

THERMOREGULATORY ADAPTATIONS OF THE WETSALTS
TIGER BEETLE, *CICINDELIDIA HAEMORRHAGICA*,
IN YELLOWSTONE NATIONAL PARK

by

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in

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DEDICATION

I dedicate this thesis to my mother, Diane Bowley, and father, Lynn Bowley, for their continued support, inspiration, and their advocacy for my pursuit of my desired study.

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ABSTRACT

The wetsalts tiger beetle, *Cicindelidia haemorrhagica* (LeConte) is found throughout Yellowstone National Park (YNP) associated with active thermal hot spring areas with surface temperatures that exceed 50 °C. Previous studies suggest that YNP *C. haemorrhagica* behaviorally thermoregulate differently than populations from Idaho not associated with hot springs. However, the cause of this behavioral difference is unknown. Therefore, our study had five objectives: 1) compare internal temperatures of YNP and Idaho adult *C. haemorrhagica* in similar environmental conditions; 2) determine if the cuticular wax influences YNP beetle internal body temperature; 3) measure and analyze venter reflectivity of YNP and Idaho beetles; 4) compare YNP and Idaho beetle abdominal structures using scanning electron microscopy; and 5) analyze thermal video recordings of YNP adults to estimate times spent on different surface temperatures. Internal temperatures of freshly killed adults were recorded using thermocouples inserted into the abdomen and were suspended over a heated water bath. Our results found a mean difference of 1.03 °C between YNP and Idaho beetle internal temperatures, suggesting that the exoskeleton reflects more heat in YNP adults. Our results suggest that the wax does not play a significant role in temperature reduction. The dissected exoskeletal ventral abdominal face experienced a mean difference of 0.58 °C compared to an imitation venter, suggesting the venter was primarily responsible for heat reflection. Spectrophotometric differences revealed greater reflective properties of YNP beetle venters. Scanning electron microscopic images indicated no visible structural differences between YNP and Idaho beetles. Thermal video analysis indicated YNP adults spent an increasing amount of time on surface temperatures greater than 20 °C, but decreasing amounts of time on surfaces temperatures greater than 40 °C. Beetles from only one YNP location were on surfaces exceeding 50 °C. Our results suggest that physical properties of the abdominal venter of YNP beetles are likely responsible for improved heat reflection from bottom-up heating of hot spring areas. Improved heat reflection resulted in lower internal temperatures of YNP beetles relative to Idaho.

CHAPTER ONE

LITERATURE REVIEW

Introduction

The wetsalts tiger beetle, *Cicindelidia haemorrhagica* (LeConte 1851), was first recorded actively hunting and scavenging on thermal soil directly adjacent to active hot springs locations in Mammoth Hot Springs, Yellowstone National Park (YNP) during an ecological survey 129 years ago (Bertholf 1979; Hubbard 1891). The environment that these beetles inhabited was projected to exceed many thermal limits for other known species of insects (Hubbard 1891). Since the original ecological survey, researchers have conducted many studies on the thermal features of YNP where this beetle resides, but there has not been a study of this insect in YNP until recently (Willemsens 2019).

The first known records of *C. haemorrhagica* are from San Diego, California (LeConte 1851), and since then its presence has been recorded in states along the west coast of the United States in addition to Utah, Idaho, Nevada, and Wyoming (Pearson et al. 2015). Tiger beetles typically spend most of their time in open landscapes and some species can inhabit—but only briefly—areas with surface temperatures exceeding 60 °C before needing to behaviorally thermoregulate, which is an atypical temperature experienced in most tolerable insect conditions (Hadley 1994). Tiger beetles when exposed to these high temperatures typically exhibit thermoregulatory behavior to actively lower their internal body temperature. This behavior can consist of moving to shade, dipping their abdomens in water to evaporatively cool, or lifting the front ends of their bodies to create an angle more perpendicular to the sun so they can reflect

more sunlight, known as stiling (Brosius & Higley 2013). Some tiger beetle species spend as much as 56% of their diurnal time actively thermoregulating and others limit their activity levels during midday to reduce the amount of exposure to direct sunlight (Pearson 1988).

However, *C. haemorrhagica* populations within YNP either do not exhibit these thermoregulatory behaviors or do so at greatly reduced frequencies when compared to a population in Idaho in a non-hot spring environment (Willemsens 2019). Adult *C. haemorrhagica* from our study location in Idaho had considerably higher proportions of diel movement and feeding activity when compared to the YNP beetle populations (Willemsens 2019). YNP beetles are likely to employ a maxi-thermal feeding strategy, which requires them to be in an environment close to their upper thermal threshold to more easily find prey items (Dreisig 1980; Morgan 1984). Previous studies of warm-adapted tiger beetles suggest increases in air temperature are directly related to the presence of beetles, indicating that some level of heat in the air is required to promote beetle activity (Knisley et al. 1990). This may indicate that *C. haemorrhagica* within YNP experience internal temperatures that keep them relatively warm, but not high enough that it would require them to frequently move between shaded areas to cool or need to compensate for increased metabolism influenced by their environment.

The Idaho population exhibited thermoregulatory behavior that was directly related to the increase in ambient air and soil temperature that occurred throughout the day. The YNP beetles behaviorally thermoregulated to warm themselves at colder temperatures but did not typically exhibit thermoregulatory behavior to cool themselves as air temperatures increased during the day (Willemsens 2019). Not only do they cope with direct sunlight, but in this ecosystem, they are continuously exposed to radiant heat from the thermally active soil and shallow water on

which they spend almost all of their time. This is contrary to what *C. haemorrhagica* in the Idaho population experience. Most of the heat they experience is from direct sunlight while the salt flat and mud they inhabit does not contribute additional heating through radiation or conduction. Often the soil where beetles are hunting, foraging, or mating is much hotter than the ambient air temperature to which they are exposed. Because *C. haemorrhagica* from within YNP do not show thermoregulatory behavior consistently during these extended periods of increased temperature from sunlight and thermally active soil, there must be some component to *C. haemorrhagica* physiology, genetic makeup, or chemical sequestration that contributes to their thermal tolerance or resistance.

Previous results of lethal thermal maxima experiments of *C. haemorrhagica* adults collected from Willcox, Arizona, a population not associated with hot springs, suggested a thermal maximum of 48 °C (Pearson & Lederhouse 1987). Similar experiments conducted with YNP and Idaho beetles suggested similar results; YNP and Idaho beetles had a lethal thermal maximum of 50.41 °C and 49.33 °C respectively (Willemssens 2019). Because of the similarity in lethal thermal maxima, experiments conducted by our lab in the summers of 2018 and 2019 in which we recorded internal body temperatures of freshly euthanized pairs of YNP and Idaho beetles suggested that there was a difference of internal temperature between the populations when individuals were ventrally heated using a water bath.

Therefore, the primary focus of my thesis research is to determine if there is a physical or physiological component to their exoskeletons that allows them to tolerate or resist the temperatures to which they are exposed without the need to exhibit typical thermoregulatory behavior.

Ecology of YNP Hot Springs with a Focus on Insects

Cicindelidia haemorrhagica forages and actively hunts for prey in many hot spring areas provided there is acceptable habitat for larval burrows (Willemssens 2019). Dragon Spring (one of my research sites which will be discussed in detail in the proposed methods below) is primarily an acidic, thermally active sulfide hot spring with multiple water sources within the lower Norris Geyser Basin. These sources flow continuously around a small peninsula of substrate and eventually combine with water flow from Reservoir Lake to flow freely through a mostly vegetation-barren landscape. At many of these hot springs' sources, it is as hot as 72 °C, and rapidly cools as water reaches 200+ cm away from the source. Microbial growth begins at temperatures of 60 °C, approximately 225 cm from the source (D'Imperio et al. 2007).

Consisting primarily of unicellular and filamentous algae, these mats are cool enough to accommodate the development of stratiomyid and ephydrid larvae. Of the 22 species of Ephydridae that are known thermophiles, only *Paracoenia bisetosa*, *P. turbida*, *P. bispinosa*, and *Ephydra bruesii*, are found within YNP (Collins et al. 1976; Kuenzel & Wiegert 1973; Foote 1995; Wickstrom & Wiegert 1980). These herbivorous thermophiles can survive in hot water areas as hot as 40 °C and can gain access to plentiful food sources by laying eggs in and feeding as an adult on the cyanobacterial mats that can be found in thermal waters (Wickstrom & Wiegert 1980). Stratiomyid larvae can also survive in this environment by surviving off the cyanobacterial mat materials in cooler portions of the water system (Boyd et al. 2009).

Cicindelidia haemorrhagica in this type of ecosystem constantly move along the water's edge in search of prey during the day. They continuously dip their mandibles in the saturated soil to locate ephydrid larvae, other recently deceased insects, and insect parts to consume. Based on

recent research conducted throughout segments of the lower Norris Geyser Basin, ephydrid larvae are the primary food source for adult *C. haemorrhagica* (Willemsens 2019). Food availability is limited due to the extreme landscape, making these cyanobacterial mats keystone resources for much of the arthropod life residing near or in Dragon Spring and other surrounding hot springs for the Norris Lower Geyser Basin. The largest population of *C. haemorrhagica* found so far in the Norris Lower Geyser Basin is located at Dragon and Grendel Springs, two adjacent hot springs that have intermingling waters that produce a peninsula of ideal habitat on what would be unsafe for large fauna foot traffic (Willemsens 2019). Beetles are found hunting on and near these microbial mats for food sources that are especially essential for female beetles so they can produce a greater number of eggs (Pearson & Knisley 1985). This egg production is crucial because, to my knowledge, the population of beetles in the Dragon Spring area is isolated from neighboring populations. Additional food sources in this ecosystem include Saldidae and various foraging ant species that may pass nearby (Resh & Barnby 1984).

In Rabbit Creek, an alkaline water system produced from a large source spring in the Midway geyser basin where the temperature changes are less intense, blue-green and orange mats consisting of cyanobacteria, chloroflexi, acidobacteria, and cytophaga-flavobacterium-bacteroides can grow continuously throughout the year without winter interruption (Weltzer & Miller 2013). These primarily cyanobacterial mats can accommodate large populations of ephydrids where the water pools throughout alkaline water systems in the Upper, Lower, and Midway Geyser Basins (Miller et al. 2009; Weltzer & Miller 2013). The blue-green and orange cyanobacterial mats within these alkaline springs provide a sustainable food source as well as shelter, much like what occurs at the Norris Geyser Basin with cyanobacterial mats (Wickstrom

& Weigert 1980). Typically, these flies are found in areas within alkaline waters of approximately 40 °C that possess surface—developing cyanobacterial mats facilitated by siliceous substrate and the water temperature (Petersen & Wiegert 1982; Kuenzel & Wiegert n.d.). Food availability in this area is not as important as it is in the more extreme environment of the Norris Geyser Basin. If conditions allow for presence of populations, *C. haemorrhagica* can be seen regularly grazing the numerous cyanobacterial mats and locating ephydrid larvae for consumption. There are competing predators in the area such as dragonflies, spiders, and wasps that also take advantage of the readily available food source in the Midway Geyser Basin unlike the almost nonexistent competition present in the Norris Geyser Basin.

Integument

The integument not only serves to protect the insect from the outside world, but also performs many other critical functions. The integument of an insect is not only colored, which can serve as a reflective and visually appealing surface, but it also can reflect, transmit, and absorb different types of light. Because most insects do not experience direct heat from thermally active surfaces, many studies have been conducted on dorsal surfaces and elytra of beetles to characterize the integument most exposed to heat and other radiation from sunlight.

An important component of the exoskeleton is the cuticular wax renewably produced by insects. This wax layer serves important roles in water loss and, in most cases, serves thermoregulatory purposes. In some instances of desert-dwelling *Tenebrio* spp., dermal glands produce excessive wax in a filamentous type layer that increases their ability to hold water and withstand greater external temperatures by producing a more reflective surface. These reflective surfaces often arise as pastel or matte colors on the beetle and provide enhanced diurnal activity

by allowing them to maintain lower body temperatures throughout the day (Hanrahan et al. 2001; McClain et al. 1985; McClain et al. 1991). Desert tenebrionid beetles also possess integuments protected by an atypical amount of long hydrocarbon chains, allowing for increased water retention that assisted in thermoregulation (Hadley 1978; Hadley 1979). These same beetles even exhibited drastic changes in color because they were exposed to and held in areas with varying levels of relative humidity. Typically, the less humid the environment, the brighter the beetle appeared. This was the result of wax production that produced a surface network of microstructures that refracted incident light, producing a pale-blue color. This was hypothesized to not only assist in water retention but also in thermoregulation of individuals (Hadley 1979). However, matte colors are uncharacteristic of tiger beetles, which are typically held in high regard for their brilliant metallic colors that are located within the integument (Pearson et al. 2015; Seago et al. 2009; Shultz & Rankin 1985; Shultz & Rankin 1985). Therefore, it is unlikely that the *C. haemorrhagica* will be the exception to this condition. Tiger beetles, like many other beetles, exhibit some form of iridescence. Specifically, tiger beetles have a multilayer reflector system in which layers of chitin that have different levels of refractive properties produce an iridescent surface (Seago et al. 2009). This multilayer system was first shown in a cross-sectional analysis conducted on *Cicindelidia formosa*, *C. splendida*, *C. scutellaris rugata*, and *C. repanda* (Shultz and Rankin 1985).

Other insects do not produce excessive wax blooms as a reflective surface, but rather they possess exocuticular microstructures that act as photonic crystals and reflect light on a microscopic scale (Seago et al 2009). These microstructures can serve multiple purposes. In the case of the stick insect, *Peruphasma schultei*, microstructures covering the entire exoskeletal

surface served the purpose of heat capture through producing a matte black color and reflecting water droplets at most impact angles. (Maurer et al. 2017). In other cases, the microstructures on elytra of beetles serve as individual reflective prisms that capture sunlight within hollow cylinders comprised of cuticular tubes. Reflective properties of elytra often limit the amount of near-infrared or infrared light that reaches the viscera and is a property directly related to internal body temperatures (Wang et al. 2021). This property makes beetles rely on a highly reflective surface for exposure to consistent direct sunlight, allowing individuals to function at the hottest times of the day (Xie et al. 2019). The reflective surfaces found on insects can vary in efficiency when quantifying the amount of light reflected. The pyramid-like microstructures that are present on *Neocerambyx gigas* offer total internal reflection when exposed to varying angles of light (Zhang et al. 2020). These notable differences in reflective performance often imply improved fitness of the insect for the more extreme environment, suggesting that the elytra play one of the key roles in thermoregulation. Elytra serve multiple purposes in insect-light relationships. Not only are they responsible for reflectivity, but they can also directly absorb certain wavelengths of light that prevent them from reaching and causing harm to the internal organs. There are, however, some wavelengths of light that transmit, or pass directly through the elytra, thus heating the rest of the body. In some cases, the elytra may be more reflective with visible light or near infrared light (NIR) while infrared light (IR) is absorbed by the elytra and ultraviolet light (UV) is transmitted through the elytra to heat the body (Alves et al. 2017; Amore et al. 2017). Proper testing of elytra involves removal from the body and performing tests with a spectrophotometer that can measure absorbance, reflectance, and transmittance for different samples (Cuesta & Lobo 2019). Because this type of testing can be done on elytra, the same

processes should apply to ventral plates of the abdomen to conduct the same measurements. This would theoretically result in an accurately measured visual representation of the differences between the two populations in terms of light interactive properties, which ultimately could explain the mechanism of thermoregulation in YNP populations.

All elytra have varying levels of transmittance, absorbance, and reflectance depending on the type of light to which they are exposed, and these characteristics can be modified or altered based on the microstructures covering the surface (Alves et al. 2017; Pavlovic et al. 2018). These measurements will almost certainly vary between beetle species, and even within a species there could be variations in levels of wax production, wax composition, or developmental differences between individuals. With many beetles coping with top-down heat from the sun, these attributes are very important in daily thermoregulation, so it is essential that these microstructures serve their purpose. There are differences in absorbance and transmittance related to habitat preference between species, but this was mostly because they were related species and not the same species as the YNP and Idaho *C. haemorrhagica* populations (Cuesta & Lobo 2019). It is likely there are differences in ventral and/or abdominal microstructures between populations that will require examination. Integument analysis of transmittance, reflectance, and absorbance may also need to be performed on both ventral and dorsal sides of beetles to ensure there is not a difference in visible light, NIR, IR, or UV light altering internal body temperatures. Other forms of differences in the integument might be wax composition and the sequestration of different materials that influence body temperature. Differences in these microstructures, if discovered, may lead to expanded research in properly defining said differences and testing synthetic replicas for certainty.

Thermoregulation

The integument is an important component for insects in thermoregulation. Diel thermoregulation for insects is very important because they cannot internally produce heat to regulate their own body temperatures. The integument also directly influences thermoregulation and thermoregulatory behavior on an insect through structure or color formation. In some cases, the microscopic photonic crystals produced by the insect integument assist in producing an iridescent surface that reflects light to maintain a lower body temperature (Seago et al. 2009). The Saharan silver ant, *Cataglyphis bombycina*, currently regarded as the most heat tolerant insect, possess integument structures that directly relate to thermoregulatory properties. These ants possess textured triangular setae that help reduce the effect of direct sunlight while the ant workers forage at near-lethal surface temperatures of approximately 56 °C, (Sherwood 1996; Shi et al. 2015; Willot et al. 2017; Willot et al. 2017). These integumental structures located on the dorsal and lateral sides of the insect in addition to their long legs that help keep the ants' bodies above the surface allow them to traverse hot surfaces by assisting in heat dissipation.

Thermoregulation is not only assisted by physiology, but it also heavily influences observed behavior. As mentioned previously, *C. haemorrhagica* within YNP do not exhibit typical thermoregulatory behavior that is seen in many tiger beetles, including that of the Idaho population (Willemssens 2019). Tiger beetles typically operate near their lethal thermal limits and exhibit thermoregulatory behavior like changing body orientation to reduce incoming heat or shuttling themselves to shaded areas to cool down (Brosius & Higley 2013; Dreisig 1980). This shade-seeking behavior is common in many arthropod species but is especially crucial for tiger beetle survival. However, this behavior is not seen when observing *C. haemorrhagica* in YNP

populations. The environment they inhabit within the Norris Geyser Basin does not have any notable vegetation, so *C. haemorrhagica* adults are completely exposed without shade throughout the entire day. Potential thermoregulatory behaviors could involve burrowing as far as 2.5 cm below the surface of the substrate, but their environment is continuously heated from underneath by active hot springs (Dreisig 1980; Willemsens 2020).

Pearson and Lederhouse (1987) recorded hourly surface temperatures of habitat in their observational work of 13 tiger beetle species including *C. haemorrhagica*. These temperature data were recorded from July to August over 15 days for three years in the non-hot spring location of Sulphur Springs Valley, Arizona. The same concept of recording surface temperatures in areas inhabited by *C. haemorrhagica* can apply to observational data recorded within YNP. Continuous measurements can be recorded throughout the day using both traditional thermocouple data loggers as well as newly introduced technology, such as thermal videography. The concept of “grab and stab” can also be modified to continuously record recently euthanized beetle internal temperatures over extended periods of time within a laboratory environment (Pearson & Lederhouse 1987). *Cicindelidia haemorrhagica* from YNP would be categorized as an extremophile when referencing the temperatures to which individuals are regularly exposed (Willemsens 2019). Studying the exoskeleton of *C. haemorrhagica* to discover how they tolerate their current environment is essential. Further study of their ecosystem including locating readily available food sources may help inform us on the resources available to enable their survival.

Objectives

This study had five objectives: 1) compare internal temperatures of YNP and Idaho adult *C. haemorrhagica* when in similar environmental conditions; 2) determine the cuticular wax's influence on YNP beetle internal body temperature; 3) measure and analyze YNP and Idaho beetle venter reflectivity; 4) compare YNP and Idaho beetle abdominal structures using scanning electron microscopy; and 5) analyze thermal video recordings of YNP adults to estimate times spent on different surface temperatures.

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CHAPTER TWO

INCREASED VENTRAL HEAT RESISTANCE BY *CICINDELIDIA*
HAEMORRHAGICA ASSOCIATED WITH HOT SPRINGS
IN YELLOWSTONE NATIONAL PARK

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Abstract

Cicindelia haemorrhagica (LeConte) is a tiger beetle species found in the western U.S., including the active thermal hot spring areas in Yellowstone National Park (YNP). However, populations of adult *C. haemorrhagica* in YNP behaviorally thermoregulate differently than beetle populations outside of YNP. Therefore, we compared internal body temperatures of adults between two thermally active hot spring locations in YNP and a non-hot spring associated Idaho location to determine causes for behavioral differences. We recorded internal body temperatures of freshly killed adults using thermocouples over a water bath. Our results indicated that YNP beetles had a lower mean internal temperature of 1.03 °C ($p < 0.001$) than Idaho beetles with water surface temperatures of 40 °C to 55 °C, suggesting a more heat reflective exoskeleton on YNP adults. We compared internal temperatures of adults with and without their cuticular wax to determine the amount the wax layer contributes to the reduced temperature. Our results suggest that cuticular wax was not a major contributor of the heat reflective phenomena that we observed. Instead, the wax less exoskeleton is primarily responsible for the internal temperature differences. We compared dissected ventral abdominal plates of YNP individuals against imitation plates and bare thermocouples. Our results suggest the venter is primarily responsible for the internal temperature reductions. Next, we measured reflective properties of the venter

with a high-performance spectrophotometer and found there were differences between YNP and Idaho beetle abdomen reflectivity. Finally, we examined the abdomens of YNP and Idaho adults with and without cuticular wax using scanning electron microscopy. We did not observe any distinct structural cuticular differences between YNP and Idaho. Consequently, we suggest that structural layering may be responsible for the observed heat resistance and reflection.

Introduction

The wetsalts tiger beetle, *Cicindelidia haemorrhagica* (LeConte) (Coleoptera: Carabidae), is a warm-adapted tiger beetle that has been recorded actively hunting and foraging in active hot springs areas of Yellowstone National Park (YNP) for more than 130 years (Bertholf 1979; Hubbard 1891). Hubbard (1891) noted that the environments these tiger beetles inhabited likely exceeded 50 °C, surpassing the upper thermal limits for many known insect species. Although there have been many research studies conducted on the thermal features of YNP, this tiger beetle in YNP has not been studied until recently (Willemsens 2019).

The wetsalts tiger beetle is found on the west coast of the United States in addition to Utah, Idaho, Nevada, and Wyoming, and is typically found on sandy, wet substrates that border saline flats or other warm stagnant water sources (Pearson et al. 2015). Warm-adapted tiger beetles often employ a maxi-thermal strategy, in which their optimal temperature is close to their upper lethal limit (Dreisig 1980). However, in YNP, *C. haemorrhagica* (which is only found in YNP in thermal areas) is active in environments with not only high surface temperatures, but also thermal waters, high concentrations of heavy metals, and alkaline or acidic pH that most vertebrates and invertebrates in YNP actively avoid (Fouke 2011; Boyd et al. 2009; Inskeep and McDermott 2005).

Insects often behaviorally thermoregulate to adjust their internal body temperatures. As ectotherms, these thermoregulatory behaviors often change as the position of the sun shifts throughout the day (Brosius and Higley 2013; Pearson and Lederhouse 1987; Schultz and Hadley 1987; Dreisig 1981; Dreisig 1980). These behaviors also change in the presence of cloud cover or precipitation, which may often result in seeking temporary shelter. Warm-adapted tiger beetles, especially species in environments similar to *C. haemorrhagica*, behaviorally thermoregulate in these warm environments by stilting, shading, and dipping their abdomens in water to evaporatively cool themselves (Willemsens 2019; Brosius and Higley 2013; Knisley et al. 1990; Dreisig 1989; Pearson and Lederhouse 1987, Morgan 1984; Dreisig 1980).

To better understand the thermoregulatory behaviors exhibited by *C. haemorrhagica* in YNP, Willemsens (2019) observed numerous adults from several populations in YNP on soil surfaces or shallow water environments where surface temperatures often exceeded 50 °C. The adults infrequently displayed cooling behaviors on these hot surfaces. These behaviors were directly compared to that of another population of *C. haemorrhagica* that resided outside of YNP on a non-hot springs salt flat environment in Idaho. YNP populations of *C. haemorrhagica* spent more of their time trying to actively warm themselves instead of cooling themselves compared to the Idaho population, even though YNP beetles were regularly exposed to full sun and on surfaces exceeding 50 °C (Willemsens 2019). YNP beetles spent time basking in full sun on hot surfaces while much of the same time spent by Idaho beetles involved higher frequencies of cooling behaviors such as stilting, shuttling between shaded areas, dipping their abdomen dipping in water, and stilting. Willemsens (2019) showed strong statistical evidence of differences in behavioral cooling and heating times between beetles from the two populations.

Willemsens (2019) then investigated the cause of the behavioral differences by first observing the critical thermal limits of both YNP and Idaho adult *C. haemorrhagica*. It was predicted that *C. haemorrhagica* would have a lethal maximum temperature of approximately $48.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, the same lethal temperature that was reported in Pearson and Lederhouse (1987). The YNP and Idaho beetles had an average critical thermal limit of 50.41°C and 49.33°C , respectively (Willemsens 2019). Both temperatures were greater than the previously reported lethal maximum for the Arizona population (Pearson and Lederhouse 1987). However, the potential overlap in critical thermal limit data between the YNP and Idaho populations suggested that there might be another mechanism or mechanisms to explain the striking thermoregulatory behavioral differences between the two populations. As a result, we conducted preliminary experiments in 2018 of internal body temperatures when adults were subjected to heating over a water bath. Dried and freshly killed specimens were used in these comparison experiments. Results suggested that YNP beetles had lower internal temperatures than Idaho beetles in both experimental procedures involving freshly killed and desiccated specimens. Consequently, results suggest that there was no liquid component involved in influencing any observed effect on internal body temperature. Therefore, the objective of this study was to identify and evaluate physiological, physical, and morphological mechanisms that may explain the observed differences in thermoregulatory behavior.

Materials and Methods

Site descriptions

Dragon Spring is a relatively well-studied acid-sulfate-chloride spring located in the Norris Geyser Basin that has high levels of As (III) and MeHg^+ levels in the source waters. This location contains *Hydrogenobaculum* and thermophilic chemolithotroph species (Boyd et al. 2009; D’Imperio et al. 2007; Fouke 2011; Inskeep et al. 2013). Between the numerous highly active thermal water sources for this spring is a peninsula primarily comprised of sulfate and calcium-based sediment that has been the primary research site for *C. haemorrhagica* since ecological research began in 2017 (Willemsens 2019). Here the beetles actively burrow, hunt, forage, mate, and oviposit while being continuously surrounded by thermal waters that directly influence ground and surface temperatures. Dragon Spring has an average measured pH of approximately 2.88 and running water with sufficiently low temperatures in some areas to allow for the development of acid-loving *Zygogonium* spp. mats that can accommodate stratiomyid and ephydrid populations (Boyd et al., 2009; D’Imperio et al. 2007; Willemsens 2019). The fly larvae that reside in these mats also provide a reliable food source for *C. haemorrhagica* adults in the summer months. In addition to these fly populations, the tiger beetles at Dragon Spring also feed on a variety of small insects, including shore bugs and ephydrid adults, but will also scavenge and feed on anything that falls into the thermal waters and is in shallow water. Most water sources cool sufficiently within a few meters from the source to allow for the growth of bacterial and archaea microbial communities, which produce a mat color that is indicative of water temperature and in some cases, pH (Inskeep & McDermott 2005). The mat colors in addition to the present archaea consistently indicate the extreme temperatures of water sources

and associated water temperature falloffs that are present in Dragon Spring. This further indicates that a variety of temperatures may be experienced by insects, both convectively and conductively, when traversing the terrain directly adjacent to the variable temperature thermal waters.

Rabbit Creek is the other hydrothermally active field research site that we selected. Unlike Dragon Spring, this thermal area contains multiple alkaline water sources located in the Midway Geyser Basin. This field research site has an average pH of 9.2. Because this area is a constantly flowing water source that moves from east to west, it produces a wide range of water temperatures that in turn accommodate several different communities with Acidobacteria and Cyanobacterial mats (Weltzer & Miller 2013; Meller et al. 2009). Rabbit Creek is the larger of the two sites, and contains a steep thermal gradient throughout the waterway, with estimated changes in water temperature of 7.5 °C for every 100 m of continuous waterway. The Cyanobacterial mats present in the waterways directly adjacent to thermal water injections stimulate the growth and development of numerous microbial and arthropod species, including thermophilic ephydriids. These ephydriid populations serve as a primary food source for *C. haemorrhagica* at Rabbit Creek.

The beetle population that we selected outside of YNP as the comparison population resides on a salt flat near the Snake River and Mountain Home, Idaho (42°56'06.5"N 115°45'00.9"W) that has summer water temperatures of approximately 30.34 °C and a pH of approximately 8.22 (Willemsens 2019). *Cicindelidia haemorrhagica* share this habitat with a much less abundant *C. punctulata* (Olivier) and seem to be the only tiger beetle species that consistently occupies the saline flat throughout days with full sun. The presence of *C.*

haemorrhagica was recorded in 2016 by J. Runyon (USFS), and Idaho Department of Fish and Game gave permission for us to collect samples as needed.

Internal temperature comparisons

To characterize potential differences in internal temperatures, we conducted a series of laboratory experiments. We collected approximately 25 beetles per site for the 2019 and 2020 field seasons to use for internal temperature comparison and other planned experiments (numbers were limited by YNP research permit requirements). We collected each beetle using a 30.5-cm diameter and 91.5-cm long aerial net during times of increased activity (BioQuip 7312NA, San Diego, CA, USA). Collected individuals were then placed in small plastic containers that contained air openings for respiration and a damp paper towel for maintained relative humidity. We placed a maximum of five beetles in each container to prevent cannibalism and each container was placed in a day- pack. Containers were then placed in a YETI Tundra 35 cooler (YETI, Austin, TX, USA) with ice packs as soon as possible after returning to the field vehicle. Coolers were unloaded on arrival at Montana State University and placed in a -20 °C freezer to quickly kill individuals and provide freshly frozen specimens for experimentation.

We created the heated water bath using a 12-L sous vide container (Rubbermaid, Wooster, OH, USA) filled with approximately 8L of water before each testing period. The temperature of the water bath was controlled and regulated by a Joule sous vide (ChefSteps, Seattle, WA, USA) that was connected to a cellphone controller via Bluetooth. We produced thermocouples were produced using 24-American Wire Gauge thermocouple wire connected to miniature T-type thermocouple connector plugs (OMEGA, Norwalk, CT, USA). Plastic straws with a diameter of 6 mm were cut into 10-cm segments to act as straight framed reinforcement

for the thermocouple wire to hang straight down, perpendicular to the water bath. The wire was bent into a 90° angle, so the exposed thermocouple wire was parallel to and 2mm above the surface of the water. Internal temperatures of the beetles were recorded using an SD-947 4-channel thermometer/SD card data logger (REED, Wilmington, NC, USA).

Beetles collected from each site within YNP were paired with an Idaho individual of similar size and same sex if available. We grouped pairs together based on the original field research site the YNP beetles were collected (Dragon Spring vs Idaho and Rabbit Creek vs Idaho). We then inserted the exposed end of the thermocouple wire into each beetle through the genital opening, running the wire up the abdomen lengthwise without breaking through exoskeleton or penetrating the thorax (Figure 1). Consequently, the thermocouple wire was positioned approximately midline between the ventral and dorsal portions of the abdomen. In later experiments, we removed every individual's legs prior to experimentation to reduce the chance of thermal conduction or evaporative cooling from the water touching specifically the hind and mid pairs of legs.

We conducted two water-bath experiments with eight treatment replicates per experiment over four different temperatures. Each replicate consisted of two beetles attached to thermocouples and one bare thermocouple control for all eight replicates. Eight beetles from Idaho were paired with eight from Rabbit Creek for the first experiment, and another eight beetles from Idaho were paired with eight from Dragon Spring for the second experiment. Beetles with their legs removed were used in the last four pairings of each YNP location and Idaho comparison experiment. We used a total of 32 adults between all three populations: eight from Dragon Spring, eight from Rabbit Creek, and 16 from the Idaho population (Table 1).

Three thermocouples were connected to the thermocouple reader at a time and held in place over the water bath by a 2.5-cm thick x 5.1-cm wide wooden plank with notches filed 2.5-cm deep into the side of the board that were designed to hold the reinforced thermocouple wire (Figure 2). One beetle from a YNP site and the Idaho site were randomly assigned to thermocouples and one bare thermocouple was used as the control. Thermocouples that were attached to beetles had alternating positions within the water bath. This ensured that temperatures experienced by the thermocouples were independent of position in the enclosed water bath. The water bath was heated initially to 40 °C for the first trials and increased to 55 °C by 5 °C increments for each trial thereafter. Once the thermocouples were in place and the water bath was heated to the correct temperature, the thermocouples were allowed to equilibrate for 2 minutes. Once the thermocouples equilibrated, we recorded internal temperatures of the beetles once per minute for 10 minutes.

Our findings from the internal temperature comparison experiments between Idaho and YNP populations suggested that there was a physical aspect responsible for the difference in internal temperatures we observed. To further understand this phenomenon, we conducted temperature comparison experiments that determined the contribution of cuticular wax to any difference in internal temperatures that YNP adult *C. haemorrhagica* experienced. Nine pairs of beetles of similar size and sex were selected from Dragon Spring and Rabbit Creek. One beetle from each pair was placed in a 20-mL scintillation vial and submerged in 15 mL of dichloromethane for 1 minute before being removed, removing the cuticular wax without compromising the exoskeleton (Table 1). We mounted both beetles above the water bath in the

same fashion as previous experiments and recorded internal temperatures for only 45 °C and 50 °C.

Abdominal plate extraction and temperature comparison

The results of the internal temperature comparison (including completely dried specimens on thermocouples) and the cuticular wax removal experiments suggested that the mechanism or mechanisms responsible for differences in internal temperatures are caused by a physical characteristic on the ventral face of the YNP beetles. Because the thermocouples were placed in the abdomen, we focused on the ventral abdominal face. The abdominal ventrites are not connate, but they remain linked together after dissection if carefully handled, forming what will hereafter be referred to as a ventral abdominal plate. Eight individuals from the summer 2020 YNP collection period were used for isolating the ventral abdominal plate. We did not use Idaho beetles because our objective was to determine if the lower internal temperatures in YNP beetles were solely attributed to the ventral abdominal plate. Once we identified and labeled specimens by sex, we removed each of their legs. We used a #2 X-Acto knife (X-Acto, Westerville, Ohio, USA) to dissect out the abdominal plate by slicing the body along the midline where the dorsal and ventral sides meet. These cuts were made on both sides of the beetle before forceps were used to lift the ventral plate from the body. The plate was then cleaned using forceps to carefully scrape away viscera and rinsed with ethanol. Once the abdominal plate was prepared, each plate was hot glued to a randomly assigned thermocouple. Another thermocouple had electrical tape cut to the approximate shape of the abdominal plate attached to it using hot glue to act as the control for each experiment. A bare thermocouple was also included to monitor air temperatures above the water bath as in previous experiments (Figure 3). We conducted eight experiments

using one thermocouple with a YNP ventral plate, one with electrical tape, and one bare thermocouple.

CO1 comparison

To verify that the beetles from YNP and Idaho were the same species, we selected individuals from both YNP and the Idaho location for cytochrome c oxidase subunit 1 (CO1) comparison testing to determine any potential genetic difference between populations. Individuals were selected at random from the collected samples from each location. We then dissected the adults to isolate viscera contained in the thorax and viscera attached to the middle pair of coxae. The isolated viscera were stored in 90% ethanol in a 5-mL Eppendorf tube before DNA extraction and PCR amplification using primers LCO 1490 and HCO 2198. Our samples were compared with the *C. haemorrhagica* JX260543 and the *C. highlandensis* JX260544 reference sequences in Genbank. Prepared samples were then shipped to Molecular Cloning Laboratories (San Francisco, California, USA) for CO1 comparison. Results confirmed the YNP and Idaho beetles were *C. haemorrhagica* (>99% match to reference sequence) (Table 2).

Data analysis

Thermocouple measurements between the YNP and Idaho *C. haemorrhagica* beetles, the wax-intact versus wax-removed YNP beetles, and the isolated abdominal plate thermocouple recordings were entered in spreadsheets. Not every thermocouple displayed the same temperature measurement as the bare thermocouple control when measuring the ambient air temperature. The difference between the control and thermocouples used for adult beetles only differed by a maximum of 0.2 °C. Therefore, thermocouples were calibrated up to ± 0.2 °C to account for the difference in measured temperature between the bare thermocouple that was used

as the control. Each thermocouple was measured against the control before recording for this reason. Corrected temperatures were then compiled into a spreadsheet based on the paired treatment of comparison experiment performed. Initial data visualizations and t-tests were performed for each group using Microsoft Excel. Temperature values of each thermocouple paired treatment with a beetle attached to it were directly compared to the bare thermocouple temperature reading for each minute of recording. This provided us with the following equation:

$$\Delta T = T_{Bare} - T_{Beetle} \quad [1]$$

where ΔT is the difference in temperature between the bare thermocouple and the thermocouple with the beetle. The ΔT values of each YNP and Idaho pairing were totaled and averaged according to the temperature of the water bath to produce one average ΔT per temperature per beetle. These average values were grouped together by location and used to calculate the standard deviation and standard error for each location. The standard error values were later incorporated into visualizations of these temperature data.

The temperature data from the Idaho and YNP comparison experiments and the cuticular wax comparisons were condensed into a single master file document that was transferred to RStudio version 4.0.2 for further data visualization and analysis (RStudio Team 2021). This document consisted of columns recording the individual number, mean ΔT values for each water bath temperature, water bath temperature, location or treatment of the beetle, the presence of legs, dried body weight, ambient air temperature outside of the water bath at the time of recording, and sex of the beetle. A linear mixed effects model was developed using the mean ΔT values as the dependent variable for each temperature a beetle that had its temperature recorded, resulting in four mean ΔT values per individual beetle in the YNP and Idaho comparisons. The

mean ΔT values for 45 °C and 50 °C in the cuticular wax comparison experiments were the only two ΔT values used for these individuals. Each beetle was assigned its treatment to differentiate between groups. Beetles from the Idaho and YNP comparison experiments had their location substituted for their treatment. Additional variables included in the model were the presence of legs, air temperature outside the water bath, sex, and dried beetle weight and each were evaluated for their potential influence on the ΔT values of each beetle. Variables that were not influential to ΔT values were excluded from the final model as they did not contribute to any additional information in analyzing the data set. Initial diagnostic figures evaluating equal variance among observations and assessing the residual normality of the data were developed for the model to prevent any analysis that carried statistical violations. The model was then evaluated for assessing differences between groups accounting for the repeated measurements conducted on each beetle.

A separate linear mixed effects model was produced for the repeated measurements in the ventral abdominal comparison experiment to assess differences between the cuticular venter and the imitation venter. These values were kept separate from the whole beetle thermocouple experiments because dry weights were negligible and ambient air temperatures were not recorded throughout experimentation. This model was only used to determine the differences in ΔT between treatments and no additional other variable.

Scanning electron microscope characterization

Results from our temperature comparison experiments justified the need for further investigation of cuticular microstructures on the abdomens of adult *C. haemorrhagica*. We first examined the exoskeleton of YNP and Idaho populations using a relatively high-powered

dissection microscope and did not see any outstanding physical differences in cuticular structure. Therefore, we next investigated physical structural differences using Scanning Electron Microscopy (SEM).

We used beetles collected in 2020 from both YNP and Idaho for SEM work because these specimens were least likely to have a brittle exoskeleton or have been damaged in the freezer over time. Each specimen was rinsed in ethanol and gently wiped clean using kimwipes (Kimtech, Roswell, GA, USA) and allowed to air dry in a petri dish for approximately 2 minutes. While the beetle was drying, SEM sample stages were prepped by layering small circles carbon tape on top of the sample stage. The beetle was then placed on the carbon tape with the venter of the abdomen on the center point of the stage. Specimen stability on the sample stages was reinforced with carbon tape strips holding down the beetle on either end of the ventral plate of the abdomen. The first piece of carbon tape generally covered the prothorax and the second piece only covered the last two abdominal segments as to not obstruct the view of first four abdominal segments, the area of interest. Once samples were secure on their respective stages, they were placed in an EMS 550 sputter coater (Electron Microscopy Sciences, Hatfield, PA, USA) and sputter-coated with 25 nm of iridium. Coated beetles were then placed back on the stage holder in their respective numbered slots, then placed in the SUPRA 55VP Field Emission Scanning Electron Microscope (Zeiss, Dublin, CA, USA) for analysis. Images recorded were at 500x, 1000x, 1500x, and 5000x, focusing on the first four abdominal segments on the ventral side.

We recorded additional SEM imaging using the same procedure on YNP beetles that had their cuticular wax removed using dichloromethane. Imaging the same area on each beetle was crucial for this process because there may be extreme differences in microstructure from one

section of an abdominal segment to another. Discerning between environmental clutter like dust or irregular sputter coating and cuticular structures was critical for determining any observed morphological differences.

To determine if there were noticeable differences in the layering of the ventral plate of YNP *C. haemorrhagica*, we dissected abdominal plates into slices along the latitudinal midline of the first abdominal segment using a #2 X-acto knife (X-Acto, Westerville, Ohio, USA). Images captured using this method of dissection were unstable in the SEM and contained too much iridium to discern any real differences in cross section. Therefore, we embedded two adult beetles in resin and polished it to 0.05 μm grit finish to produce a smoother area of interest and a more stable sample for cross sectional analysis. This helped us record more refined and stable images that required less iridium to stabilize, so more comparative images were recorded using this method of sample preparation.

Spectrophotometric measurements

Six adult beetles were analyzed the Materials Research Laboratory at the University of Illinois at Urbana-Champaign for spectrophotometric performance of the abdominal plate using spectrophotometry. Three beetles from YNP and three from Idaho were selected at random for the observational analysis. Each beetle had its ventral abdominal plate dissected from its body with any remaining viscera carefully scraped away to not damage any of the area of interest. Baselines were then established on a Cary 5000 UV-VIS-NIR spectrophotometer (Agilent Technologies, Santa Clara, California, USA). We placed each abdominal plate carefully in receptacles, so they were not accidentally damaged between observational periods. Each plate was placed on a fabricated piece of sheet metal that was specially made for accommodating the

passage of light through the abdominal plate (Figure 4). The abdominal plate was also suspended on a glass pillar throughout the diffuse absorption recording to ensure all reflected and transmitted light was captured by the equipment's sensors. Each beetle was recorded using the same baselines established for diffuse reflection, diffuse absorption, and diffuse transmittance. The numerical measurements of each spectrophotometric property were entered in a spreadsheet and each file was reformatted for easier data analysis and visualization in RStudio. Reformatted data from each of the three beetles from each location were combined into mean reflectance measurements and visualized in RStudio.

Results and Discussion

YNP vs Idaho internal temperature comparisons

Our COI comparative analysis strongly suggested that the YNP and Idaho populations are the same species (Table 2). The simulated environmental conditions of the water bath comparison experiments were carried out from 40 °C through 55 °C to compare internal abdominal body temperatures of Idaho and YNP populations. Our results indicated for the YNP versus Idaho *C. haemorrhagica* comparison experiments that YNP beetles had a lower internal temperature than Idaho beetles. To determine the validity of our results, we produced initial diagnostics figures examining equal variance and residual normality suggested our data contained no statistical violations and the data set did not need to be transformed. The linear mixed effects model directly compared the Idaho and YNP beetle thermocouple groups. We found little evidence to suggest that the presence of legs ($p = 0.068$), ambient air temperature at the time of recording ($p = 0.295$), sex of the beetle ($p = 0.751$), and dry weight of the beetle ($p = 0.49$) had any influence on the ΔT values recorded in the internal temperature comparison

experiments conducted with Idaho and YNP beetles. Therefore, these variables were excluded from the final model because they did not contribute to strengthening the predictive power of the model. This meant that whatever biological differences we were observing between the YNP and Idaho beetles was a difference purely attributable to the location where the beetles were sampled from or the treatment that was applied to the YNP beetles in wax presence comparisons.

Knowing all collected beetles are the same species from each population, it makes these temperature measurements even more important because there are fewer confounding variables influencing the recorded temperatures, leaving only the location, cuticular wax, and the cuticular structures of the beetle primarily responsible for any differences we observed. Direct comparisons between location in Table 3 ($p = 159$) and the Tukey group comparison in Table 4 ($p = 0.61$) suggest that YNP populations are not sufficiently different from one another to be considered different population measurements, effectively simulating a uniform YNP group of ΔT values.

Because our results show the YNP populations are not significantly different from one another, the main comparison between YNP and Idaho beetles remains as the primary comparison of interest in determining differences in internal body temperatures between populations. Direct comparisons between Idaho ΔT values in Table 3 ($p < 0.001$) to the intercept of Dragon Spring provide us with very strong evidence that these groups have different ΔT values. In the Tukey comparison in Table 4 comparing Idaho to both Rabbit Creek ($p < 0.001$) and Dragon Spring ($p = 0.002$), there is very strong evidence that these population ΔT values are different from one another. This relationship was consistent for all temperatures of the water bath experiments that we conducted with an overall mean difference in temperature of $1.03\text{ }^{\circ}\text{C}$ (Table

1). The largest mean difference between internal body temperatures recorded was a difference of 1.63 °C at 55 °C between the Idaho individuals and individuals from Rabbit Creek. Both the linear mixed effects model and the Tukey group treatment comparison suggest that there are statistical differences between internal body temperatures of YNP and Idaho beetles.

Our results further suggest that YNP adults have a greater heat reflection mechanism along the ventral face of the abdomen than Idaho beetles. Because we did not observe differences in this heat reflective effect between freshly killed and completely dried specimens, our results suggest that a liquid milieu in the body or physiological process is not involved in this heat reflection. This implies the lower internal body temperatures for adult YNP *C. haemorrhagica* are most likely produced by a physical or morphological mechanism.

Cuticular wax internal temperature comparisons

Microstructures made of chitin and wax secretions have been implicated in improving the survivability of an insect. Several studies have explored the effects that chitin and wax secretion on light and heat dissipation or water loss issues with insects (Dreisig 1979; Hadley 1978; Hadley 1979; Hadley and Schultz 1987; Schultz and Hadley 1987; McClain et al. 1991; Hanrahan et al. 2001; Seago et al. 2009; Shi et al. 2015; Amore et al. 2017; Alves et al. 2018; Pavlovic et al. 2018; Cuesta and Lobo 2019; Xie et al. 2019; Zhang et al. 2020). Individuals with their cuticular wax intact had mean internal temperatures of 33.8 °C at 45 °C and 36.5 °C at 50 °C surface temperatures. Individuals with their cuticular wax removed had mean internal temperatures of 34.5 °C at 45 °C and 37.2 °C at 50 °C surface temperatures. The bare thermocouple mean control values were 36.3 °C at 45 °C and 39.8 °C at 50 °C surface temperatures. Using these control values in determining ΔT we found that individuals with their

cuticular wax had mean differences in temperature of 2.48°C at 45 °C and 3.30 °C at 50 °C between the control bare thermocouple, and individuals with wax removed had a mean difference in temperature of 1.77 °C at 45 °C and 2.64 °C at 50 °C.

However, our Tukey comparison model suggests that there no statistical differences between the mean ΔT values of the cuticular wax comparison groups with and without their cuticular waxes ($p = 0.193$) (Table 4). This means there is likely little contributed by the wax in the reflective heat effect we were observing with just the Idaho and YNP comparison measurements. Additionally, there is likely no difference between the Rabbit Creek and the individuals with their cuticular wax intact ($p = 0.799$), and Dragon Spring and the intact cuticular wax beetles ($p = 0.999$) (Table 4). These data suggest that the structure of the cuticle itself is responsible for the observed difference between YNP and Idaho internal body temperatures.

Ventral abdominal plate temperature comparisons

Because we determined that the wax was only responsible for minor differences in internal body temperatures between the YNP and Idaho *C. haemorrhagica* populations when exposed to high surface temperatures, we needed to isolate the structure of the exoskeleton to determine if we could continue to observe the temperature differences. This was done by dissecting the ventral abdominal plate in preparation for more thermocouple comparison measurements. To ensure that overall shape of the beetle was not responsible, and to determine if contact with the thermocouple created a difference in the temperature reading, we compared the ventral abdominal plate to an imitation plate and the bare thermocouple in proceeding comparison experiments. The ΔT results from thermocouple measurements from eight ventral plates at water bath temperatures of 45 °C and 50 °C suggest there were statistical differences

between thermocouples that had an abdominal plate attached, the electrical tape imitation plate, and the bare thermocouple ($p = 0.037$) (Table 5). This further suggests that contact with the thermocouple was not influencing temperature recordings because the ventral abdominal plate group had greater ΔT values than the imitation plate group. Building off what we observed in our previous experiments, we know that YNP beetles show increased heat resistance and the component responsible is not only present in living or freshly thawed YNP beetles, but desiccated specimens as well. We also know that the cuticular wax is not statistically responsible for this difference in ΔT measurements. Based on our results, we determined there may be a difference between the microstructures on the ventral face of the abdomen of YNP beetles that may help reduce incoming heat from their environmental surfaces rather than a difference in temperature caused by the shape of the beetle or the presence of cuticular wax.

Structural features that aid in thermoregulation are known in insects. The Saharan silver ant, *Cataglyphis bombycina* (Hymenoptera: Formicidae), is the most heat-tolerant insect currently known. These ants possess microscopic triangular setae that aid in light reflection that have shown improved performance in dissipating heat from incoming sunlight (Shi et al. 2015). The micro setae on the elytra of *Neoceramyx gigas* (Coleoptera: Cerambycidae) possess corrugated facets on one side, improving spectrophotometric performance and reducing incoming heat on the elytra by providing total internal reflection of light when subjected to various angles of sunlight (Zhang et al. 2020). The mechanism or structure may be something more discrete such as micro-pyramid structures on the ventral face of the abdomen, much like that of *Rosalia alpina* (Coleoptera: Cerambycidae), which possess these pyramid-shaped structures on the elytra (Pavlovic et al. 2018). These structures improve radiative heat exchange

between the beetle and its environment, making it possible for the insect to survive in areas with more intense sunlight for longer periods of time (Pavlovic et al. 2018).

Spectrophotometry and scanning electron microscopic analysis

The suggestion that the mechanism for temperature differences is physical or morphological caused us to next employ spectrophotometric measurements on the ventral abdominal plate of *C. haemorrhagica*. The diffuse reflectance properties in the ultraviolet (UV), visible, and near infrared (NIR) light wavelength ranges of the six selected individuals from YNP and Idaho were recorded. Our results indicated that YNP beetles had a greater reflectance from the 750 nm to 2000 nm wavelength range (Figure 7). This range lies primarily in the NIR to IR spectrum. These results support our internal temperature results and suggest that YNP beetles reflect more light that is influential on body temperature and may possess structures that can offload this light by reflecting it towards the environment. Reflective properties of the exoskeleton are important strategies in insect thermoregulation. Both chitinous and wax reflective properties can contribute to improved reflection which help reduce body temperatures (McClain et al. 1991; Seago et al. 2009; Shi et al. 2015; Alves et al. 2018; Pavlovic et al. 2018; Cuesta and Lobo 2019; Wang et al. 2021).

We could not observe physical differences between *C. haemorrhagica* populations in thorough examinations using a relatively high-powered dissection microscope (Willemssens 2019). Therefore, we believed the best way to approach isolating any physical structures that may exist on the ventral abdominal plate surface was to use scanning electron microscopic analysis. We selected both Idaho and YNP adults for comparison work with the Scanning

Electron Microscope (SEM) and analyzed during the same sessions to ensure the same settings were used while imaging individuals from both locations.

Images of ventral abdominal plates with and without the cuticular waxes removed suggested that there were no obvious structural differences between beetles from either location (Figures 8, 9). The ventral surface appears as an arrangement of polygonal chitin tiles (Figure 9). These structures are unlike those found on the identified heat reflective structures on *Cataglyphis bombycina*, *Neoceramyrx gigas*, and *Goliathus goliatus*, which rely on setae-like structures on the dorsal abdominal surface of each species to improve the reflective properties of their exoskeletons from solar insolation (Shi et al. 2015; Xie et al. 2019; Zhang et al. 2020).

Cicindelidia haemorrhagica is subject to near-infrared and mid-infrared radiation in YNP that is both generated by and reflected off the substrate, affecting internal body temperatures. Because there are virtually no setae directly on the venter of the abdomen, setae are not responsible for the heat reflectance we observed. Because we did not observe obvious structural differences on the ventral abdominal plates between YNP and Idaho beetles, the heat resistance mechanism might involve layering of the cuticle. Structural layering can be responsible for coloring of the insect cuticle, which may in turn affect the reflective and thermoregulatory properties of the exoskeleton (Shultz and Hadley 1987). However, results of our SEM cross-sectional comparative analysis of individuals from YNP and Idaho suggest no obvious differences in either the thickness or layering of the insect cuticle. More refined imaging may be required in future observations for cross-sectional analysis. This does not mean that structural layering is not responsible for the observed heat reflectance differences. The instrumentation

used for this analysis did not provide us with an elemental and chemical composition of the individual cuticle layers, which could be used in more thorough analyses of the cuticle layering.

The geography encompassing Yellowstone National Park is a relatively young and diverse environment that rests on the volcanic Yellowstone Plateau (Pierce 1979; Fournier 1989; Fouke 2011). It was formed by the recession of the Pinedale Glacial Icecap approximately 14,000 years ago, which was followed by periodic volcanic eruptions that shaped many geological features (Pierce 1982; Fournier 1989). This volcano continues to heat the ground beneath the territory, producing constantly changing environments and providing the heat necessary to warm the active hot springs within YNP (Fouke 2011).

The recession of the Pinedale Icecap only 14,000 years ago indicates there were no insects present until the icecap had receded. This allowed only a relatively short time for *C. haemorrhagica* to establish in YNP, meaning there could have only been a maximum of 14,000 generations (assuming one year per generation, but it may be two years per generation) separating past YNP *C. haemorrhagica* populations from the present. In this relatively short time of perhaps only 7,000 years, individuals living in YNP seemingly have adapted to the bottom-up heat associated with active hot springs. The YNP beetles, consequently, have diverged from other populations of the same species by developing distinct thermoregulatory behaviors (Willemssens 2019) that may be the result of enhanced heat reflectance. This increased heat reflectance seems to be caused by a physical feature as part of the ventral abdominal plate of the exoskeleton, which likely protects the beetle by serving as a heat shield.

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Table 1: Summary information of thermocouple experiments. Differences between the ΔT values that are displayed in Figure 5. (Ex. Dragon Spring YNP ΔT vs. Idaho ΔT). Differences in ΔT values are average values from the eight pairs of beetles used in that temperature for the labeled experiment.

Experiment	Pairs of beetles	Temperature (°C)	Differences in ΔT (°C)
Dragon Spring YNP vs. Idaho	8	40	0.76
Dragon Spring YNP vs. Idaho	8	45	0.78
Dragon Spring YNP vs. Idaho	8	50	1.25
Dragon Spring YNP vs. Idaho	8	55	1.23
Rabbit Creek YNP vs. Idaho	8	40	0.69
Rabbit Creek YNP vs. Idaho	8	45	0.88
Rabbit Creek YNP vs. Idaho	8	50	1.22
Rabbit Creek YNP vs. Idaho	8	55	1.63
Cuticular wax intact vs. removed	9	45	0.71
Cuticular wax intact vs. removed	9	50	0.66
Plate vs. imitation plate	8	45	0.61
Plate vs. imitation plate	8	50	0.56

Table 2: Results of the COI analysis containing individuals from both YNP and Idaho populations of *Cicindelia haemorrhagica*. Direct comparisons to the reference gene sequences and one another in match percentage values show that all specimens were 99% or greater match to the *C. haemorrhagica* reference gene sequence.

	JX260544 (<i>Cicindela</i> highlandensis)	Idaho 1 COI	YNP R3 COI	Idaho 2 COI	YNP4 R4 COI	Idaho 3 COI	Idaho 4 Col	JX260543.1 (<i>Cicindela</i> haemorrhagica)
JX260544 (<i>Cicindela</i> highlandensis)		94	93	93	92	93	93	91
Idaho 1 COI	94		100	100	99	100	100	100
YNP R3 COI	93	100		100	100	99	99	100
Idaho 2 COI	93	100	100		100	100	100	100
YNP R4 COI	92	99	100	100		99	99	100
Idaho 3 COI	93	100	99	100	99		100	100
Idaho 4 COI	93	100	99	100	99	100		100
JX260543.1 (<i>Cicindela</i> haemorrhagica)	91	100	100	100	100	100	100	

Table 3: Linear mixed effects model. The residual standard deviation value of 0.957 suggests the model does not deviate from our mean ΔT values recorded. Other variables were not included as they did not carry the same predictive weight as the location variable.

Response Variable	Value	Standard Error	DF	p-value
Intercept (Dragon)	-1.2620229	0.6242426	113	0.0456
Location Idaho	-0.8660937	0.2320032	45	0.0005
Location Rabbit-YNP	0.3840625	0.2678942	45	0.1586
Location YNP-No Wax	-0.6250694	0.2935698	45	0.0387
Location YNP-Wax	0.0588194	0.2935698	45	0.8421
Random effects from repeated measures on individual:	Intercept	Residual		
Standard Deviation	0.246	0.957		

Table 4: Tukey contrast comparison. All treatments (location and wax) were incorporated to compare all recorded difference in temperature values with whole beetles. The p-values suggest statistical differences between YNP and Idaho populations while determining statistical differences between cuticular wax groups. YNP and Idaho groups had 40 °C - 55°C ΔT values included while cuticular wax groups only had 45 °C and 50 °C ΔT values included.

Location Comparison	Estimate	Standard Error	p-value
Idaho - Dragon-YNP	-0.866	0.232	0.002
Rabbit-YNP - Dragon-YNP	0.384	0.268	0.601
YNP-No Wax - Dragon-YNP	-0.625	0.294	0.204
YNP-Wax - Dragon-YNP	0.059	0.294	0.999
Rabbit-YNP - Idaho	1.25	0.232	< 0.001
YNP-No Wax - Idaho	0.241	0.261	0.886
YNP-Wax - Idaho	0.925	0.261	0.004
YNP-No Wax - Rabbit-YNP	-1.009	0.294	0.005
YNP-Wax - YNP-No Wax	0.684	0.317	0.193
YNP-Wax - Rabbit-YNP	-0.325	0.294	0.799

Table 5: Linear mixed effects model that provides comparative p-values for the ventral abdominal plate thermocouple experiments. The low standard error of the random effects suggests little influence on the ΔT values of the ventral abdominal plate thermocouples over time. This table shows the effect of the repeated measures while accounting for difference between variable groups.

Response Variable	Value	Standard Error	DF	p-value
Intercept (Imitation)	0.7517187	2.2198397	15	0.7396
TreatmentPlate	0.5509375	0.2386398	14	0.0367
Random effects from repeated measures on individual:	Intercept	Residual		
Standard Deviation	0.152	0.639		



Figure 1: *Cicindelidia haemorrhagica* adult with a thermocouple inserted into its abdomen in preparation for water bath experiment. Photo by RKD Peterson.

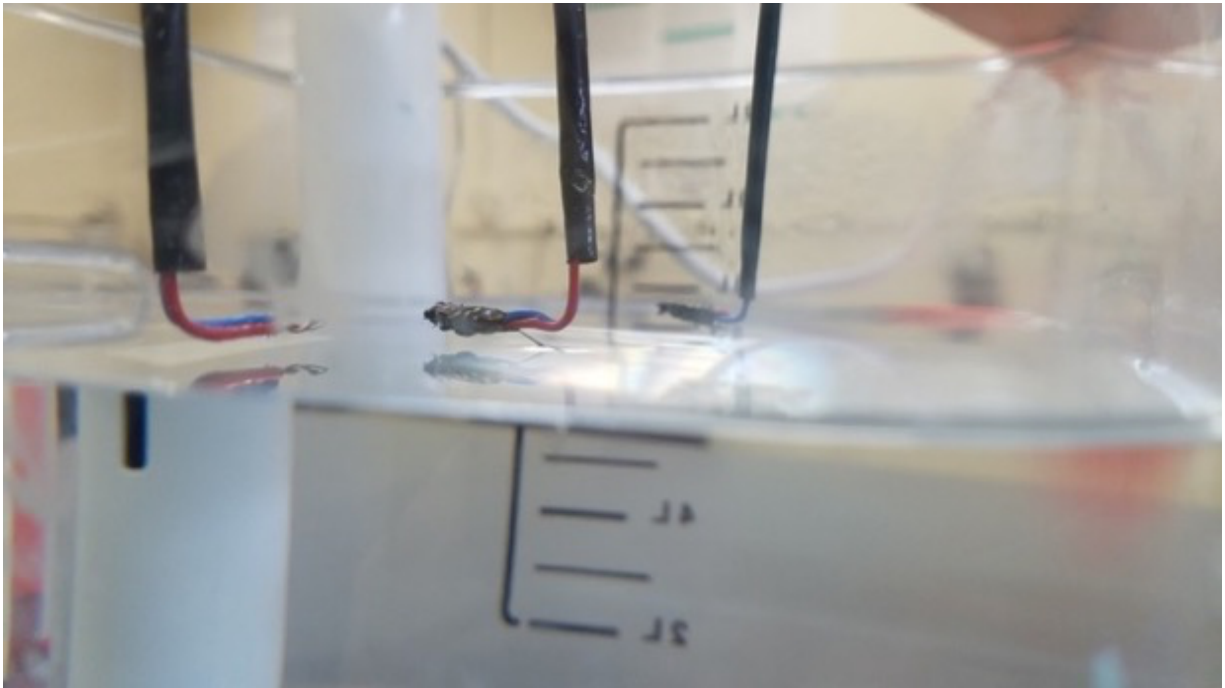


Figure 2: *Cicindelidia haemorrhagica* adults having their internal temperatures recorded. Thermocouple wires were suspended above the water bath in which the temperature was regulated by the sous vide (left) and recorded using the thermocouple logger/recorder (not shown). Photo by JL Bowley.

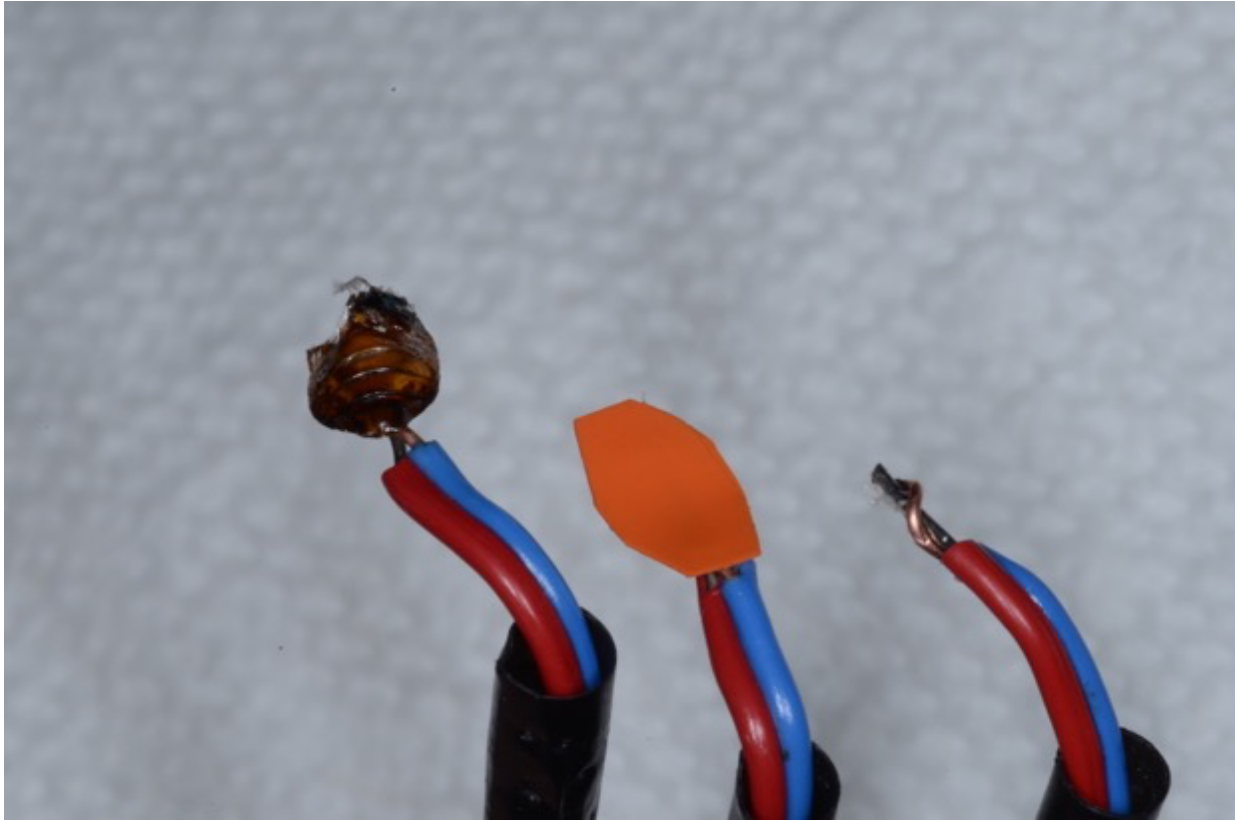


Figure 3: The three types of thermocouples used for the abdominal plate comparison. The dissected ventral abdominal plate of *Cicindelidia haemorrhagica* (left), the similar-sized electrical tape control (center), and the bare thermocouple used for recording ambient air temperatures (right). Photo by JL Bowley.

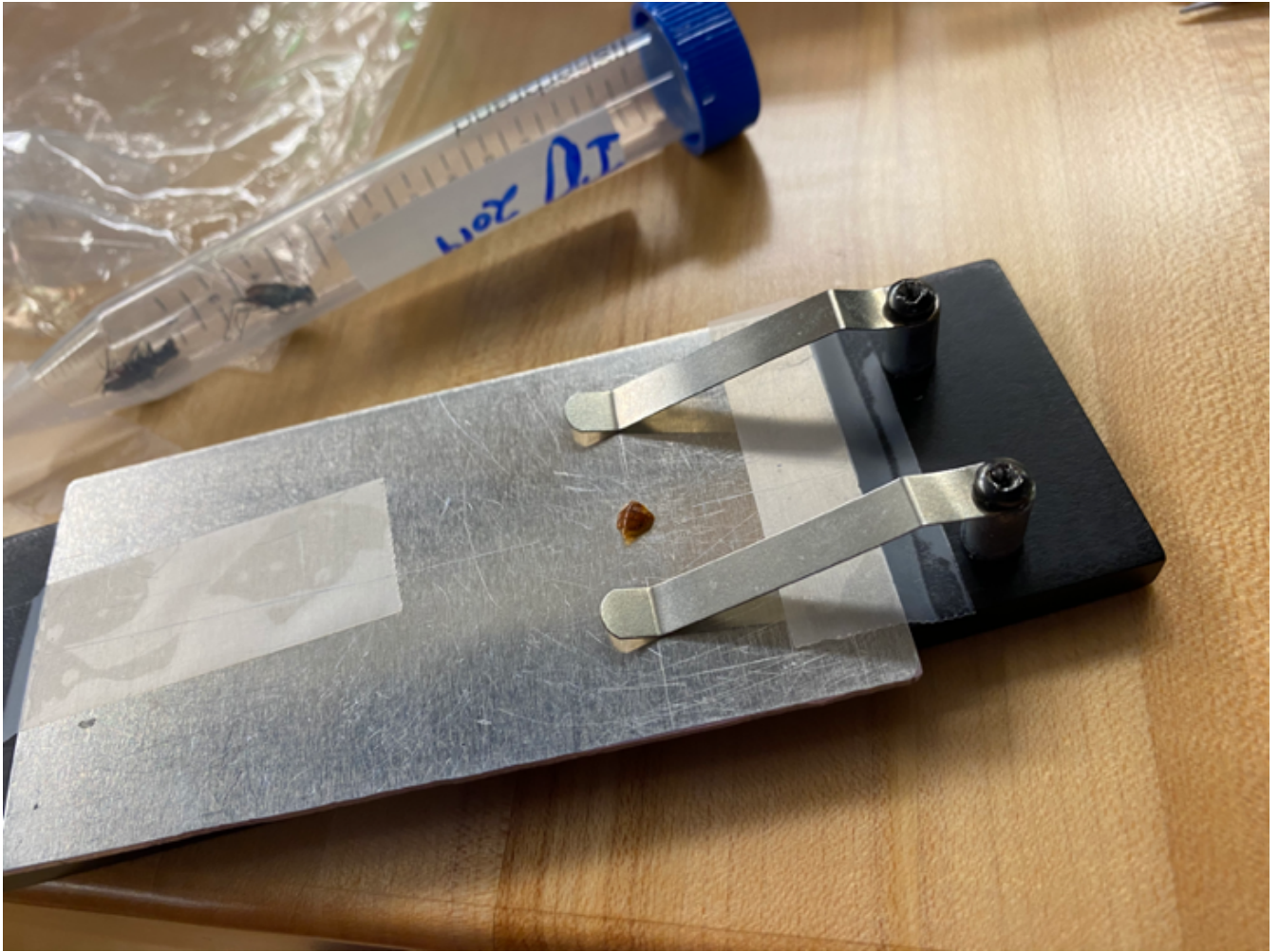


Figure 4: A dissected ventral abdominal plate of *Cicindelidia haemorrhagica* used in the spectrophotometric analysis. It was positioned on this plate after dissection in preparation for recording spectrophotometric measurements in the Cary 5000 UV-Vis-NIR spectrophotometer. Photo by JL Bowley.

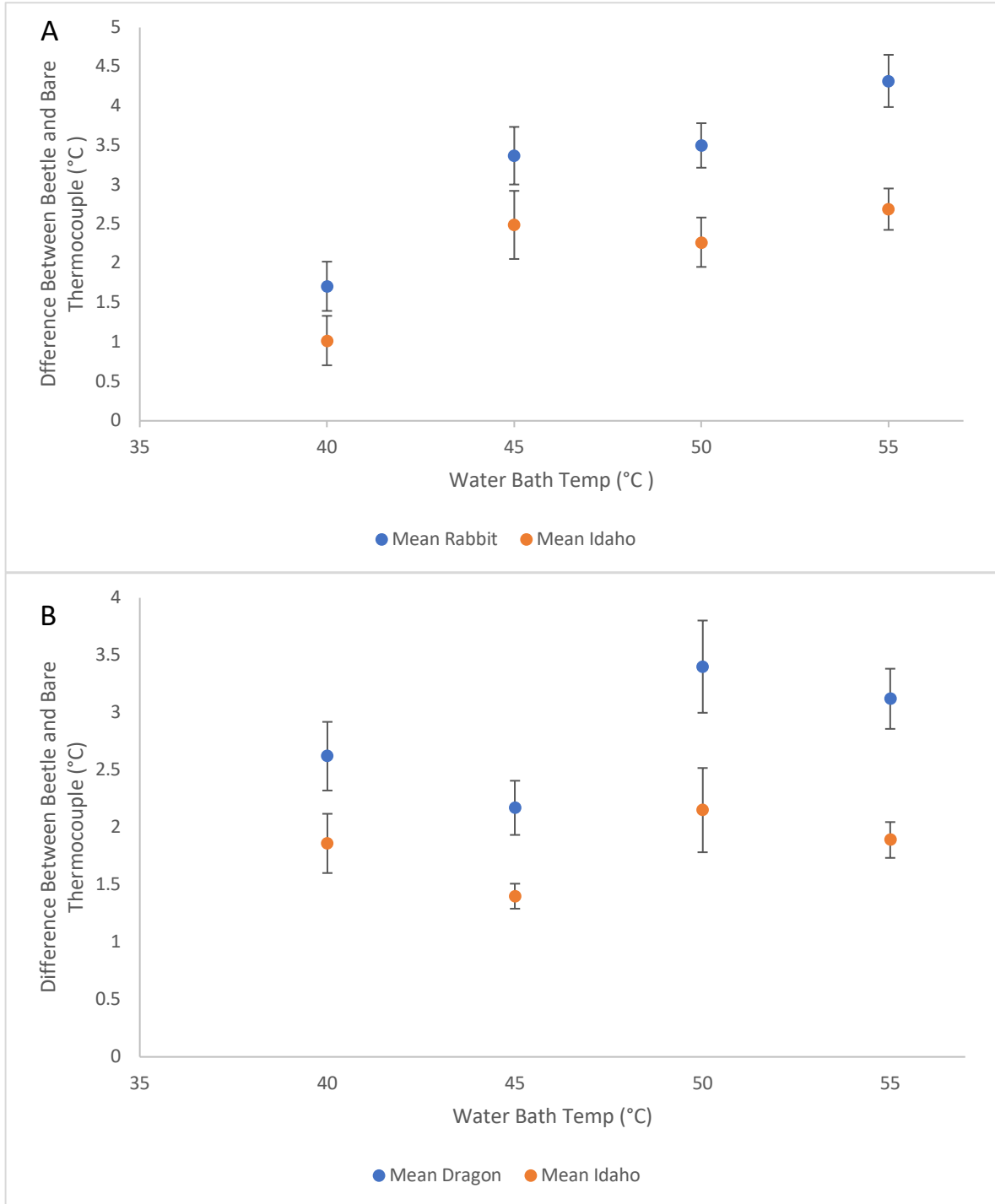


Figure 5: Results of the thermocouple comparison experiments between YNP and Idaho populations. The difference in temperature between the beetle and bare thermocouple used as the control is the displayed variable. Rabbit Creek (A) and Idaho beetles had mean internal temperature differences of 0.69 °C at 40 °C, 0.88 °C at 45 °C 1.22 °C at 50 °C , and 1.63 °C at 55 °C. Dragon Spring (B) and Idaho beetles also had a mean difference in internal temperatures

of 0.76 °C at 40 °C , 0.78 °C at 45 °C 1.25 °C at 50 °C, and 1.23 °C at 55 °C. YNP beetles have greater differences of internal temperature measurements between beetle and bare thermocouple than the Idaho populations for every water bath temperature.

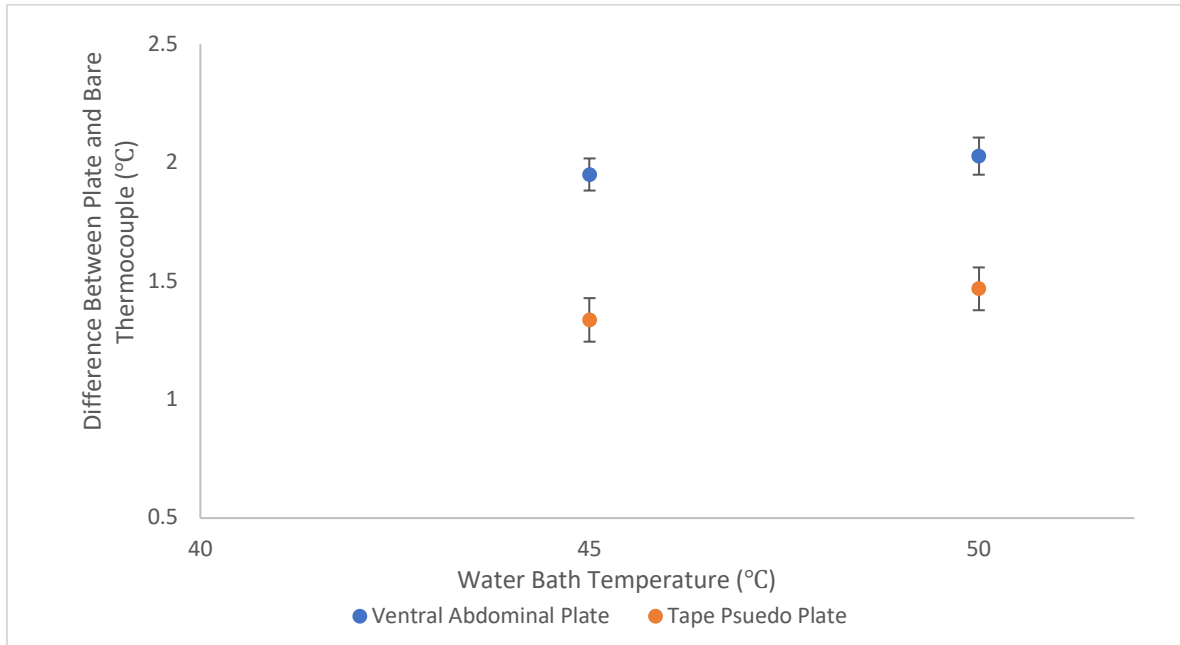


Figure 6: Results of the thermocouple comparison experiments between the ventral abdominal plate and the electrical tape imitation plate between the bare thermocouple control. Differences between the ventral abdominal plate and the imitation plate are 0.61 °C at 45 °C ($p = 0.002$) and 0.56 °C at 50 °C ($p = 0.005$).

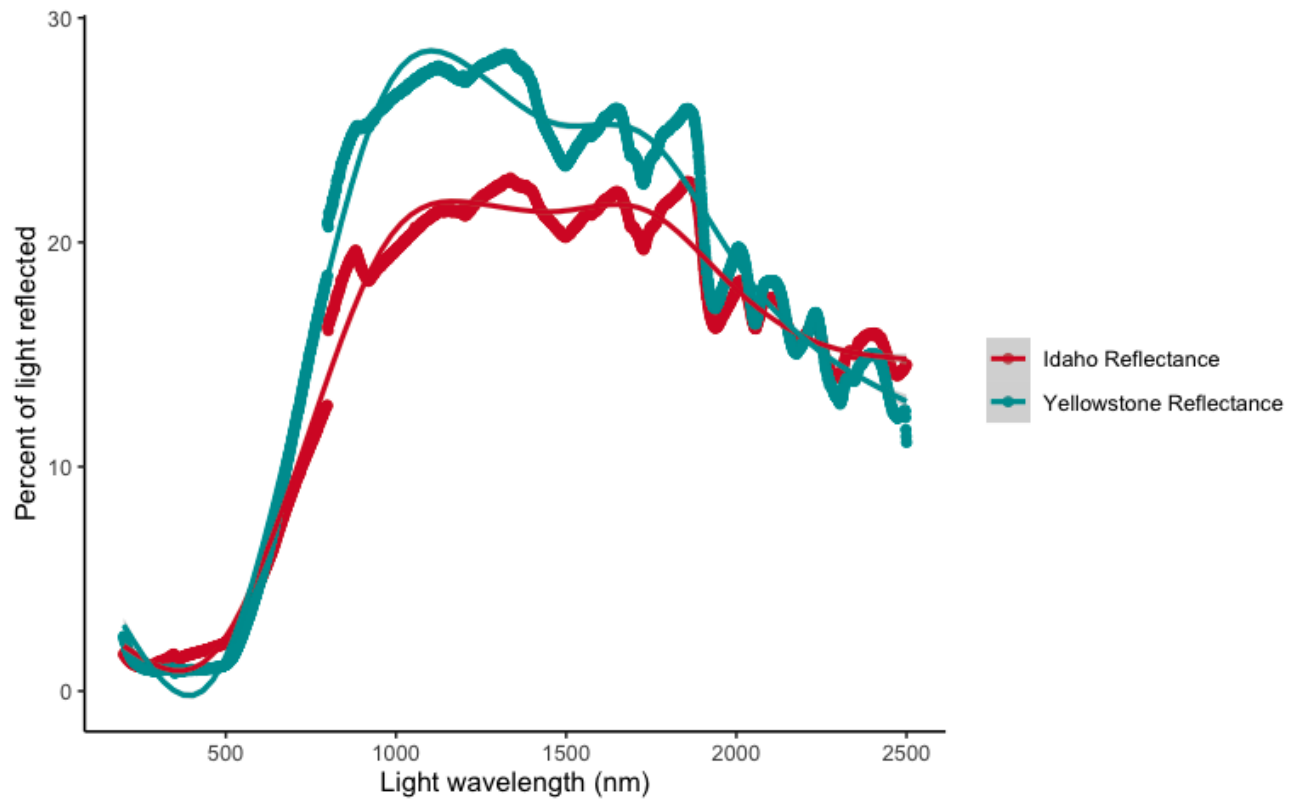


Figure 7: Average reflectance across ultraviolet, visible, and near infrared (NIR) light wavelength range of Yellowstone National Park (YNP) and Idaho *Cicindelidia haemorrhagica* beetles. This figure emphasizes the difference between Idaho and YNP beetle ventral abdominal plates and the reflective properties for radiation in the NIR and infrared (IR) range. The bold line represents raw data with the thin line acting as the reflection data trendline.

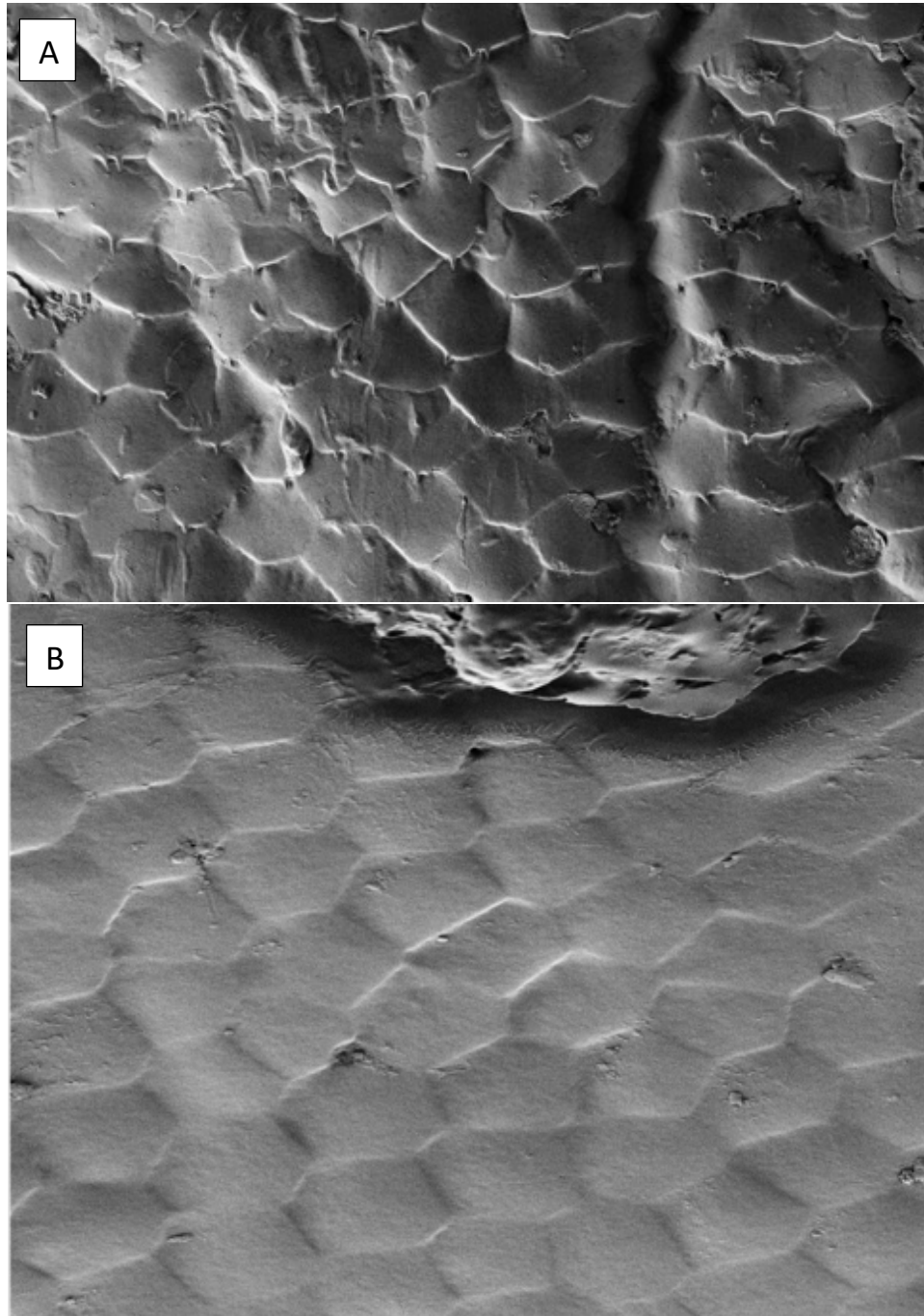


Figure 8: Difference between a beetle with its cuticular wax intact (A) at 1 KX and another with its cuticular wax removed (B) at 1.5 KX magnification of a Yellowstone National Park (YNP) beetle. Both images are at the approximate same location on two separate individuals. Similar differences were observed in Idaho beetles when comparing beetles with and without their cuticular waxes intact.

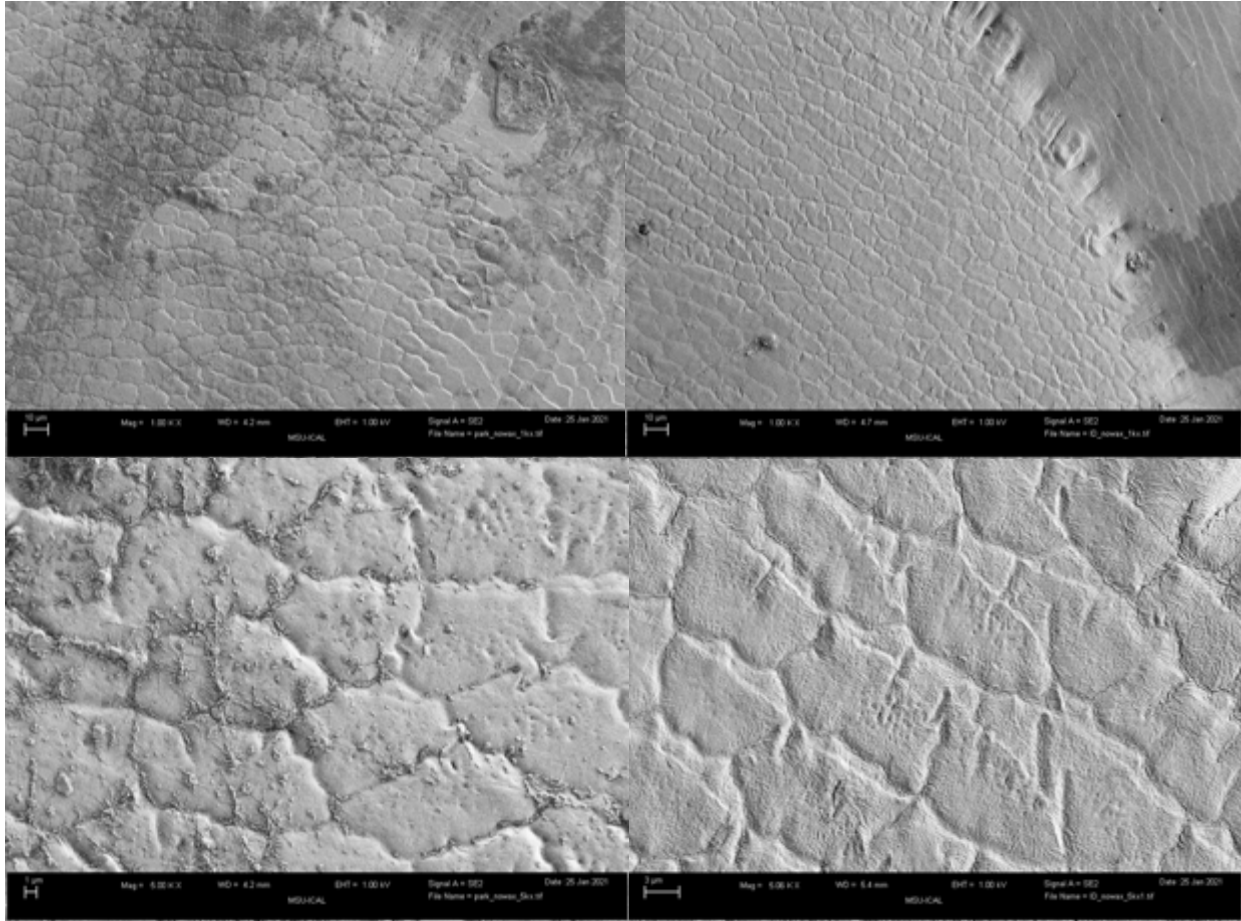


Figure 9. Ventral abdominal plates of YNP (top and bottom left) and Idaho (top and bottom right) ventral abdominal plates taken at 1KX and 5KX magnification respectively.

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CHAPTER THREE

THERMAL PROFILES OF WETSALTS TIGER BEETLE, *CICINDELIDIA*
HAEMORRHAGICA, ACTIVITY IN HOT SPRINGS IN
YELLOWSTONE NATIONAL PARK

Contribution of Authors and Co-Authors

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Contributions: Wrote manuscript for journal submission, conceived and designed experiments, recorded and processed experimental data, analyzed and interpreted results, and organized information for interpretation.

Co-Author: Chelsea Heveran

Contributions: Provided intellectual input for analysis and edited succeeding drafts of the manuscript.

Co-Author: David K. Weaver

Contributions: Provided intellectual input for analysis and edited succeeding drafts of the manuscript.

Co-Author: Leon G. Higley

Contributions: Provided intellectual input for analysis and edited succeeding drafts of the manuscript.

Co-Author: Robert K. D. Peterson

Contributions: conceived and designed experiments, provided input regarding procedures and statistical analysis, and aided in manuscript preparation through editing and writing material.

Manuscript Information

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CHAPTER THREE

THERMAL PROFILES OF WETSALTS TIGER BEETLE, *CICINDELIDIA*
HAEMORRHAGICA, ACTIVITY IN HOT SPRINGS IN
YELLOWSTONE NATIONAL PARK

Abstract

The wetsalts tiger beetle, *Cicindelia haemorrhagica* (LeConte), is found in several active thermal hot spring areas in Yellowstone National Park (YNP) where substrate surface temperatures can exceed 50 °C. However, relationships between surface temperatures and the time adults spend on them remain poorly understood. Therefore, we characterized thermal profiles of Dragon Spring and Rabbit Creek, two thermally active research sites containing *C. haemorrhagica* in YNP, to quantify the time adults spend at different surface temperatures. We took 58 thermal video recordings of adults over six total days of observation ranging from 10-15 minutes for each adult. Thermal video analysis results indicated a positive relationship between total time adult beetles spent on surface temperatures from Dragon Spring and Rabbit Creek as temperatures increased from 20 °C. Once surface temperatures exceeded 40 °C, the total time spent at those surface temperatures declined. Adults were recorded on substrates exceeding 50 °C at only one of the two research locations. Rabbit Creek had substantially more instances of adults with surface temperatures exceeding 40 °C, including one individual on a surface temperature of 61.5 °C. There were three instances of beetles spending more than four minutes at a particular surface temperature, all within the preferred range of 30 °C to 40 °C. Our thermal profile results and previous behavioral observations suggest that adults may be resistant to the

heat produced from the thermal waters that influence the substrate temperatures but may not be subject to high surface temperatures as previously reported.

Introduction

The wetsalts tiger beetle, *Cicindelidia haemorrhagica* (LeConte), has been observed actively hunting and foraging in the hot springs of Yellowstone National Park (YNP) for more than 100 years (Bertholf 1979; Hubbard 1891). Although Hubbard (1891) noted that the environments these beetles inhabited likely exceeded the upper thermal limits for most known insect species, his paper was only an ecological survey for species identification within YNP and he did not study their thermal tolerances.

Outside of YNP, *C. haemorrhagica* is rarely found in environments other than saline flats and sandy, wet substrate. However, in YNP this species thrives in environments with thermal waters, high concentrations of heavy metals, and alkaline or acidic pH that most vertebrates and invertebrates in YNP actively avoid (Fouke 2011; Boyd et al. 2009; Inskeep and McDermott 2005). *Cicindelidia haemorrhagica* is only found in YNP associated with thermal areas.

As ectotherms, insects often behaviorally thermoregulate to adjust their internal body temperatures. Tiger beetles, including species in environments similar to *C. haemorrhagica*, behaviorally thermoregulate in warm conditions by stilting, shading, and dipping their abdomens in water to evaporatively cool themselves (Willemssens 2019; Brosius and Higley 2013; Knisley et al. 1990; Dreisig 1989; Pearson and Leaderhouse 1987, Morgan 1984; Dreisig 1980).

Willemssens (2019) studied several aspects of the ecology of adult *C. haemorrhagica* in YNP.

Adults were observed for several minutes on shallow water and soil surfaces where temperatures exceeded 50 °C. In addition, adults seemed to infrequently engage in cooling behaviors on the

hot surfaces. Willemssens (2019) also compared behaviors between YNP *C. haemorrhagica* and a population of the same species at an Idaho salt flat without hot springs. She observed that YNP populations of behaved differently than the Idaho population. This was especially apparent with thermoregulatory behavior. The YNP beetles spent significantly more time warming and significantly less time cooling than the Idaho beetles. Specifically, there were lower frequencies of shading, posture adjustment (i.e., stiling), and abdomen dipping in water for evaporative cooling were lower (Willemssens 2019). Although Willemssens (2019) observed *C. haemorrhagica* on heated substrates, the measurements of surface temperatures were imprecise because she relied on readings from an IR gun during or immediately after behavioral observations. Consequently, our objective in this study was to characterize more accurate and precise thermal profiles and timespans that individual adult *C. haemorrhagica* spend at different surface temperatures using thermal videography and direct temperature measurement.

Materials and Methods

Site description

Dragon Spring is a relatively well-studied acid-sulfate-chloride spring located in the Norris Geyser Basin that has high levels of As (III) and MeHg⁺ levels in the source waters contains *Hydrogenobaculum* and thermophilic chemolithotroph species (Boyd et al. 2009; D’Imperio et al. 2007; Fouke 2011; Inskeep et al. 2013). Between the numerous highly active thermal water sources for this spring is a peninsula primarily comprised of sulfate and calcium-based sediment that has been the primary research site for *C. haemorrhagica* since ecological research began in 2017 (Willemssens 2019). Here the beetles actively burrow, hunt, forage, mate, and oviposit while being continuously surrounded by thermal waters that directly influence

ground and surface temperatures. Dragon Spring has an average measured pH of approximately 2.88 and running water with sufficiently low temperatures in some areas to allow for the development of acid-loving *Zygogonium* spp. mats that can accommodate stratiomyid and ephydrid populations (Boyd et al., 2009; D’Imperio et al. 2007; Willemsens 2019). The fly larvae that reside in these mats also provide a reliable food source for *C. haemorrhagica* adults in the summer months. In addition to these fly populations, the tiger beetles at Dragon Spring also feed on a variety of small insects, including shore bugs and ephydrid adults, but will also scavenge and feed on anything that falls into the thermal waters and is in shallow water. Most water sources cool sufficiently within a few meters from the source to allow for the growth of bacterial and archaea microbial communities, which produce a mat color that is indicative of water temperature and in some cases, pH (Inskeep & McDermott 2005). The mat colors in addition to the present archaea consistently indicate the extreme temperatures of water sources and associated water temperature falloffs that are present in Dragon Spring. This further indicates that a variety of temperatures may be experienced by insects, both convectively and conductively, when traversing the terrain directly adjacent to the variable temperature thermal waters.

Rabbit Creek is the other hydrothermally active field research site that we selected. Unlike Dragon Spring, this thermal area contains multiple alkaline water sources located in the Midway Geyser Basin. This field research site has an average pH of 9.2. Because this area is a constantly flowing water source that moves from east to west, it produces a wide range of water temperatures that in turn accommodate several different communities with Acidobacteria and Cyanobacterial mats (Weltzer & Miller 2013; Meller et al. 2009). Rabbit Creek is the larger of

the two sites, and contains a steep thermal gradient throughout the waterway, with estimated changes in water temperature of 7.5 °C for every 100 m of continuous waterway. The Cyanobacterial mats present in the waterways directly adjacent to thermal water injections stimulate the growth and development of numerous microbial and arthropod species, including thermophilic ephydriids. These ephydriid populations serve as a primary food source for *C. haemorrhagica* at Rabbit Creek.

Thermal video

Previous infrared thermography studies conducted on insects established protocols for analyzing thermally induced stress in insects and is typically used when recording lower thermal limits (Gallego et al. 2015; Verdú et al. 2012; Palmer et al. 2004). However, no arthropod-related studies using infrared thermal imaging focused on recording the environment directly associated with the study species. Therefore, infrared thermal imaging and videography may serve as a valuable tool to evaluate the microclimates associated with *C. haemorrhagica* in the YNP hot springs ecosystems.

We recorded thermal video at each of the two YNP sites 15-21 July 2020. Recordings were taken using a FLIR T1020 thermal camcorder (FLIR, Wilsonville, OR USA) with a stock 28° lens while mounted on a carbon fiber tripod (Swarovski, Cranston, RI USA). Video files were stored on two 32GB SDHC memory cards.

Recordings took place over three days at each research site. Each recording focused on one beetle at a time to capture the interaction the individual had with its environment (Figure 1). The primary observer with the FLIR recorded from a minimum distance of 5 m from any observed beetle to avoid influencing its behavior during the observation period (Figure 2). The

number of individuals observed per recording varied as there were individuals that flew or ran away and were lost by the primary observer. Beetle thermal recordings were taken twice per hour from 10:00 - 15:00 for a 10 to 15-minute period of observation for each beetle. A total of 28 beetles were observed from Dragon Spring and 30 beetles from Rabbit Creek over the three-day period at each research site. After each day in the field, stored recordings were transferred to a BarraCuda 1TB solid state storage drive (Seagate, Cupertino, CA, USA) and the files on the memory cards were erased after each trip to the field to make room for the proceeding day of recording.

Data analysis

Recordings were analyzed using the FLIR Video Analysis software provided with the T1020 camcorder. Video files were analyzed frame-by-frame using the point temperature-reading tool in the software to follow the individual throughout the entirety of the observation period. Surface temperature values directly below the beetle-with an accompanying timestamp from the video files were recorded in an Excel spreadsheet each time the beetle moved to a new area with different surface temperatures. The point directly below the beetle gave us the most accurate temperature, unobstructed by any solar reflection. Excel spreadsheets were analyzed and manipulated to obtain total time spent at any given temperature throughout the video file using duplicate temperature values and the timestamps that provide aggregate temperature data. An Excel Masterfile was then transferred to RStudio version 4.0.2 for further analysis (RStudio Team 2021). Once the data were condensed into a single data set that could be analyzed using RStudio, diagnostic visualizations of the data assessing statistical assumptions of normality and residual equal variance were performed to determine the shape of the data and assess the

presence of influential points using Cook's distance. Any observation with a Cook's distance greater than 0.5 was considered influential and would have been removed from the analysis. However, no observations surpassed this value so all data points were included in the analysis. The data were initially separated and analyzed by field research site before determining the possibility of a day effect.

Using RStudio with the ggplot, lmer, ggthemes, and sjPlot packages, we produced data visualizations of each day of observational data, compiled data from each site, temperature regression curves from each site to characterize a relationship between temperature and time for an average summer day in July and compared temperature measurement figures to determine the accuracy of the FLIR camcorder.

To accompany these data, we recorded surface soil and water temperatures using an SD-947 4-channel thermometer/SD card data logger (REED, Wilmington, NC, USA). These temperature data were taken and used as a standard for comparison with the thermal video to gauge the accuracy of the temperatures recorded with the FLIR camcorder (Figure 3). Two of the four thermocouples were placed on the water's surface near the most beetle-rich area at each field research site. The remaining two were placed on the soil's surface near the water's edge. The logger was set to record four temperatures, one from each thermocouple, every five minutes from 10:00-15:00 for two of the three days at both Rabbit Creek and Dragon Spring. Additional temperature recordings were also taken with an IR gun (Fluke, Everett, WA USA) in a grid pattern across both research sites every other hour to obtain temperature readings from a third source. These measurements were used to produce a temperature regression curve for the

warmest time period of day at each site while exposed to direct sunlight in the thermal environment.

Results

Compiling the individual observations using time stamps with accompanying temperatures resulted in total time spent at each surface temperature for each of the three days of observation at each research site. There were 3,414 observations of temperature–total time change instances recorded across the 58 total beetle thermal recordings. We found that *C. haemorrhagica* spent an average of 1.41 minutes at a mean surface temperature value of 36.4 °C. The mean value differed between research sites ($p < 0.001$). Beetles spent a mean of 1.45 minutes at a mean surface temperature of 38.9 °C at Rabbit Creek compared to 1.38 minutes at 33.1 °C at Dragon Spring.

There were few instances where beetles at Rabbit Creek were on surfaces exceeding 50 °C. The maximum surface temperature on which an adult beetle resided was 61.2 °C for 1.5 seconds (Figure 4 and 5). The greatest amount of time an adult spent at a temperature greater than 50 °C was 28.2 seconds at 55.1 °C (Figure 4 and 5). This single observation was the second highest surface temperature recorded. In addition, there were instances of adults on surfaces with two observations at 53.1 °C for a cumulative time of 28.1 seconds, a single observation at 51.7 °C for 17.6 seconds, and a single observation at 50.9 °C for 12 seconds (Figure 4 and 5). All observations of adults on surfaces exceeding 50 °C were from Rabbit Creek. Beetles at Dragon Spring spent a maximum cumulative time of 7.43 minutes at 32.9 °C. Individuals at Rabbit Creek spent a maximum cumulative time of 6.32 minutes at 32.6 °C.

Dragon Spring surface temperatures below the beetles were, on average, lower than those of Rabbit Creek based on the FLIR camcorder measurements (Figure 4 and 5). Variance in temperature data collected throughout each observational period may have been due to cloud cover, soil saturation, and camera angle throughout the recording. However, there is considerable overlap in the data, suggesting that there may be no statistical difference between the two sites. However, our linear model using temperature as a dependent variable of day of observation and research site suggests that there was a substantial statistical difference between research site within YNP ($p < 0.001$).

A polynomial regression curve was fit to the data for each research location to describe the relationship between total time spent and surface temperature (Figure 6). The temperature-total time relationship regression curves for both research locations contain the estimated values with a 95% confidence interval for the true mean value for total time spent at the 20 °C to 60 °C temperature range. The apex of the relationship curve at approximately 38 °C at Rabbit Creek indicated a larger amount of time spent at higher temperatures than at apex of approximately 32 °C at Dragon Spring (Figure 6).

We compared the temperature values recorded with the FLIR camcorder to the direct thermocouple measurements (Figure 7). We estimated the substrate surface temperatures using two labeled thermocouples while measuring subsurface water temperatures with two additional thermocouples. These measurements, used as the standard for comparison for the FLIR data, recorded temperature measurements from the same height and had similar sun exposure to what the beetles experience. The majority of the FLIR data were similar to the temperature measurements we observed with the thermocouples. Any differences were likely because the

beetles were seeking out warmer surfaces whereas the thermocouples were measuring background surface temperatures.

Discussion

Cicindelia haemorrhagica adults spend a considerable amount of time on surfaces with increased surface temperatures in YNP relative to the environments where this species is typically found. Moreover, they spend much of their adult lives (four to eight weeks) on these relatively high temperature surfaces seemingly without the need to behaviorally cool, and therefore seem different than many other insects. Previous research suggested that YNP beetles are on surfaces exceeding 50 °C (Willemssens 2019). Our results reveal that beetles are on surfaces that exceed these temperatures, but they do not remain on these surfaces for long periods.

There are many tiger beetle species that can survive in thermally extreme environments, but they typically prefer cooler areas where food items are more likely to be present (Brosius and Higley 2013; Pearson and Leaderhouse 1987). These species primarily remain in areas between 30 °C and 40 °C, where they are more likely to mate, forage, and hunt without the risk of overheating (Pearson and Leaderhouse 1987; Dreisig 1981; Dreisig 1980). Therefore, we would expect *C. haemorrhagica* populations outside of YNP and other warm- adapted tiger beetle species to behaviorally thermoregulate by constantly shifting microhabitats, stilting, and basking to regulate their internal body temperatures in YNP conditions (Brosius and Higley 2013; Pearson and Leaderhouse 1987; Schults and Hadley 1987; Dreisig 1981; Dreisig 1980). These thermoregulatory behaviors in extreme conditions are not limited to tiger beetles alone. The Saharan silver ant, *Cataglyphis bombycina* (Hymenoptera: Formicidae), is currently the

most heat tolerant insect known. Workers of this species have been observed on desert substrates that exceed 60 °C (Shi et al. 2015). When on these surfaces, workers behaviorally thermoregulate by stilting, basking, and climbing vegetation to get away from the high surface temperatures of their environment. Body orientation and scaling vegetation is also an important tool for thermoregulation in species such as *Melanoplus sanguinipes* (Orthoptera: Acrididae), which prefers temperature ranges between 37.4 °C and 40.5 °C and must behaviorally thermoregulate throughout the day to remain in this preferred temperate zone (O'Neill and Rolston 2007). Other beetle species continuously move with shaded areas throughout the day to reduce their direct exposure to the sun (Alves et al. 2018).

Much like *C. bombycina*, we predict that YNP *C. haemorrhagica* can resist high surface temperatures for extended periods of time. However, unlike *C. bombycina*, which possess lightly colored triangular setae that provide increased reflectance from the sun and black body radiation from the sand on which it forages (Shi et al. 2015), we do not yet know the exact mechanism for *C. haemorrhagica* (Bowley 2021).

Because *C. haemorrhagica* spends little time behaviorally cooling themselves, as compared to another population of the same species in Idaho, there is likely some mechanism that is responsible for resistance to bottom-up heating. Willemssens (2019) showed that YNP *C. haemorrhagica* adults might have a slightly greater critical thermal limit than the adults of the Idaho population, but the explanation seems to be more physical than physiological. We know that exoskeletal structures may influence internal body temperatures from previous research but isolating and identifying the mechanism responsible can be difficult (Amore et al. 2017). The mechanism might be a physical structure similar to that of *Rosalia alpina* (Coleoptera:

Cerambycidae), which uses pyramid shaped microstructures on its elytra to improve their radiative heat exchange with their environment (Pavlovic et al. 2018). The mechanism may also contain multiple structures that work together to produce improved thermal tolerance or resistance, or both.

For instance, *Neocerambyx gigas* (Coleoptera: Cerambycidae), possess reflective micro setae with corrugated facets on one side. Cross sectional analysis of these setae revealed that the corrugated facets work with the three-dimensional pyramidal shape of the setae to provide total internal reflection along the elytra, lowering the overall internal temperature of the beetle (Zhang et al. 2020). Structures working together to reduce temperature increases are also found in the multiple hollow cylindrical structures found on the elytra of *Goliathus goliatus* (Xie et al. 2019). However, the structure on *C. haemorrhagica* in YNP is more likely to be present on the venter as opposed to the dorsal face of the beetle (Bowley 2021). *Cicindelidia haemorrhagica* experiences much of its environmental heat from conductively heated surfaces as opposed to the direct heat from the sun experienced by *N. gigas* and *G. goliatus*. Bowley (2021) demonstrated that the mechanism is most likely associated with the physical structure of the abdominal ventrites and is not associated with the cuticular wax structure or composition. This suggests the mechanism may lie within the structural layering of the cuticle in *C. haemorrhagica*, which has been known to play a role in heat transfer with other tiger beetle species (Seago et al. 2008; Schultz and Hadley 1987). However, to date, more refined structural explanations have not been elucidated. We used thermal video of *C. haemorrhagica* to capture adults interacting with surface temperatures of conductively heated substrate in thermal hot spring areas of YNP. Previous behavioral thermoregulation data of these beetle populations indicated that they were active in

environments with surface temperatures exceeding 50 °C (Willemssens 2019). A thermal profile was developed for two research sites that focused on recording substrate surface temperatures directly underneath adults to further understand the conditions these populations experience throughout the summer. Our results showed that *C. haemorrhagica* spent a large portion of their time on substrates with surface temperatures exceeding those experienced by many insects. There were multiple instances where adults were recorded on surfaces that exceeded 50 °C surface temperatures. One individual was observed running on an area with a surface temperature over 60 °C for 1.5 seconds. Another individual remained on substrate with surface temperatures exceeding 55 °C for nearly 30 seconds. This suggests that *C. haemorrhagica* might exhibit a physical and morphological mechanism associated with exoskeleton (Bowley 2021). This mechanism will need to be explored to better understand the heat-resistant capabilities of *C. haemorrhagica* within YNP.

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Table 4. Summary statistics of the linear model describing temperature with respect to the research location and day of observation. Predicted values are provided using day one of observation at Dragon Spring as the base intercept for the linear model. P values for each day and site are presented in addition to 95% confidence intervals for each predicted value of the model with an R^2 value of 0.5.

Temperature (°C)			
<i>Predictors</i>	<i>Estimates</i>	<i>95% Confidence Intervals</i>	<i>p</i>
Dragon Spring, Day 1 (Intercept)	30.61	30.33 – 30.88	<0.001
Day 2	2.32	1.98 – 2.66	<0.001
Day 3	3.39	3.07 – 3.71	<0.001
Site [Rabbit Creek]	7.88	7.60 – 8.15	<0.001
Total Observations	3414		
R^2 / R^2 adjusted	0.505 / 0.504		

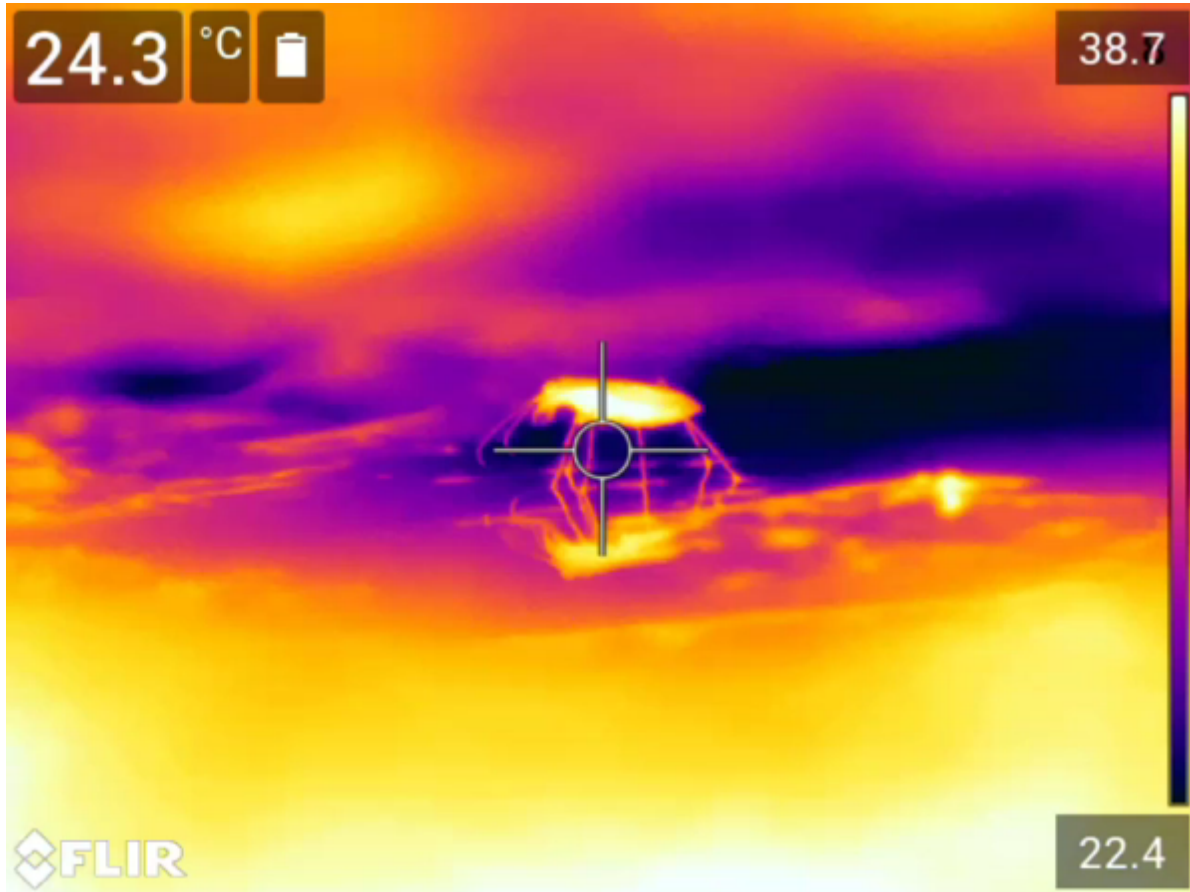


Figure 1. A still image from the thermal video recording. The reflection of the beetle on the substrate is the more accurate surface temperature of the substrate not influenced by solar reflection and was the target area for measurement when analyzing the temperature-time relationship data in the recordings. Video still by JL Bowley.



Figure 2. Thermal recording equipment on-site at Dragon Spring. The thermocouple reader/recorder can be seen in the background recording substrate and water temperatures while adult *Cicindelidia haemorrhagica* traverse the area.



Figure 3. Thermocouple reader/recorder recording temperatures at Dragon Spring. Two thermocouples were positioned near the surface of the water. The other two (not shown) were recording substrate surface temperature.

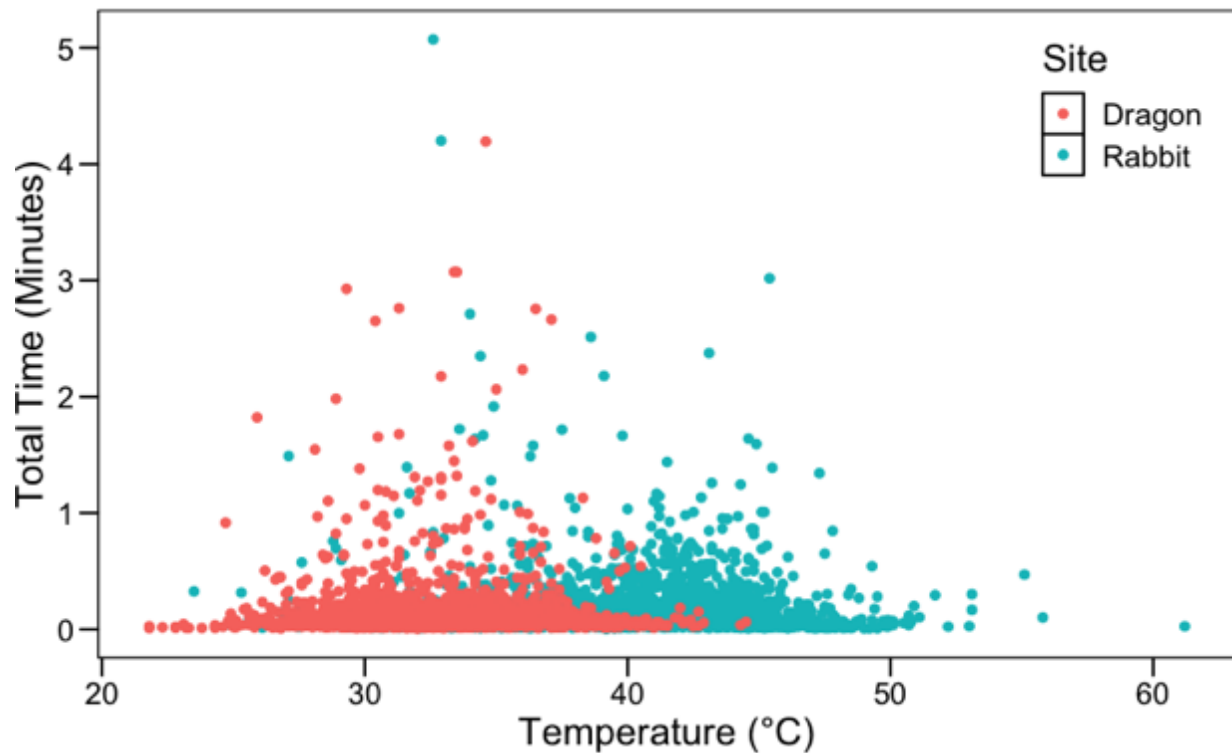


Figure 4. Surface temperatures for each research site. Each point represents the time spent by one adult *Cicindelidia haemorrhagica* at a specific surface temperature.

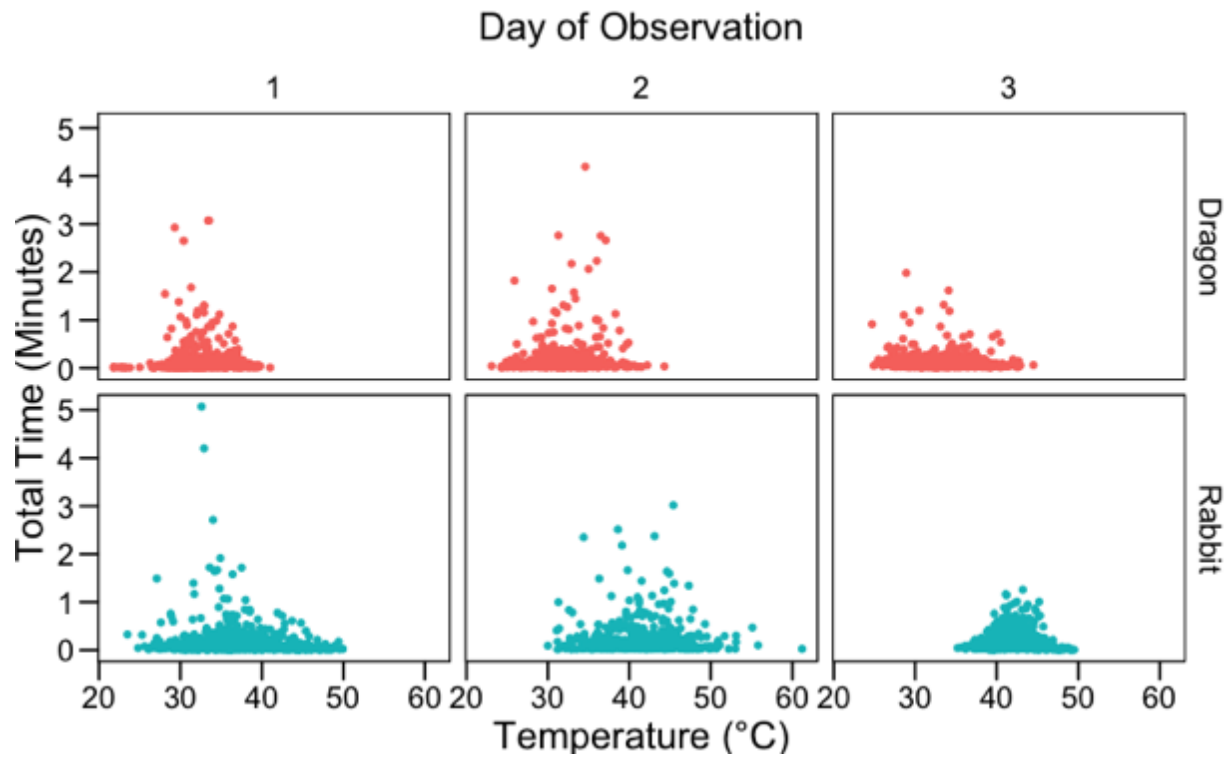


Figure 5. Surface temperatures for each day of recording from Dragon Spring and Rabbit Creek. Each point represents the time spent by one adult *Cicindelidia haemorrhagica* at a specific surface temperature.

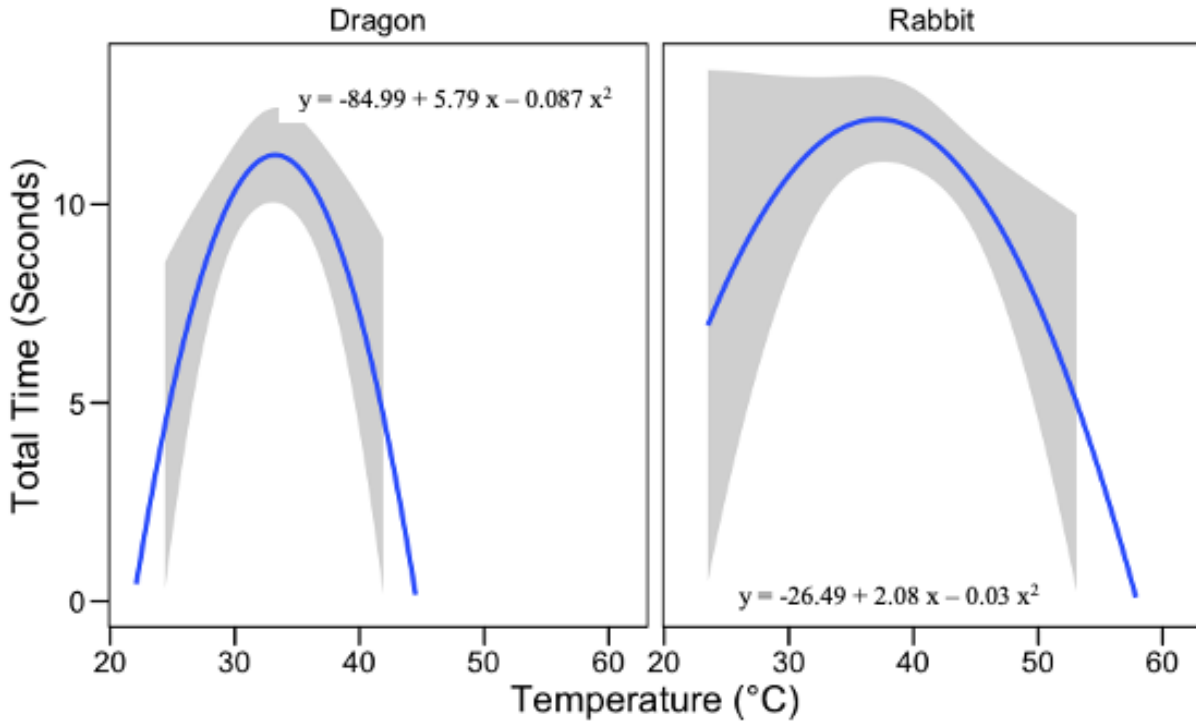


Figure 6. Regressions of the relationship between substrate surface temperature from each research site and the time adult *Cicindelidia haemorrhagica* spent at those temperatures. The blue line is the fitted values using the total time averages from each research site. The grey shaded area represents the 95% confidence interval for the average value.

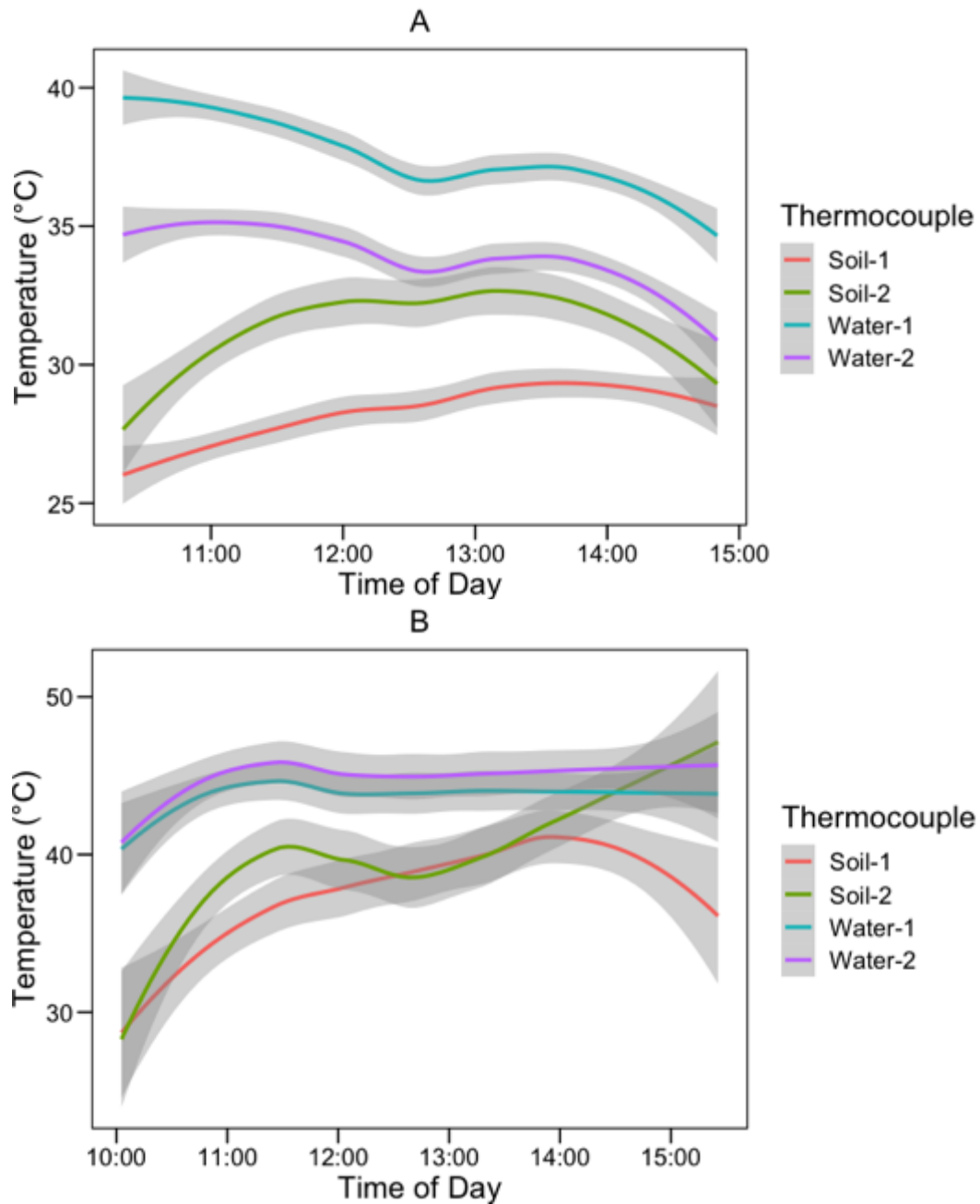


Figure 7. A) Average temperatures during periods of observation at Dragon Spring recorded by thermocouples. B) Average temperatures during periods of observation at Rabbit Creek recorded by thermocouples.

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CHAPTER FOUR

CONCLUSION

Results from our thermocouple experiments comparing Yellowstone National Park (YNP) and Idaho populations of *Cicindelidia haemorrhagica* suggest that there are differences between internal temperatures when they are subjected to the same environmental conditions. We have determined that the primary cause of this observed difference is not improved thermal tolerance nor is it a liquid component interacting with the internal anatomy of YNP beetles. The difference between them is likely some form of physical or structural mechanism that acts as an near-infrared radiation reflective exoskeleton on the ventral face of the abdomen of YNP beetles.

Future cross-sectional comparisons between the exoskeletons of YNP and Idaho beetles need to be conducted using more refined imaging tools and procedures to better understand the cuticular layering of the ventral abdominal plate. Perhaps the differences between the YNP and Idaho exoskeletons lie in the composition of YNP beetle cuticle and the reflective capabilities are consequences of living in their thermally active environments. A molecular composition analysis of the layering would help us better understand the potential of cuticular layering in any future comparison experiments with YNP beetles and other potential comparison populations.

The emergence of these thermoregulatory adaptations in YNP populations of *C. haemorrhagica* that we observed are relatively recent. The Pinedale Icecap was more than 1,200 meters thick in some areas 17,000 years ago in what is now YNP (Pierce 1982). Assuming populations of *C. haemorrhagica* moved to the newly emerged thermally active areas as soon as the Pinedale Icecap receded starting 14,000 years ago, there have only been relatively few generations to adapt to the hot springs environments of YNP.

However, in the relatively short time that they have inhabited YNP thermally active environments, we have observed the effects of the YNP hot springs. YNP beetles are likely to continue to adapt in the thermally active environments, which may, over time, lead to further diversification between populations both in and outside of YNP.

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