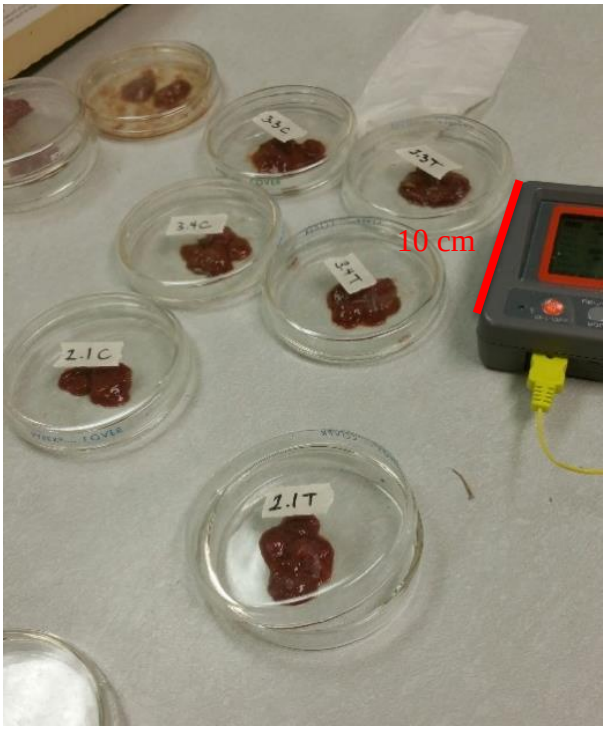


Three separate trials were conducted out of three different generations of flies. Within each trial, two jars containing immatures of the estimated same stadia were taken and approximately ten larvae pulled from both jars (for a total of 20) and identified to instar. For *C. macellaria* 1st instar larvae were identified by absence of visible prothoracic/anterior spiracles, 2nd and feeding 3rd instar larvae were identified by posterior spiracular slits (2 and 3 respectively) as per Liu & Greenberg (1989). Post-feeding 3rd instar larvae were identified by 3 posterior spiracular slits, absence of fresh liver in the gut, wandering behavior and presence of uneaten liver and puparia in the jars. For *C. rufifacies* 1st instar larvae were identified by absence of anterior spiracles and fleshy protuberances along the body, 2nd and feeding 3rd instar larvae were identified by posterior spiracular slits (2 and 3 respectively) as per Liu & Greenberg (1989). Post-feeding 3rd instar larvae were identified by 3 posterior spiracular slits, and presence of uneaten liver and puparia in the jars (observation of fresh liver in the gut was not used as an indication of instar for this species due to their thick cuticle, and wandering behavior was not considered as this species is often observed pupariating on food sources). All identified larvae were offered beef liver immediately before experimentation, and any suspected post-feeding 3rd instar larvae observed feeding on the liver was replaced with a true post-feeding 3rd instar larvae.

For 1st, 2nd, feeding 3rd and post-feeding 3rd instar – ten larvae were taken randomly from the previously collected group of 20 with a paintbrush or forceps and placed in an empty control petri dish, another ten were taken and placed on the stage in the water bath. For some 1st instar larvae, it was necessary to wet a paintbrush to

transfer the maggots to the stage without sticking and desiccating on the aluminum immediately. These larvae were allowed to move about until the small amount of water had evaporated. No larvae of any stage with an excessive amount of liquid from the liver on their cuticle was placed on the stage. Two petri dishes were set aside for post-experimental control and temperature treatment larvae, each containing the same amount of beef liver (2.5g for 1st instar, 5 g for 2nd, feeding 3rd and post-feeding 3rd instar) (Figure 11).

Figure 11: Petri dishes set aside with beef liver for post-experimental control and treatment larvae.



Once both the control and trial maggots were in place the water bath was set to 65˚C (the stage started at 20˚C (+/- 1˚C)) and time was recorded until the stage reached 60˚C (+/- 1˚C). Larvae on the stage were constantly monitored during temperature ramping until individual knockdown was observed, and then immediately moved to the post-experimental temperature treatment petri dish containing beef liver. Knockdown in this experiment was considered the point at which the larvae were no longer capable of moving their body below the head, even when agitated (head twitching was not considered body movement). Once suspected critical thermal maximum was reached, maggots were rolled to test that no righting response was exhibited, and knockdown was reached. This rolling test ensured that no maggots were temporarily 'frozen in place', after which the maggot continued to move around the plate. This behavior was sometimes exhibited after encounters with other larvae or at stressful temperatures. Temperature was recorded for each knockdown of the ten maggots. Once the last larvae had been knocked down, removed from the stage and placed into the post-experimental temperature treatment petri dish, time was recorded and all of the control maggots were taken from their petri dish and moved to the post-experimental control petri dish with beef liver. The two post-experimental petri dishes with liver were placed in room temperature (recorded low 16.6˚C and high 20.5˚C) for a 24-hour recovery period. After the 24 hours (+/- 2 hrs) had elapsed, the control and temperature treatment larvae were checked, and survival recorded (any indication of life was considered survival). Experimentation for both species was repeated six times per instar (twice during each

generation), for a total of 60 temperature treatment and 60 control larvae for each instar of *C. rufifacies* and *C. macellaria*.

Analyses

The analyses for this objective followed those outlined by Rusch & Angilletta (2017). A mixed effects modeling technique was used in the platform R, version 3.5.1 (R Core Team 2015) with libraries nlme (Pinheiro et al. 2012) and lme4 (Bates et al. 2015), testing for differences in means of the dependent variables – mean knockdown temperature of larvae exposed to extreme temperatures across different stadia. Within these models, species, life stage and generation were modeled as fixed effects, and group was treated as a random effect. In addition to this, a pairwise Tukey analysis was run in R Studio 3.5.1 with the library emmeans (Lenth 2019) to determine the significance of results between instars within a species and between species. To assess larval survival, 3 and 2-way ANOVAs and pairwise Tukey analyses were run using libraries DHARMa (Hartig 2017) and car (Fox et al. 2012).

Results

Three separate models (Table 2) were analyzed using a mixed effect analysis to determine what had the strongest effect on the data. Model 1 was run using a generalized linear model (glm) with a gaussian distribution comparing only the fixed

effects of species, stage and generation. A similar Model 2 was run using a linear mixed effects model (lme) with a gaussian distribution due to the addition of the group random effect. Model 3 was run with the same factors as Model 2, but with a generalized linear mixed effects model (glmer) with a gamma distribution due to the slightly nonlinear distribution of the data. The Akaike weights (AIC scores) were used to estimate the likelihood of a model better describing the data than another, the lower the score the better. Model 2 was the best model with the lowest AIC score (Table 2). Stage was found to be the highest weighted factor across species (Tables 3 & 4).

Table 2: Results of mixed effects models used to assess effect sizes of factors contributing to larval knockdown.

Model #	Model	DF	AIC	ΔAIC	Error Distribution	Effects
M2	lme	15	2058.244	0	Gaussian	Species*Stage+Gen+Group
M1	glm	14	2170.627	112.383	Gaussian	Species*Stage+Gen
M3	glmer	15	2189.491	131.247	Gamma	Species*Stage+Gen+Group

Table 3: Factor weights for *C. rufifacies*.

Model	K	logLik	AIC_c	ΔAIC_c	Weight
1. Stage	9	-430.5	879.7	0	0.82
2. Stage + Generation	11	-429.8	882.7	2.97	0.18
3. Null	6	-472.0	956.4	76.67	0.00
4. Generation	8	-471.8	960.3	80.56	0.00

Table 4: Factor weights for *C. macellaria*.

Model	K	logLik	AIC_c	ΔAIC_c	Weight
1. Stage	9	-447.92	914.62	0	0.77
2. Stage + Generation	12	-445.82	917.02	2.4	0.23
3. Generation	9	-478.64	976.07	61.45	0.00
4. Null	6	-484.17	980.69	66.07	0.00

Table 5: Significance of fixed effects, species by stage plus generation.

	Estimate	Std Error	DF	p val
(Intercept)	42.89053	1.119337	425	0
Species 2	-2.63075	1.188761	41	0.0325
Stage 2	8.20017	0.915951	41	0
Stage 3	11.69381	0.872467	41	0
Stage 4	11.605	0.915372	41	0
Generation 3	-0.72117	1.102202	41	0.5166
Generation 4	-1.10987	0.807192	41	0.1766
Generation 5	-1.37015	0.793112	41	0.0916
Generation 6	-1.12478	1.429594	41	0.4359
Generation 7	-0.80528	1.121629	41	0.4769
Species2:Stage2	0.74005	1.394151	41	0.5984
Species2:Stage3	2.05857	1.354494	41	0.1362
Species2:Stage4	1.41327	1.382364	41	0.3126

A fixed effects model was evaluated to determine the significance of species, stage and generation (Table 5). In addition to the mixed effects models, a pairwise Tukey analysis was also used to determine the significance of knockdown temperatures between different stages of larvae within and between species (Table 6).

Table 6: Pairwise analysis of species by life stage (1, = *C. rufifacies*: 2, = *C. macellaria*: ,1 = 1st instar: ,2 = 2nd instar: ,3 = feeding 3rd instar: ,4 = post-feeding 3rd instar).

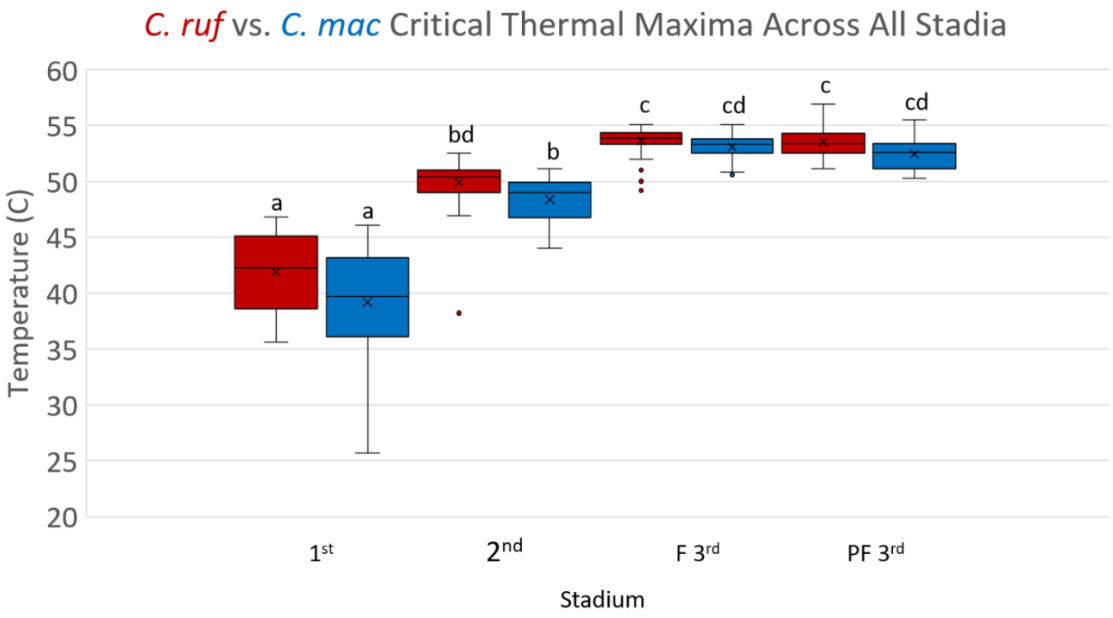
Contrast	Estimate	SE	DF	t ratio	p val
1,1 - 2,1	2.63075	1.188761	41	2.213	0.3655
1,1 - 1,2	-8.20017	0.915951	41	-8.953	<.0001
1,1 - 2,2	-6.30948	1.120277	41	-5.632	<.0001
1,1 - 1,3	-11.6938	0.872467	41	-13.403	<.0001
1,1 - 2,3	-11.1216	1.106247	41	-10.053	<.0001
1,1 - 1,4	-11.605	0.915372	41	-12.678	<.0001
1,1 - 2,4	-10.3875	1.106053	41	-9.392	<.0001
2,1 - 1,2	-10.8309	1.189642	41	-9.104	<.0001
2,1 - 2,2	-8.94023	1.051043	41	-8.506	<.0001
2,1 - 1,3	-14.3246	1.172539	41	-12.217	<.0001
2,1 - 2,3	-13.7524	1.036076	41	-13.274	<.0001
2,1 - 1,4	-14.2357	1.188761	41	-11.975	<.0001
2,1 - 2,4	-13.0183	1.035869	41	-12.567	<.0001
1,2 - 2,2	1.890697	1.121211	41	1.686	0.6957
1,2 - 1,3	-3.49364	0.873018	41	-4.002	0.0057
1,2 - 2,3	-2.92146	1.107194	41	-2.639	0.1717
1,2 - 1,4	-3.40483	0.915951	41	-3.717	0.0128
1,2 - 2,4	-2.18735	1.106999	41	-1.976	0.5097
2,2 - 1,3	-5.38434	1.103048	41	-4.881	0.0004
2,2 - 2,3	-4.81216	0.87315	41	-5.511	0.0001
2,2 - 1,4	-5.29552	1.120277	41	-4.727	0.0007

Table 6: Continued

Contrast	Estimate	SE	DF	t ratio	p val
2,2 - 2,4	-4.07805	0.87241	41	-4.674	0.0008
1,3 - 2,3	0.572179	1.088797	41	0.526	0.9994
1,3 - 1,4	0.088811	0.872467	41	0.102	1
1,3 - 2,4	1.306289	1.088599	41	1.2	0.9273
2,3 - 1,4	-0.48337	1.106247	41	-0.437	0.9998
2,3 - 2,4	0.734111	0.816938	41	0.899	0.9845
1,4 - 2,4	1.217478	1.106053	41	1.101	0.953

In Figure 12 we can see that average knockdown temperature increases along with life stage. First instars tend to knockdown in the upper 30s and mid-40s°C. Second instars knocked down in the upper 40s and early 50s°C. Third instars regardless of stage tended to knockdown in the early to mid-50s°C. All three instars within a species had significantly different knockdowns. Feeding and post-feeding third instars did not show any significant differences. *Chrysomya rufifacies* as a whole, tended to knockdown at slightly higher temperatures than *C. macellaria*. As instar increased across species, the range of knockdowns also tended to decrease.

Figure 12: Larval critical thermal maxima. Measured by knockdown across species and life stage.



Survival 24 hours after experimentation was analyzed separately to determine whether or not species, stage, or exposure to critical thermal maxima had a significant effect on the data. A 3-way ANOVA was used to compare species, treatment and stage. Because we want to determine what proportion of individuals survived, the data was transformed with the arcsine square root transformation. When looking at Table 7, we can see that treatment (exposure to critical thermal maximum vs control) followed by stage of the larvae have the most significant effects on the data. During data collection, it was very apparent that the survival rates of the control and treatment larvae were very different, and with confirmation from the 3-way ANOVA that this was true, data

Table 7: 3-way ANOVA for larvae 24 hours post-experimentation.

Factor	DF	Sum Sq	Mean Sq	p value
Species	1	0.007	0.007	0.70743
Treatment	1	17.831	17.831	<2E-16
Stage	3	4.787	1.596	2.31E-14
Species: Treatment	1	0.476	0.476	0.00198
Species:Stage	3	0.528	0.176	0.01353
Treatment:Stage	3	3.621	1.207	7.92E-12
Species: Treatment:Stage	3	0.122	0.041	0.45779

from the knockdown and control larvae were analyzed again separately with a 2-way ANOVA and pairwise Tukey so as not to skew interpretations of the data.

When evaluating results from the treatment group in Table 8 we see that species is slightly significant, and stage has a large effect on the survival of the larvae. In Figure 13 it is apparent that across species, larvae that are older have a higher chance of survival after knockdown. None of the life stages between species have significantly

Table 8: 2-way ANOVA without control larvae (treatment).

Factor	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Species	1	0.297	0.2971	4.784	0.0346
Stage	3	8.273	2.7575	44.392	8.48E-13
Species:Stage	3	0.537	0.1789	2.879	0.0478

different chances of survival, except in the 2nd instar. *Cochliomyia macellaria* has a 28.33% higher average of survival than *C. rufifacies* at this stage, with *C. macellaria* exhibiting an average of 30% survival and *C. rufifacies* exhibiting 1.67%. It is also interesting that while feeding 3rd and post-feeding 3rd instars of both species knockdown at similar temperature (Figure 12), the post-feeding 3rd instar survival after knockdown is quite a bit higher than feeding 3rd instar, the difference was significant in *C. macellaria*. *Cochliomyia macellaria* exhibited 88.33% average survival in the post-feeding 3rd instar stage and 43.33% average survival in the feeding 3rd instar stage. *Chrysomya rufifacies* exhibited 75% average survival in the post-feeding 3rd instar stage and 56.67% average survival in the feeding 3rd instar stage, though this difference was not significant.

Figure 13: Percent survival of larvae after a 24-hour recovery period once exposed to their critical thermal maxima.

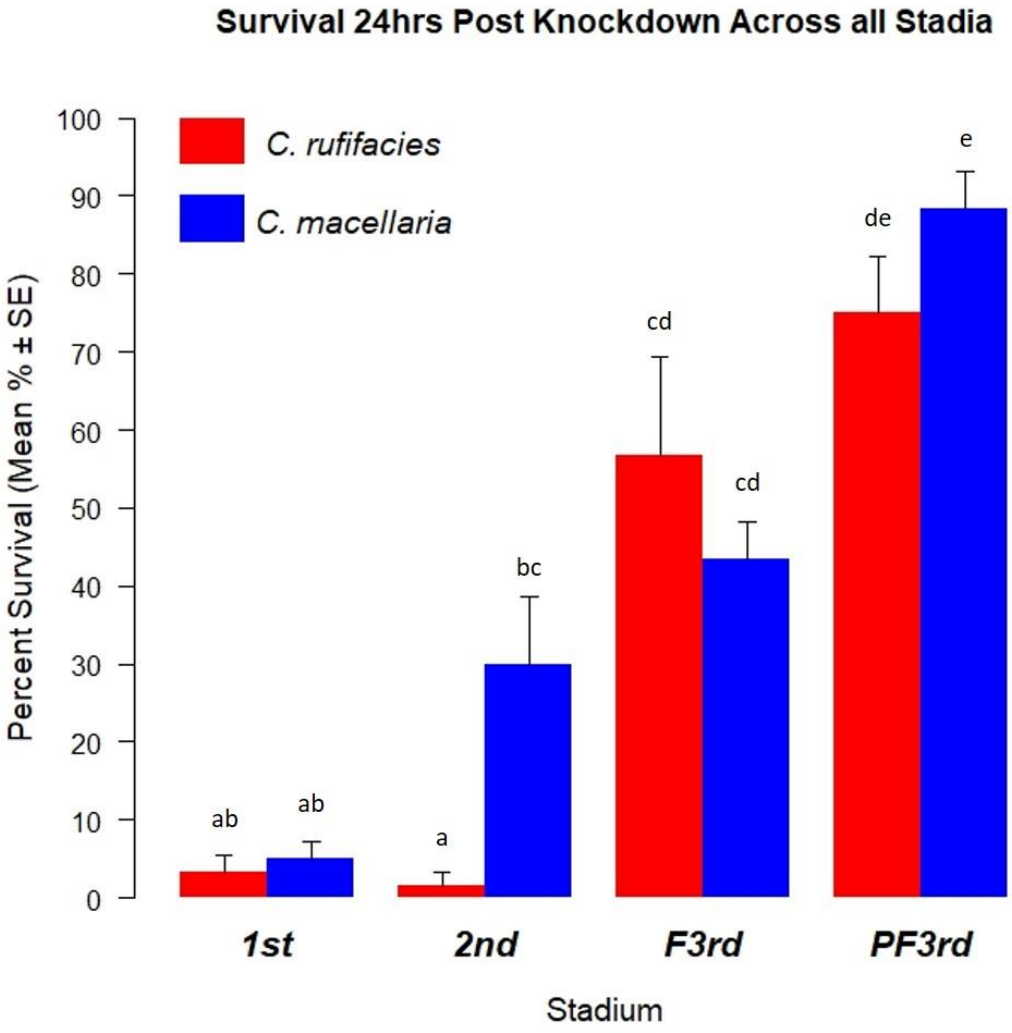


Table 9: 2-way ANOVA without treatment larvae (control).

Factor	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Species	1	0.1851	0.18513	6	0.0188
Stage	3	0.1355	0.04516	1.464	0.2389
Species:Stage	3	0.1136	0.03786	1.227	0.3124

Table 10: 2-way ANOVA of treatment larvae accounting for control mortality.

Factor	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Species	1	0.481	0.4813	7.516	0.0091
Stage	3	9.127	3.0423	47.506	2.99E-13
Species:Stage	3	0.564	0.1879	2.935	0.0449

The results from the control group are quite different. In Table 9 we see that species is a slightly significant factor, while stage isn't significant at all. In Figure 14 it is clear that the control larvae all had very high rates of survival. Across life stages, *C. macellaria* had slightly lower average rates of survival than *C. rufifacies*, except in the 2nd instar when they performed slightly better by an average of 3.33%. However, none of these differences proved significant.

To account for the mortality of the control larvae, the data was analyzed again dividing proportion of survival of the treatment larvae over proportion of survival of the control larvae. Table 10 shows the 2-way ANOVA for survival of treatment larvae corrected with the control mortality data. Stage is still the most significant factor affecting survival rates, and species has increased in significance. When looking at Figure 15, it is apparent it looks very similar to Figure 13. The pairwise comparison between species and stages even resulted in the same significance. The biggest difference we see between graphs is an increase in the adjusted *C. macellaria* post-feeding 3rd instar average survival. Any adjusted value in this stage of *C. macellaria* that was above 100% (no other stage or species exceeded 100%), was changed to 100% to allow for the same arcsine square root transformation. In some instances, the heat treated post-feeding 3rd instar *C. macellaria* performed better than the control.

Figure 14: Percent survival of the control larvae after a 24-hour recovery period (reference legend in Figure 13).

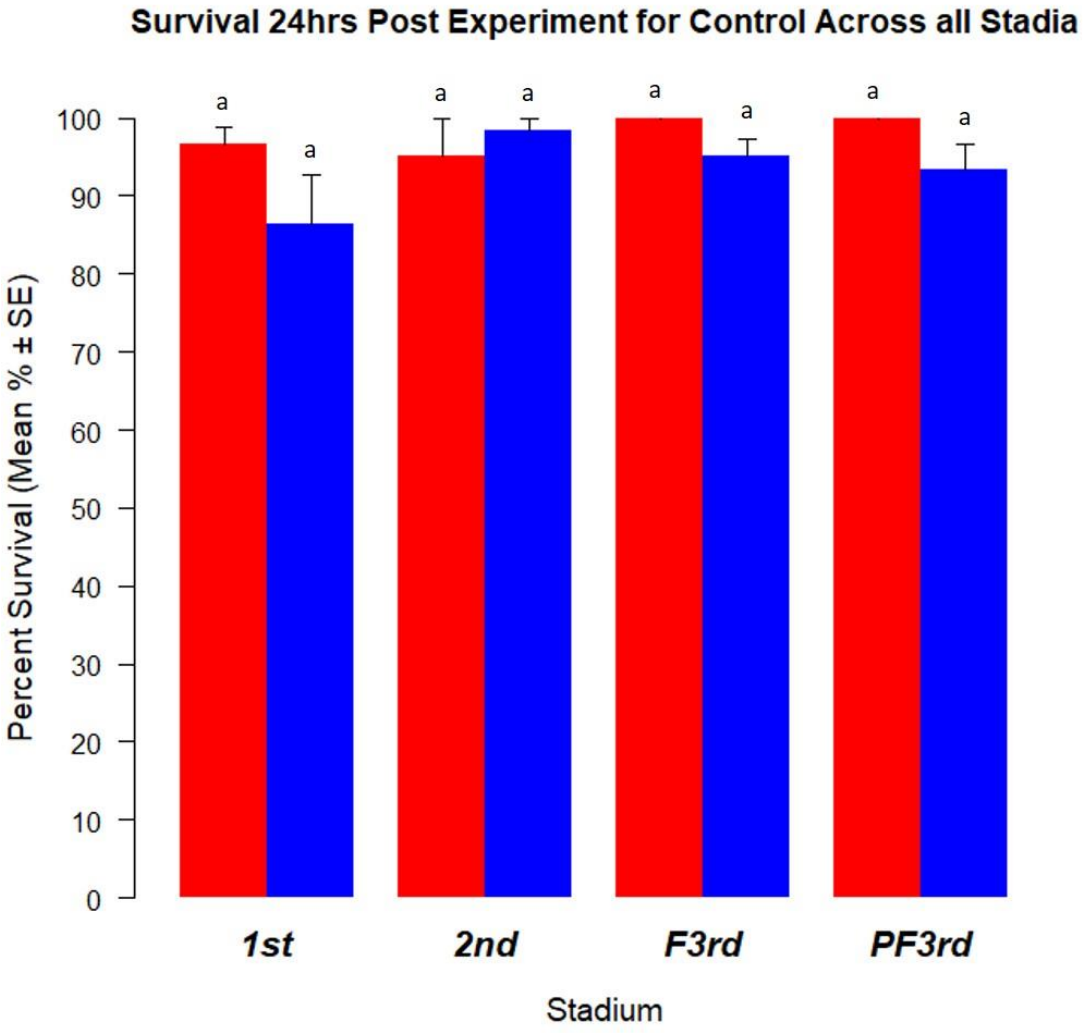
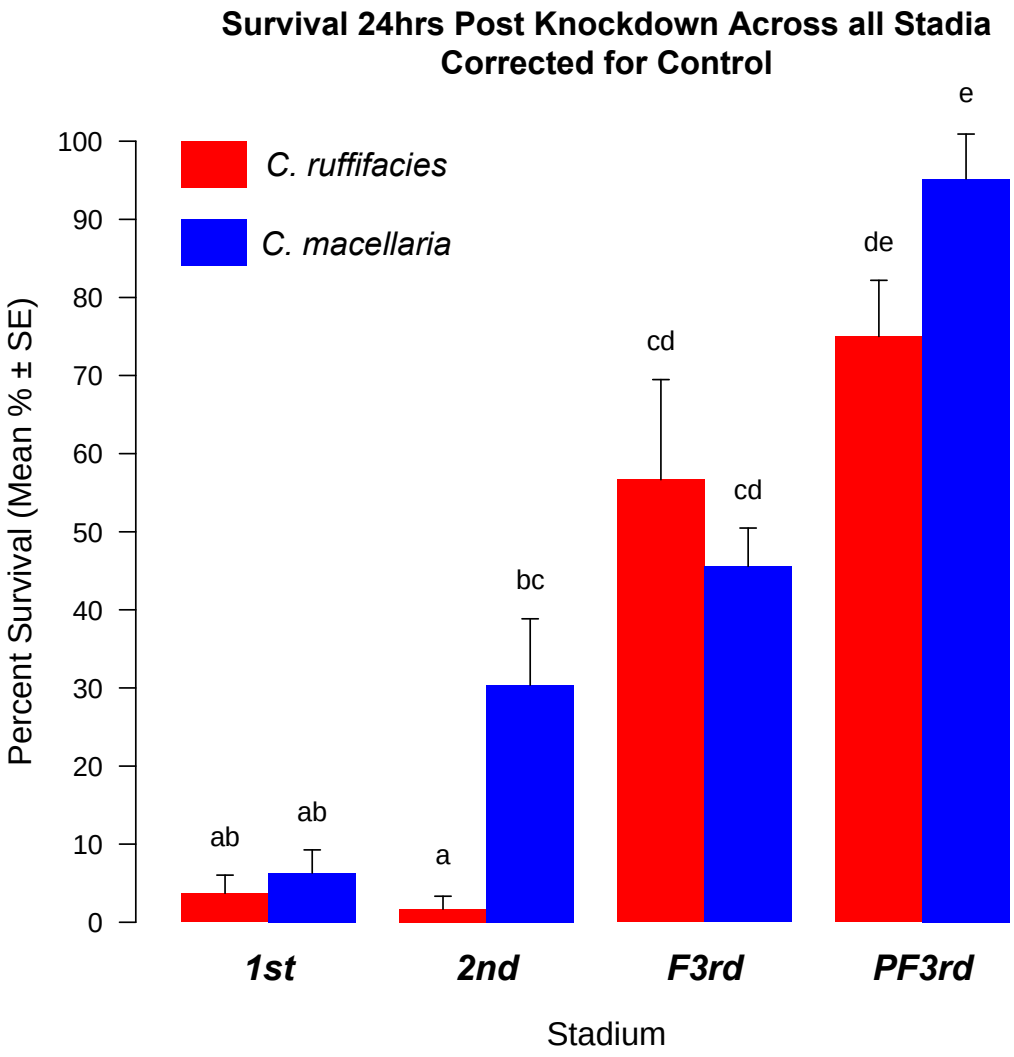


Figure 15: Percent survival of the treatment larvae after a 24-hour recovery period adjusted for the control mortality. Any value over 100 was changed to 100 to allow for transformation.



Discussion

Temperature Effects on Knockdown and Survival

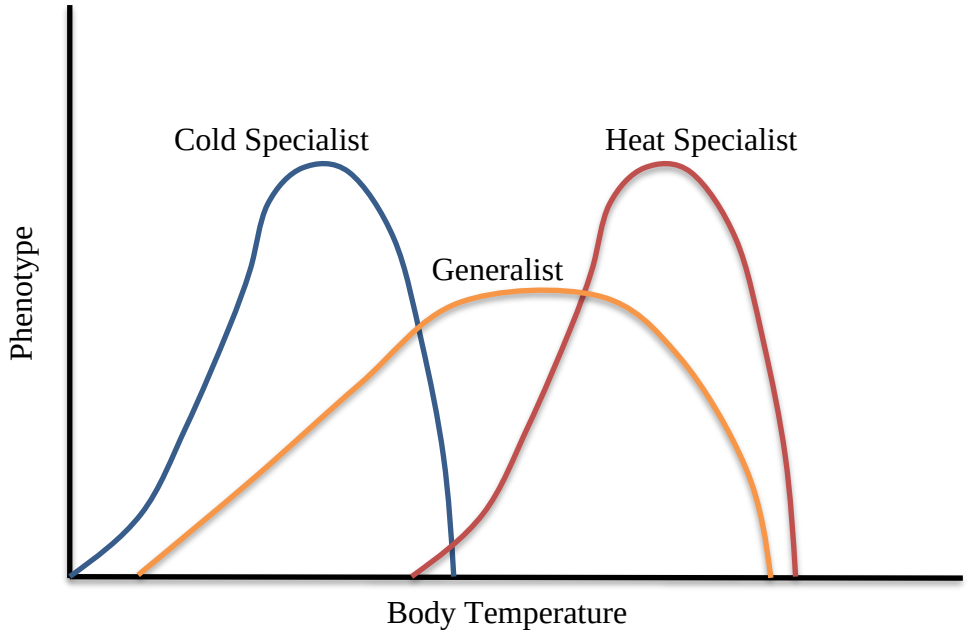
The goal of this Chapter was to determine critical thermal maxima and proportion of survival for individual maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria* by recording knockdown temperatures during a constant ramp in temperature, and survival after 24 hours. Thermal biology is important to all flies in that it dictates all forms of activity, including reproduction and development. In forensic entomology, we rely on flies of forensic importance and their surrounding temperatures to produce a timeline of development that can aid a medical examiner in determining the time of death. In this study we determined the average knockdown and proportion of survival for larvae exposed to a ramp in temperature. Species and stage were the most significant factors.

The native geographic distribution of species is one explanation for the variance in data between *C. rufifacies* and *C. macellaria*. *Chrysomya rufifacies* is an Old-World species originating in the Australasian regions (Baumgartner 1993, Byrd & Castner 2010), while *C. macellaria* is native to the Americas (Byrd & Castner 2010). The tropics tend to be consistently warmer, and this could have an impact on the life history of *C. rufifacies*. Thermal acclimation is the ability to increase thermal tolerance that enhance performance after exposure to extreme or moderate temperatures. One type of thermal acclimation is referred to as developmental acclimation, where organisms

irreversibly respond to temperature variance during an early life stage to alter performance later in life, or where parents respond to alter the performance of their offspring (Angilletta 2009). *Chrysomya rufifacies* is considered a thermal specialist and does not perform as well in cooler temperatures (Figure 16). It has been recorded as far north as southern Canada, but it is thought to make its way up during the summer months, only to disappear again in the winter (Rosati & VanLaerhoven 2007). Thermal plasticity evolves in thermally variable environments (DeWitt & Scheiner 2004), for example in 2017 in College Station, Texas the monthly average temperatures ranged from 10.0°C to 30.4°C (where the generalist, *C. macellaria* is native) ("Climate College Station – Texas", n.d.), and the average historical monthly temperatures for Malaysia ranged from 24.9°C to 25.9°C (where the specialist, *C. rufifacies* is native) ("Malaysia", n.d.). It is possible that *C. rufifacies* is thermally acclimated to consistently warmer temperatures where historical monthly averages vary by 1°C, resulting in the slightly higher knockdown temperatures visible in Figure 12.

One factor that could contribute to the species and stage effect is the thickness of the cuticle. As larvae age their cuticle thickens. This can help prevent water loss as well as protect the internal organs from damage. *Chrysomya rufifacies* larvae are also darker, more sclerotized and difficult to dissect than the smoother, opaque *C. macellaria* larvae. At high temperatures on the heat plate, larvae would often 'roll around' frantically minutes before reaching CT_{max} and would sometimes end up on their backs at knockdown. This could be to protect their ventral side from becoming too damaged

Figure 16: Variations in body temperature affect the phenotype of specialists and generalists differently. Adapted from Angilletta 2009 (Fig. 1.3 a).



during the process, as this is the side consistently exposed to direct heat while they navigate the plate. If older maggots have thicker cuticles, as do larvae of the species *C. rufifacies* like it is presumed, they would be better protected against desiccation and thermal damage. Different species would have different cuticle compositions as well, including different lipid ratios. Perhaps *C. rufifacies* has membranes composed of more saturated fatty acids, which perform better at higher temperatures (Angilletta 2009). Records of cuticle thickness and composition are unpublished and could be a future area of interest.

Another factor that could influence the knockdown and survival of the larvae is body size. As larvae feed and molt, they grow larger and their surface area to volume ratio decreases. As surface area to volume ration decreases organisms are less prone to desiccation, an example of this being an experiment conducted by Pellegrino in 1984 on several species of crab, in which smaller specimens had higher rates of desiccation. This stage dependent change in size results in a reduction of water loss through the cuticle, making the larvae less prone to desiccation. When exposed to the heated aluminum plate with no access to water, the maggots are at great risk of water loss.

One of the most notable differences in survival rate is the comparison of survival between feeding third and post-feeding third instars. During knockdown, both species had comparable critical thermal maxima for both feeding and post-feeding third instars. After 24 hours however, post-feeding third instars had higher percentages of survival. *Cochliomyia macellaria* in particular performed very well, having a significantly different average survival. Although technically in the same life stage as feeding third instars, post-feeding third instars (the wandering stage) are preparing for and nearing pupariation. During the wandering stage the body segments withdraw and invaginate, the posterior spiracles collapse, and the cuticle darkens and becomes more sclerotized (Barros-Cordeiro et al. 2016). While knockdown temperatures may be similar during the feeding 3rd instar stage, these structural differences could aid in the protection of the internal organs from heat damage. There are also potentially different metabolites present in a post-feeding 3rd instar, as they are preparing for pupariation. If these

metabolites have different thermal limitations, it is possible that this would affect knockdown and survival of the post-feeding 3rd instar.

Another possible explanation for the high survival rate of post-feeding third instar larvae is a difference in bacterial gut content. Blow fly larvae are known to be associated with many different species of bacteria (Tomberlin et al. 2017), and there is evidence to suggest that some flies may associate with various bacteria that is beneficial at different life stages (Zurek et al. 2000). Since bacteria are separate organisms, they do not necessarily have the same CT_{max} as their hosts. If a maggot experienced temperatures at which the bacteria in its gut beneficial at later stages could no longer withstand, it would have the potential to affect larval survival, even if the maggot itself had the potential to recover. This means that if the bacterial flora were eradicated at a certain point; feeding, development, etc. may not be able to be carried out effectively after knockdown.

A second notable difference in the data is the significantly higher rate of survival post-knockdown in 2nd instar *C. macellaria*. One logical explanation for the lower survival rate of 2nd instar *C. rufifacies* would be the fact that they are knocking down at higher temperatures than *C. macellaria*. The stressful temperatures reached by *C. rufifacies* larvae upon reaching CT_{max} could be too damaging for a delicate 2nd instar maggot to recover from. As mentioned above, certain bacteria beneficial at this life stage could also be wiped out at these higher temperatures.

This data set is important to the field of forensic entomology and cases where temperatures may reach high extremes. We now know a range of temperatures in which

to expect knockdown from larvae of different species and various life stages, as well as the likelihood of survival after exposure to these temperatures. This could also give insight as to why the predatory *C. rufifacies* has not yet displaced the native *C. macellaria*. There could be coexistence on a body with various internal temperature readings. There also may be periods in the year (especially colder months) in which *C. macellaria* is able to withstand a wider range of temperatures, and *C. rufifacies* is not.

CHAPTER III

THERMAL TOLERANCES OF THE HAIRY MAGGOT BLOW FLY, *CHRYSOMYA RUFIFACIES* (MACQUART), AND THE SECONDARY SCREWWORM, *COCHLIOMYIA MACELLARIA* (FABRICIUS) (DIPTERA: CALLIPHORIDAE)

Introduction

As mentioned in Chapters I and II, temperature plays a major role in the work of a forensic entomologist. Knowing upper thermal limits is important for calculating development time and is a possible explanation of a maggot mass die off. In Chapter II, a range of critical thermal maxima were determined for two species of blow flies (*Chrysomya rufifacies* and *Cochliomyia macellaria*) at four different life stages: 1st, 2nd, feeding 3rd and post-feeding 3rd instar. Determining CT_{max} gives us a better estimate of a hard cap for development at higher temperatures. However, duration of time spent at these high temperatures is just as important as the temperature readings themselves. It is possible to be exposed to lethal temperatures for a brief amount of time and survive, especially after immediate removal from the stressful environment post-knockdown as in Objective I. While the temperature of the water bath used in Objective I was constantly climbing, larvae were not spending a significant amount of time at each degree change. Based on the rate of temperature change for the aluminum stage in the water bath from 20˚C to 60˚C, larvae were spending just over a minute at every degree increase. In the wild it is unlikely that maggots would be exposed to quickly increasing temperatures

until reaching CT_{max}. In addition, it is also unlikely that the larvae will be immediately removed from that stressful environment. The data collected in Chapter II may be a bit of an exaggeration when compared to naturally occurring stressful temperatures.

It is also possible to be knocked down or die at sub-lethal temperatures for longer durations of time. As observed in preliminary studies and Objective I, it is obvious that the maggots become distressed well before their knockdown temperatures (exaggerated movements, frantically fast pace). It is likely that prolonged exposure to stressful temperatures below the recorded critical thermal maxima could be lethal. In nature it is unlikely that an organism will be exposed to its CT_{max}, be removed from the stressful environment and be able to recover. It is more probable that organisms, when nearing CT_{max} will remain in that environment until death occurs. However, in rare instances maggots have been observed writhing in direct sunlight on top of a body, succumbing to knockdown, and then rolling off of the body and into a shaded area (Rusch, Tarone; personal observations). If larvae were to be immediately removed from the thermally stressful environment like this (landing on the cooler ground, potentially shaded by grass or brush), it is possible that the larvae could recover and return to feed.

The final thing that needed to be addressed in Chapter III was access to food. *Chrysomya rufifacies* and *C. macellaria* larvae typically spend one generation, or their entire immature stages feeding on their source of food before pupariation (Rivers & Dahlem 2014). The body utilized as the food source will usually provide enough moisture to prevent desiccation. Larvae exposed to high stressful temperatures on the heat plate in Chapter II did not have access to fresh liver, and therefore were more

susceptible to desiccation, especially at such high temperatures. Younger larvae seemed especially prone to quick water loss, some even sticking to the plate and knocking down within minutes near room temperature (1st instar). To eliminate lack of food/water source as a missing factor in the determination of larval upper thermal tolerance, larvae tested in this Chapter were given access to fresh beef liver. In experiment 3 of Rusch et al. 2019 nutrient availability had a strong effect on the probabilities of knockdown and survival of adult flies exposed to stressful temperatures for 1, 2, 4 or 6 hours. At two of the three tested temperatures (42 and 44°C) the availability of nutrients (water or food and water) improved the thermal tolerance of flies. This could imply that the critical thermal maxima determined in Chapter II could be too conservative. Thus, because the results of the last chapter have the potential to be either exaggerated or too conservative, these additional factors were added to Chapter III to quantify the importance of time and food on larval responses to stressful temperatures. As with the comparison of species in the previous chapter, the responses of two different species originating from separate hemispheres (*C. rufifacies* and *C. macellaria*) will be compared.

The objective of this chapter is as follows:

II. To observe the likelihood of knockdown and survival of maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria*

- Record proportion knockdown and survival post-24 hours for maggots of all instars of both species at different temperatures for varying amounts of time with and without food

H_0: Likelihood of knockdown and survival of larvae at various temperatures between species, stadia, duration of exposure and access to food will be the same

H_a: Likelihood of knockdown and survival of larvae at various temperatures between species, stadia, duration of exposure and access to food will differ

Materials and Methods

This experiment took place in the F.L.I.E.S. facility between September 12th 2018 and July 4th 2019, and is of a novel experimental design. Voucher specimens (739) were submitted to the Texas A&M University Insect Collection (Whitworth 2006).

Source of Blow Fly Larvae

Collection of adults and rearing techniques of larvae follow that outlined in Chapter II.

Source of Animal Diet

Blow fly larvae for this experiment were fed on beef liver for the entirety of their immature stadia. Beef liver and blood were collected and purchased from the Rosenthal Meat Science and Technology Center. Beef liver was also occasionally purchased from Readfield Meats and Deli (Bryan, TX) and HEB (San Antonio, TX). No vertebrate animals were killed for the purpose of this experiment, and appropriate forms have been completed and approved by Texas A&M for the purchase and use of vertebrates in this study.

Experimental Design

For all larval stages of both *Chrysomya rufifacies* and *Cochliomyia macellaria*, incubators (Percival Scientific, Perry, IA) were set to 25 (control), 35, 45 and 50˚C (+/- 5˚C) at 50% humidity (+/- 10%) (Percival 2019). Forty-eight total 8oz. glass mason jars (Ball® Corporation, Broomfield, CO) and forty-eight total bath cups (3 oz Great Value ™ Bath Plastic Cups, Wal-Mart Stores, Inc, Bentonville, AR) were set out and prepped for each larval stage of both species tested in this experiment (1^{st}, 2^{nd}, feeding 3^{rd} and post-feeding 3^{rd} instar). Half of the bath cups (24) contained a small amount of beef liver (~ 0.5g), while the other half remained empty. These cups were placed into the mason jars at the start of the experiment, and a single layer of Wypall (Kimberly-Clark

Worldwide Inc., Roswell, GA) was screwed onto the top of all jars to allow for oxygen flow.

Larvae were taken from their colony jars held at 23.89°C/75°F, identified to 1st, 2nd, feeding 3rd and post-feeding 3rd instar and offered food prior to experimentation as outlined in Chapter II, Objective I. Larvae ten generations or less out of the wild will be used for this experiment. Once the temperature and humidity in the incubators were ready, ten larvae of the same species and stadia were placed in each bath cup with or without beef liver (Figure 17). Each of these bath cups were covered with half of a Kimwipe (Kimberly-Clark Worldwide Inc., Roswell, GA), using a rubber band to secure it in place. This was to prevent larvae from escaping the cup. Each cup was then placed in an 8oz. glass mason jar, which was secured with half of a Wypall and lid. For this experiment, it was most efficient to run two temperatures at a time (24 total jars, 12 to an incubator, 6 with and 6 without liver).

All jars were placed randomly on the incubator racks in a timely fashion to avoid a large time gap between any of the treatments (Figure 18). After 12 jars were placed in the first incubator, timing began. The second set of 12 jars was added to its respective incubator in a staggered fashion (~15 minutes after) to allow for data collection between treatments. Four jars (2 with and 2 without liver) from each temperature treatment were removed after half an hour had elapsed. This was repeated after one and two hours. This same pattern was followed for all temperature treatments and all larval stages of both species. Immediately after removal from the incubators, knockdown was recorded

for each maggot. Three separate trials for each species and larval instar were conducted out of three different generations of flies.

Figure 17: Bath cups each containing 10 1st instar *C. macellaria* larvae prepared for the temperature:time treatments. With (right) and without (left) beef liver.

Figure 18: Incubator with 12 mason jars containing bath cups for one temperature treatment.

Once knockdown was recorded, all ten larvae from each cup (with and without liver) were transferred into a new bath cup lined with half of a Kimwipe dampened with several drops of RO water, and a fresh piece of beef liver (~ 0.5g) (Figure 19). These cups were also covered with half of a Kimwipe, which was secured in place with a rubber band. Each of these cups were then replaced into their mason jars (for jars coming out of 45 and 50°C incubators, new room temperature jars were used), and

Figure 19: Bath cups prepared with half of a dampened Kimwipe and beef liver for the 24-hour recovery period.

topped with half of a Wypall and lid. These jars were then placed into an incubator set at 25°C for a 24-hour recovery period. After the 24 hours had elapsed (+/- 6 hours) survival was recorded. As mentioned earlier, trials for both species will be repeated three times per larval stage for a total of 1440 larvae of each stage (360 for each temperature treatment) for *C. rufifacies* and 1440 larvae of each stage (360 for each temperature treatment) for *C. macellaria.*

Analysis

A generalized linear model was implemented in the platform R, version 3.5.1 (R Core Team 2015) with library scales (Wickham 2018) to test for the significance of the interactions of factors affecting the dependent variables – proportion of knockdown and survival after exposure to extreme temperatures for different durations of time across different stadia. The factors considered in this objective were; species, temperature, duration of exposure, food treatment, and life stage.

Results

The knockdown data was analyzed using a generalized linear model to determine the factors affecting the data. The Akaike weights (AIC scores) were used to estimate the likelihood of a model better describing the data than another, the lower the score the better. The full model including all five interacting factors (species, temperature, duration of exposure, food treatment, life stage) was found to have the best fit. An ANOVA was used to compare null, non-interacting, and interacting models (Table 11). A summary of the accepted model can be seen in Table 12. All five factors show significance, as well as the majority of interactions among them.

Table 11: Comparisons of generalized linear models of knockdown using ANOVA.

a. Descriptions of glm models used to assess factor significance and interactions contributing to larval knockdown.

Number	Model
M1	Null
M2	Species+Temp+Duration+Food.Treatment+Stage
M3	Species*Temp*Duration*Food.Treatment*Stage

b. Analysis of Deviance Table for M1 showing significance for M2 and M3.

Model	Resid. Df.	Resid. Dev.	Df	Deviance	Pr(>Chi)
M1	1151	11045.1			
M2	1146	4092.9	5	6952.2	<2.2e-16
M3	1136	3304.1	15	7741	<2.2e-16

c. Analysis of Deviance Table for M2 & M3 showing significance.

Model	Resid. Df.	Resid. Dev.	Df	Deviance	Pr(>Chi)
M2	1146	4092.9			
M3	1136	3304.1	10	788.87	<2.2e-16

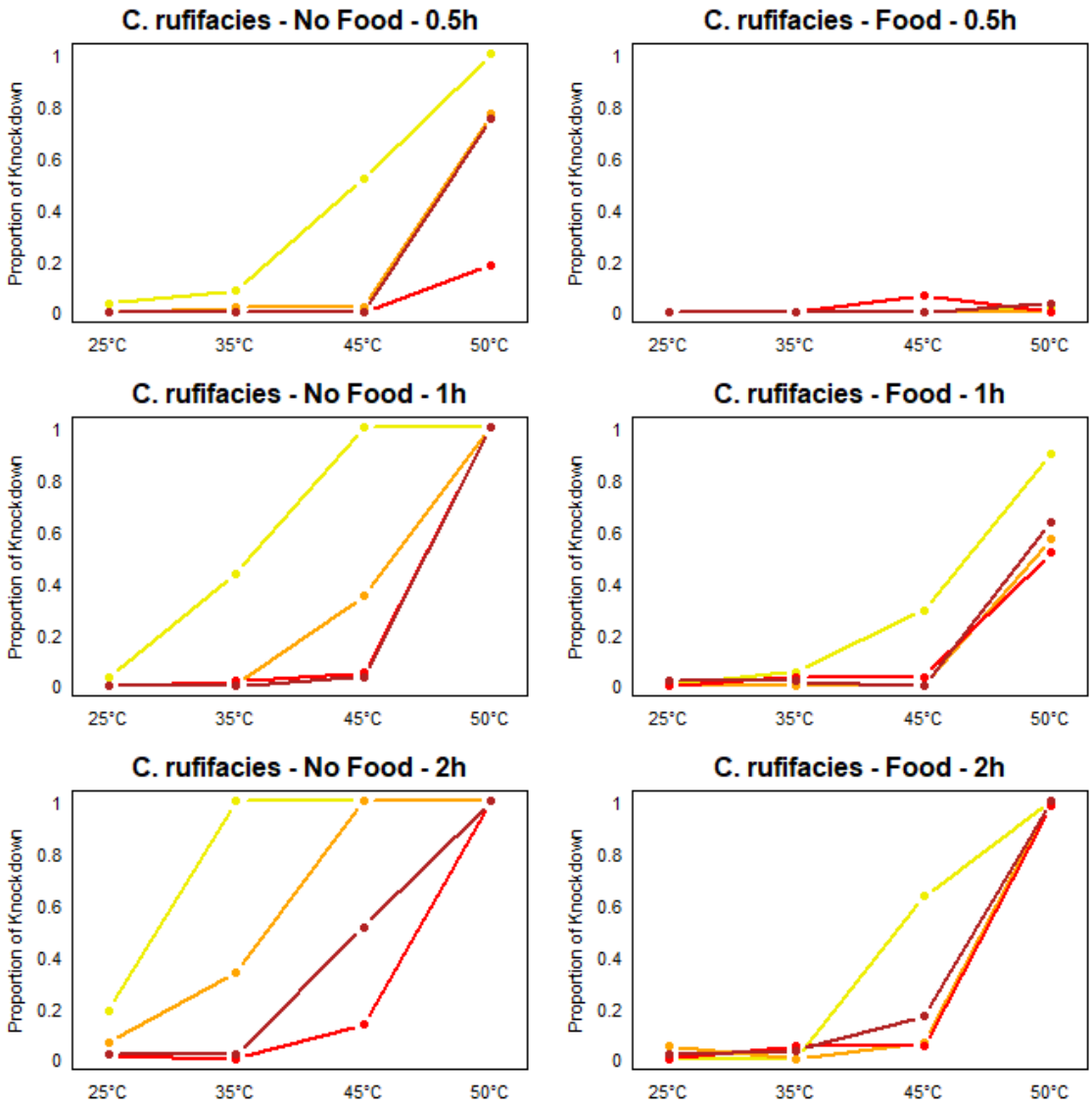
Table 12: Summary of the full model used to assess factor significance and interactions contributing to larval knockdown.

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	6.62016	3.50311	1.890	0.058785 .
Species	-31.07545	4.56025	-6.814	9.47e-12 ***
Temp	-0.32698	0.07321	-4.467	7.95e-06 ***
Duration	-8.79222	2.05449	-4.280	1.87e-05 ***
Food.Treatment	-17.23246	7.21773	-2.388	0.016963 *
Stage	-27.56290	3.03772	-9.074	< 2e-16 ***
Species*Temp	0.67019	0.09693	6.914	4.71e-12 ***
Species*Duration	20.61953	2.85011	7.235	4.67e-13 ***
Temp*Duration	0.24993	0.04636	5.392	6.99e-08 ***
Species*Food.T	2.37342	12.81946	0.185	0.853118
Temp*Food.T	0.26329	0.15267	1.725	0.084599 .
Duration*Food.T	-7.54637	6.28531	-1.201	0.229892
Species*Stage	25.64749	3.53484	7.256	4.00e-13 ***
Temp*Stage	0.54864	0.06132	8.947	< 2e-16 ***
Duration*Stage	10.40360	1.64974	6.306	2.86e-10 ***
Food.T*Stage	24.61871	4.01673	6.129	8.84e-10 ***
Species*Temp*Duration	-0.47784	0.06416	-7.447	9.52e-14 ***
Species*Temp*Food.T	-0.10436	0.26592	-0.392	0.694743
Species*Duration*Food.T	-0.68648	9.05313	-0.076	0.939556
Temp*Duration*Food.T	0.16389	0.13723	1.194	0.232365

Table 12: Continued

	Estimate	Std. Error	z value	Pr(>|z|)
Species*Temp*Stage	-0.53169	0.07206	-7.379	1.60e-13 ***
Species*Duration*Stage	-16.73259	2.18439	-7.660	1.86e-14 ***
Temp*Duration*Stage	-0.21961	0.03395	-6.468	9.91e-11 ***
Species*Food.T*Stage	-16.83493	5.31587	-3.167	0.001541 **
Temp*Food.T*Stage	-0.48793	0.08231	-5.928	3.07e-09 ***
Duration*Food.T*Stage	-8.51302	2.69318	-3.161	0.001573 **
Species*Temp*Duration *Food.T	0.05917	0.19335	0.306	0.759602
Species*Temp*Duration*Stage	0.35703	0.04585	7.787	6.86e-15 ***
Species*Temp*Food.T*Stage	0.36243	0.10928	3.317	0.000911 ***
Species*Duration*Food.T *Stage	11.49488	3.62354	3.172	0.001512 **
Temp*Duration*Food.T*Stage	0.17344	0.05687	3.050	0.002288 **
Species*Temp*Duration *Food.T*Stage	-0.25215	0.07641	-3.300	0.000967 ***

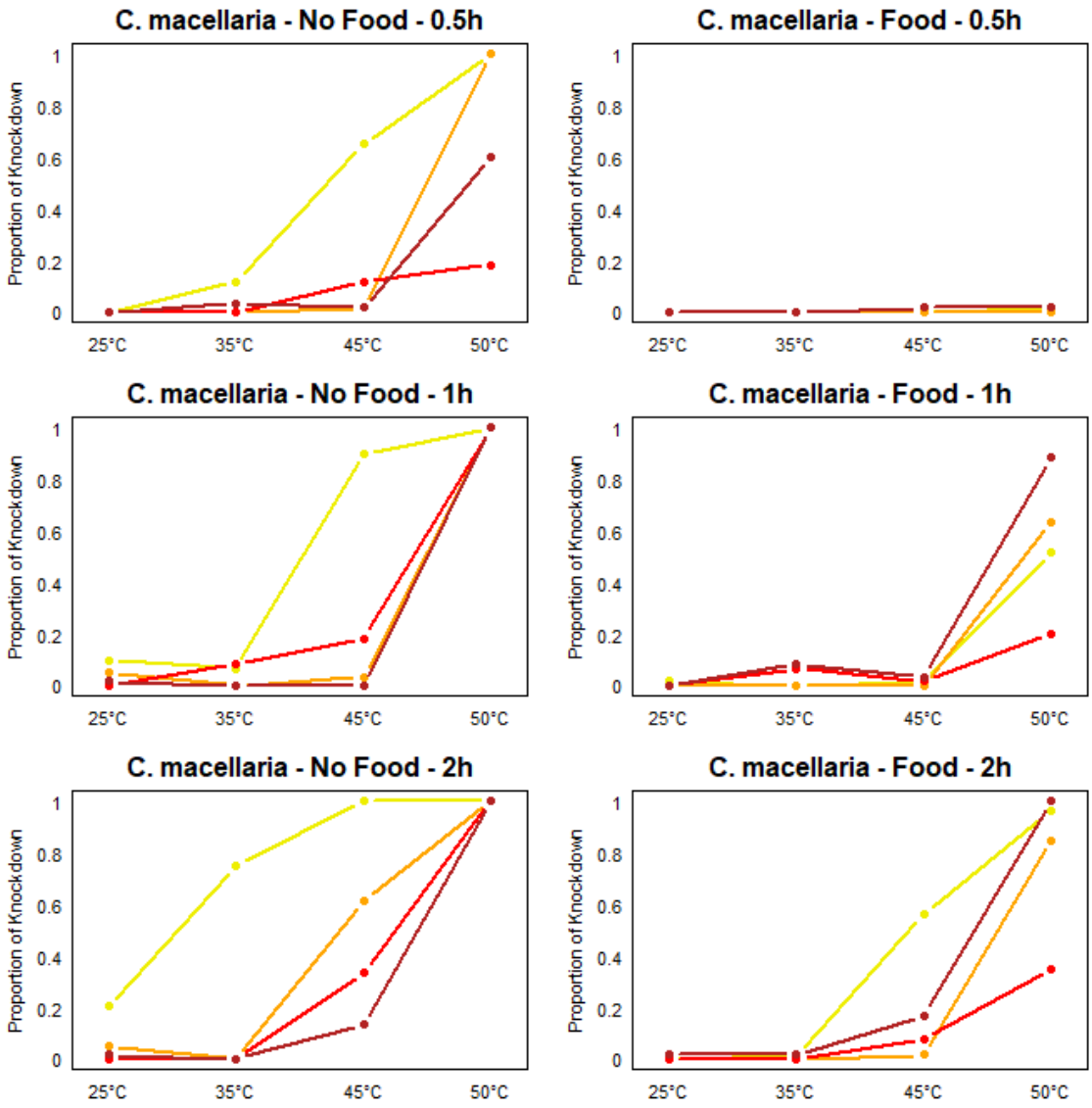
Figure 20: Proportion of knockdown for *C. rufifacies* at each of the four tested temperatures; 25, 35, 45 and 50°C. First instars are represented in yellow, second instars in orange, feeding third instars in red and post-feeding third instars in brown.



Visual representations of the knockdown data were also created in R (Figures 20 & 21). It is apparent that younger larvae are more susceptible to knockdown. Higher temperatures, longer durations and lack of food also result in a larger proportion of knockdown across species and stage.

The survival data was analyzed separately using a generalized linear model to determine the factors affecting the data. The AIC scores were used to estimate the likelihood of a model better describing the data than another, the lower the score the better. The full model including all five interacting factors (species, temperature, duration of exposure, food treatment, life stage) was found to have the best fit.

Figure 21: Proportion of knockdown for *C. macellaria* at each of the four tested temperatures; 25, 35, 45 and 50°C. First instars are represented in yellow, second instars in orange, feeding third instars in red and post-feeding third instars in brown.



An ANOVA was used to compare null, non-interacting, and interacting models (Table 13). A summary of the accepted model can be seen in Table 14. Species, temperature and duration were all significant factors. However, food treatment and stage individually were not. Roughly half of the interactions shown in the table also proved to be significant.

Table 13: Comparisons of generalized linear models of survival using ANOVA.

a. Descriptions of glm models used to assess factor significance and interactions contributing to larval survival after temperature treatments.

Number	Model
M1	Null
M2	Species+Temp+Duration+Food.Treatment+Stage
M3	Species*Temp*Duration*Food.Treatment*Stage

b. Analysis of Deviance Table for M1 showing significance for M2 and M3.

Model	Resid. Df.	Resid. Dev.	Df	Deviance	Pr(>Chi)
M1	1151	10409			
M2	1146	5245	5	5163.9	<2.2e-16
M3	1120	4403	31	6005.9	<2.2e-16

Table 13: Continued

c. Analysis of Deviance Table for M2 & M3 showing significance.

Model	Resid. Df.	Resid. Dev.	Df	Deviance	Pr(>Chi)
M2	1146	5245			
M3	1120	4403	26	842.03	<2.2e-16

Table 14: Summary of the full model used to assess factor significance and interactions contributing to larval survival.

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-1.196168	1.915412	-0.624	0.532302
Species	6.191873	2.590854	2.390	0.016853 *
Temp	-0.172745	0.043859	-3.939	8.19e-05 ***
Duration	-6.965192	1.505615	-4.626	3.73e-06 ***
Food.Treatment	2.154111	2.898177	0.743	0.457322
Stage	-1.542320	1.208303	-1.276	0.201802
Species*Temp	-0.170634	0.059199	-2.882	0.003947 **
Species*Duration	-1.183978	1.944344	-0.609	0.542568
Temp*Duration	0.051427	0.037108	1.386	0.165789
Species*Food.T	-13.144194	3.817995	-3.443	0.000576 ***

Table 14: Continued

	Estimate	Std. Error	z value	Pr(>|z|)
Temp*Food.T	-0.007517	0.065454	-0.115	0.908572
Duration*Food.T	1.429492	2.259406	0.633	0.526939
Species*Stage	-1.265915	1.474644	-0.858	0.390641
Temp*Stage	0.044065	0.025915	1.700	0.089057 .
Duration*Stage	7.562851	1.096105	6.900	5.21e-12 ***
Food.T*Stage	-2.799683	1.509439	-1.855	0.063627 .
Species*Temp*Duration	0.102025	0.046936	2.174	0.029726 *
Species*Temp*Food.T	0.332675	0.086479	3.847	0.000120 ***
Species*Duration*Food.T	7.272617	2.865170	2.538	0.011140 *
Temp*Duration*Food.T	0.016795	0.053214	0.316	0.752296
Species*Temp*Stage	0.033225	0.031858	1.043	0.296984
Species*Duration*Stage	-2.930564	1.302196	-2.250	0.024419 *
Temp*Duration*Stage	-0.143879	0.023768	-6.053	1.42e-09 ***
Species*Food.T*Stage	5.621187	1.874946	2.998	0.002717 **
Temp*Food.T*Stage	0.054665	0.032820	1.666	0.095790 .
Duration*Food.T*Stage	-0.763517	1.438989	-0.531	0.595701
Species*Temp*Duration *Food.T	-0.224741	0.066899	-3.359	0.000781 ***
Species*Temp*Duration*Stage	0.044154	0.028338	1.558	0.119198

Table 14: Continued

	Estimate	Std. Error	z value	Pr(>|z|)
Species*Temp*Food.T*Stage	-0.135307	0.041011	-3.299	0.000969 ***
Species*Duration*Food.T *Stage	-3.085359	1.693457	-1.822	0.068466 .
Temp*Duration*Food.T*Stage	0.002135	0.031339	0.068	0.945684
Species*Temp*Duration *Food.T*Stage	0.086310	0.036996	2.333	0.019649 *

Visual representations of the survival data were also created in R (Figures 22 & 23). Higher temperatures and longer durations result in a smaller proportion of survival across species and stage.

Figure 22: Proportion of survival after 24 hours for *C. rufifacies* at each of the four tested temperatures; 25, 35, 45 and 50°C. First instars are represented in yellow, second instars in orange, feeding third instars in red and post-feeding third instars in brown.

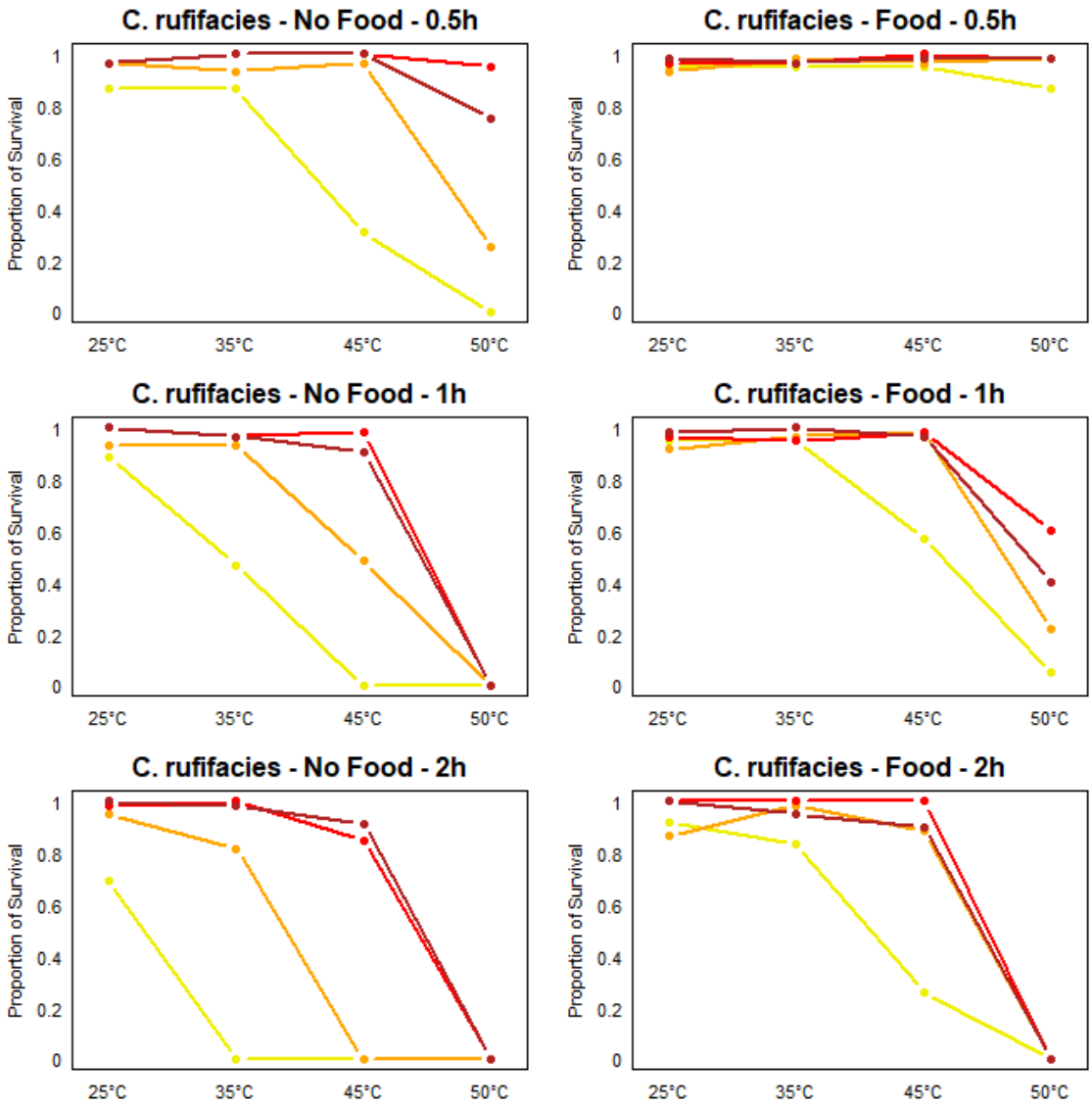
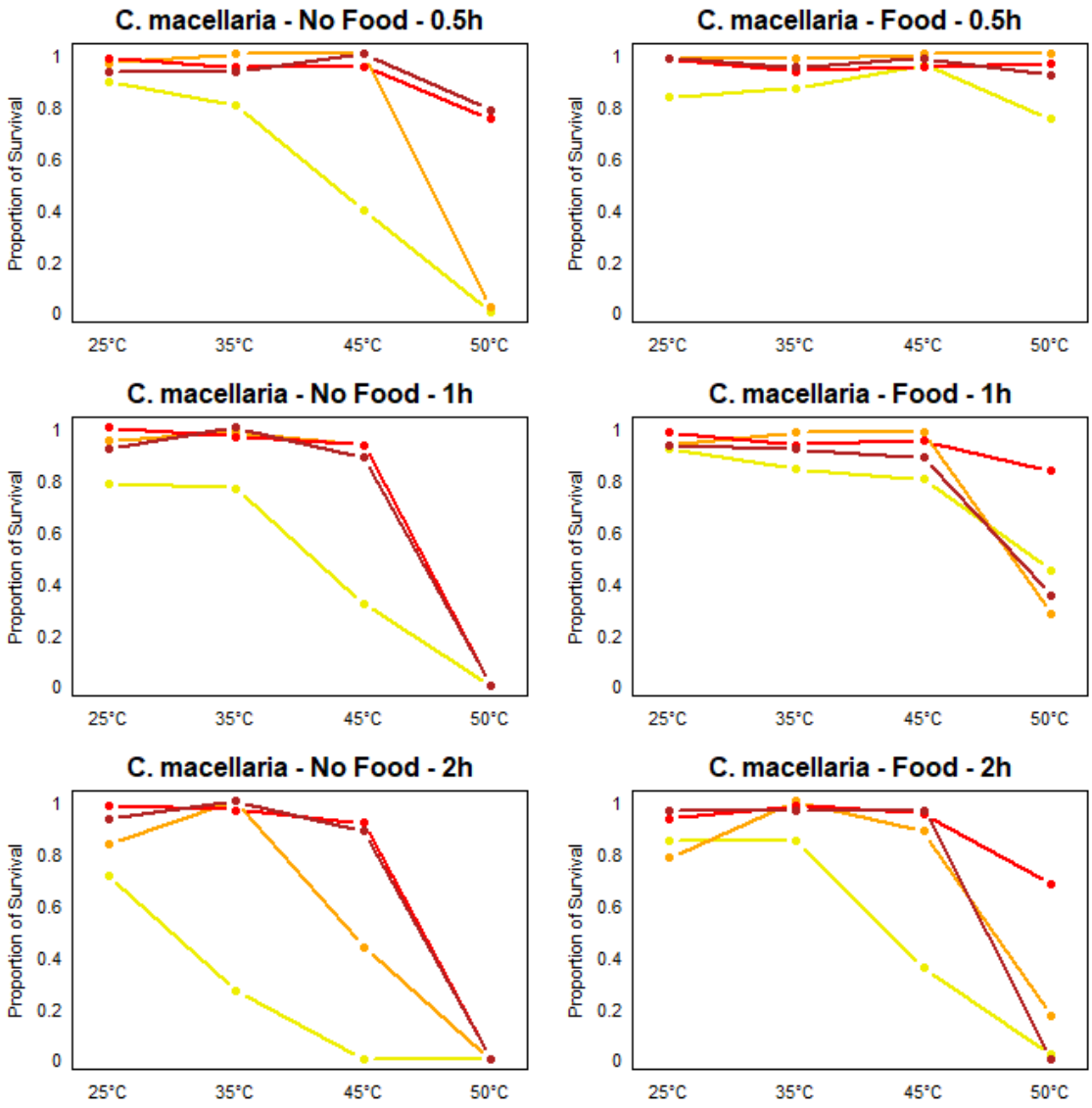


Figure 23: Proportion of survival after 24 hours for *C. macellaria* at each of the four tested temperatures; 25, 35, 45 and 50°C. First instars are represented in yellow, second instars in orange, feeding third instars in red and post-feeding third instars in brown.



Discussion

Temperature and Time Effects on Larval Thermal Tolerance

The goal of this Chapter was to determine likelihood of knockdown and survival post 24 hours for maggots of the species *Chrysomya rufifacies* and *Cochliomyia macellaria* by recording knockdown and survival after exposure to various temperatures for different lengths of time. As discussed in Chapter II, thermal biology is critical when it comes to their activity. Forensic entomologists rely on the temperature dependent development and activities of larvae to estimate forensically important timelines (Byrd & Castner 2010, Gennard 2012). In this study, the likelihood of knockdown and survival after exposure to stressful temperatures were determined for different stages of larvae. Species, temperature, duration of exposure, food treatment and life stage were all significant factors.

In Chapter II, reasons for variability in data between species were discussed in detail. Consistently warm foreign origins ("Malaysia", n.d.) of *C. rufifacies* could account for different responses to temperature in comparison to the thermally variable origins ("Climate College Station – Texas", n.d.) of *C. macellaria. Chrysomya rufifacies* in general performed better than *C. macellaria* at the half hour time exposure. However, in this experiment *C. rufifacies* tends to knockdown at higher rates, and survive at lower rates than *C. macellaria* at the one and two hour time exposures. One drastic example can be seen in Figure 22 "*C. rufifacies* – Food - 2h" vs Figure 23 "*C.*

macellaria – Food – 2h". *Chrysomya rufifacies* has no survival for any stage at 50°C, while *C. macellaria* has around 70% survival for feeding third instar, and almost 20% survival for second instar at the same temperature. This could be attributed to the potentially thermal generalist nature of *C. macellaria,* having a broader range of survivable temperatures. *Chrysomya rufifacies* could still be the hot thermal specialist, possibly out-performing *C. macellaria* at slightly lower temperatures. It must also be considered that survival post 24 hours also does not ensure survival to pupariation or emergence. In addition to this, it has been shown that exposure to extreme temperatures without an acclimation period can negatively affect survivorship (Chen et al. 1987). This should be considered in the interpretation of this data and future work.

Duration of exposure was one of the reasons to experiment with the static method of measuring thermal tolerance after using the ramping method in Chapter II. Larvae from the previous chapter were only exposed to each degree increase for very brief amounts of time, which is an unrealistic expectation. In the wild, larvae can be exposed to extreme heat on a body for hours at a time (Villet et al. 2009). Thus, a maggot that is knocked down is likely to experience some period of time after the knockdown event at elevated temperatures. For a body to reach the critical thermal maxima (CT_{max}) recorded in Chapter II, temperatures would slowly increase throughout the day, potentially exposing larvae to slightly sub- CT_{max} temperatures for extended amounts of time. It is therefore possible that larvae would knockdown before true CT_{max} is reached. For example, in Figure 20 "*C. rufifacies* – No Food – 2h" and "*C. rufifacies* – Food – 2h" knockdown at 50°C is effectively 100%. In Chapter II, recorded CT_{max} for this species

was always above 50°C with the exception of 2 outliers. In Figure 22 "*C. rufifacies* – No Food – 2h" and "*C. rufifacies* – Food – 2h" survival after exposure to 50°C is 0%. In Chapter II, survival after exposure to CT_{max} never drops to 0% for any instar - for feeding and post-feeding third instar, the average survival is between 55 and 75%. In this case we must also still consider that survival post 24 hours does not ensure survival to sexual maturity.

Availability of food was another reason to utilize the static method of measuring thermal tolerance. In Chapter II, beef liver (our standard food substrate) was not available to the larvae during the temperature ramp to determine CT_{max}. Ideally, a body will provide blow fly larvae with a food source needed for one complete generation, until they are ready for pupariation (Rivers & Dahlem 2014). During the temperature ramp it was speculated that some larvae (especially the younger instars) were desiccating due to the extreme heat before an accurate CT_{max} could be measured. This issue was addressed in this experiment, where half of all larvae were given access to beef liver throughout the entire treatment. One example where we can see the effect of food on the data set is in Figure 21 "*C. macellaria* – No Food – 0.5h" and "*C. macellaria* – Food – 0.5h". A drastic increase in proportion of knockdown can be seen without access to food even at 35 and 45°C. At 50°C 100% knockdown for first and second instar is seen. With access to food minimal knockdown was recorded for all instars. It was also noted that knockdown and survival graphs for both species at "No Food – 0.5h" and "Food – 2h" looked quite similar. It is possible that access to food provides an hour and a half buffer before knockdown or death. This extra time would be crucial for a feeding

maggot on a body, considering it would have an hour and a half at a stressful temperature to seek thermal refuge before risking knockdown and possible death. Larvae that have fallen off of a body or wandered away would be much more at risk at high temperatures.

Life stage was also a significant factor affecting thermal tolerance of the larvae. In Figure 20 "*C. rufifacies* – No Food – 2h" a large spread of knockdown is seen among larval life stages. First instar larvae jump to 100% knockdown by 35°C, followed by second instar at 45°C. Feeding and post-feeding third instar larvae slowly increase proportion of knockdown along with heat treatments, until 100% knockdown is reached for both at 50°C. As discussed in Chapter II, this could be impacted by cuticle thickening as the maggots age, or a change in bacterial gut content. Body size is another factor to consider as the larvae progress through their life stages. As body size increases, surface area to volume ratio decreases, meaning the organism is less prone to desiccation. Older larvae have also had more time to feed and have accumulated more energy and fat storage. Significant amounts of energy are required to combat heat stress, and later stages of larvae have more to spare than younger instars.

This data set is important to the field of forensic entomology and cases where temperatures may reach high extremes. We now know roughly when to expect knockdown and death of larvae below potential CT_{max}. If dead maggots are found in the field, temperature data can be traced back and tracked over time to determine when larvae of various ages may have succumbed to the heat. This information could also give insight to the coexistence of the invasive predatory *C. rufifacies* and the native *C.*

macellaria. Differences in knockdown and survival after extreme heat exposure could be supported by the segregation of masses of these species occupying various parts of a body (Brundage et al., 2014) with different internal temperatures.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

In Chapter I, Figure 6 showed remains reaching temperatures of 60°C and above. There is a significant amount of anecdotal evidence that exists to suggest that in Texas, maggots on a body exposed to extreme heat can die. Larval death at high temperatures has also been documented in literature (Donovan et al. 2006). There is also evidence here in Chapters II and III to back that up. Stage plays a significant role in larval response to heat. It is no surprise that first instar larvae are the most susceptible. Younger, smaller larvae have a larger surface area to volume ratio and are more prone to desiccation – this could highlight the need for effective maternal choice for oviposition sites in the warmer summer months. Without direct access to food, first instar larvae have been observed knocking down at temperatures barely above ambient. It was expected that older larvae would be able to withstand higher temperatures as they are less prone to desiccation, have thicker cuticles, are more mobile and have more stored energy to combat stressful environments.

It is also apparent in Chapter II that second instar *Cochliomyia macellaria* larvae survive better than second instar *Chrysomya rufifacies* larvae when exposed to knockdown at stressful temperatures. *Cochliomyia macellaria* has also shown higher survival rates at older instars like feeding and post-feeding third instar than *C. rufifacies*. These higher rates of survival could be due to their slightly lower knockdown

temperatures. If *C. macellaria* will almost always reach their critical thermal maximum a degree or two before *C. rufifacies*, they are not being exposed to quite as extreme temperatures. This could protect crucial gut bacterium or prevent irreversible cellular damage.

Thermal life histories also could play a significant role in response to temperature. Slightly different responses between species could be attributed to their differing origins. The variance (or lack thereof) in temperatures both *C. macellaria* and *C. rufifacies* have been exposed to in their native ranges is likely to have shaped them to be thermal generalists and specialists, respectively.

It is encouraged for a forensic entomologist to collect larvae of various stages on a body at a crime scene (Byrd & Castner 2010) to ensure a fully representative sample has been taken. This is reasonable considering that there is a minimum and maximum development rate for all stages of larvae, which could paint a more complete picture of mPMI/PIA/TOC (Amendt et al. 2007, Tomberlin et al. 2011, Tarone & Sanford 2017). The data from Chapter II shows that there are limitations to development at high temperatures for each instar. It is possible for a heat wave to kill all of the first or second instars feeding on a body, leaving only the oldest, most thermally tolerant larvae. This still tells a story and could provide important insight to the life histories of the older maggots collected on site. In the event of a heat wave, it must also be considered that the surviving larvae on a corpse may represent a biased sample compared to evidentiary samples with less extreme thermal profiles. High temperatures may cause feeding larvae to abandon a food source, ceasing development, in search of thermal refuge. It is

possible that survival at different instars after extreme heat exposure is linked to specific temperatures. Linking each individual with its knockdown temperature and tracking that for survival would give a more complete data set. For this experiment however, individuals were not followed to survival after 24 hours. Knowing if survival to pupariation and sexual maturity after exposure to CT_{max} is possible or normal would also be a beneficial addition to this data set. The research from Chapter II is the first step to enable forensic entomologists to consider CT_{max} for various species as a limitation when considering forensically important timelines.

In Chapter II, knockdown was assessed using the ramping method. As discussed in Chapter I, there are two methods in which to determine knockdown or CT_{max} – the ramping and the static method (Lighton & Turner 2004, Lutterschmidt & Hutchison 1997). There were two issues that needed to be addressed once objective I was completed for a fuller understanding of these species' thermal tolerances - these were access to food, and duration of time spent at stressful temperatures. Both of these factors are known to influence upper thermal limits aside from absolute temperature (Rivers et al. 2011). First instars were very sensitive to the ramping method, often being knocked down shortly above room temperature, and reviving quickly when placed on liver. This was thought to be due to lack of access to water or moisture, which is present on their food source of beef liver. With the resources available, assessing knockdown utilizing the ramping method with access to food proved difficult. In an ecological sense, the ramping method also has a limitation in that once an organism is knocked down it is immediately removed from the stressful environment. In the wild, if a maggot is

knocked down on a body it is unlikely that it will be 'rescued' and taken to a thermal refuge. By utilizing the static method in Chapter III, access to food and longer durations of heat exposure were both addressed. Survival to pupariation and sexual maturity would also be a beneficial addition to this data set.

This thesis is concerned with the upper thermal limits of blow fly larvae that are important to a plethora of ecological services and applications. In Chapter II *C. rufifacies* reached higher average knockdown (CT_{max}) temperature at every larval stage tested. The findings from Chapter III indicated that *C. rufifacies* often had higher rates of knockdown than *C. macellaria* after longer durations of exposure (1 and 2 hours). This indicates that *C. rufifacies* may be able to withstand higher temperatures for brief amounts of time, but *C. macellaria* may be able to withstand longer periods of time at stressful temperatures below CT_{max}. Survival data from both Chapters II & III show that *C. macellaria* often had better survival rates than *C. rufifacies*. This research still seems to follow the assumption that *C. rufifacies* is a thermal specialist, reaching higher extremes, and that *C. macellaria* is a specialist, having higher rates of survival across temperatures and durations. Larvae of both species have extremely high CT_{max} if they are reached quickly, and can even survive if immediately removed, but both will knockdown at lower temperatures when exposed for longer periods of time. Knockdown below CT_{max} was exacerbated by lack of food. These stressful high temperature environments are definitely achievable in the field during the summer months in Texas.

These differences in thermal tolerances between species could help explain some of the separation we see when *C. rufifacies* and *C. macellaria* are feeding on a body.

Maggot masses of both species are typically segregated when feeding – *C. rufifacies* is generally at the soil/body interface, while *C. macellaria* feeds up within the body (Pimsler et al. 2019). There could be many reasons driving the separation; temperatures within the body and at the soil interface could be ideal for different species, or *C. macellaria* could be avoiding *C. rufifacies* because of predation. Research has shown that *C. macellaria* has the greatest rate of survival when colonizing more than two days before or after *C. rufifacies* (Brundage et al. 2014). This is thought to be advantageous to *C. macellaria* in that they are able to exploit a food source and complete development free of competition or predation. *Cochliomyia macellaria* had a sharp decrease in survivorship when colonizing within two days of C. *rufifacies* (Brundage et al. 2014). This left feeding *C. macellaria* larvae potentially vulnerable to predatory second and third instar *C. rufifacies*. *Chrysomya rufifacies* on the other hand displayed its lowest rates of survival when arriving four days ahead of *C. macellaria* (Brundage et al. 2014). This is thought to be due to missed opportunities to predate on *C. macellaria* or inability of first instars to join mixed species larval masses, which can enable improved feeding on a food source (Rivers et al. 2011). The thermal tolerance data collected in Chapters II and III could shed light on these unknown factors contributing to preferred colonization time, and segregation of *C. macellaria* from the predatory stages of *C. rufifacies*. *Cochliomyia macellaria* could be utilizing thermal zones (cooler or warmer) within a body that are unsuitable for the more specialized *C. rufifacies*. *Cochliomyia macellaria* could also be capitalizing on earlier colonization time in order develop to more thermally

tolerant stages and 'heat out' any potential competitors or predators that may not be able to withstand the high temperatures of a feeding third instar mass at younger stages.

To further complete these data sets, future work is needed to determine CT_{max} of *C. rufifacies* and *C. macellaria* with access to food and tracking individual knockdown temperatures to pupariation, eclosion and sexual maturity if possible. Allowing for a gradual cool down from heat treatments could also produce more accurate data, knowing that there are negative effects of placing a heat shocked individual directly into a cooler environment (Chen et al. 1987). It should be noted again that collection of wild individuals and experimentation took place within College Station, Texas. Texas is unique in that it contains ten different ecoregions within nearly 270,000 square miles of land (Texas Parks and Wildlife, n.d.). These regions all have different temperature variances and can be expected to reach different maximum and minimum temperatures throughout the year. One goal of this research was to demonstrate variation between species with different thermal life histories, so it is important that this data be applied cautiously to closely related species, or even the studied species collected from other states or ecoregions.

In conclusion this thesis was designed to address the following questions: At what upper thermal limit do larvae of various stadia knockdown (CT_{max}), potentially disrupting related ecological processes and forensic timelines? What are the rates of survival after knockdown? Do the thermal tolerances of larvae change between stadia? What is the probability of knockdown and survival after exposure to other stressful temperatures for different durations of time? Does access to food change the response?

Do the responses to extreme temperatures differ between species? This thesis answers all of these questions. All of these factors considered appear to be important in their effect on larval knockdown and survival, as well as the interactions among the factors studied. The details of these interactions can be found above and in the previous chapters. The take home message from this research is that temperatures in the 40s and 50s°C (and even 30s°C for first instars) are stressful for the larvae of *C. rufifacies* and *C. macellaria*, at biologically relevant exposure times and physiological states. These temperatures can also be reached in ecologically pertinent conditions. Therefore, thermal stress may be an important factor in blow fly related ecological processes and derived applications, such as forensic entomology.

REFERENCES

Amendt, J., Campobasso, C. P., Gaudry, E., Reiter, C., Leblanc, H. N., & Hall, M. J. R. (2007). Best practice in forensic entomology – standards and guidelines. *International Journal of Legal Medicine, 121*(2), 90-104.

Ames C., & Turner B. (2003). Low temperature episodes in development of blowflies: implications for postmortem interval estimation. *Medical and Veterinary Entomology, 17*(2), 178-186.

Anderson, D. L., Sedgley, M., Short, J. R. T., & Allwood, A. J. (1982). Insect pollination of mango in northern Australia *Mangifera indica*, includes *Apis mellifera*. *Australian Journal of Agricultural Research, 33*(3), 541–548.

Anderson, G. S., & Vanlaerhoven, S. L. (1996). Initial studies on insect succession on carrion in southwestern British Columbia. *Journal of Forensic Sciences, 41*(4), 617-625.

Angilletta, M. J. (2009). *Thermal adaptation a theoretical and empirical synthesis.* New York, New York: Oxford University Press.

Asgari, S., Hardy, J. R., Sinclair, R. G., & Cooke, B. D. (1998). Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia. *Virus research, 54*(2) 123-132.

Bakken, G. S. (1976). A heat transfer analysis of animals: Unifying concepts and the application of metabolism chamber data to field ecology. *Journal of Theoretical Biology, 60*(2), 337-384.

Bala, M., & Singh, D. (2015). Development of two forensically important blowfly species (*Chrysomya megacephala* and *Chrysomya rufifacies*) (Diptera: Calliphoridae) at four temperatures in India. *Entomological Research, 45*(4), 176-183.

Barros-Cordeiro, K. B., Pujol-Luz, J. R., Name, K. P. O., & Báo, S. N. (2016). Intra-puparial development of the *Cochliomyia macellaria* and *Lucilia cuprina* (Diptera, Calliphoridae). *Revista Brasileira de Entomologia*, *60*(4), 334-340.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R.H.B., Singmann, H., Dai, B., Grothendieck, G., Eigen, C. & Rcpp, L. (2015). Package 'lme4'. *Convergence*, *12*(1).

Baumgartner, D. L. (1993). Review of *Chrysomya rufifacies* (Diptera: Calliphoridae). *Journal of Medical Entomology, 30*(2), 338-352.

Baumgartner, D. L., & Greenberg, B. (1984). The Genus *Chrysomya* (Diptera: Calliphoridae) in the New World. *Journal of Medical Entomology, 21*(1), 105-113.

Becker, C. D., & Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes, 4*(3), 245-256.

Beers, J. M., & Sidell, B. D. (2011). Thermal Tolerance of Antarctic Notothenioid Fishes Correlates with Level of Circulating Hemoglobin. *Physiological and Biochemical Zoology, 84*(4), 353-362.

Berrigan, D., & Hoffmann, A. A. (1998). Correlations between measures of heat resistance and acclimation in two species of *Drosophila* and their hybrids. *Biological Journal of the Linnean Society, 64*(4), 449-462.

Boatright, S. A., & Tomberlin, J. K. (2010). Effects of temperature and tissue type on the development of *Cochliomyia macellaria* (Diptera: Calliphoridae). *Journal of Medical Entomology, 47*(5), 917-923.

Bogert, C. M. (1949). Thermoregulation in reptiles, a factor in evolution. *Evolution, 3*(3), 195-211.

Brett, J. R. (1971). Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *American Zoologist, 11*(1), 99-113.

Brodie, B. S., Smith, M. A., Lawrence, J., & Gries, G. (2015). Effects of floral scent, color and pollen on foraging decisions and oocyte development of common green bottle flies. *Plos One, 10*(12), e0145055.

Brundage, A., Benbow, M. E., & Tomberlin, J. K. (2014). Priority effects on the life-history traits of two carrion blow fly (Diptera, Calliphoridae) species. *Ecological Entomology, 39*(5), 539-547.

Byrd, J. H., & Butler, J. F. (1996). Effects of temperature on *Cochliomyia macellaria* (Diptera: Calliphoridae) development. *Journal of Medical Entomology, 33*(6), 901-905.

Byrd, J. H., Castner, J. L. (2010). *Forensic entomology: the utility of arthropods in legal investigations*. Boca Raton, Florida: CRC Press.

Carter, D. O., Yellowlees, D., & Tibbett, M. (2007). Cadaver decomposition in terrestrial ecosystems. *Naturwissenschaften, 94*(1). 12-24.

Charabidze, D., Bourel, B. & Gosset, D. (2011). Larval-mass effect: Characterisation of heat emission by necrophageous blowflies (Diptera: Calliphoridae) larval aggregates. *Forensic Science International, 211*(1-3), 61-66.

Chen, C., Denlinger, D. L., & Lee, R. E., Jr. (1987). Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiological Zoology, 60*(3), 297-304.

Chen, L., Onagbola, E. O., & Fadamiro, H. Y. (2005). Effects of temperature, sugar availability, gender, mating, and size on the longevity of phorid fly *Pseudacteon tricuspis* (Diptera: Phoridae). *Environmental Entomology, 34*(2), 246-255.

Cianci, T. J., & Sheldon, J. K. (1990). Endothermic generation by blow fly larvae *Phormia regina* developing in pig carcasses. *Bulletin of the Society for Vector Ecology*, *15*(1), 33-40.

Climate College Station – Texas. (n.d.). Retrieved May 23, 2019, from https://www.usclimatedata.com/climate/college-station/texas/united-states/ustx2165.

College Station Summary: August 2017. (n.d.). Retrieved May 2, 2019, from http://climatexas.tamu.edu/products/college-station-summaries/august-2017.html.

College Station Summary: July 2017. (n.d.). Retrieved May 2, 2019, from http://climatexas.tamu.edu/products/college-station-summaries/july-2017.html.

Cowles, R. B., & Bogert, C. M. (1944). A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the American Museum of Natural History*, *83*(5), 265-296.

DeWitt, T. J., & Scheiner, S. M. (2004). *Phenotypic plasticity functional and conceptual approaches.* New York, New York: Oxford University Press.

Donat, M. G., Alexander, L. V., Yang, H., Durre, I., Vose, R., Dunn, R. J. H., Willett, K. M., Aguilar, E., Brunet, M., Caesar, J., Hewitson, B., Jack, C., Klein Tank, A. M. G., Kruger, A. C., Marengo, J., Peterson, T. C., Renom, M., Oria Rojas, C., Rusticucci, M., Salinger, J., Elrayah, A. S., Sekele, S. S., Srivastava, A. K., B. Trewin, B., Villarroel, C., Vincent, L. A., Zhai, P., Zhang, X., & Kitching, S. (2013). Updated analyses of temperature and precipitation extreme indices since the beginning of the twentieth century: The HadEX2 dataset. *Journal of Geophysical Research: Atmospheres*, *118*(5), 2098-2118.

Donovan, S. E., Hall, M. J. R., Turner, B. D., & Moncrieff, C. B. (2006). Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures. *Medical and Veterinary Entomology*, *20*(1), 106-114.

Erzinçlioğlu, Y. Z. (1988). The larvae of the species of *Phormia* and *Boreellus*: Northern, cold-adapted blowflies (Diptera: Calliphoridae). *Journal of Natural History*, *22*(1), 11-16.

Falk, B., & Dotan, R. (2008). Children's thermoregulation during exercise in the heat – a revisit. *Applied Physiology, Nutrition, and Metabolism, 33*(2), 420-427.

Fey, S. B., Siepielski, A. M., Nusslé, S., Cervantes-Yoshida, K., Hwan, J. L., Huber, E. R., Fey, M. J., Catenazzi, A., & Carlson, S. M. (2015). Recent shifts in the occurrence, cause, and magnitude of animal mass mortality events. *Proceedings of the National Academy of Sciences, 112*(4), 1083-1088.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., Firth, D., Friendly, M., Gorjanc, G., Graves, S. & Heiberger, R. (2012). Package 'car'. *Vienna: R Foundation for Statistical Computing*.

Fry, F. E., & Hart, J. S. (1948). Cruising speed of goldfish in relation to water temperature. *Journal of the Fisheries Research Board of Canada, 7b*(4), 169-175.

Gabre, R. M., Adham, F. K., & Chi, H. (2005). Life table of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae). *Acta Oecologica, 27*(3), 179-183.

Gennard, D. (2012). *Forensic entomology: an introduction*. Chichester, England, UK: John Wiley & Sons.

Gessner, M. O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H., & Hättenschwiler, S. (2010). Diversity meets decomposition. *Trends in Ecology & Evolution, 25*(6), 372-380.

Google Earth Pro V7.3.2.5776 (September 7, 2017). College Station, Texas. 30.573103, -96.360912, Eye alt 16.5 mi. Landsat/Copernicus 2018. Retrieved May 10, 2019.

Grassberger, M., & Reiter, C. (2001). Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. *Forensic Science International, 120*(1-2), 32-36.

Greenberg, B. (1991) Flies as forensic indicators. *Journal of Medical Entomology, 28*(5), 565-577.

Hall, M., & Wall, R. (1995). Myiasis of humans and domestic animals. *Advances in Parasitology Volume 35, 257–334.*

Hanks, J., & Ritchie, J. T. (1991). *Modeling plant and soil systems.* Madison, Wisconsin: American Society of Agronomy.

Hartig, F. (2017). DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models. *R package version 0.1, 5*.

Hättenschwiler, S., Tiunov, A. V., & Scheu, S. (2005). Biodiversity and litter decomposition in terrestrial ecosystems. *Annual Review of Ecology, Evolution, and Systematics, 36*(1), 191-218.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology, 57*(1), 19-42.

Hazel, J. R., & Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research*, *29*(3), 167-227.

Hazell, S. P., & Bale, J. S. (2011). Low temperature thresholds: Are chill coma and CT_{min} synonymous? *Journal of Insect Physiology, 57*(8), 1085-1089.

Hochachka, P. W., & Somero, G. N. (2002). *Biochemical adaptation: Mechanism and process in physiological evolution.* New York, New York: Oxford University Press.

Horton, B. J., Corkrey, R., & Doughty, A. K. (2018). Sheep death and loss of production associated with flystrike in mature Merino and crossbred ewes. *Animal Production Science, 58*(7), 1289-1296.

Huey, R. B., & Stevenson, R. (1979). Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *American Zoologist, 19*(1), 357-366.

Hutchison, V. H. (1961). Critical thermal maxima in salamanders. *Physiological Zoology, 34*(2), 92-125.

Jirón, L. F. (1989). On the calliphorid flies of Costa Rica (Diptera: Cyclorrhapha). *Brenesia, 16*, 221-223.

Joern, A., & Chapman, R. F. (1990). *Biology of grasshoppers.* New York, New York: John Wiley & Sons.

Johnson, A. P., Wighton, S. J., & Wallman, J. F. (2014). Tracking movement and temperature selection of larvae of two forensically important blow fly species within a "maggot mass". *Journal of Forensic Sciences, 59*(6), 1586-1591.

Jürgens, A., & Shuttleworth, A. (2015). Carrion and dung mimicry in plants. In: *Carrion ecology, evolution, and their applications* (ed. by Benbow, M. E., Tomberlin, J. K., & Tarone, A. M.), pp. 361-386. Boca Raton, Florida: CRC Press.

Kumar, S., Joshi, P. C., Nath, P., Singh, V. K., & Mansotra, D. K. (2016). Role of insects in pollination of mango trees. *International Research Journal of Biological Sciences, 5*(1), 64-67.

Lactin, D. J., Holliday, N. J., Johnson, D. L., & Craigen, R. (1995). Improved rate model of temperature-dependent development by arthropods. *Environmental Entomology, 24*(1), 68-75.

Lenth, R. (2019). Emmeans: Estimated marginal means, aka least-squares means. *R package version, 1.4*

Licht, P. (1967). Thermal adaptation in the enzymes of lizards in relation to preferred body temperatures. *Molecular mechanisms of temperature adaptation.* (ed. by Prosser, C. L.) pp. 131-45. Pub. 84, *American Association for the Advancement of Science.*

Lighton, J. R., & Turner, R. J. (2004). Thermolimit respirometry: An objective assessment of critical thermal maxima in two sympatric desert harvester ants, *Pogonomyrmex rugosus* and *P. californicus*. *Journal of Experimental Biology, 207*(11), 1903-1913.

Liu, D., & Greenberg, B. (1989). Immature stages of some flies of forensic importance. *Annals of the Entomological Society of America*, *82*(1), 80-93.

Lord, W. D., & Stevenson, J. R. (1986). Directory of forensic entomologists, 2nd ed., Pest Management Information Analysis Center, Walter Reed Army Medical Center, Washington, DC.

Lutterschmidt, W. I., & Hutchison, V. H. (1997). The critical thermal maximum: History and critique. *Canadian Journal of Zoology, 75*(10), 1561-1574.

Makovetskaya, K., & Verves, Y. G. (2018). First records of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) in Belarus with analysis of distribution of this species in Europe. *Dipteron: Bulletin of the Dipterological Section of the Polish Entomological Society, 34*, 60-67.

Malaysia. (n.d.) Retrieved May 23, 2019, from https://climateknowledgeportal.worldbank.org/country/malaysia.

Martin, T., & Huey, R. (2008). Why "suboptimal" is optimal: Jensen's inequality and ectotherm thermal preferences. *The American Naturalist, 171*(3), 102-118.

Moore, J. A. (1939). Temperature tolerance and rates of development in the eggs of Amphibia. *Ecology, 20*(4) 459-478.

Morris, M. C. (2000). Ethical issues associated with sheep fly strike research, prevention, and control. *Journal of Agricultural and Environmental Ethics, 13*(3), 205-217.

Osilla, E. V., & Sharma, S. (2019). Physiology, Temperature Regulation. In *StatPearls [Internet]*. StatPearls Publishing. Retrieved September 20, 2019, from https://www.ncbi.nlm.nih.gov/books/NBK507838/

Owings, C. G., Spiegelman, C., Tarone, A. M., & Tomberlin, J. K. (2014). Developmental variation among *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) populations from three ecoregions of Texas, USA. *International Journal of Legal Medicine, 128*(4), 709–717.

Pace, R. C., Talley, J. L., Crippen, T. L., & Wayadande, A. C. (2017). Filth fly transmission of *Escherichia coli* O157: H7 and *Salmonella enterica* to Lettuce, *Lactuca sativa*. *Annals of the Entomological Society of America, 110*(1), 83-89.

Parmenter, R. R., & MacMahon, J. A. (2009). Carrion decomposition and nutrient cycling in a semiarid shrub-steppe ecosystem. *Ecological Monographs, 79*(4), 637-661.

Payne, J. A. (1965). A summer carrion study of the baby pig *Sus scrofa* Linnaeus. *Ecology, 46*(5), 592–602.

Pellegrino, C. R. (1984). The role of desiccation pressures and surface area/volume relationships on seasonal zonation and size distribution of four intertidal decapod Crustacea from New Zealand: Implications for adaptation to land. *Crustaceana, 47*(3), 251-268.

Percival. (2019). Retrieved on May 2, 2019, from https://www.percival-scientific.com/product/i-36llvl/

Pimsler, M. L., Sze, S. H., Saenz, S., Fu, S., Tomberlin, J. K., & Tarone, A. M. (2019). Gene expression correlates of facultative predation in the blow fly *Chrysomya rufifacies* (Diptera: Calliphoridae). *Ecology and Evolution, 9*(15), 8690-8701.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & Team, R. C. (2012). nlme: linear and nonlinear mixed effects models. *R Package Version, 3*, 103.

R Core Team (2015). *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/.

Riback, T. I. S., & Godoy, W. A. C. (2008). Fecundity, body size and population dynamics of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae). *Brazilian Journal of biology, 68*(1), 123-128.

Rivers, D. B., & Dahlem, G. A. (2014). *The science of forensic entomology*. Hoboken, New Jersey: John Wiley & Sons.

Rivers, D. B., Thompson, C., & Brogan, R. (2011). Physiological trade-offs of forming maggot masses by necrophagous flies on vertebrate carrion. *Bulletin of Entomological Research, 101*(05), 599–611.

Rosati, J. Y., & VanLaerhoven, S. L. (2007). New record of *Chrysomya rufifacies* (Diptera: Calliphoridae) in Canada: Predicted range expansion and potential effects on native species. *The Canadian Entomologist, 139*(5), 670-677.

Rusch, T. W., Adutwumwaah, A., Beebe, L. E. J., Tomberlin, J. K., & Tarone, A. M. (2019). The upper thermal tolerance of the secondary screwworm, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae). *Journal of Thermal Biology, 85*, 102405.

Rusch, T. W., & Angilletta, M. J. (2017). Competition during thermoregulation altered the body temperatures and hormone levels of lizards. *Functional Ecology, 31*(8), 1519-1528.

Schoenly, K., & Reid, W. (1983). Community structure of carrion arthropods in the Chihuahuan Desert. *Journal of Arid Environments, 6*(3), 253-263.

Seebacher, F., & Franklin, C. E. (2005). Physiological mechanisms of thermoregulation in reptiles: a review. *Journal of Comparative Physiology B, 175*(8), 533-541.

Simmons, T., Adlam, R. E., & Moffatt, C. (2010). Debugging decomposition data - comparative taphonomic studies and the influence of insects and carcass size on decomposition rate. *Journal of Forensic Sciences, 55*(1), 8–13.

Slone, D. H., & Gruner, S. V. (2007). Thermoregulation in larval aggregations of carrion-feeding blow flies (Diptera: Calliphoridae). *Journal of Medical Entomology, 44*(3), 516-523

Smith, J., & Curnow, M. (2017). Managing Flystrike in Sheep. Retrieved May 8, 2019, from https://www.agric.wa.gov.au/livestock-parasites/managing-flystrike-sheep.

Smith, K. G. V. (1986). *A manual of forensic entomology*. London, England, UK: The Trustees of the British Museum (Natural History).

Sugiura, N. (1996). Pollination of the orchid *Epipactis thunbergii* by syrphid flies (Diptera: Syrphidae). *Ecological Research, 11*(3), 249–255.

Sulaiman, S., Sohadi, A. R., & Jeffery, J. (1989). Human helminth parasite burdens on cyclorrhaphan flies (Diptera) trapped at an aboriginal settlement in Malaysia. *Bulletin of Entomological Research, 79*(4), 625-629.

Sulaiman, S., Sohadi, A. R., Yunus, H., & Iberahim, R. (1988). The role of some cyclorrhaphan flies as carriers of human helminths in Malaysia. *Medical and Veterinary Entomology, 2*(1), 1-6.

Sung, I. H., Lin, M. Y., Chang, C. H., Cheng, A. S., Chen, W. S., & Ho, K. K. (2006). Pollinators and their behaviors on mango flowers in southern Taiwan. *Formosan Entomologist, 26*, 161-170.

Szpila, K., Matuszewski, S., Bajerlein, D., & Konwerski, S. (2008). *Chrysomya albiceps* (Wiedemenn,), a forensically important blowfy (Diptera: Calliphoridae) new for the Polish fauna. *Polish Journal of Entomology, 77*(4), 351-355.

Tarone, A. M., & Sanford, M. R. (2017). Is PMI the hypothesis or the null hypothesis? *Journal of Medical Entomology, 54*(5), 1109-1115.

Terblanche, J. S., Hoffmann, A. A., Mitchell, K. A., Rako, L., Roux, P. C., & Chown, S. L. (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *Journal of Experimental Biology, 214*(22), 3713-3725.

Texas Climate Report: July 2017. (n.d.). Retrieved May 2, 2019, from http://climatexas.tamu.edu/products/texas-climate-bulletins/july-2017.html.

Texas Extremes. (n.d.). Retrieved May 2, 2019, from http://climatexas.tamu.edu/products/texas-extremes/index.html.

Texas Parks and Wildlife. (n.d.) Retrieved December 2, 2017, from https://tpwd.texas.gov/education/hunter-education/online-course/wildlife-conservation/texas-ecoregions.

Tomberlin, J. K., Barton, B. T., Lashley, M. A., & Jordan, H. R. (2017). Mass mortality events and the role of necrophagous invertebrates. *Current Opinion in Insect Science, 23*, 7-12.

Tomberlin, J. K., Crippen, T. L., Tarone, A. M., Chaudhury, M. F., Singh, B., Cammack, J. A., & Meisel, R. P. (2017). A review of bacterial interactions with blow flies (Diptera: Calliphoridae) of medical, veterinary, and forensic importance. *Annals of the Entomological Society of America*, *0*(0), 1-18.

Tomberlin, J. K., Mohr, R., Benbow, M. E., Tarone, A. M., & Vanlaerhoven, S., (2011). A roadmap for bridging basic and applied research in forensic entomology. *Annual Review of Entomology, 56*(1), 401-421.

Triplehorn, C. A., & Johnson, N. F. (2005). *Borror and DeLong's introduction to the study of insects, 7th edition*. Belmont, California: Brooks/Cole

Van Dine, D. L. (1908). Insects affecting livestock in Hawaii. In: *Hawaii Livestock Breeders Association Proceedings 5th Annual Meeting, 1987, Honolulu*. pp. 10-70.

Villet, M. H., Richards, C. S., Midgley, J. M. (2009). Contemporary precision, bias and accuracy of minimum post-mortem intervals estimated using development of carrion-feeding insects. In: *Current concepts in forensic entomology* (ed. by Amendt, J., Goff, M., Campobasso, C., Grassberger, M.) pg. 118, Fig. 7.4. Springer Science+Business Media BV.

Waterhouse D, F, (1947). The relative importance of live sheep and of carrion as breeding grounds for the Australian sheep blowfly *Lucilia cuprina. Council for Scientific and Industrial Research,* Bulletin 217.

Went, F. W., & Stark, N. (1968). Mycorrhiza. *Bioscience, 18,* 1035-1039.

Whitworth, T. (2006). Keys to the genera and species of blow flies (Diptera Calliphoridae) of America north of Mexico. *Proceedings of the Entomological Society of Washington, 108*(3), 689-725.

Zinn, K. E., Tunc-Ozdemir, M., & Harper, J. F. (2010). Temperature stress and plant sexual reproduction: Uncovering the weakest links. *Journal of Experimental Botany, 61*(7), 1959-1968.

Zurek, L., Schal, C., & Watson, D. W. (2000). Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. *Journal of Medical Entomology, 37*(6), 924–928.

Supplemental Sources Consulted

Caughley, G. (1994). Directions in conservation biology. *The Journal of Animal Ecology, 63*(2), 215-244.

Li, Z., & Srivastava, P. (2003). Heat-Shock Proteins. *Current Protocols in Immunology*. A. IT.1-A.IT6

Lindquist, S. (1986). The Heat-Shock Response. *Annual Review of Biochemistry*, *55*(1), 1151–1191.

Solaimanian, M., & Kennedy, T. W. (1993). Predicting maximum pavement surface temperature using maximum air temperature and hourly solar radiation. In: *Transportation Research Record Publication*, Pub. 1417, pp. 9-19. Washington, D. C.: National Academy Press.

Vereecken, N., & McNeil, J. N. (2010). Cheaters and liars: Chemical mimicry at its finest. *Canadian Journal of Zoology*, *88*(7), 725-752.