ESTABLISHING AGE-BASED COLOR CHANGES FOR THE AMERICAN BURYING BEETLE, NICROPHORUS AMERICANUS (OLIVIER)

By

ROBERT SHANE MCMURRY

Bachelor of Science in Horticulture

Oklahoma State University

Stillwater, OK

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2022

ESTABLISHING AGE-BASED COLOR CHANGES

FOR THE AMERICAN BURYING BEETLE,

NICROPHORUS AMERICANUS (OLIVIER)

Thesis Approved:

Dr. Wyatt Hoback

Thesis Adviser

Dr. Justin Talley

Dr. Andrine Shufran

ACKNOWLEDGEMENTS

I would like to thank the following people: Dr. Wyatt Hoback for his continued support and encouragement of me to pursue this degree, Dr. Andrine Shufran for allowing me to work for the Insect Adventure for so many years, Dr. Justin Talley for allowing me this opportunity in the Department of Entomology and Plant Pathology at Oklahoma State University, Dr. Michael Cavallaro for performing the statistical analysis and his helpful insights throughout this project, and Lauren Osborn for encouragement, editing, and grammatical assistance. I would also like to thank Daniel Koch, Glenn Frei, and Kayla Garcia, at the St. Louis Zoo for providing invaluable husbandry advice. Most importantly, I would like to thank my partner Sunny Evans, without whose assistance, support, and patience this endeavor would not have been possible.

Name: ROBERT SHANE MCMURRY

Date of Degree: DECEMBER, 2022

Title of Study: ESTABLISHING AGE-BASED COLOR CHANGES FOR THE AMERICAN BURYING BEETLE, *NICROPHORUS AMERICANUS* (OLIVIER)

Major Field: ENTOMOLOGY

Abstract:

The American Burying Beetle, *Nicrophorus americanus* (Olivier) is the largest North American species of carrion beetle (Silphidae). Due to prey loss and 90% reduction in historic range is federally listed as threatened at this time. Recovery of the species requires observation and cataloging of individuals captured in the wild. Research has been conducted to determine the lifespan, habitat, and range of the beetle; however, it is difficult to determine age in the field beyond teneral (young) and senescent (old). Since a demonstrable color difference was observed under laboratory conditions, we propose that a color gradient exists and can be used estimate the age of an individual beetle. From data of four beetles over their lifetime, the color of the pronotum and elytral markings were shown to darken in a gradual, predictable manner with ageing. The elytral markings darkened at a slower rate than the pronotum. Applications to field research are discussed as well as potential complicating variables including pigment involved, temperature, and other environmental factors.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Arthropods are among the most diverse animals in regard to lifespan. The longest lifespan of any insect currently recorded belongs to the ant species *Lasius niger* (Hymenoptera: Formicidae) at 28.75 years in captivity, while female mayflies (*Dolania americana*) live only five minutes after emerging into adulthood (Zeng, 1995). Beetles are variable in lifespan with male *Lucanis cervis* (Coleoptera: Lucanidae) living only 19 days as an adult (Crowson, 2013). *Nicrophorus americanus,* the American Burying Beetle (ABB) are known to be univoltine, where new adults emerge and overwinter, then reproduce the following year before senescing and dying (Smith, 2002). When collecting these beetles in the field, it is important to determine the age of the beetle because it affects how many broods they can produce and the likelihood of successful breeding. Survival of *Nicrophorus* beetles is dependent on many factors including size, overwintering conditions, and age (Bedick et al, 1999). However, little published data exists on how long *Nicrophorus* beetles, specifically the ABB, survive in captivity.

The ABB is the largest carrion beetle in North America with individuals reaching up to 35 mm in length (Raithel, 1991; Holloway & Schnell, 1997). ABBs and other

Nicrophorus are beneficial to the environment by reducing fly breeding resources, decomposing organic matter, cycling nutrients, and increasing soil fertility (Hoback et al, 2020) through burying carrion and rearing their young together—a rare trait among invertebrates that sometimes contributes to greater longevity (Carey 2001). Like other *Nicrophorus* species, the male and female ABB will find carrion of appropriate size using chemosensors on their antennae and can travel long distances in their search (Bedick et al, 1999). They assess the substrate and, if necessary, move the carrion to a suitable location to bury it—removing the hair or feathers and molding the carcass into a ball (Scott, 1998). While cleaning the carcass, burying beetles also cover it in oral and anal secretions that are anti-bacterial and delay the decomposition process (Hoback et al, 2020). Burial chambers can be as deep as 60 cm underground (Pukowski, 1933; Wilson & Fudge, 1984). Eggs are then laid around the brood ball, which has been prepared by opening a small depression at the top treated with the anti-bacterial oral fluids. The larvae feed after hatching (Pukowski, 1933) by either receiving food from the parents or feeding directly from the brood ball (Scott, 1998). Under laboratory conditions the larvae are fed and cared for by one or both parents for approximately 10 days at which point the larvae disperse and enter the pupal stage for approximately 50 days.

The ABB range was previously known from across eastern North America and into parts of Canada (Ratcliffe, 1996; Lomolino et al, 1995). However, numbers and distribution have declined—as much as 90%—with remaining populations currently recorded in only six states: Rhode Island, South Dakota, Nebraska, Kansas, Oklahoma, and Arkansas (Anderson, 1982; Lomolino et al, 1995). In 1991, a population of ABB was discovered at

Cherokee Wildlife Management Area/Camp Gruber in Muskogee and Cherokee counties in eastern Oklahoma, which provided data on the ABB's habitat usage in Oklahoma as the population inhabited a relatively undisturbed area (Creighton et al, 1993). From these data, researchers discovered that undisturbed areas had higher numbers of ABB versus post-agricultural fields. Further, ABBs were found to be most abundant in hardwood forests—a more typical habitat for the beetle's range before its decline. Creighton et al (1993) also suggested that ABB may be more like *N. tomentosus* in relation to habitat preferences than to other species of *Nicrophorus* beetles specifically in Oklahoma. This illustrates the variability of ABB's habitat preference and the complex factors involved in their range loss. Because of its decline, the ABB was listed as federally endangered in 1989 (Bedick et al, 1999) but later reclassified as threatened in 2020 by the Fish and Wildlife Service based on information that suggested a diminished threat of extinction in its current range (U.S. Fish and Wildlife Service, 2020).

Attempts to restore the ABB populations to areas where it once occurred have been underway since 1991 with varying success (U.S. Fish and Wildlife Service, 2019). Because of these restoration efforts, it is important for scientists to identify the age of wild-caught beetles—as age is an integral factor that influences success in rearing lab colonies to release in the wild. Creighton et al (2009) discovered that resource allocation of female *Nicrophorus* beetles increased with age and shortened their lifespan after breeding. Older females allocated less of the carrion to their own body mass, reserving the bulk of the resource for their offspring. Further, females were found to produce larger broods and larger eggs when the females were 65 days old (Creighton et al, 2009). Age-

grading has been utilized by Bedick et al (1999) to document the ABB's life history by noting the clypeal membrane hue and brightness. The clypeal membrane hue and brightness have been associated with immune response in *N. pustulatus* and was found to be positively correlated with age; the more red (lower hue values) and darker (lower brightness values) membranes indicated an increased immune response in both sexes (Wormington & Luttbeg, 2018). However, there has yet to be a study examining ABB pronotum color and its association with age. The objective of this study is to determine if the ABB's pronotum becomes darker with age in a measurable progression.

CHAPTER II

REVIEW OF THE LITERATURE

Age Grading in Insects

Arthropods are among the most diverse animals in regards to lifespan. Currently, the longest recorded lifespan of any insect belongs to the species *Lasius niger* (Hymenoptera: Formicidae) at 28.75 years in captivity (Walker, 1996). By contrast, female *Dolania americana* mayflies live only five minutes after emerging into adulthood—within which time she mates, lays eggs, and dies (Walker, 1996). Despite these examples science has barely begun to discover the vast diversity in aging patterns and mechanisms of insects. For entomologists, accurate age grading is an invaluable contribution to the understanding of insect ecology and behavior. Age grading aids researchers in determining the number of viable broods an insect can produce, probability of successful breeding, survival rates in captivity, and how long an insect has been in a specific location. Further, knowledge of a population age structure makes it possible to draw conclusions about population changes over time (Hayes & Wall, 1994).

However, age grading is often a complicated task as age-related fitness traits are sensitive to a range of environmental factors including temperature, humidity, diet, and oxygen

levels (Promislow, 2022). Research on aging typically relies on experimental cohorts of individuals of known age, or on physiological, morphological, or behavioral traits assumed to vary with age (Hartmann et al 2019). Yet, lab-grown specimens can differ greatly from those caught in the wild, adding another complication to insect aging research. Multiple studies have been conducted to determine the best indicator for insect aging with varying results (e.g., Carey, 2001; Keller & Genoud, 1997; Partridge, 1986). Here I discuss how insect age is currently assessed, its limitations, and how insect color may be used as an indicator for insect aging.

Insect Age Grading Methods

Chronological Age Grading

Chronological age grading refers to the practice of measuring the time between an insect's emergence to the end of its last larval instar (Lehane, 1985). Examples of this method include determining the age of the female by calculating her rate of reproductive development through dividing her previous ovipositions by the temperature measured in degree days (Saunders, 1962; Vogt *et al*., 1974; Wall *et al*., 1991). Degree days refers to the number of days above a threshold average daily temperature that it takes for an insect to mature. These degree days vary across species. While this is among the most common form of age grading, all chronological age grading techniques rely on assessing physiological changes in the insect, and thus may be subject to interference from outside factors–leading to imprecise data (Hartmann, 2019). For example, in addition to

temperature, nutrition and light-intensity have been found to alter insect physiology across species (Robson et al, 2006). Consequently, no methods, with the exception of those based on cuticular growth layers, directly measure chronological age as they all rely on behavioral and physiological history of the individual (Hayes 1999).

Another form of chronological age grading is assessing age through Near-Infrared Spectroscopy (NIRS). A study by Perez-Mendoza et al (2004), tested if NIRS could be used to assess chronological age in adults of the stored-product beetles *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Tribolium castaneum* (Coleoptera: Curculionidae, Bostrichidae, and Tenebrionidae, respectively) through analyzing cuticular lipids, with additional objectives to assess whether NIR wavelengths absorbed by water influenced age-grading. This study found NIRS to be valuable in determining age in weevils through analyzing water content and cuticular lipids. The benefits of NIRS include not having dependence on adult sex or temperature. Further, NIRS is a non-destructive technique which can assess large populations at a time. However, it is expensive as NIRS requires a spectrophotometric system with a fiber optic probe that requires careful calibration (Perez-Mendoza et al, 2002).

Reproductive-Based Age Grading

Typically, insect reproduction requires a male and female. The female possesses ova that require fertilization by male sperm. If the eggs are successfully fertilized, the female becomes gravid, and the eggs will develop inside of her. The female will then deposit the

eggs in a suitable location where they hatch. During the reproduction process, the female's reproductive system undergoes permanent morphological changes, which are the basis for reproductive-based age grading techniques (Hayes, 1999). Such age grading techniques can be valuable when reproductive information about the insect is important, and often is used in relation to pest management. Additionally, because this technique is applied to a single individual, it has the potential to be highly accurate (Hayes, 1999). However, there are many drawbacks. First, reproductive-based age grading is a destructive technique, requiring sacrifice of a live individual for dissection to observe internal reproductive morphology. Second, numerous factors can influence the morphological variability of individuals. Protein intake, previous mating experience, and dehydration can impact ovulation and ovarian development (Vogt & Walker, 1987; Stoffolano et al, 1995; Spradbery & Vogt, 1993). Additionally, the data collected from reproductive age grading are only applicable to the females of the species. In practicality, these techniques require lab technology–making them unviable for most field research and subject to error.

Somatic-Based Age Grading

Somatic-based age grading refers to assessing age related to an insect's physiological features. One example is age grading based on cuticular deterioration. As insects age, they undergo irreversible damage to their exoskeletons. This damage can be caused by predation, normal activity, or abrasion from the environment (Hayes, 1999). Damage to the wings, or "wing fray", is a commonly analyzed somatic character for age grading. A

study by Jackson (1946) revealed that damage to wings had about a 95% correlation with chronological age. Wing fray-grading's popularity can be attributed to its simplicity and applicability to both male and female insects. Additionally, it is low-cost and can be easily performed in the field. Other forms of exoskeleton damage such as that on the tibia, mandibles, abdominal sternites, ctenidia, and setae have successfully been used as a basis to determine age (Tyndale-Biscoe et al, 1981; Butterfield, 1996; Kosminsky, 1960; Corbet, 1960). Similar to "wing fray", assessment of exoskeleton damage is independent of sex. However, other environmental factors such as protein availability and temperature could influence the rate of insect activity–leading to variations in accuracy (Hayes, 1999).

One subset of somatic changes is based on the accumulation of pteridine pigments in the compound eyes of various insect species. Pteridines fluoresce under UV light and accumulation can be quantified using a fluorescence spectrometer (Mail et al, 1983). Pteridine pigments are created through the breakdown of purine and have been shown to increase in a linear fashion with age for many, but not all fly species studied (Dimitriv et al, 2020; Lardeux et al, 2000) and have begun to be explored for age grading in other insects, including ants and bed bugs (Hartmann et al, 2019; Kremenova et al, 2020).

Color in Insects

Insect colors visible to humans and instrumentation are broadly classified as either pigmentary or structural. Structural coloring comes from diffraction or interference of light across microscopic structures found on insect cuticles. Colors that are often

structural include white/UV and iridescent blues, greens, and purples, although some reds and oranges can be non-pigmentary as well (Seago et al, 2009). White and UV on beetle exoskeletons are always structural, created from the scattering of light by structures like microtrichomes on the cuticular surface (Seago et al, 2009). Iridescence is common in beetles and frequently results from cuticular structures in different shapes or layers (e.g., "thin films") so that the hue changes with the angle of viewing as light is reflected at different wavelengths from longer or shorter microstructures (Mason, 2002; Seago et al, 2009). Because of their structural nature, these colors typically last across an insect's lifespan and well after death, resisting change except under extreme stress or deformation (Seago et al, 2009).

Pigment, structural, and combined color types provide many useful functions. For structural color, functionality is often for inter- or intra-specific signaling. Inter-specific functions include camouflage from predators for greens and other background matching patterns, and aposematic warnings of distastefulness, stinging, or other predatory defense mechanisms (Badejo et al, 2020). Other interspecific signals have a function in heterospecific competition interactions, possibly reducing costs of direct conflict (Caro & Allen, 2017). Intraspecific signals are typically sexually-selected through attractiveness to mates, age-related changes to signal reproductive readiness, or defensive colors and patterns to look bigger to competitors. Colors can be useful also in thermoregulation, e.g., structural white helps some tiger beetles (Carabidae) to forage longer in the heat of day than darker morphs (Hadley et al, 1992) while darker colors can help poikilothermic insects to warm faster in cooler parts of the day or season (Brakefield & Willmer, 1985).

Pigments can be grouped based on either derivation, or precursors, i.e., those that are synthesized by the insect *de novo* or those acquired from the diet. Within pigments, the carotenoids are often classified the most common red/orange pigment, but in animals these almost always must be diet-based (Moller et al, 2000). Rare exceptions are found in aphids which have acquired *de novo* carotenoid synthesis ability through horizontal transfer from fungi (Moran & Jarvik, 2010), and whiteflies which produce carotenoids through association with their endosymbiotic bacteria (Sloan & Moran, 2012). Hill (1996) proposed that red carotenoids were both rarer in diets and more costly than yellow or orange. Functionally, carotenoids are useful as antioxidants (Moriyama, 2021) as well as being used as signals of fitness to potential mates and predators, as seen in the ladybeetle *Harmonia axyridis* (Bezzerides et al, 2007). Carotenoid expression is often reduced under heavy parasite pressure, making this an honest signal for fitness (Moller et al, 2000).

Flavonoids, or flavins, are another group of pigments that must be obtained from the diet by an insect. Like carotenoids, these come only from plants or microorganisms and require phenylalanine as a precursor (Futahashi & Osanai-Futahashi, 2021). Most commonly found in Lepidoptera, they have also been identified in some plant-feeding members of Orthoptera, Hymenoptera, and Coleoptera (Futahashi & Osanai-Futahashi, 2021). Flavonoid pigments sequestered by the insect may appear different in ultraviolet spectra, and function for sexual fitness-signaling as documented in the common blue, *Polyommatus icarus* (Burghardt et al, 2000; Knuttel & Fiedler, 2001).

Ommochromes are another pigment class found in insects. They have been found to produce the colors red, brown, orange, yellow and purple. They are most frequently found in eye pigments but sometimes also in wings and bodies (Futahashi & Futahashi, 2021). Chemically, ommochromes require the precursors tryptophan and sometimes sulfur from cysteine and are able to shift in color based on whether they are in a reduced (reddish) or oxidized (yellow) form (Futahashi & Futahashi, 2021; Insausti & Casas, 2008). These redox reactions can be driven by temperature changes and sexual maturity within an individual, which has been observed in some dragonflies (Futahashi et al, 2012), or between individuals in a population with seasonal morphs for multivoltine species as observed with the buckeye butterfly *Junonia coenia* (Nijhout, 1997). The reversible color change in crab spiders (Thomisidae) from white to yellow is also a result of ommochrome pigments, which cause the red stripe in some species (Insausti & Casas, 2008). Ommochromes in eyes function as light collectors for sight, but also antioxidative molecules that can bind with the free radicals produced by invertebrate eye exposure to blue light photodamage, similar to melanin in vertebrate eyes (Dontsov et al, 2020; Insausti & Casas, 2008). Ommochromes found on external integument have been hypothesized to function in signaling and crypsis, like other surface pigments (Insausti & Casas, 2008).

Pterins or pteridines are pigments found in most insects, and can be yellow, orange, white, and red, as well as fluorescent (Badejo et al, 2020), although many do not act as an external pigment but are instead functional in other physiological processes (Ziegler & Harmsen, 1970). As discussed previously, pteridine concentration in the eye has been

used as an age grading tool in multiple insect orders. They are chemically related to purine and uric acid and found in many animals as well as some plants (Futahashi & Futahashi, 2021). The name refers to their discovery as the pigments responsible for the orange, yellow, and white on the wings of Pierid butterflies (Pieridae) (Ziegler & Harmsen, 1970). They have been found to be responsible for the yellow and red patterning in some true bugs and are believed to function as aposematic coloration (Krajicek et al, 2014). Some pterins, like erythropterin, have been investigated in their capacity to change color with insect age. Niva and Takeda (2002) found that the sternum of the true bug *Halyomorpha brevis* changed from ivory to red as the insects aged, although this was more apparent for males than females and did not occur when reared at photoperiods corresponding to diapause induction. Despite some documented other cases, their primary function as a pigment appears to be as a screening tool in the eye similar to ommochromes (Ziegler & Harmsen, 1970). When they appear on the integument, they are usually located in the epidermal cells, unlike melanin which is found in the cuticle (Ziegler & Harmsen, 1970).

Melanins are perhaps the most common pigment associated with insect coloration. Melanin has two main classes: eumelanin which is black or brown, and pheomelanin which is reddish yellow (Futahashi & Osanai-Futahashi, 2021). Melanins are derived from tyrosine but have several alternative pathways to pigment production (Whitten & Coates, 2017). Functionally, melanins are fundamental to many physiological processes including sclerotization and strengthening of the exoskeleton, innate immune response, clot formation, and organogenesis (Whitten & Coates, 2017). Melanin-based variations in

color within a species have been linked to thermal adaptations. For instance, the darker, melanistic variants of the ladybeetle *Adalia bipunctata* have an advantage in early spring activity in the Netherlands (Brakefield & Willmer, 1985), and the darkness of the ground cricket *Allonemobius socius* increases with the growing coldness of the latitude where they are found (Fedorka et al, 2013b). Interestingly, in these crickets the higher cuticular melanin correlated with greater immune ability, suggesting a multiple pressures on melanin selection (Fedorka et al, 2013a). For the reddish pheomelanin, function is often signaling-based. Black, paired with red, orange, or yellow, creates high contrast and the markings are meant to be highly visible. Aposematism is used to discourage would-be predators by announcing distastefulness and being very memorable for faster learning (Prudic et al, 2006). The orange-red of aposematic coloration in velvet ants (Mutillidae) and bumblebees (Apidae) is from pheomelanin (Hines et al, 2017).

The function of pigment deposition in insect cuticles overlaps with structural color. Pigmentary color is used in many forms of signaling between organisms of the same or different species. Sexual selection is still a vital function, but compared with structural color, most pigments must be synthesized from molecular precursors and are metabolically "expensive" to produce, thus consistent with the handicap principle (Zahavi, 1975) and the signaling of fitness (Weaver et al, 2017). Parasitism load has been shown to reduce the use of carotenoids in sexual fitness signaling in diverse taxa (Moller et al, 2000). Pigments that must be obtained via the diet in most animals, such as carotenoids and flavonoids, are good indicators of foraging ability. Melanin also has a direct role for immune response in insects and therefore is a good immune indicator (Futahashi & Futahashi, 2021).

Aposematism displays commonly use orange or red paired with black, as these colors show strong chromatic contrast against one another and their background and help with predator learning (Lindstrom et al, 2004; Prudic et al, 2007) *Nicrophorus vespilloides* exudes distasteful anal secretions that deterred predatory ants, while their markings were very visually apparent to avian models (Lindstedt et al, 2017). It seems reasonable that when ABB arrive at a carcass, they might be exposed to potential predators that are also attracted to carrion, like crows or nocturnal scavengers like opossum (Jurzenski & Hoback, 2011). However, in a free-choice study on avian predation of beetles, three orange and black *Nicrophorus* spp. were not fed on, lending support to their ability to avoid being accepted as food (Jones, 1932). Generally, there is support that the coloration and markings of *Nicrophorus* spp. function at least in part as aposematic warning (Lindstedt et al, 2017).

The source of color on the ABB pronotum or elytra is unknown, and to date, no study found had determined if the color in any *Nicrophorus* spp. is a pigment. Although some studies have measured hues from ABB and related species (e.g., Wormington & Luttbeg, 2017), none have identified the source of color on a physical or molecular level. Like many *Nicrophorus* spp., ABB have distinctive orange/red and black markings. The red colors occur in similar places on the body for most *Nicrophorus* spp.: the antennal club; less ubiquitously, the clypeus; in mostly separate patches on the anterior and posterior of the proximal elytra, as well as the distal margin; and, somewhat distinctively for the ABB, across the pronotal shield (Haarstad, 1985). Functionally, these colors have been

shown to provide several benefits, including aposematic coloration (Lindstedt et al, 2017) and sexual signaling of immune function (Wormington & Luttbeg, 2017).

Several laboratories with colonies have noted the progressive change in color of the orange/red patches over time. Immediately after pupal eclosion, the patches appear bright orange, then incrementally shift toward a darker red until death. After death, they quickly turn to a dark brown with decomposition. This occurs within both wild-caught and lab-reared individuals, irrespective of diets fed. In the lab, they occur in both individuals that are used for breeding that burrow into a substrate, as well as those never given access to a soil-based substrate. In this study, I determined the rate of change in color and assessed its potential use as a means of non-destructive age grading. I hypothesized that the rate of change was related to age and that the progression was predictable.

CHAPTER III

METHODOLOGY

Rearing

All ABB were the first generation of wild caught parents and were reared in a basement at OSU's Insect Adventure where the colony is kept at 24.4-25.5 °C. Brood chambers were constructed using 19-liter buckets with 4 mm holes drilled into the bottom to allow water drainage and 140 mm holes cut into the lid for air flow. Both the inside bottom and inside lid hole were covered using a fine metal screen and hot glue to prevent escape. The outside of the lid hole was covered in synthetic fine-mesh organdy cloth attached with hot glue to prevent nuisance fly access. Brood chamber substrate was composed of 3 parts Greensmix® sphagnum peat moss to 1 part sandy loam topsoil from the Cimarron river in Oklahoma. The substrate was completely saturated using hot tap water and allowed to drain and settle for seven days. Once the substrate had sufficiently drained, a previously

frozen quail weighing approximately 110g was thawed then placed on top and in the center of the substrate. One male and one female ABB were introduced onto the substrate and the lid was closed. Buckets were checked once a day for three days. If the quail carcass was completely or partially buried, both beetles were left in the bucket and allowed to continue their mating behaviors for ten days, at which time the male was removed, and the female was left with the larvae. At 15 days since introduction, the female was removed and a visual search for larvae was conducted. If larvae were found, they remained undisturbed for 50 days since introduction and then checked daily until new adult beetles emerged at the surface. New beetles were sexed, weighed, and pronotal width measured, before they were separated into individual containers. Throughout the experiment, the beetles were kept individually in semi-translucent plastic containers (76mm x 78mm depth) with a damp paper towel. They were fed three times a week (Monday, Wednesday, and Friday) with two mealworms and two waxworms. All procedures were adapted from unpublished care instructions received from the insectarium of the St. Louis Zoo in St. Louis, Missouri.

Digital Imaging

To determine the color difference of the ABB pronotum over time, photographs were taken of four live ABBs from the lab colony over the course of their lifespan—from emergence until death. The four beetles chosen for this experiment were divided evenly between sex. Pictures were taken three times a week (Monday, Wednesday, and Friday) with a MotoGPower2022 android smartphone 50MP camera, in the same staging area on

a white laminated work bench. The beetles were photographed in the dorsal view so the pronotum, elytra and head were visible. The photographs also included the individual ID, sex, and date recorded on a laminated card, in-frame, so that the number of days since emergence could be determined. After the pictures were taken, the beetles were placed back into their containers until the next experimental date.

To collect the Red, Green, Blue (RGB) values the Fiji/ImageJ computer software was used. Each image was uploaded and the rectangle tool was used to draw a 25x25 pixel square that was positioned consistent locations on the beetle body. For the pronotum, the non-black portion adjacent to the center of the head was selected (Figure 1). This location was chosen to avoid glare from the camera flash. For the elytral RGB values, the same 25x25 pixel square was moved to the top left portion of the upper elytron when viewed dorsally. RGB values were collected by generating a color histogram for each location and recording in a spreadsheet. This was done for every photograph taken throughout the individual beetle's life.

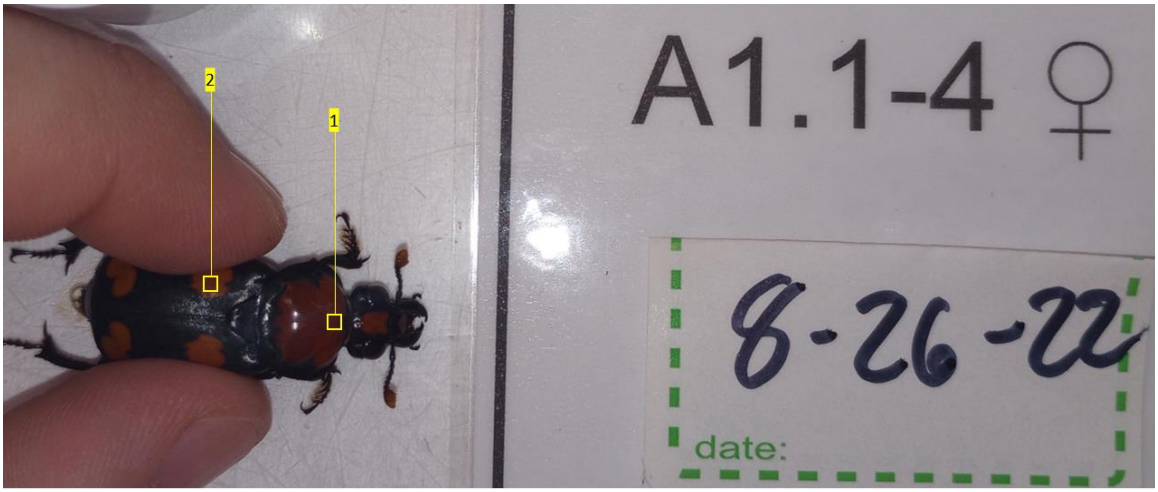

Figure 1. RGB 25x25 pixel capture locations. 1) Pronotum 2) Elytral spot

Data analyses

Statistical analyses were conducted in R version 4.1.1. A series of linear mixed-effects models (LMMs package lme4; Bates et al, 2015) were used to assess the change in the red, green, and blue color channels over time from distinct markings on pronotum and elytron of four individual *N. americanus*, two males and two females. Model set used to determine the change in color channels over time included the fixed effect of beetle age (days). The model used to characterize the comparative change in distinct markings from the pronotum and elytra included the fixed effects of beetle age + beetle marking. The comparative model set was also evaluated with an interaction term of beetle age * beetle marking. Random effects were weighed *a priori*, where the variance of camera identity, photography technique, beetle identity, and beetle sex were considered. Camera identity consistently contributed the greatest source of variation in the null model and was used for all subsequent model sets. Using a chi-square distribution, a direct comparison with likelihood ratio tests (LRT) among models determined the significant variation over time ($p \leq 0.05$).

CHAPTER IV

FINDINGS

The four beetles lived 76, 90, 97, and 99 days (mean= 90.5, range=76-99, SD= 2.495). From measurements taken of each beetle's pronotum and elytron on the same three days every week, there was a significant change in color over time.

Values in the red color channel significantly decreased over time in the pronotum (β ± SE = -13.5 ± 0.9, $p < 0.001$) and elytron markings (β ± SE = -12.0 ± 1.3, $p < 0.001$), displaying the most precipitous decrease among all color channels (Table 1). Compared to the null model, red color models observed the greatest ΔAIC and adj. R^2 values among all color channels, which further supports the best fit in the data series over time. The random effect (camera identity) generated the greatest variance in red color among the pronotum and elytron models with a variance ± standard deviation of 761 ± 28 and 431 ± 21, respectively (Table 1). The adjusted R^2 for the pronotum and elytral red color null models were 0.77 and 0.65, respectively. Green color also significantly decreased over time in the pronotum (β ± SE = -4.9 ± 0.9, $p < 0.001$) and elytron marking (β ± SE = -3.9 ± 0.9, $p < 0.001$). However, the change in green color was less steep than the red color; and was not significantly different over time ([LRT]; $\chi2(1) = 0.0$, $p > 0.05$). Blue color on the pronotum did not change over time (β ± SE = -0.10 ± 0.8, $p = 0.91$), whereas the elytral blue color measurements exhibited a significant increase over time (β ± SE = 1.30

± 0.5, $p = 0.005$); this change was the only positive increase in color value. Blue channels were the least influenced by the camera.

Table 1. Results of liner mixed models (LMM) assessing the change in the red, green, and blue color channels (response variable) from distinct color markings on the pronotum and elytron over the lifespan of each beetle (fixed effect).

Beetle marking	Model parameters[a]							
Pronotum	AIC	ΔAIC	R^2	β	SE	$\chi 2$	p	σ^2_i
Red	1071.1	124.9	0.90	-13.5	0.9	193.6	<0.001	761 ± 28
Green	1032.3	29.1	0.71	-4.90	0.9	32.8	<0.001	118 ± 11
Blue	1081.3	0.75	<0.10	-0.10	0.8	0.01	0.91	1.6 ± 1.3
Elytra	AIC	ΔAIC	R^2	β	SE	$\chi 2$	p	σ^2_i
Red	1158.1	68.7	0.78	-12.0	1.3	85.9	<0.001	431 ± 21
Green	1060.8	15.5	0.46	-3.90	0.9	18.2	<0.001	26.8 ± 5.2
Blue	980.1	3.40	0.49	1.30	0.5	7.8	0.005	0.0 ± 0.0

[a]Model parameters are listed by column: Akaike's Information Criterion (AIC), change in AIC (ΔAIC) from the intercept-only model, adjusted coefficient of determination (R^2), beta coefficients (β), standard error of β, chi square ($\chi 2$), probability value (p), and variance of random effect (σ^2_i). Covariate in model structure: Beetle age. Random effect: camera identity.

Comparative change in color channels from the pronotum and elytron markings were significantly different for each color (Table 2). The interaction term, (beetle age * beetle marking), performed better than the additive model and was retained ([LRT]; $\chi 2(1) = 5.3$, $p = 0.02$). Decreases in the red, green, and blue color values over time were significantly less in the pronotum than the elytron. Green color had the greatest measured difference between beetle markings, decreasing nearly 20% faster in the pronotum (β ± SE = -0.11 ± 0.03, $p < 0.001$). Similar to the marking-specific models, the camera generated the greatest variance in the red color values.

Table 2. Results of liner mixed models (LMM) assessing the change in the red, green, and blue color channels (response variable) explicitly testing the interaction of distinct color markings on the pronotum and elytron over time (i.e., Beetle age * location of distinct beetle marking).

Response variable	Model results[a]							
	AIC	ΔAIC	R^2	β[b]	SE	χ2	*p*	σ^2_i
Red	2250.3	295.9	0.87	-0.09	0.04	5.3	0.02	586 ± 24
Green	2114.3	94.9	0.64	-0.11	0.03	11.8	<0.001	60 ± 7.7
Blue	2088.1	-10.3	0.02	-0.06	0.03	4.4	0.04	0.38 ± 0.6

[a] Model parameters are listed by column: Akaike's Information Criterion (AIC), change in AIC ΔAIC) from the intercept-only model, coefficient of determination (R^2), beta coefficients (β), standard error of β, chi square (χ2), probability value (*p*), and variance of random effect (σ^2_i). Covariate in model structure: Beetle age. Random effect: camera identity.
[b] Beta coefficients reflect the comparative estimates in the pronotum (i.e., the color channels measured on the pronotum decreased more rapidly than on the elytra)

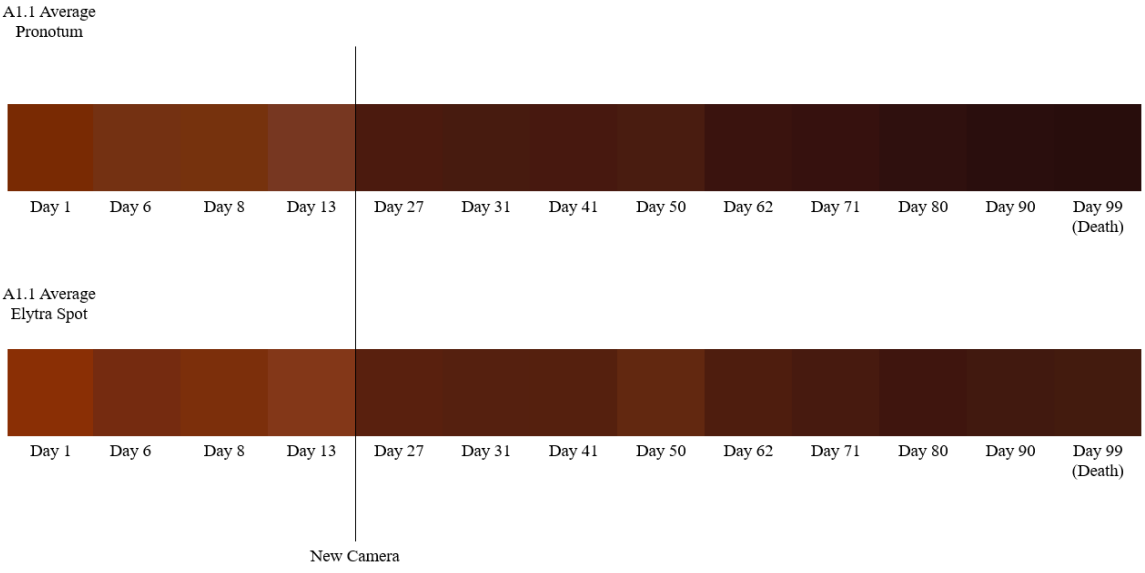

Figure 2. Average RGB values of pronotum and elytron spot over the lifespan of the ABB.

CHAPTER V

CONCLUSION

My study supported the observation that the orange-colored pronotal and elytral markings on ABB darken over time under controlled environmental conditions. This occurred in a gradual fashion across the lifespan of the beetle and did not differ by sex. The rate of darkening differed between body part sampled, with pronotal markings becoming darker faster than elytral markings. Over the course of our study, the beetles darkened with age at a predictable rate. As the ABBs aged, their pronotum color transformed from orange to a darker red. Both the red and green channels decreased in value significantly over time. Interestingly, while the change in pronotal blue channel did not differ across age of insect, it increased in a small but significant way in the elytron.

These results were obtained from beetles bred and reared under laboratory conditions, a way to measure this response under controlled environmental conditions that establishes a base inherent rate of change without the variation that natural environmental and behavioral conditions might induce. The beetles were reared under a stable day/night photoperiod and temperature regimen, 12L/12D and nearly constant temperature 25.5℃ (78°F). Bedick et al. 1999 found that ABB in Nebraska were active at temperatures between 12.7℃ (54.86°F) and 24℃ (75.2°F), while adults in diapause presumably

experience winter temperatures very near freezing, although diapausing adults in northern Nebraska were found to dig below the frostline to avoid colder surface temperatures (Hoback & Conley, 2014). The upper limit may be a consequence of ABB susceptibility to desiccation, as found for *N. marginatus* (Bedick, 1997).

If ABB are particularly susceptible to desiccation and this involves the structure of their cuticle, variation in aridity could play a part in the appearance of their colored spots. Under controlled conditions, beetles were only subjected to direct fluorescent lighting during the brief period when they were photographed and fed, around 3 minutes total weekly. Under natural conditions these largely nocturnal beetles would rarely be exposed to sunlight, but even brief exposure to UV light can affect color through the breaking of chemical bonds.

Reproductive environment was also controlled in the lab. Beetle cuticles are very tough partly because they must sustain abrasion in the wild. ABB males typically fight other intraspecific males at a carcass site (Ratcliffe, 1996), and must contend with interspecific competition from other attracted invertebrates (Howard & Hall, 2019) while escaping predation risk from vertebrates (Jurzenski & Hoback, 2011). Both sexes that engage in mating have to bury their brood ball by pushing through the soil substrate, frequently hard and full of dense grass roots in areas where many *Nicrophorus* spp. must partition habitat (Scott, 1998). ABBs can utilize variable soil textures for brood rearing but appear to show preference for high proportions of sand (Lomolino et al, 1995), possibly lowering bulk density for faster burial (Scott ,1998) and/or increasing oxygen diffusion.

Moving through sand in particular could accumulate many small or large abrasions, causing changes to perceived color on their external markings. Additionally, ABB is host to phoretic mites on their cuticle that can behave in a mutualistic manner by reducing competing phoretic carrion populations in brood balls (Wilson & Knollenberg, 1987). These mites may also cause abrasive wear from their active movements and high densities. Observations made in the field study by Bedick et al, 1999 show that, contrary to the lab reared beetles, the senescent beetles had markings that faded to a pale color and showed obvious signs of damage.

Darkening with age could aid in mate choice. A darker pronotum might signify an older female beetle, potentially making being more attractive to a mate as age has been shown to be correlated with offspring provisioning in *Nicrophorus orbicollis* (Creighton et al, 2009). Because ABB are exposed to many microbes through carcass interaction, color could also indicate immune competency (Wormington & Luttbeg, 2018).

The gradual and irreversible changes indicate the colors are most likely pigmentary rather than structural. Although it is possible that microstructures on the cuticle could change color for a burrowing species through surface abrasion, the color shift also occurs on individuals never given access to soil-like substrate. The rapid color change at death likely indicates a catabolic change to pigmentary molecular structure, although desiccation has been found to reversibly change structural color in tortoise beetles (Seago et al, 2009).

Within pigmentary candidates for ABB orange color, some seem more likely than others. Although carotenoids are often used by diverse species in color signaling, there is a significant obstacle against their use by carrion beetles. ABB are necrophores and insect predators, and therefore not likely to frequently acquire these molecules through their diet. Thus, the pigments responsible for the orange to red patches on ABB are unlikely to be carotenoids unless they are found to have formed them through microbial synthesis via symbionts as in the plant-feeding hemipterans (Moran & Jarvik, 2010; Sloan & Moran, 2012). Being similarly plant-diet restricted, flavonoids are also unlikely to be the pigment type present (Badejo, 2020). Pterins are synthesized without the requirement for plant diets, but they have yet to be identified as a pigment in coleopteran elytra (Futahashi & Futahashi, 2021). More likely candidates include ommochromes, which have been shown to change from a more yellow to red form across an individual dragonfly's lifespan (Futahashi et al. 2012), and melanin.

Melanin in the form of pheomelanin seems the most likely candidate for ABB orange-red pigmentation. Wormington & Luttbeg (2017) researched the correlation between clypeal color and immune function of *Nicrophorus pustulatus* by injecting them with sandpaper in their abdominal cavity to trigger an immune response, then analyzing the color change of the clypeus. Their study found that manually triggering *N. pustulatus*'s immune response resulted in a significantly darker clypeus color, leading the authors to suggest the possibility that the measured pigment was pheomelanin. The darkening by age could be a result of sequential immune challenges if these occur consistently and regularly across individuals. Symbiotic microbes from a carcass-visiting lifestyle could potentially

play this role with the host. Determining the actual identity of the pigment in question may help to illuminate some of the selection pressures faced by the ABB.

Our findings have the potential to be a useful tool for categorizing relative age of unknown ABB. Age grading is a very important tool in population studies. Bedick et al (1999) suggested that senescent adults, identified by their pale color, might be past reproductive capability and therefore prove useful for destructive sampling in molecular analysis although unsuitable for establishing captive breeding colonies. The ability to split a population into age groupings also has wide application in demographic studies. Life table analyses can indicate which stages experience high mortality rates, and further studies can focus on identifying those mortality factors with clear application to conservation efforts (Carey, 2001). Our study produced a color gradient that shows a "baseline" of RGB value by age. This chart could be further validated for field use by comparing the colors created from lab beetles to wild beetles released after age identification via mark-recapture or field cage enclosures to determine the extent of color changes as influenced by environmental variables. Ultimately, a print or electronic color chart could be created to allow researchers and conservationists to easily estimate adult beetle age from encountered individuals in a nondestructive manner.

REFERENCES

Aguiar, A. P. (2005). An accurate procedure to describe colors in taxonomic works, with an example from Ichneumonidae (Hymenoptera). *Zootaxa*, *1008*(1), 30-38.

Amaral, M., Kozol, A., & French, T. (1997). Conservation status and reintroduction of the endangered American burying beetle. *Northeastern Naturalist,* 121-132.

Anderson, R. S. (1982). On the decreasing abundance of *Nicrophorus americanus* Olivier (Coleoptera: Silphidae) in eastern North America. *The Coleopterists' Bulletin*, 362-365.

Badyaev, A. V., & Hill, G. E. (2000). Evolution of sexual dichromatism: contribution of carotenoid-versus melanin-based coloration. *Biological journal of the Linnean Society*, *69*(2), 153-172.

Bedick, Jon Charles. "Distribution and ecology of the American burying beetle (*Nicrophorus americanus*, Olivier) in south-central Nebraska" Thesis (M.S.)--University of Nebraska--Lincoln, 1997.

Bedick, J. C., Ratcliffe, B. C., Hoback, W. W., & Higley, L. G. (1999). Distribution, ecology and population dynamics of the American burying beetle [*Nicrophorus americanus* Olivier (Coleoptera, Silphidae)] in south-central Nebraska, USA. *Journal of Insect Conservation*, *3*(3), 171-181.

Badejo, O., Skaldina, O., Gilev, A., & Sorvari, J. (2020). Benefits of insect colours: a review from social insect studies. *Oecologia*, *194*(1), 27-40.

Barek, H., Sugumaran, M., Ito, S., & Wakamatsu, K. (2018). Insect cuticular melanins are distinctly different from those of mammalian epidermal melanins. *Pigment Cell & Melanoma Research*, *31*(3), 384-392.

Bates D., Mächler M., Bolker B. & Walker S., Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw 67*:1-48 (2015).

Bezzerides, A. L., McGraw, K. J., Parker, R. S., & Husseini, J. (2007). Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behavioral Ecology and Sociobiology*, *61*(9), 1401-1408.

Bishop, A. A., Hoback, W. W., Albrecht, M., & Skinner, K. M. (2002). GIS reveals niche partitioning by soil texture among carrion beetles. *Transactions in GIS*, *6*, 457-470.

Brakefield, P. M., & Willmer, P. G. (1985). The basis of thermal melanism in the ladybird *Adalia bipunctata*: differences in reflectance and thermal properties between the morphs. *Heredity*, *54*(1), 9-14.

Burghardt, F., Knüttel, H., Becker, M., & Fiedler, K. (2000). Flavonoid wing pigments increase attractiveness of female common blue (Polyommatus icarus) butterflies to mate-searching males. *Naturwissenschaften*, *87*(7), 304-307.

Butterfield, J. (1996) Carabid life-cycle strategies and climate change: a study on an altitude transect. *Ecological Entomology*, *21*, 9–16.

Carey, J. R. (2001). Insect biodemography. *Annual review of entomology*, *46*(1), 79-110.

Caro, T., & Allen, W. L. (2017). Interspecific visual signalling in animals and plants: a functional classification. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1724), 20160344.

Crawford, P. H., & Hoagland, B. W. (2010). Using species distribution models to guide conservation at the state level: the endangered American burying beetle (*Nicrophorus americanus*) in Oklahoma. *Journal of Insect Conservation*, *14*(5), 511-521.

Creighton, J. C., Vaughn, C. C., & Chapman, B. R. (1993). Habitat preference of the endangered American burying beetle (*Nicrophorus americanus*) in Oklahoma. *The Southwestern Naturalist*, *38*(3), 275-277.

Creighton, J. C., Heflin, N. D., & Belk, M. C. (2009). Cost of reproduction, resource quality, and terminal investment in a burying beetle. *The American Naturalist*, *174*(5), 673-684.

Crowson, R. A. (2013). *The biology of the Coleoptera*. Academic press.

Corbet, P. S. (1960). Recognition of nulliparous mosquitoes without dissection. *Nature*, 187(4736), 525-526.

Dimitrov, R. E., Amendt, J., Rothweiler, F., & Zehner, R. (2020). Age determination of the adult blow fly *Lucilia sericata* (Diptera: Calliphoridae) through quantitative pteridine fluorescence analysis. *Forensic Science, Medicine and Pathology*, *16*(4), 641-648.

Dontsov, A. E., Sakina, N. L., Yakovleva, M. A., Bastrakov, A. I., Bastrakova, I. G., Zagorinsky, A. A., ... & Ostrovsky, M. A. (2020). Ommochromes from the

compound eyes of insects: physicochemical properties and antioxidant activity. *Biochemistry (Moscow), 85*(6), 668- 678.

Fedorka, K. M., Copeland, E. K., & Winterhalter, W. E. (2013a). Seasonality influences cuticle melanization and immune defense in a cricket: support for a temperature dependent immune investment hypothesis in insects. *Journal of Experimental Biology*, *216*(21), 4005-4010.

Fedorka, K. M., Lee, V., & Winterhalter, W. E. (2013b). Thermal environment shapes cuticle melanism and melanin-based immunity in the ground cricket Allonemobius socius. *Evolutionary Ecology*, *27*(3), 521-531.

Ferrari, T. N. (2014). *Seasonal dynamics of the American burying beetle (Nicrophorus americanus) in eastern Oklahoma* (Doctoral dissertation, Oklahoma State University).

Futahashi, R., & Osanai-Futahashi, M. (2021). Pigments in insects. *Pigments, Pigment Cells and Pigment Patterns*, 3-43.

Futahashi, R., Kurita, R., Mano, H., & Fukatsu, T. (2012). Redox alters yellow dragonflies into red. *Proceedings of the National Academy of Sciences*, *109*(31), 12626-12631.

Haarstad, J. A. (1985). *Ecological Relationships Among Eight Species of Coexisting Burying Beetles (Coleoptera--Silphidae; Nicrophorus) in East-central Minnesota*. University of Minnesota.

Hadley, N. F., Savill, A., & Schultz, T. D. (1992). Coloration and its thermal consequences in the New Zealand tiger beetle *Neocicindela perhispida*. *Journal of Thermal Biology*, *17*(1), 55-61.

Hartmann, C., Heinze, J., & Bernadou, A. (2019). Age-dependent changes in cuticular color and pteridine levels in a clonal ant. *Journal of insect physiology*, *118*, 103943.

Hashimoto, H., Goda, M., Futahashi, R., Kelsh, R., & Akiyama, T. (Eds.). (2021). Pigments, pigment cells and pigment patterns. Singapore: Springer.

Hayes, E. J., & Wall, R. (1999). Age-grading adult insects: a review of techniques. *Physiological Entomology*, *24*(1), 1-10.

Heath, J. J., Cipollini, D. F., & Stireman III, J. O. (2013). The role of carotenoids and their derivatives in mediating interactions between insects and their environment. *Arthropod-Plant Interactions*, *7*(1), 1-20.

Hoback, W. W., Bishop, A. A., Kroemer, J., Scalzitti, J., & Shaffer, J. J. (2004). Differences among antimicrobial properties of carrion beetle secretions reflect phylogeny and ecology. *Journal of chemical ecology*, *30*(4), 719-729.

Hoback, W. Wyatt and Conley, Adrienne, "Overwintering Biology and Tests of Trap and Relocate as a Conservation Measure for Burying Beetles" (2014). Nebraska Department of Transportation Research Reports. 175.

Hoback, W. W., Freeman, L., Payton, M., & Peterson, B. C. (2020). Burying beetle (Coleoptera: Silphidae: *Nicrophorus Fabricius*) brooding improves soil fertility. *The Coleopterists Bulletin, 74*(2), 427-433.

Howard, D. R., & Hall, C. L. (2019). Examining the management of rare insects through the lens of biotic interactions: a comparative case study of *Nicrophorus americanus* (Coleoptera: Silphidae) and *Gryllotalpa major* (Orthoptera: Gryllotalpidae). *Annals of the Entomological Society of America*, *112*(3), 158-168.

Hill, G. E. (1996). Redness as a measure of the production cost of ornamental coloration. *Ethology Ecology & Evolution, 8*(2), 157-175.

Hines, H. M., Witkowski, P., Wilson, J. S., & Wakamatsu, K. (2017). Melanic variation underlies aposematic color variation in two hymenopteran mimicry systems. *PloS one*, *12*(7), e0182135.

Holloway, A. K., & Schnell, G. D. (1997). Relationship between numbers of the endangered American burying beetle *Nicrophorus americanus* Olivier (Coleoptera: Silphidae) and available food resources. *Biological Conservation*, *81*(1-2), 145-152.

Insausti, T. C., & Casas, J. (2008). The functional morphology of color changing in a spider: development of ommochrome pigment granules. *Journal of Experimental Biology*, *211*(5), 780-789.

Jackson, C. H. N. (1946). An artificially isolated generation of tsetse flies (Diptera). *Bulletin of Entomological Research, 37*(2), 291-299.

Jones, F. M. (1932). Insect coloration and the relative acceptability of insects to birds. *Transactions of the Royal Entomological Society of London*, *80*(2), 345-371.

Jurzenski, J., & Hoback, W. W. (2011). Opossums and leopard frogs consume the federally endangered American burying beetle (Coleoptera: Silphidae). *The Coleopterists Bulletin*, *65*(1), 88-90.

Jurzenski, J. D. (2012). *Factors affecting the distribution and survival of endangered American burying beetles, Nicrophorus americanus Olivier* (Doctoral dissertation, The University of Nebraska-Lincoln).

Keller, L., & Genoud, M. (1997). Extraordinary lifespans in ants: a test of evolutionary theories of ageing. *Nature, 389*(6654), 958-960.

Knuttel, H., & Fiedler, K. (2001). Host-plant-derived variation in ultraviolet wing patterns influences mate selection by male butterflies. *Journal of Experimental Biology*, *204*(14), 2447-2459.

Kosminsky, R. B. (1960). On the methods to determine the age of fleas *Leptopsylla segnis* and *Leptopsylla taschenbergi* and the age analysis of the population of *L. segnis* [in Russian]. *Medskaya Parazit., 29*, 590-594.

Krajíček, J., Kozlík, P., Exnerová, A., Štys, P., Bursová, M., Čabala, R., & Bosáková, Z. (2014). Capillary electrophoresis of pterin derivatives responsible for the warning coloration of Heteroptera. *Journal of Chromatography A, 1336*, 94-100.

Křemenová, J., Balvín, O., Otti, O., Pavonič, M., Reinhardt, K., Šimek, Z., & Bartonička, T. (2020). Identification and age-dependence of pteridines in bed bugs (*Cimex lectularius*) and bat bugs (*C. pipistrelli*) using liquid chromatography-tandem mass spectrometry. *Scientific reports*, *10*(1), 1-14.

Lardeux, F., Ung, A., & Chebret, M. (2000). Spectrofluorometers are not adequate for aging *Aedes* and *Culex* (Diptera: Culicidae) using pteridine fluorescence. *Journal of Medical Entomology*, *37*(5), 769-773.

Leasure, D. R., & Hoback, W. W. (2017). Distribution and habitat of endangered American burying beetle in northern and southern regions. *Journal of Insect Conservation, 21*(1), 75-86.

Lehane, M. J., & Mail, T. S. (1985). Determining the age of adult male and female *Glossina morsitans morsitans* using a new technique. *Ecological Entomology, 10*(2), 219-224.

Lindstedt, C., Boncoraglio, G., Cotter, S., Gilbert, J., & Kilner, R. M. (2017). Aposematism in the burying beetle? Dual function of anal fluid in parental care and chemical defense. *Behavioral Ecology*, *28*(6), 1414-1422.

Lindström, L., Alatalo, R. V., Lyytinen, A., & Mappes, J. (2004). The effect of alternative prey on the dynamics of imperfect Batesian and Müllerian mimicries. *Evolution, 58*(6), 1294-1302.

Lomolino, M.V., Creighton, J.C., Schnell, G.D. and Certain, D.L. (1995) Ecology and conservation of the endangered American burying beetle (*Nicrophorus americanus*). *Conserv. Biol. 9*, 605–614.

Looney, C., Caldwell, B. T., Hatten, T. D., Lorion, C., & Eigenbrode, S. D. (2004). Potential Habitat Factors Influencing Carrion Beetles Communities of Palouse Prairie Remnants. *Proceedings of the North American Prairie Conferences.* 75

Mail, T. S., Chadwick, J., & Lehane, M. J. (1983). Determining the age of adults of *Stomoxys calcitrans* (L.)(Diptera: Muscidae). *Bulletin of Entomological Research*, *73*(3), 501-525.

Mason, C. W. (2002). Structural colors in insects. II. *The Journal of Physical Chemistry*, *31*(3), 321-354.

McGraw, K. J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants?. *Animal Behaviour*, *69*(4), 757-764.

Moller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N., & Surai, P. F. (2000). Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability?. *Poultry and Avian Biology Reviews*, *11*(3), 137-160.

Moran, N. A., & Jarvik, T. (2010). Lateral transfer of genes from fungi underlies carotenoid production in aphids. *Science*, *328*(5978), 624-627.

Moriyama, M. (2021). Physiological and Biochemical Mechanisms of Insect Color Change: Towards Understanding Molecular Links. In *Pigments, Pigment Cells and Pigment Patterns* (pp. 451-472). Springer, Singapore.

Nguyen, C. V., Lovell, D. R., Adcock, M., & La Salle, J. (2014). Capturing natural-colour 3D models of insects for species discovery and diagnostics. *PloS one*, *9*(4), e94346.

Nijhout, H. F. (1997). Ommochrome pigmentation of the linea and rosa seasonal forms of *Precis coenia* (Lepidoptera: Nymphalidae). *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, *36*(3), 215-222.

Niva, C. C., & Takeda, M. (2002). Color changes in *Halyomorpha brevis* (Heteroptera: Pentatomidae) correlated with distribution of pteridines: regulation by environmental and physiological factors. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *132*(3), 653-660.

Okude, G., & Futahashi, R. (2021). Pigmentation and color pattern diversity in Odonata. *Current Opinion in Genetics & Development, 69*, 14-20.

Partridge, L. (1986). Sexual activity and life span. In *Insect Aging* (pp. 45-54). Springer, Berlin, Heidelberg.

Perez-Mendoza, J., Dowell, F. E., Broce, A. B., Throne, J. E., Wirtz, R. A., Xie, F., & Baker, J. E. (2002). Chronological age-grading of house flies by using near-infrared spectroscopy. *Journal of medical entomology*, *39*(3), 499-508.

Perez-Mendoza, J., Throne, J. E., Dowell, F. E., & Baker, J. E. (2004). Chronological age-grading of three species of stored-product beetles by using near-infrared spectroscopy. *Journal of Economic Entomology, 97*(3), 1159-1167.

Popadić, A., & Tsitlakidou, D. (2021). Regional patterning and regulation of melanin pigmentation in insects. *Current Opinion in Genetics & Development*, *69*, 163-170.

Promislow, D. E., Flatt, T., & Bonduriansky, R. (2022). The biology of aging in insects: from Drosophila to other insects and back. *Annual Review of Entomology*, *67*, 83-103.

Prudic, K. L., Skemp, A. K., & Papaj, D. R. (2007). Aposematic coloration, luminance contrast, and the benefits of conspicuousness. *Behavioral Ecology*, *18*(1), 41-46.

Pukowski, E. (1933). Ökologische Untersuchungen an *Necrophorus* F. *Z Morphol Ökol Tiere. 27*: 518–586.

R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/ (2021).

Ratcliffe, B.C. (1996) The carrion beetles (Coleoptera: Silphidae) of Nebraska. *Bulletin of the University of Nebraska State Museum 13*, 1–100.

Robson, S.K.A., Vickers, M., Blows, M.W., Crozier, R.H., (2006). Age determination in in-dividual wild-caught *Drosophila serrata* using pteridine concentration. *J. Exp. Biol. 209*, 3155–3163.

Rozen, D. E., Engelmoer, D. J. P., & Smiseth, P. T. (2008). Antimicrobial strategies in burying beetles breeding on carrion. *Proceedings of the National Academy of Sciences*, *105*(46), 17890-17895.

Santos, L. V. (2020). *The American Burying Beetle (Nicrophorus americanus) Population at Camp Gruber, OK Breeding and Overwintering Survival* (Doctoral dissertation, Oklahoma State University).

Schnell, G. D., Hiott, A. E., Creighton, J. C., Smyth, V. L., & Komendat, A. (2008). Factors affecting overwinter survival of the American burying beetle, *Nicrophorus americanus* (Coleoptera: Silphidae). *Journal of Insect Conservation*, *12*(5), 483-492.

Scott, M. P. (1994). Competition with flies promotes communal breeding in the burying beetle, *Nicrophorus tomentosus*. *Behavioral Ecology and Sociobiology*, *34*(5), 367-373.

Scott, M. P. (1998). The ecology and behavior of burying beetles. *Annual review of entomology*, *43*(1), 595-618.

Seago, A. E., Brady, P., Vigneron, J. P., & Schultz, T. D. (2009). Gold bugs and beyond: a review of iridescence and structural colour mechanisms in beetles (Coleoptera). *Journal of the Royal Society Interface*, *6*(2), S165-S184.

Simmonds, M. S. (2003). Flavonoid–insect interactions: recent advances in our knowledge. *Phytochemistry*, *64*(1), 21-30.

Sloan, D. B., & Moran, N. A. (2012). Endosymbiotic bacteria as a source of carotenoids in whiteflies. *Biology letters*, *8*(6), 986-989.

Smith, R. J. (2002). Effect of larval body size on overwinter survival and emerging adult size in the burying beetle, *Nicrophorus investigator*. *Canadian Journal of Zoology*, *80*(9), 1588–1593.

Southwood, T.R.E., (1978). The construction, description and analysis of age-specific life-tables. In: Southwood, T.R.E. (Ed.), *Ecological Methods*. Springer Netherlands, Dordrecht, pp. 356–387.

Spradbery, J.P. & Vogt, W.G. (1993) Mean life expectancy of Old World screwworm fly, *Chrysomya bezziana*, inferred from the reproductive age-structure of native females caught on swormlure-baited sticky traps. *Medical and Veterinary Entomology*, *7*(2), 147–154.

Springett, B. P. (1968). Aspects of the relationship between burying beetles, *Necrophorus* spp. and the mite, *Poecilochirus necrophori* Vitz. *The Journal of Animal Ecology*, 417-424.

Stelbrink, P., Pinkert, S., Brunzel, S., Kerr, J., Wheat, C. W., Brandl, R., & Zeuss, D. (2019). Colour lightness of butterfly assemblages across North America and Europe. *Scientific reports*, *9*(1), 1-10.

Stoffolano Jr, J. G., LI, M. F., Sutton Jr, J. A., & YIN, C. M. (1995). Faeces feeding by adult *Phormia regina* (Diptera: Calliphoridae): Impact on reproduction. *Medical and Veterinary Entomology*, *9*(4), 388-392.

Timmons, C., Hassell, A., Lauziere, I., & Bextine, B. (2011). Age determination of the glassy- winged sharpshooter, *Homalodisca vitripennis*, using wing pigmentation. *Journal of Insect Science, 11*(1), 78.

Trumbo, S. T. (1992). Monogamy to communal breeding: exploitation of a broad resource base by burying beetles (Nicrophorus). *Ecological entomology, 17*(3), 289-298.

Tyndale-Biscoe, M., Wallace, M. M. H., & Walker, J. M. (1981). An ecological study of an Australian dung beetle, *Onthophagus granulatus* Boheman (Coleoptera: Scarabaeidae), using physiological age-grading techniques. *Bulletin of Entomological Research, 71*(1), 137-152.

Tyndale-Biscoe, M., (1984). Age-grading methods in adult insects: a review. *Bull. Entomol. Res. 74*, 341.

U.S. Fish and Wildlife Service (2020). Endangered and Threatened Wildlife and Plants; Reclassification of the American Burying Beetle From Endangered to Threatened With a Section 4(d) Rule. *Federal Register 85*:65241–65261

U.S. Fish and Wildlife Service (2019). Species Status Assessment Report for the American Burying Beetle (*Nicrophorus americanus*). Southwest Regional Office, Albuquerque, New Mexico

Vogt, W. G., & Walker, J. (1987). Influences of temperature, fly size and protein-feeding regime on ovarian development rates in the Australian bush fly, *Musca vetustissima*. *Entomologia experimentalis et applicata, 44*(2), 101-113.

Weaver, R. J., Koch, R. E., & Hill, G. E. (2017). What maintains signal honesty in animal colour displays used in mate choice?. *Philosophical Transactions of the Royal Society B: Biological Sciences, 372*(1724), 20160343.

Whitten, M. M., & Coates, C. J. (2017). Re-evaluation of insect melanogenesis research: Views from the dark side. *Pigment cell & melanoma research, 30*(4), 386-401.

Willemssens, K. A. (2015). *Soil preferences of Nicrophorus beetles and the effects of compaction on burying behavior*. (Masters thesis, University of Nebraska).

Wilson, D. S., & Fudge, J. (1984). Burying beetles: intraspecific interactions and reproductive success in the field. *Ecological Entomology, 9*(2), 195-203.

Wormington, J. D., & Luttbeg, B. (2018). Red clypeal membrane color predicts immune function in a burying beetle (Coleoptera: Silphidae). *Journal of Zoology, 304*(4), 284-292.

Zeng, Y. (1995). University of Florida Book of Insect Records Chapter 12 Longest Life Cycle.

Ziegler, I., & Harmsen, R. (1970). The biology of pteridines in insects. In *Advances in insect physiology* (Vol. 6, pp. 139-203).

APPENDICES

Daily ABB care procedures

The beetles were kept individually in semi-translucent lidded plastic containers (76mm x 78mm depth) with a damp paper towel under a 12:12 photoperiod cycle at 24.4-25.5 °C. They were fed three times a week (Monday, Wednesday, and Friday) with two mealworms and two waxworms. Each container with ABB was secured by one 5mm rubber band to prevent escape. At each feeding all uneaten food was removed and the paper towel substrate was used to wipe out the enclosure before being replaced with a new paper towel, dampened with excess water squeezed out by hand. Every beetle was cataloged with relevant information being recorded on a label attached to the individual's container and later added to an Excel spread sheet. Table 3 shows information recorded for the A1.1 brood from which the photograph models belonged.

Table 3. ABB brood A1.1 colony data

ID	Emergence date	Gender	Pronotal width (mm)	Weight (g)	Death Date
A1.1-1	29-Jun-22	M	11.2	1.404	23-Sep-22
A1.1-2	29-Jun-22	M	10.48	1.483	23-Sep-22
A1.1-3	29-Jun-22	M	10.7	1.283	23-Sep-22
A1.1-4	29-Jun-22	F	10.99	1.334	12-Sep-22
A1.1-5	29-Jun-22	M	11.56	1.437	26-Sep-22
A1.1-6	29-Jun-22	M	11.68	1.615	23-Sep-22
A1.1-7	29-Jun-22	M	11.16	1.239	12-Sep-22
A1.1-8	29-Jun-22	M	11.2	1.458	23-Sep-22
A1.1-9	29-Jun-22	F	10.87	1.334	12-Sep-22
A1.1-11	29-Jun-22	F	11.41	1.481	23-Sep-22
A1.1-12	29-Jun-22	F	11.54	1.452	27-Jul-22
A1.1-13	29-Jun-22	M	10.56	1.111	23-Sep-22
A1.1-14	29-Jun-22	F	12	1.48	3-Oct-22
A1.1-15	29-Jun-22	M	11.71	1.533	5-Oct-22

Data were collected for each brood attempt. Table 4 shows information collected for various pairings as well as each number of offspring. The A1.1 Brood is highlighted because it was used for this experiment.

Table 4. ABB brood data

Bucket ID	Intro date	Food type	weight (g) corpse	Burial	Offspring Emerge	# of Offspring
AT.1	1/25/2022	Rat	179.722	No	N/A	N/A
BT.1	1/25/2022	Rat	178.753	Yes	3/16/2022	2
AT.2	2/1/2022	Quail	112.093	Partial	N/A	N/A
CT.1	2/1/2022	Quail	112.137	Yes	3/25/2022	7 Live Beetles
AT.3	2/22/2022	Quail	109.52	Yes	4/16/2022	7 Live Beetles
DT.1	2/22/2022	Quail	110.49	Yes	4/16/2022	22 Live Beetles
BU.1	4/5/2022	Quail	114.183	Yes	5/24/2022	19 Live Beetles
CU.1	4/5/2022	Rat	75.227	Yes	5/23/2022	1
A1.1	5/9/2022	Quail	120.017	Yes	6/29/2022	15 Live Beetles
D1.1	5/9/2022	Rat	95.413	Yes	N/A	N/A
E1.1	5/9/2022	Quail	129.551	Yes	N/A	N/A
D1.2	5/27/2022	Quail	117.467	Yes	N/A	N/A
E1.2	5/27/2022	Rat	96.337	Yes	N/A	N/A

A secondary experiment was conducted to test if brood soil temperature influenced ABB reproduction from Oklahoma and Nebraska. Table 5 shows the information collected on brood buckets kept at three different temperatures: 15 °C, 20 °C, and 26 °C. Breeding attempts without a successful brood are marked N/A.

Table 5. ABB brood soil temperature experiment

Bucket ID	Intro date	Temp °C	Food type	weight (g) corpse	Burial	Offspring Emergence	# of Offspring
BXN.1	6/24/2022	15	Quail	114.79	Yes	9/16/2022	18
CXN.1	6/24/2022	15	Quail	116.229	Yes	9/16/2022	19
FXN.1	6/24/2022	20	Quail	122.136	Yes	8/19/2022	25
GXN.1	6/24/2022	20	Quail	117.215	Yes	8/24/2022	12
HXN.1	6/24/2022	26	Quail	110.353	Yes	N/A	N/A
IXN.1	6/24/2022	26	Quail	108.743	Yes	8/10/2022	11
JXO.1	6/24/2022	15	Quail	114.539	Yes	9/16/2022	5
KXO.1	6/24/2022	15	Quail	113.434	Yes	N/A	N/A
LXO.1	6/24/2022	20	Quail	119.09	Yes	N/A	N/A
MXO.1	6/24/2022	20	Quail	111.922	Yes	N/A	N/A
NXO.1	6/24/2022	26	Quail	110.762	Yes	8/10/2022	16
OXO.1	6/24/2022	26	Quail	118.991	Yes	8/10/2022	16

Table 6. Successful ABB broods with number of individuals and average measures

Brood ID	# Total	# male	# Female	Avg pronotal width (mm)	Avg weight (g)
A1.1	15	9	6	11.23	1.402
AT.3	7	4	3	9.92	0.892
BT.1	2	1	1	10.73	1.418
BU.1	18	10	8	10.62	1.187
BXN.1	8	2	6	8.78	0.723
CT.1	7	2	5	11.17	1.601
CXN.1	13	2	11	8.94	0.742
DT.1	22	12	10	10.92	1.204
FXN.1	25	7	18	8.87	0.814
GXN.1	10	8	2	10.23	1.028
IXN.1	12	5	7	11.21	1.462
NXO.1	16	6	10	10.75	1.224
OXO.1	16	11	5	9.82	0.815

VITA

Robert Shane McMurry

Candidate for the Degree of

Master of Science

Thesis: ESTABLISHING AGE-BASED COLOR CHANGES FOR THE AMERICAN BURYING BEETLE, NICROPHORUS AMERICANUS (OLIVIER)

Major Field: Entomology

Biographical:

Education:

Completed the requirements for the Master of Science in Entomology at Oklahoma State University, Stillwater, Oklahoma in December 2022

Completed the requirements for the Bachelor of Science in Horticulture at Oklahoma State University, Stillwater, OK in 2013.

Experience:

1st place Graduate Poster, Entomological Society of America SWB 2013
"Identification and manipulation of natural enemies of key arthropod pests in Oklahoma vineyards"

Wentz Research Scholarship Recipient
2011-2012
"Evaluating Trap Crop Effectiveness to Control Thrips Populations"

Professional Memberships:

Entomology Society of America 2013-2016, 2019
Coleopterist Society 2014