

BOLD OR BOXED?: TESTING FOR MORPHOLOGICAL AND PHYSIOLOGICAL
DIFFERENCES BETWEEN EASTERN BOX TURTLES (*TERRAPENE CAROLINA*)
WITH INDIVIDUAL VARIATION IN BOLDNESS

by

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ABSTRACT

Consistent individual variation in behavior has become a well-known and recognized phenomenon across animal taxa and is commonly referred to as animal personality. As the number of animal species and populations exhibiting personality continues to grow, many researchers have turned their attention towards studying the proximate causes and fitness implications of personality traits. In behavioral ecology and evolution consistent differences in individual state (intrinsic and/or extrinsic) are often theorized to co-vary with animal personality traits. Relatedly, some research has suggested that animal personality may involve individual differences in coping styles (e.g., proactive vs reactive), pace-of-life, and behavioral plasticity. The eastern box turtle, *Terrapene carolina*, is a long-lived reptile that is relatively easy to track and recapture and may therefore present an interesting and accessible vertebrate model for studies of animal personality traits in the wild. Past studies on this species have suggested it exhibits boldness personality and that this trait may interact with temperature and shell damage but not sex, age, or morphology. This study sought to further explore boldness as a personality trait in wild *T. carolina* and these previously studied interactions in addition to body condition, pinch force, innate immunity, plasma triglycerides, steroid hormone concentrations, and gastrointestinal nematode loads. The results indicate that eastern box turtles display consistent bold personalities across individuals and suggests that less bold individuals tend to display higher levels of plasticity in their bold responses (emergence from the shell) than bolder turtles. Moreover, box turtles appear to have the ability to habituate to repeated handlings which may increase their likelihood to behave boldly in subsequent tests. Turtles that are consistently proactive in their use of active defenses

during a potentially threatening encounter may be more vulnerable to predation as they appear less likely to tightly close the shell (had lower pinch force values). However, turtles with the inability to fully close the shell (regardless of boldness) may also suffer similar consequences. Interestingly, boldness appears to be largely independent of the short-term physiological variables considered in this study, although there was a negative trend between average eye emergence and body condition, suggesting turtles that emerge quicker (bolder) may have higher body conditions. Additionally, boldness appeared to be dependent on some short-term environmental conditions, such as cloud coverage and immediate shell temperature, but appears to be largely uncoupled from the daily temperatures experienced across several days. Lastly, daytime temperatures differed between sexes and negatively correlated with age, suggesting that turtles of these distinctions may thermoregulate differently. Daytime temperatures also exhibited nearly significant positive trends with shell injury scores and body condition meaning there could be differential consequences for thermoregulators and thermoconformers. Further studies are needed to better understand the implications of these interactions and their possible correlation with other potential personality traits like aggression and exploration.

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INTRODUCTION

A. Introducing Animal Personality

What is animal personality?

In recent decades, there has been increasing interest in measuring and describing behavioral variations that exist across individuals of animal species and populations. A growing number of these studies have placed particular focus on behavioral traits expressed with consistent and stable individual variation. These traits are commonly referred to as personality traits while species expressing such traits are said to exhibit ‘animal personality’, a term adopted from traditional studies of psychology involving individual differences in the behavioral tendencies of humans (Carter *et al.*, 2010; Kaiser & Müller, 2021; Réale *et al.*, 2007, 2010a, b; Stamps & Groothuis, 2010; Wolf & Weissing, 2012). Following early recognition of consistent individual variation in certain behaviors of non-human animals (e.g., see Gosling, 2001; Wilson, 1998; Wilson *et al.*, 1993), many researchers of the behavioral sciences sought to describe these underlying personality traits. This has since been reflected by a growing number of animal personality studies in vertebrates and more recently, invertebrates (examples: insects: Tremmel & Müller, 2013; arachnids: Kwek *et al.*, 2021; and mollusks: Dahirel *et al.*, 2021; also see Kralj-Fišer & Schuett, 2014; Modlmeier *et al.*, 2015). As such, animal personality is now a well-documented phenomenon in several major animal phyla (Kralj-Fišer & Schuett, 2014; Sih *et al.*, 2004a; Sih *et al.*, 2015; Wolf & Weissing, 2012). Today, there continues to be an increase in identified animal personality traits and recognized species with personality in general. Some of the most frequently cited personality traits relate to forms of aggression, exploration, boldness, and sociability,

however dozens of others have also been described (see reviews Gosling, 2001; Kaiser & Müller, 2021; Réale *et al.*, 2007).

Why study animal personality?

Although further describing personality traits across animal taxa remains a major goal today, many researchers within these fields have turned their attention to exploring the underlying causative effects, ecological relevance, and evolutionary implications. (Carter *et al.*, 2010; Koolhaas *et al.*, 2007; Réale *et al.*, 2010a, b; Stamps & Groothuis, 2010; Wolf & Weissing, 2012). For behavioral ecologists, this often involves detailing the physiological and/or environmental factors that directly influence or interact with the expression of personality traits (e.g., Dall *et al.*, 2012; Koolhaas *et al.*, 2007; Sih *et al.*, 2015). Research into these areas may shed light on the trade-offs associated with personality traits by demonstrating how the underlying behaviors interact with other phenotypic characteristics or change across ecological and developmental contexts (Carter *et al.*, 2010). Indeed, studies have demonstrated animal personality traits as heritable (Ariyomo *et al.*, 2013; Dingemanse *et al.*, 2002) and capable of effecting survivability (Broecke *et al.*, 2021; Hall *et al.*, 2017a; Smith & Blumstein, 2008) and reproductive success (Ariyomo & Watt, 2012; Both *et al.*, 2005; Vargus *et al.*, 2018). The trade-offs of personality traits are therefore important to consider empirically given that they may influence fitness of the individual and their offspring.

Descriptions of the fitness implications of personality traits within the framework of selection theories may, for instance, help explain why individual members of a species or population will consistently differ in their methods and tendencies for aggression (e.g.,

Kwek *et al.*, 2021), territoriality (e.g., Amy *et al.*, 2010), courtship (e.g., Chen *et al.*, 2018; Li *et al.*, 2021; Munson *et al.*, 2020; Pogány *et al.*, 2018), sociality (e.g., Bergmüller & Taborsky 2010; Bull *et al.*, 2017; Gartland *et al.*, 2022), parenting (e.g., Lou *et al.*, 2021), feeding (e.g., Neave *et al.*, 2018), and dispersion (e.g., Cote & Clobert, 2007), among many other ecologically relevant behavioral phenotypes (also see Wolf & Weissing, 2012). If personality traits are dependent on differences in individual state (e.g., physiology, morphology, social status) and therefore interact with the individual's intrinsic and extrinsic environments, then further explaining how these traits perform in certain contexts may reveal more about when they are advantageous or harmful (Dall *et al.*, 2004; Dall *et al.*, 2012; Koolhaas *et al.*, 1999; Réale *et al.*, 2010a, b; Sih, *et al.*, 2015; Wolf & Weissing, 2012).

Approaches to studying animal personality

There are several different approaches to studying animal personality (Table 1). Although some studies choose to compare personality traits across species of varying relatedness (e.g., turtle species of different families in Ibáñez *et al.*, 2018), most personality studies to date instead highlight comparisons within a single species. In addition, such intraspecific studies might choose to investigate personality traits within or across populations depending on the aims of the study. For example, the within-population approach may be valuable to understanding aspects of individual coping styles under similar environmental contexts, whereas the across population approach might detail differences in niche specialization that occur across populations living under different environmental contexts (e.g., see Dall *et al.*, 2012). The within-species approach

also has additional options for study, such as within or across different sexes, developmental stages, age groups, or morphs of polymorphic species. In all regards, studies may conduct their behavioral tests under natural or laboratory conditions, or both (e.g., Wilson *et al.*, 1993). Each of these methods present potential advantages and limitations that will likely depend on the study organism, duration of study, and behavioral traits of interest. However, studies of personality traits within species under largely natural conditions can be especially valuable when considering the ecological implications and evolution of personality (Kaiser & Müller, 2021). For this study, the within-species approach was used to study adult male and female box turtles of a Middle Tennessee population in their natural forested wetland habitat.

Table 1. Various approaches to studying animal personality traits.

First Level of Approach	Second Level of Approach	Recent Example Sources
I. The Across-Species Approach	A. Across Distantly Related Species (e.g., different family, order, class, phylum)	Rey <i>et al.</i> (2021); White <i>et al.</i> (2019)
	B. Across Closely Related Species (e.g., same family or genus)	Carter & Feeney (2012); Ibáñez <i>et al.</i> (2018)
II. The Within-Species Approach (May also investigate within- or across sexes, age, polymorphs, etc.)	A. Across Populations	Brand <i>et al.</i> (2021); Michelangeli <i>et al.</i> (2019)
	B. Within-Populations	Jolles <i>et al.</i> (2019); Kwek <i>et al.</i> (2021); Pogány <i>et al.</i> (2018); This study

Evolutionary and ecological perspectives of animal personality

A stumbling block from the evolutionary view of animal personality results from its puzzling origins and persistence throughout Animalia despite having obvious fitness

costs that could instead be mitigated by behavioral plasticity, in which the behavioral repertoires are flexible across contexts and adjust to better fit ecological and physiological demands (see Dingemanse *et al.*, 2010; Wolf *et al.*, 2008). In other words, when considering evolution by selection, the existence of personality within a group of animals might seem non-intuitive in comparison to more flexible behavior. It is possible that personality traits could impede the benefits otherwise offered by behavioral plasticity (e.g., see Dall *et al.*, 2012; Carter *et al.*, 2012). A central question might be why personality traits would evolve across so many taxa if suitable responses to environmental changes could instead be effectively assumed by all members of the group via behavioral plasticity? Would these personality differences not cause some individuals to be less suited to certain environmental circumstances than others and thus experience reduced fitness in those contexts?

Several studies of animal personality have begun to address this seemingly detrimental effect of personality traits and more critically examine the adaptive roles and evolutionary origins of both behavioral plasticity and animal personality (e.g., Carter *et al.*, 2012). This has led to more careful consideration of the underlying interactions important to personality and plasticity development and expression. This includes (although not limited to): (1) the possible correlations between consistent individual variation in state with consistent variation in behavior (Niemelä & Dingemanse, 2018; Pich *et al.*, 2019; Sih *et al.*, 2015), (2) the effects of past experiences (Frost *et al.*, 2007; Sih *et al.*, 2015) and early development (Rödel & Monclús, 2011; Trillmich *et al.*, 2018), (3) the reliability of environmental cues and how they might influence the evolution in behavioral flexibility (Carter *et al.*, 2012), and (4) where the underlying behaviors may

fall in terms of a “plasticity-personality continuum” (Carter *et al.*, 2012). The latter may be especially important to address, as there are misconceptions which frame personality and plasticity as strictly opposing traits of animal behavior. However, studies suggest that species and populations could exhibit behavioral traits that consistently differ across personality types (among individuals) while also exhibiting behavioral plasticity within individuals (e.g., Carter *et al.*, 2012; Hall *et al.*, 2017b). Furthermore, because the degree and/or direction of behavioral plasticity can consistently vary among individuals, personality and plasticity may often co-vary. (e.g., Carter *et al.*, 2012, Dingemanse & Wolf, 2013; Hall *et al.*, 2017b; Wolf *et al.*, 2008). For example, bolder individuals of some fish species have been shown to exhibit less flexibility in their bold behaviors across repeated tests in comparison to their shier counterparts (Jolles *et al.*, 2019; Kareklas *et al.*, 2016; Magnhagen & Bunnefeld, 2009).

In behavioral and evolutionary ecology, a popular theory is that animal personality is an adaptive response to consistent differences in individual state, coping style, or pace-of-life (Dall *et al.*, 2004; Dall *et al.*, 2012; Koolhaas *et al.*, 1999; Réale *et al.*, 2010a, b; Sih *et al.*, 2015; Wolf & Weissing, 2012; although see Niemelä & Dingemanse, 2018). These individual-specific states are also likely to be shaped by early experiences and include variables that might be environmental, morphological, physiological, behavioral, or social in nature (Sih *et al.*, 2015). Specific differences in personality may then allow for a behavioral adaptation for one state or another and therefore be tightly linked to these other traits (cross-trait) or contexts (cross-context) (Pich *et al.*, 2019; Sih *et al.*, 2015; Sih *et al.*, 2010). Discovering these interactions may help explain the trade-offs of certain personality traits. For example, while being bold

may be highly adaptive in one context, it may be maladaptive in another (Carter *et al.*, 2010, 2012). Therefore, individuals that associate with the first context may tend to be consistently bolder than those that associate with the second, creating consistent individual variation across the group. This may be especially true when the onset of- or switch between- contexts is unpredictable (i.e., the environmental cues are unreliable), reducing the reliability of plasticity, and possibly providing an evolutionary advantage for populations that consistently produce various individuals suited for either context (Carter *et al.*, 2012; Sih *et al.*, 2004a, 2010). Moreover, a primary behavior underlying personality differences could be tightly linked to other beneficial secondary traits (cross-trait) and therefore be selected for when the advantages of this relationship outweigh any direct disadvantages (Pich *et al.*, 2019). In fact, ‘behavioral syndromes’, defined when a “suite of correlated behaviors” is expressed with consistent individual variation, are forms of cross-trait interactions that are frequently considered in animal personality studies (Bell, 2007; Sih *et al.*, 2004a, b; Sih *et al.*, 2010; Wolf *et al.*, 2010).

B. Study Design Introduction: Boldness Personality of Box Turtles

Personality in the eastern box turtle, Terrapene carolina

Personality, in which a set of related measurable behavior(s) exhibit consistently high variation between individuals and low variation within individuals, has been shown to exist in several non-mammalian vertebrates, including fish (e.g., Vargus *et al.*, 2018; Jolles *et al.*, 2019), frogs (e.g., Videlier *et al.*, 2018), salamanders (e.g., Moore & Nicholson, 2021), lizards (e.g., Carter *et al.*, 2012; Ward-Fear *et al.*, 2018), turtles (e.g., Allard *et al.*, 2019; Carter *et al.*, 2016; Ibáñez *et al.*, 2014), and birds (e.g., Hall *et al.*,

2017a; Pogány *et al.*, 2018). The eastern box turtle (*Terrapene carolina*) of the family Emydidae exhibits intraspecific and intrapopulation behavioral variations between individuals in risk-taking behaviors (i.e., “boldness”) which have shown both temporal stability and contextual consistency within individuals (e.g., Carlson & Tetzlaff, 2020; Kashon & Carlson, 2018; Pich *et al.*, 2019; Preston *et al.*, 2020).

Aggression and exploration are often associated with boldness in vertebrates, including reptiles (e.g., Allard *et al.*, 2019; Carter *et al.*, 2010), and are also loosely reported in box turtles (Dodd, 2001; although see Stickel, 1989). Dodd (2001) states that “aggressive tendencies [in box turtles] seem associated with certain individuals”, and explains that while some individuals remain docile, others are consistently prepared to fight. A study on the social and spatial aspects of *T. carolina* suggested that these turtles may also rely on neighbor recognition, stating that in general turtles tended to exhibit more aggressive and/or exploratory behavior towards unfamiliar conspecifics (Davis, 1981 unpublished dissertation; Dodd, 2001). Based on these few accounts, aggressive and exploratory behaviors could possibly represent personality traits in eastern box turtles and may be integral aspects of their social ecology. If this is true, aggressive and exploratory personality traits may also be correlated with boldness within a behavioral syndrome, as is often the case with these types of behaviors in other vertebrates (Kendall *et al.*, 2018; Mazué *et al.*, 2015).

Box turtles as a model for studying bold personality

Eastern box turtles (*Terrapene carolina*) are often long-lived, remain largely terrestrial, and typically exhibit small home ranges throughout life (Dodd, 2001; West &

Klukowski, 2016). Given that *T. carolina* is also relatively easy to handle, observe, and track in the wild, they might offer an exemplary vertebrate study system for animal personality research under natural testing conditions. The latter, trackability, is essential to personality research, as being able to resample the behavior of the animal is critical to testing the individual repeatability of behavior (Dingemanse & Wright, 2020). Indeed, box turtles have been successfully tracked via threading and telemetry methods (reviewed in Dodd, 2001) and because of their small home-ranges are relatively easy to recapture across days, seasons, or even years.

Recently, measures of boldness have become popular in personality studies of wild and captive turtles of various species (Allard *et al.*, 2019; Cassola *et al.*, 2020; Kashon & Carlson, 2018; Ibáñez *et al.*, 2014, 2018). This is particularly interesting in species of *Terrapene*, as these turtles possess a shell with reduced plastral buttresses and a well-developed plastral hinge, which together give these turtles the ability to completely close the shell via a process known as “shell kinensis” (Cordero *et al.*, 2018; Dodd, 2001). Therefore, these turtles can voluntarily withdraw their head and limbs within the shell and completely shut the anterior and posterior openings (although exceptions are observed, with some individuals seemingly being unable to fully withdraw and/or completely close the shell). This ability of *Terrapene* is believed to have evolved independently from other Emydids in concurrence with a shift to an increasingly terrestrial lifestyle by ancestral *Terrapene*. As such, this level of shell kinensis is not seen in other Emydids, all of which have remained primarily aquatic (Cordero *et al.*, 2018; Dodd, 2001). Even among extant turtles (Testudines) as a whole, this degree of shell kinensis is rare, with only two known genera, *Terrapene* and *Cuora* (of Geoemydidae),

possessing the ability to completely close the shell (Cordero *et al.*, 2018; Dodd, 2001). Given that these two genera collectively consist of roughly 20 species, the eastern box turtle is one of few turtles to have evolved this level of shell kinesis. Could it be possible that changes in behavioral phenotypes coincided with its evolution?

Boldness of turtles is often operationally defined by one or more of the following measures during or immediately after an encounter with an investigator or simulated predator: (1) the time it takes for the turtle to begin retreating into and/or close the shell, (2) the degree of head and limb retraction and shell closure, (3) the time that it takes, once withdrawn, to emerge and begin moving, (4) the distance the turtle moves over a certain time frame, and (5) the number of active defenses deployed (e.g., bites, urination/defecation, flee attempts) during or shortly after handling (see Carlson & Tetzlaff, 2020; Ibáñez *et al.*, 2014, 2018; Kashon & Carlson, 2018; Pich *et al.*, 2019; Preston *et al.*, 2020). Using these measures, studies are just beginning to explore the variations and fitness implications of boldness behaviors and personality types that exist within and across Chelonian species and populations.

What trade-offs might be associated with boldness in box turtles?

There are several possible trade-offs associated with boldness to consider in turtles, many of which might be expected for ectotherms in general. For example, while “bolder” individuals may be able to better secure territories, mates, food, or basking sites over shier counterparts, they may also be more likely to suffer from exposure-, predator- and/or combat-related injuries or infections by parasites (e.g., Carter *et al.*, 2010; Patrick *et al.*, 2017; Ward-Fear *et al.*, 2018). Such trade-offs involving reproduction and

survivability are consistent with predictions of the pace-of-life syndrome hypothesis, which would suggest that bolder individuals adopt a “live-fast, die young” pace of life (Broecke *et al.*, 2021; Jacques-Hamilton *et al.*, 2017; Réale *et al.*, 2010b). As for box turtles in particular, Kashon & Carlson (2018) found that boldness in an Indiana population of *T. carolina* correlated with body temperature and shell injuries, in that turtles that tended to emerge quicker from the shell following confinement tended to have higher average body temperatures in comparison to “shier” individuals, but also tended to have higher (although non-significant) levels of shell damage. These findings indicated that while bold turtles may acquire the advantages offered from thermoregulation more so than their shy counterparts, they might also suffer higher levels of shell damage, the latter supposedly due to increased encounters with conspecifics and/or predators (Kashon & Carlson, 2018). If true, this could demonstrate some potential trade-offs for boldness in eastern box turtles. However, more recent follow-up studies from the same research group concluded that while temperature may influence emergence, it does not appear to be a strong determinant of the behavior (Carlson & Tetzlaff, 2020; Pich *et al.*, 2019). Additionally, several other studies centered on the thermoregulation of box turtles suggest that they are largely thermoconforming rather than active regulators of body temperature (Parlin *et al.*, 2018). In fact, investigations by Parlin *et al.* (2018) suggest that eastern box turtles, at least in their northern range of southwest Ohio, are “broad environmental generalists”, with activity and movement patterns that are largely “independent of environmental conditions”. Therefore, thermoregulation does not appear to be an integral advantage tied to boldness in *T. carolina*, and other benefits from boldness should be considered. Moreover, while increased conspicuousness and

predation are reasonable and likely costs of boldness behaviors by box turtles, others could also exist (e.g., parasitism). Because boldness behaviors of box turtles can be easily observed and measured in the wild, these traits should be further compared along with physiological (e.g., hormones, immune measures), and ecological (e.g., temperature) variables to elucidate any cross-trait or cross-context advantages and costs of bold personalities.

Steroid hormone variation as possible correlate of boldness

Steroid hormones often play critical roles in the development, expression, and life-history trade-offs of various traits in vertebrates, including turtles (reviewed in Blanvillain *et al.*, 2011). Corticosterone, the major stress hormone in reptiles, is involved in the adaptive response to long-term stressors, but also tends to exert numerous effects on development, activity, energy allocation, immune function, and overall health (Blanvillain *et al.*, 2011). Boldness personality and stress (short- and long-term) could therefore be linked in many ways. Indeed, studies have suggested that bolder, more “proactive” (characterized by active fight-or-flight behaviors during stressful situations) individuals exhibit different physiological patterns of stress, such as lower hypothalamic-pituitary-adrenal axis activity, when compared to more timid, “reactive” individuals (Atwell *et al.*, 2012; Koolhaas, 2008; Koolhaas *et al.*, 2007; Øverli *et al.*, 2007; Thomson *et al.*, 2011). Measures of plasma corticosterone could indicate whether turtles with differing degrees of boldness differ in their stress physiology (e.g., Clary *et al.*, 2014; although see Fu *et al.*, 2021).

In addition to corticosterone, I also chose to investigate circulating levels of testosterone in males and progesterone in females. These sex steroids are likely to have important roles in mating and nesting behaviors by *T. carolina* during the period of this study (West & Klukowski, 2018). For example, testosterone is a well-studied hormone across reptilian (particularly lizards) taxa and in addition to its anabolic effects on growth, it also has many sex-specific functions such as the expression of male-typical secondary sexual characteristics and sexual-related behaviors (Hews *et al.*, 2012; Hews & Moore, 1995; Hillsman *et al.*, 2007; also see Blanvillain *et al.*, 2011). Secondly, several studies have suggested testosterone to have an immuno-suppressing effect in at least some avian and non-avian reptiles, which could have far-reaching implications on infection (e.g., parasite loads) and overall health status (e.g., Belliure *et al.*, 2004; Cox & John-Alder, 2007; Duffy *et al.*, 2000; Tripathi & Singh, 2014). Although the effects of testosterone have not been well studied or demonstrated in Testudines, it is assumed to exhibit similar effects as observed in other reptilian and avian groups. Progesterone plays key roles in regulating uterine growth, calcification of shells during egg development, and reproductive cycles in females of oviparous reptiles (Custodia-Lora & Callard, 2002; Custodia-Lora *et al.*, 2004a, b; Kawazu *et al.*, 2014; Blanvillain *et al.*, 2011). Specifically, progesterone tends to have a periovulatory increase in oviparous reptiles, falling just after ovulation during the shelling process (Blanvillain *et al.*, 2011). This increase in progesterone may assist in inhibiting further estrogen-induced vitellogenesis (regulating the reproductive cycle), stimulation of the final processes for follicular maturation and ovulation, and possibly slowing oviductal contractions for shelling deposition to occur (Custodia-Lora & Callard, 2002; Custodia-Lora *et al.*, 2004a, b;

Blanvillain *et al.*, 2011). Testosterone also appears to play important reproductive roles in female turtles from regulation of reproductive cycles (Custodia-Lora *et al.*, 2004a; Blanvillain *et al.*, 2011) to physiological constraints on egg size (Bowden *et al.*, 2004), however testosterone was not measured in females of this study.

Immunity, parasites, and fat mobilization as possible correlates of boldness

Because boldness behaviors might be associated with locomotor activity (Fu *et al.*, 2021; Wilson & Godin, 2010), exploration (Michelangeli *et al.*, 2019; Patrick *et al.*, 2017), and dispersion (Fraser *et al.*, 2001), there may be several indirect (e.g., via steroid hormones, acquired parasites) effects of boldness on immune function and energy mobilization (e.g., fats) in box turtles (West & Klukowski, 2018). One aspect of the innate immune response in reptiles involves the lysis of foreign and potentially pathogenic antigens by natural antibodies and the complement system (Baker *et al.*, 2019; Zimmerman, 2020; Stromsland & Zimmerman, 2017). This aspect of immunity can be quantitatively assessed via a hemolysis immunoassay in which foreign red blood cells are mixed with the plasma of the vertebrate subject for subsequent scoring of lysis (Baker *et al.*, 2019; West & Klukowski, 2018). Given that parasitism could interact with immunity (e.g., Stromsland & Zimmerman, 2017), stress (e.g., Raouf *et al.*, 2006), and locomotion (Binning *et al.*, 2017), it too may be a consequence of boldness in reptiles. Although wild eastern box turtles are rarely observed to be parasitized by ectoparasites (e.g., ticks or mites), endoparasites such as gastrointestinal nematodes are commonly reported (albeit in low prevalence (Moraga *et al.*, 2012; Rose *et al.*, 2011)) and may therefore influence the health and behavior of host turtles when under high loads of parasitism (Barber &

Dingemanse, 2010). Finally, because individuals with differing levels of boldness may differ in their levels of anabolic steroid hormones, daily activity, and metabolic rate (Hau & Goymann, 2015; Koolhaas *et al.*, 1999; Mathot *et al.*, 2018; Careau *et al.*, 2008), the concentration of plasma lipids, such as triglycerides, could provide insight into whether bolder turtles are mobilizing energy differently from less bold individuals during this active season (West & Klukowski, 2018). This could be especially important in female box turtles as plasma triglycerides are known to increase during vitellogenesis and nesting in other turtle species (Duggan *et al.*, 2001; Hamann *et al.*, 2002; West & Klukowski, 2018; Stickel, 1950).

Further investigation of temperature as a correlate of boldness

Lastly, while studies of box turtle thermoregulation have largely suggested these turtles to be thermoconforming, few studies have investigated the links between temperature and behavior in *T. carolina*. As ectotherms, many turtles rely on thermoregulation by behavioral means. Indeed, thermoregulation likely serves several functions in turtles, including (but not limited to) locomotion and righting ability (Elnitsky & Claussen, 2006), effectiveness of innate and adaptive immune responses (Merchant *et al.*, 2012; Palackharry *et al.*, 2017; Zimmerman, 2020; Zimmerman *et al.*, 2017) behavioral defenses, such as biting (Vervust *et al.*, 2011), and the induction of “behavioral fevers” (Monagas & Gatten Jr, 1983; Rakus *et al.*, 2017). It is also possible that individual turtles differ consistently in their preferred temperatures or temperature sensitivity, which could be linked to personality traits (Michelangeli *et al.*, 2017). For example, the Australian delicate skink (*Lampropholis delicata*) exhibits consistent

“thermal types” (on a cold-hot continuum) based on preferred temperatures, and these thermal types relate to consistent individual variation in activity, exploration, and boldness (Michelangeli *et al.*, 2017, 2019; Goulet *et al.*, 2017a, b). Given that the activity required for bold, exploratory, and aggressive behaviors should be dependent on environmental temperatures in turtles, an interaction between thermal type and personality traits could exist.

Thermal habitat selection should also be important for nesting and ovipositing females not only for its effects on offspring survival (Refsnider *et al.*, 2022) but also because *T. carolina*, like most turtles, exhibit temperature-dependent sex determination (TSD), meaning the environmental temperature is a critical driver for sexual differentiation of developing embryos (Dodd, 2001; Ewert & Nelson, 1991). Although the pivotal temperature(s) at which sexual differentiation transitions to male or female bias have not been well documented for box turtles, one source states that *T. carolina* eggs incubated in the lab under constant temperatures below ~28°C produced primarily males (although ratios become slightly less male biased at even cooler temperatures of ~22.5°C but remain above 70% male) whereas eggs incubated at 30°C produced exclusively females (Dodd, 2001). This suggests *T. carolina* likely has a single transition zone for biasing one sex or another somewhere between 28 and 30°C (Dodd, 2001), however, more work on TSD in box turtles is needed to determine the exact pivotal temperature(s) and pattern of sexual differentiation.

As suggested in Kashon and Carlson (2018), bolder box turtles may thermoregulate at a higher average temperature than their shy counterparts, which could

provide bold individuals with any of the above-mentioned benefits. I sought to further test the potential interaction between temperature and boldness behaviors of box turtles by collecting continuous temperature data from the carapaces of turtles and nearby reference sites in the field to then calculate each turtle's differential temperature from the reference sites. Additionally, I examined potential interactions between thermoregulation and nematode loads, shell damage, or plasma steroid concentrations.

Study objectives

My study had two major objectives: (1) to test for the existence of boldness personalities in wild box turtles of a middle TN population using boldness measures comparable with those of previous studies (Kashon & Carlson, 2018), and (2) This study attempted to identify traits correlated with boldness in adult *T. carolina* turtles through investigations of thermoregulation, shell damage, immunoassays, parasite loads, triglyceride levels, body condition, and steroid hormone concentrations. When applicable, these variables are discussed in terms of their cross-trait relationships with boldness and how such interactions might explain the trade-offs of boldness personality types in box turtles. In general, this study aims to shed light on the potential evolutionary advantages and costs presented by bold personalities in this chelonian species.

MATERIALS AND METHODS

Study site and subjects

In this study male and female adult eastern box turtles (*T. carolina carolina*) were sampled from a 23.5 ha forested wetland preserve in Murfreesboro, Tennessee, USA (West & Klukowski, 2016). Four sets of seven turtles (28 total) were captured and tested in behavioral assays between May and mid-August of 2021. Subjects were tested 5 times over an average of 17.9 days (range: 15 – 30 d) to evaluate consistencies in their behavior during and immediately following handling. A minimum 3-day recovery period occurred between behavioral assays to reduce the likelihood that turtles would habituate to interactions with the investigator (similar to Kashon & Carlson, 2018). All capture and behavioral assays occurred between 0900 and 15:30 CDT. In an attempt to prevent the spread of ranavirus (Preston *et al.*, 2020), fresh nitrile gloves were used when handling each turtle and all equipment was cleaned between use by applying a 5% bleach solution for 60 sec.

Initial capture and assay conditions

Following initial capture, each turtle was placed individually into a plastic bucket, transported to the MTSU vivarium, and kept overnight (< 24 hours) within their respective buckets at ~22°C. Turtles were kept on a 12:12 h day/night cycle of incandescent lighting while in captivity. During this time the turtles were physically and visually secluded from other turtles captured that day, however they could likely hear and smell one another. Turtles were only handled during their overnight stays to capture photographs and fecal samples as well as to attach a radio transmitter (RI-2B Holohil

Systems, Ontario Canada) and a Thermochron® iButton temperature data logger (DS1921G, Maxim Integrated, San Jose, CA) (see details below). In less than 24 h from their initial capture, turtles were returned to their capture sites (± 2 m) to undergo the first behavioral assay and subsequent release. Following the first assay, tracking and behavioral testing commenced for 4 additional captures with at least a 3-day recovery period between each. Recovery periods were often scheduled so that turtles would have two 3-day recovery periods and two 4-day recovery periods in-between tests, although several turtles experienced 5-to-6-day recovery periods and one turtle (which moved onto private property) had 22 days between two consecutive tests (average: 4.3 days; range: 3 - 22 days). This latter individual was only tested for a total of 4 assays rather than five. Measures of assay conditions were also collected for each capture event including an immediate infrared temperature reading (Etekcity Infrared Thermometer 749) from the carapace at the anterior-most vertebral scute, the time of day, and a ranking of sky conditions between 0 and 3 using the following criteria: 0 = mostly clear skies, 1 = partly cloudy or variable skies, 2 = cloudy overcast with few if any breaks between clouds, and 3 = rainy. Middle scores were sometimes given for sky conditions that were intermediate, such as a 2.5 for a cloudy day with light rain.

Behavioral assays

Behavioral assays were based largely on methods established by Kashon & Carlson (2018), albeit with slight modifications to stimulus delivery and further inclusion of additional behavioral responses. To begin an assay the turtle was picked up and handled for 3 minutes at the site of capture. During handling I would conduct pseudo-

measurements of the carapace and plastron using calipers to measure carapace length, width, plastron width, and plastron length in this order and repeating until the end of the 3-minute period (typically 6 to 8 cycles, but this varied depending on how active a turtle was during handling). Over this handling period I took note of any active defenses deployed by the turtle, which included the number of snaps, bites, and events of urination, defecation, and air walking (see Table 2 for more details). Additionally, turtles were assigned a score from 0-5 during each handling period based on the time it spent closed up in the shell. A turtle was assigned 0 if it remained completely in the shell over the entire handling period, with each increase in score corresponding to roughly 36 secs out of the shell up to approximately 180 sec (entire handling period) for a score of 5. Half scores were also given to turtles that had intermediate times out of the shell. Finally, if the subject was not fully closed by the 3-minute mark of handling, I would poke the turtle on the skin of its front legs or snout until nearly full closure occurred. Some turtles appeared incapable of completely closing the anterior and posterior openings simultaneously, having to sacrifice exposing gaps on one end to close the other. For these turtles, I settled for a state of mostly closed to begin the observation period, prioritizing closure on the anterior side.

Once at least mostly closed and by the end of the 3-min handling period, the subject was gently placed on the ground under partial or full shade and within 1.0 – 1.5 m of available shelter (e.g., thick vegetation or creek embankment) near the last site of capture. I then rapidly retreated approximately 10 m away where I quietly, with minimum body movement, observed and recorded the turtle's subsequent behavior for 10 mins with the aid of binoculars. Over this observation period, the occurrence of behaviors and their

timing to the nearest sec were noted: (1) the eye emergence latency, corresponding to the time at which the turtle extended its head so that the eyes surpassed the anterior margin of the carapace (Kashon & Carlson, 2018); (2) the wrist emergence latency, determined as the time at which the wrists of the front limbs extended out from their tucked position between the carapace and plastron; and (3) the 1 m movement latency, determined as the time at which the turtle reached 1 m in distance after opening its shell. Typically, turtles would conduct these latency behaviors in this order (if at all). If one of these behaviors did not occur during the 10-min observation period, it was given a latency score of 600 sec.

Table 2: Behaviors recorded during each 3-minute handling period that occurred prior to the start of an observation period. The first 3 behaviors were considered “active defenses” while hisses were considered as a separate behavior possibly relating to rapid withdrawal into the shell.

Snaps/Bites	The turtle attempts to or successfully bites the investigator
Urination/Defecation	There is an event of urination and/or defecation by the turtle
Air Walking	The turtle kicks its legs back and forth (typically all 4 legs)
Hisses	An audible hissing noise is made by the turtle

Boldness classification

Two methods of boldness categorization were used, (1) a simple method and (2) a compiled behavior method. In the former, eye emergence latency, a behavioral measure that had high repeatability (see results), was averaged and turtles that emerged in ≤ 100 seconds were classified as ‘bolder’ while turtles with average eye emergence times > 100

seconds were classified as ‘less bold’. This cut-off was chosen because of a distinct shift in among assay variance between individuals with either average eye emergence before or after 100 sec, with the latter exhibiting higher variance across assays. In the compiled behavior method, individual turtles were classified as either ‘bolder’ or ‘less bold’ by ranking five behavioral responses across each of the 5 tests and taking the sum of these scores over the total possible. These five behaviors were ranked as either 0, 0.5, or 1 (for the three timed/latency behaviors), or as 0 or 0.5 (for the two yes/no behaviors) (see Table A1). Because the yes/no behaviors carry less information about the turtle’s response to the handling, I weighed them with less contribution to the final points than the timed/latency behaviors. After combining the points possible from these five behaviors, each turtle was assigned a score between 0 and 4 for each test. This was then averaged across tests so that each turtle had an average value for the compiled behavior method which could be compared with results from the simple method. Following Cassola *et al.* (2020) in this scoring system, with lower scores corresponded to the ‘less bold’ category (final boldness score < 2) and higher scores corresponded to the ‘bolder’ category (final boldness score ≥ 2). Bolder individuals emerge from their shell and begin moving quicker than their shier counterparts (Kashon & Carlson, 2018; Pich *et al.*, 2019) following handling, confinement, or simulated predator encounters, and quicker emergence positively correlates with higher deployment of active defenses (e.g., bites, urination, defecation, and flee attempts) during simulated predation attempts (Pich *et al.*, 2019).

Because the two above methods agreed in boldness categorization of individuals for all but one turtle, analyses between ‘bold’ and ‘less bold’ categories were based solely

on the placement of individuals by the simpler method of eye emergence latency.

However, the five key behaviors for the more complicated, compiled behavior method and the placement of turtles by this method can be found in the appendix section (Table A1, Fig. A1).

Demographics, morphometrics, and pinch force

Individual turtles were sexed by presence or absence of plastron indentations. Male eastern box turtles typically possess a deep concavity of the plastron that assists in mounting and copulation, whereas females have a flat or very shallow indentation. This character was distinct for 25 out of 28 of our turtles. Because the other 3 turtles possessed slight concavities and plasma testosterone concentrations consistent with other males, I also categorized them as males. The age of individual turtles was estimated by counting annuli (rings) across 3 carapace scutes and then taking the average of these counts. Each turtle was also placed into an age class based on age ranges, following West & Klukowski (2016) although excluding juvenile ranges. Therefore, adult turtles were placed into one of three age classes: 10-14 yrs, 15-19 yrs, or 20+ yrs.

Upon initial capture, the turtles were immediately measured for their morphometrics using a spring scale for weight and 200 mm calipers for shell dimensions. The latter included straight-line carapace length (CL), maximum carapace width (CW), and maximum shell height (SH). Shell dimensions were primarily used to calculate a volumetric body condition index (vBCI) for each individual turtle, estimated as: $vBCI = \text{Weight} / (\pi \times CL \times SH \times (CW/6000))$ (Ashton *et al.*, 2015; Preston *et al.*, 2020). Lastly, each turtle was tested for its maximum pinch force in lbs. (subsequently converted to kg)

just prior to its final release. This was conducted by inserting a pinch gauge dynamometer (Baseline® 30 lb Pinch Gauge, Catalog no. 12-0200, Fabrication Enterprises Inc. White Plains, NY) between the carapace and plastron at the anterior gap of the shell while poking and prodding the turtle on the snout and front limbs to elicit closure onto the gauge.

Attachment and removal of radio transmitters and temperature loggers

Radio transmitters and iButtons® were attached onto the posterior end of the carapace, oriented largely on each of the posterior-most costal scutes, just above the posterior marginal scutes. The side of the iButtons exposed to the environment were covered with a black rubber coating (Plasti Dip®, Blaine, MN) while the underside of both transmitters and iButtons were attached directly to the carapace by a thin layer of Epoxy (Loctite®, Westlake, OH). Immediately after the final test and bleeding procedure, iButtons and transmitters were removed using a pocketknife and any additional epoxy was carefully scraped off from the turtle's shell. No obvious alterations to the underlying shell were caused by the adhesive. Additionally, while turtles traversed a variety of microhabitats, such as creeks, horse pastures, mudflats, and forests, all iButtons and transmitters remained functional and firmly attached to subjects over the study duration (average: 18 days; range: 15 - 30 days).

Temperature data collection and differential estimates

In addition to the iButtons attached to subjects, two environmental stations consisting of 4 iButtons each were established at separate locations on the field site. Each environmental station was placed in a forested area with high shade throughout the day.

Environmental iButtons were completely coated in Plastidip and placed near a tree as follows: (1) buried 10 cm under the soil surface, (2) directly on the ground but under leaf litter, (3) 5 cm suspended off the ground, and (4) 1 m suspended off the ground. Shell and environmental iButtons were programmed to simultaneously record temperatures every 30-minutes from the shell (hereafter called T_{Shell}) or the environment (air or surface, hereafter collectively called T_{E}), respectively.

Temperature readings from the two environmental surface iButtons (hereafter called T_{ESurface}) were averaged and used to calculate each turtle's temperature differential. A turtle's full study temperature differential (FTD) was calculated by subtracting the average T_{Shell} from the average T_{ESurface} over the turtle's involvement in the study. Specifically, the FTD spanned from ~30 min after the first behavioral observation to ~30 min before the final behavioral observation. Additionally, each turtle's average daytime and nighttime temperature differentials (DTD and NTD, respectively) were calculated as the difference between the T_{ESurface} and T_{Shell} recorded during each day (0900-1700 h) and night (2300-0500 h) across the turtle's involvement in the study.

Blood collection and processing

Immediately following their final behavioral assay, blood was collected from each subject at the subcarapacial sinus using a 25-gauge needle and 1.0 mL syringe (Preston *et al.*, 2020). All blood collection occurred from morning to mid-day between 1020 and 1450 hrs. The time to bleed ranged between 90 and 443 s (mean: 197 s). An average of 830 μL (range: 230 – 2000 μL) of whole blood was collected from each turtle and placed into heparinized microcapillary tubes which in turn were immediately placed on ice

within a sealed thermos. These samples were then taken to the lab within 5 h of collection where they were immediately centrifuged at 1000 g for 10 minutes. The resulting blood plasma was carefully separated from the red blood cells and frozen at -80°C. Plasma samples were later used to obtain hemolysis immunoassay scores and levels of plasma triglycerides and steroid hormones (see below). Furthermore, 9 of 28 turtles were housed in the vivarium a second time following their final behavioral test and initial blood collection. These turtles remained in their respective buckets ~20-to-22 h and were then returned to their last site of capture in the field where they were bled a second time (between 0900 – 1345 h) and released. The time to bleed during this 2nd bleeding event ranged from 131 to 595 sec (mean: 314 sec). The second blood sample from these 9 turtles was used to test their corticosterone stress response to ~ 22 h confinement.

Shell damage estimates

Shell damage was estimated for the carapace and plastron of each turtle by dividing the shell component into 4 quadrants and ranking each quadrant's damage between 0 and 3 before taking the sum of the four quadrants of both elements for a final shell damage estimate. Following Saumure *et al.* (2007), the damage of each quadrant was determined as 0 = no damage, 1 = little superficial damage to the scute layer, 2 = deep or substantial damage to the scute layer or margins of the shell, but no damage to the underlying bone, or 3 = damage to the scute layer and the underlying bone.

Fecal collection and parasite counts

Fecal samples were collected when first noticed upon defecation by a turtle when housed in their individual buckets. Fecal samples were preserved in 5% formalin (2 g of

feces per 2 mL of formalin) for up to 45 days maximum (average: 36 d; range: 23-45 d). Counts of fecal nematodes were accomplished by manual counts of eggs/oocysts from collected fecal matter *via* the Mini-FLOTAC® technique (e.g., see Marangi *et al.*, 2020). For this study, 40 ml of an FS7 (zinc sulfate; specific gravity = 1.350) flotation solution was homogenized in the Fill-FLOTAC (size #2) device with 2 g of fecal matter per individual turtle and preserved in 2 ml of 5% formalin. This mixture was then squeezed directly from the Fill-FLOTAC device into the Mini-FLOTAC disc chambers as described in the manufacturer's instructions (see Cringoli *et al.*, 2017). By turning the “key” of the Mini-FLOTAC disc, a thin layer containing the floating parasitic elements was separated from the remaining solution and most of the fecal debris. This was then visually scanned for 1-side of the disc (24 grids, 1 ml homogenized fecal fluid) beneath a light microscope and the number of parasitic elements were counted. Suspected parasitic elements were often difficult to identify and discern from artifacts. As a result, I chose to prioritize and report only on counts of nematode ova which were more easily discernable (Wolf *et al.*, 2014). One nematode of potential interest was the ova of microcapillarids (Fig. 8), which appeared to be very common in the box turtles of this study. However, microcapillarids are not well documented in the literature, and are thought to be nematodes of unknown pathogenicity (Klingenberg, 2016). For this reason, I used nematode counts that included and excluded microcapillarids for any analyses involving parasites.

Individual turtles were also visually scanned on the skin and shell during the initial and final captures for the presence of ectoparasites, such as ticks, mites, and leeches. Although ectoparasites appear to be largely absent in *T. carolina* (Moraga *et al.*,

2012; Rose *et al.*, 2011), few if any studies have considered the presence of ectoparasites from wetland populations of *T. carolina* in Middle Tennessee.

Hemolysis immunoassays

To investigate differences in innate immunity between turtles, a hemolysis immunoassay was conducted in which the plasma of each subject was mixed with diluted rabbit red blood cells (RBC's) in a 96-well plate to observe the subsequent lysis of the blood cells by the turtle's natural antibodies and complement system (Baker *et al.*, 2019; Ferronato *et al.*, 2009; Matson *et al.*, 2005; Merchant *et al.*, 2006; West & Klukowski, 2018). In this assay, rabbit blood in Alsever (Lampire, #7206403) was washed 4X in phosphate-buffered saline (PBS) to achieve a dilution of 1% RBC's. Freshly thawed turtle plasma (15 μ l) was diluted in 15 μ l of PBS (0.01M) and further serially diluted by homogenizing and transferring 15 μ l of the resulting mixture to subsequent columns already containing 15 μ l of PBS (0.01M). This resulted in a dilution of 1:2 in the first column to a dilution of 1:2048 in the final column. Next, 15 μ l of 1% RBC's were added to all wells and the hemolysis plates were allowed to sit at 24°C for 3 hours before being scored. Lysis was determined by comparing turtle samples to a positive control row containing RBC's in deionized water and a negative control column containing RBC's in PBS solution only. Scoring was conducted by estimating the titer as the $\log_2$ of the highest dilution factor of plasma that showed lysis and could range from a score of 1 to 11 (following Matson *et al.*, 2005; West & Klukowski, 2018). Half scores were given for titers that appeared at intermediate levels of lysis. Turtles with higher hemolysis scores

were considered to have better lysing capabilities than those with lower scores.

Hemolysis scoring was conducted blind to the boldness categorization.

Plasma triglyceride concentrations

Plasma triglyceride levels were also estimated for each turtle using the same plasma sample collected immediately following the final behavioral assay. This was done by adding 10 μ l of recently thawed plasma to 1 mL of a pre-warmed triglyceride reagent (No. T7532, Pointe Scientific, Inc., Canton, MI) and incubating at 37°C for 20 min (following West & Klukowski, 2018). Following incubation, the triglyceride concentration was estimated from each sample by recording their absorbance values along with that of a 200 mg/dL triglyceride standard using spectrophotometry at 500 nm.

Steroid hormone assays

Plasma corticosterone (No. K014-H1, Arbor Assays, Ann Arbor, MI), testosterone (No. ADI-900-065, Enzo Life Sciences, Inc., Farmingdale, NY), and/or progesterone (No. ADI-900-011, Enzo Life Sciences, Inc., Farmingdale, NY) concentrations were measured via separate competitive enzyme-linked immunosorbant assays (ELISA). All turtles were tested for plasma CORT concentrations ($n = 28$; intra-assay CV = 5.6% (Preston *et al.*, 2020)) whereas only males were tested for testosterone ($n = 18$; intra-assay CV = 7.8%) and females for progesterone ($n = 10$; intra-assay CV = 4.9%). Comparisons between boldness categories in plasma testosterone was for males only and in plasma progesterone for females only due to the respective sex-specific functions and concentrations of these hormones in most vertebrates. Plasma CORT was also measured a second time in nine turtles which underwent a ~22 hr period of

confinement following their initial bleeds to then be bled a second time in the field before being released.

Statistical analyses

Data was statistically analyzed within the statistics program R (R Core Team, 2014) while graphs and tables were constructed using Microsoft© Excel. Normality of the distribution for samples was determined *via* the Shapiro-Wilk normality test (Shapiro & Wilk, 1965) with a significance cut-off of $\alpha = 0.05$. Samples determined to be normally distributed were analyzed by parametric tests while non-normally distributed samples were analyzed by their analogous non-parametric versions. When necessary, the variance equality between variables was determined using the Levene's test (Levene, 1960). Significance was determined by evaluation of confidence intervals (CIs) for generalized linear mixed models or p-values for linear regression, student T, Wilcoxon rank-sum, and correlation analyses. In all cases, significance was determined when CIs did not include zero or if p-values were less than 0.05.

Individual repeatability of eye emergence and 1 m movement latency were individually assessed using generalized linear mixed models (GLMM) fit with maximum likelihood (following Kashon & Carlson, 2018). These GLMMs were modeled with and without the effects of either assay conditions or non-behavioral phenotypic differences as fixed effects. Individual turtle identity was considered a random effect in all models. To first gain estimates of effect by random and fixed effects, the GLMM was computed using the *glmer* function from the “lmer” R package. Next, the *rpt* function from the “rptR” package was utilized to obtain link-scale estimates of repeatability (*r*) and 95%

confidence intervals (CI) via non-parametric bootstrapping of 1000 iterations to evaluate individual repeatability of the behaviors and their significance, respectively. Lastly, the significance of fixed effects from GLMMs accounting for assay conditions or non-behavioral phenotypic characteristics were similarly assessed by obtaining 95% CIs via non-parametric bootstrapping of 1000 iterations using the *confint* function. For GLMMs modeling the effects of assay conditions, infrared shell temperature, sky condition, and time of day were included as fixed effects, and for those modeling the effects of non-behavioral phenotypic differences, sex, best count age, carapace length, and vBCI were included as fixed effects. In the latter, best count age and carapace length were log transformed to re-scale and normalize their values.

Linear regressions or generalized linear regression (GLR; non-parametric) were conducted to test the predictive effect of emergence behavior, body condition, age, sex, and/or temperature on the following ecological outcome variables: full temperature differential (FTD), daytime temperature differential (DTD), nighttime temperature differential (NTD), and shell injury score. First, average eye emergence was tested as a predictor variable on FTD, DTD, and NTD in separate simple linear regression models. This was conducted to test if turtles that emerge quicker on average regulate their body temperature or sustain shell damage differently from those that emerge more slowly. Second, to test for the effects of age, sex, and body condition on temperature, multiple linear regression analyses were conducted with FTD, DTD, or NTD as the outcome variable and age (best count) + sex + vBCI as predictor variables. Lastly, to test the effects of eye emergence, temperature, body condition, and age on injury scores, average eye emergence, FTD, DTD, NTD, vBCI, and best count age were used as predictor

variables in simple or multiple GLRs with injury scores as the outcome variable. In cases where several simple and multiple GLRs were tested, the best fit model was determined by comparing all models and their residual deviances within a Chi-Square test, using the *anova* function with the command *test = "Chisq"* in R. Results for linear regression and GLRs are reported with a β and a p-value for each predictor variable to represent the estimated effect and significance, respectively. The R^2 values are also reported for linear regression models to show the percent explained by the respective variables.

To determine if any differences in behavioral, physiological, and ecological measures existed between the sexes and boldness categories (based on average eye emergence), I conducted either Two-Sample Student T-tests or Wilcoxon rank-sum tests in R. Choosing between these tests was dependent on whether the sampled data for the variables being compared were normally or non-normally distributed, respectively, which was determined by the Shapiro-Wilk's test. More specifically, these tests were used to compare male and female, or bold and less bold, individuals in regard to their morphometrics, vBCI, temperature (FTD, DTD, & NTD), steroid hormones, triglycerides, hemolysis scores, injury scores, and nematode counts. These tests were input with equal variance as true when a Levene's test provided $p > 0.05$ (*leveneTest* function of the R package "Car"). Aside from testosterone and progesterone, the sexes were pooled for comparisons between boldness categories when no difference between the sexes was indicated for that variable. However, if a difference between the sexes was indicated, they were tested separately for comparisons between boldness categories. Finally, a single paired T-test was run to analyze the change in plasma CORT between the first (baseline) and second (post-22-hour confinement) bleeds.

Lastly, several correlation analyses were conducted using the *cor.test* function in R *via* either the Pearson or Spearman method depending on if the two variables were normally or non-normally distributed, respectively (as determined by the Shapiro-Wilk test). This was conducted within and between behaviors recorded during the 3-min handling and 10-min observation periods, as well as within and between morphological and physiological measures. Furthermore, correlations were conducted to investigate the relationship between hormone measures and the date bled (using the Julian calendar) as well as the relationship between CORT values of consecutive bleeds. For correlation results, R_P and R_S delineate direction and strength of the relationship as either Pearson correlation or Spearman rho values, respectively. Positive or negative R_P or R_S values indicate negative or positive relationships, respectively, while p-values determine significance of the relationship ($p < 0.05$ considered significant).

RESULTS

Demographics

Sexing of individuals by intensity of the plastron concavity revealed 18 of our 28 turtles (~64%) were males and the remaining 10 (~36%) were females (Table 3). The majority (~64%) of our adult turtles captured were between 15-19 yrs in age, with a quarter (25%) of subjects falling into the younger age class of 10-14 yrs, and even fewer (~11%) falling into the older 20+ age class (Table 3). The presence of three age classes was found to be similar between the sexes ($W = 70.5$, $p = 0.28$) and between boldness categories ($W = 73.5$, $p = 0.20$; Fig. 1).

Table 3: The basic demographics of turtle subjects in this study, including number of individuals in each sex and age class, as well as carapace length and body weight averages with ranges included.

Variable	Category	Sample size (n) or mean (with range)
Sex	Males	$n = 18$
	Females	$n = 10$
Age class	10-14	$n = 7$
	15-19	$n = 18$
	20+	$n = 3$
Carapace Length (mm)		130.9 (120 - 145)
Weight (g)		415.8 (295 - 535)

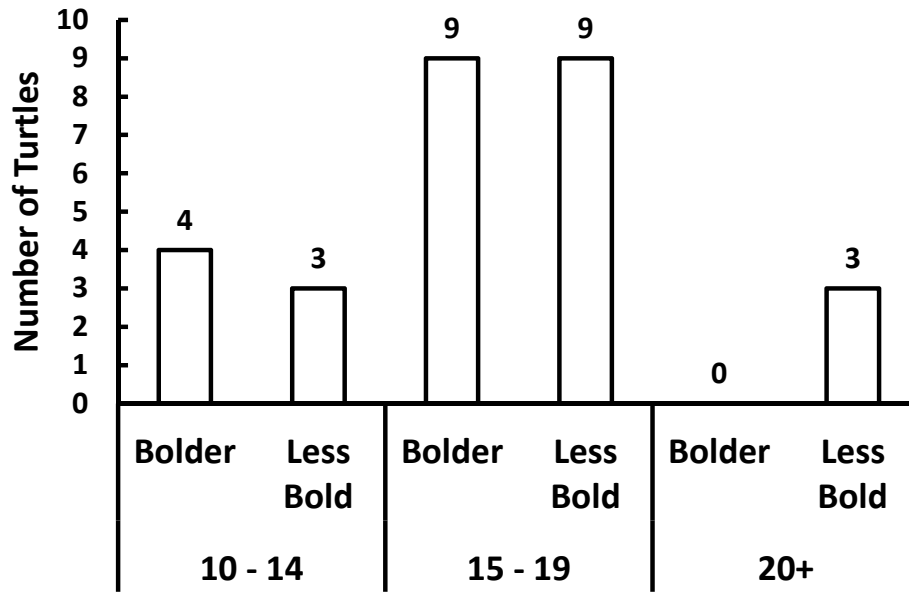


Figure 1: The number of adult eastern box turtles (*T. carolina*) in this study (total $n = 28$) are shown for each boldness category and within their respective age groups. The number of individuals within each category is displayed above their respective bars.

Behavioral observations and repeatability

The time it took to extend the eyes past the anterior margin of the shell (eye emergence latency) following the start of a behavioral trial was found to exhibit high repeatability within individual turtles ($r = 0.77$, 95% CI = [0.614, 0.856]; Fig. 2), as indicated by the CI that does not include zero. This repeatability in eye emergence latency remained after accounting for both assay conditions ($r = 0.82$, 95% CI = [0.68, 0.891]) and non-behavioral phenotypic characteristics ($r = 0.75$, 95% CI = [0.516, 0.813]). Similarly, the latency to move 1 meter following the start of a behavioral trial also showed significant individual repeatability ($r = 0.58$, 95% CI = [0.356, 0.699]), although to a lesser extent than eye emergence (wider CI and lower r value). This repeatability remained relatively stable for 1 meter movement latency after accounting

for assay conditions ($r = 0.62$, 95% CI = [0.412, 0.746]) and non-behavioral phenotypic characteristics ($r = 0.56$, CI = [0.279, 0.661]).

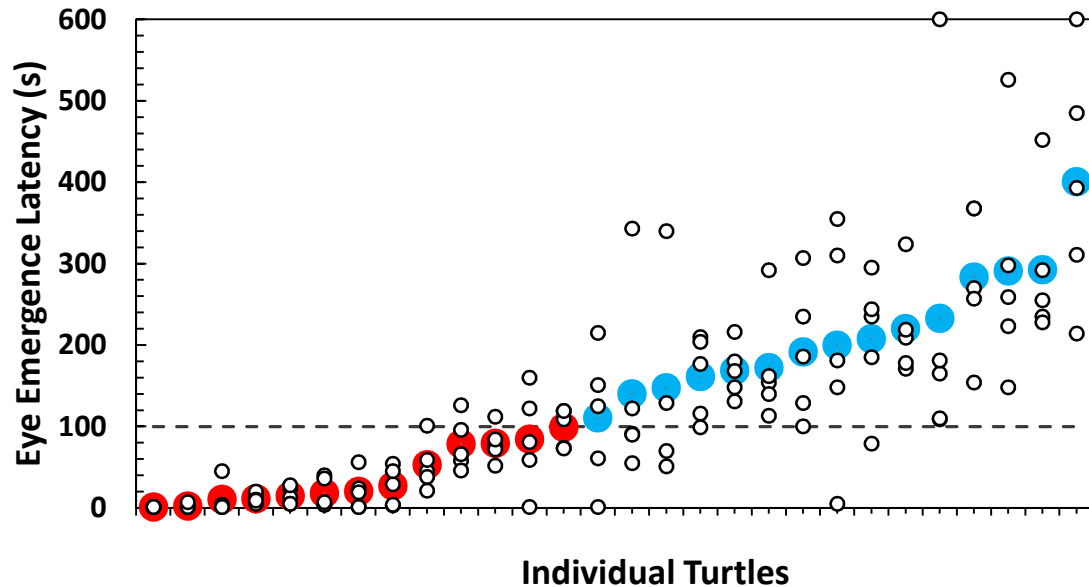


Figure 2: Eye emergence latency across all 28 turtle subjects. Open circles indicate eye emergence values from individual assays and colored circles indicate the average of those assays. Eye emergence latency was found to exhibit significant repeatability across behavioral assays within individuals ($r = 0.77$, 95% CI = [0.614, 0.856]). The first 13 turtles with average eye emergence values ≤ 100 sec (dashed line) were considered ‘bolder’ (red circles) and the last 15 turtles with average eye emergence values > 100 sec were considered ‘less bold’ (blue circles).

Based on the GLMMs, 3 of the 4 assay conditions considered had significant negative effects on eye emergence (Table 4). This includes the test number ($\beta = -0.068$, CI = [-0.079, -0.056]), immediate infrared shell temperature ($\beta = -0.062$, CI = [-0.069, -0.054]), and sky code ($\beta = -0.048$; CI = [-0.066, -0.030]). Meanwhile, the time of day tested had no significant effects on eye emergence in this model ($\beta = 0.007$, CI = [-0.007, 0.019]). It should be noted that immediate infrared shell temperature and sky code were found to be significantly negatively correlated ($R_s = -0.241$, $p = 0.0045$) and it may

therefore be redundant to include both within this model. On the other hand, none of the non-behavioral phenotypic characteristics had significant effects on eye emergence in the GLMMs (CIs all include zero; Table 4).

Table 4: Results of the effects of assay conditions and non-behavioral characteristics on eye emergence of individual turtles from generalized linear mixed models. Predicted effect of the variable is indicated by β , with directionality indicated by the presence or absence of a negative sign. Significant fixed effects are in bold, as determined by the 95% confidence intervals (CI) that do not include zero.

	β	95% CI
Assay conditions		
Time of Day	0.007	(-0.007, 0.019)
Sky Code	-0.048	(-0.066, -0.030)
IR Shell Temp (°C)	-0.062	(-0.069, -0.054)
Test Number	-0.068	(-0.079, -0.056)
Non-behavioral characteristics		
Sex	0.487	(-0.751, 1.586)
Age Best Count	1.700	(-2.085, 4.980)
Carapace Length	-4.449	(-15.794, 7.166)
vBCI	-3.095	(-10.014, 3.254)

A multiple linear regression with average eye emergence latency as the outcome variable (rather than eye emergence for each assay) indicated that neither age, sex, nor vBCI significantly predicted average eye emergence ($p > 0.1$ for all). However, when tested separately in a simple linear regression model, vBCI was nearly a significant negative predictor for average eye emergence ($p = 0.076$, $R_p = -0.34$, $R^2 = 0.082$), but this explained very little of the variation in average eye emergence.

Correlation analyses indicated significant positive correlations between time spent closed during handling, eye emergence, wrist emergence, and 1 m movement latencies at the level of average behaviors across the 28 individual turtles ($n = 28$; Table 5).

Furthermore, correlation analyses revealed that the total defenses employed during handling were negatively correlated with eye emergence, wrist emergence, and 1 m movement latency (Table 5). Therefore, the individual turtles that were out and actively employing defenses against the investigator during handling were also those that emerged and began moving the quickest during the subsequent observation period.

The most common active defense employed by turtles was air kicking, which occurred in 11 (out of 28) turtles and across 33 (out of 139) behavioral assays. Snaps and bites were employed by only a single turtle, but this subject was consistent, employing snaps and bites for 4 of its 5 behavioral assays (total: 18). Urination and/or defecation events were employed by only four turtles, with two employing this defense during only one of their 5 behavioral assays and the other two turtles employing it across 2 separate assays. One of these latter turtles was also the same subject that employed snaps and bites. Although it was not considered an active defense in this study, hissing was well noted across the majority of turtles (23/28) and behavioral assays (76/139). Often hisses accompanied rapid movements of the head and/or limbs into the shell and may therefore be a side effect of these rapid body movements rather than an active defense.

Table 5: Correlation results between average behaviors recorded during the 3-min handling period (total defenses, score closed up) and the 10-min observation period (eye emergence, wrist emergence, and 1 m movement latencies) across individual turtles ($n = 28$). All correlations involving score closed up or total defenses were conducted using the Spearman rho's test, whereas correlations between other behaviors were conducted using the Pearson's correlation. All behaviors were significantly correlated with p-values less than 0.05. Negative relationships are distinguished from positive ones by a preceding negative (–) sign for the rho value.

Behavioral Variables	Rho (R_s or R_p)	p-value
Total Defenses x Eye Emergence	-0.81	< 0.0001
Total Defenses x Wrist Emergence	-0.77	< 0.0001
Total Defenses x Latency 1 m Movement	-0.71	< 0.0001
Score Closed Up x Eye Emergence	0.74	< 0.0001
Score Closed Up x Wrist Emergence	0.65	< 0.0001
Score Closed Up x Latency 1 m Movement	0.58	< 0.001
Eye Emergence x Wrist Emergence	0.84	< 0.0001
Eye Emergence x Latency 1 m Movement	0.83	< 0.0001
Wrist Emergence x Latency 1 m Movement	0.94	< 0.0001

Behavioral categorization

Thirteen turtles were categorized as bolder with average eye emergence latencies of ≤ 100 secs whereas the remaining 15 turtles were classified as 'less bold' with average eye emergence latencies > 100 secs. The average standard deviation in eye emergence across assays for the bolder and less bold groups were 20.83 sec (range: 0 – 60.71) and 102.07 sec (range: 32.46 – 207.68), respectively. Therefore, eye emergence exhibited higher variability across assays in less bold turtles than bolder turtles. The variability in eye emergence was relatively similar between sexes, with an average standard deviation of 61.48 for males (range: 2.68 – 207.68) and 69.51 sec for females (range: 0 – 142.63).

Morphology

Female turtles were significantly heavier on average than males ($t = 2.29$, $df = 26$, $p = 0.03$; Fig. 3). However, no weight differences were detected between boldness categories in males ($t = 0.84$, $df = 16$, $p = 0.42$) or females ($t = -1.24$, $df = 8$, $p = 0.25$). Carapace length was similar between sexes ($t = -0.35$, $df = 26$, $p = 0.73$) and boldness categories ($t = -0.87$, $df = 26$, $p = 0.40$). Likewise, the calculated volumetric body condition was similar between sexes ($t = 1.73$, $df = 26$, $p = 0.096$) and between bolder and less bold turtles of pooled sexes ($t = 1.16$, $df = 26$, $p = 0.26$).

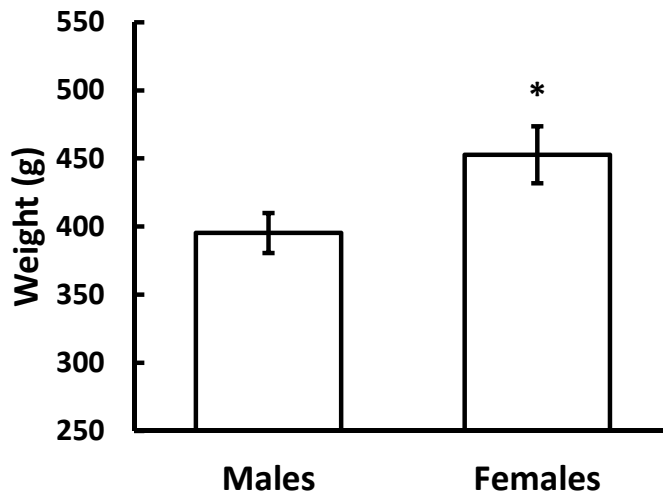


Figure 3: Average weight ($\pm$ SE) of adult male ($n = 18$) and female ($n = 10$) turtles of this study. On average, females were significantly heavier than males ($t = 2.29$, $df = 26$, $p = 0.03$).

Pinch force

Interestingly, three turtles were noted as seemingly incapable of completely closing both the anterior and posterior gaps of the shell simultaneously, often leaving one end open at the expense of closing the other (one bolder, two less bold; one 15-19 y.o.

female, one 15-19 y.o. male, one 20+ male). Additionally, six others (all bolder; two 10-14 y.o. females, two 15-19 y.o. females, two 15-19 y.o. males) were consistently reluctant to close, requiring several pokes and prods by the investigators to get the near complete closure necessary to obtain a pinch force reading. Because those either incapable or largely unwilling to fully close the shell ($n = 9$) would be expected to have lowered pinch force, I looked for differences in pinch force between them and the remaining turtles ($n = 19$). As expected, turtles noted as incapable or unwilling to close the shell had significantly lower mean pinch force than those without this distinction ($t = 2.82$, $df = 26$, $p\text{-value} = 0.0091$; Fig. 4A). When tested across all individuals ($n = 28$), including those noted as incapable or unwilling to close, there was no difference between sexes in pinch force ($t = 0.85$, $df = 26$, $p = 0.41$). However, after excluding those noted as incapable or unwilling to close ($n = 19$), the difference in pinch force between sexes was nearly significant ($t = 1.99$, $df = 17$, $p = 0.063$; Fig. 4B). Average eye emergence and pinch force were positively correlated, with turtles emerging slower on average predicted to have a slightly higher pinch force ($R_p = 0.38$, $p = 0.045$; Fig. 5). Despite this, ‘bolder’ and ‘less bold’ turtles had similar pinch force, although the mean was higher in the less bold group both when including all turtles ($t = -1.69$, $df = 26$, $p = 0.103$) and after excluding those incapable or unwilling to close ($t = -0.018$, $df = 17$, $p = 0.99$).

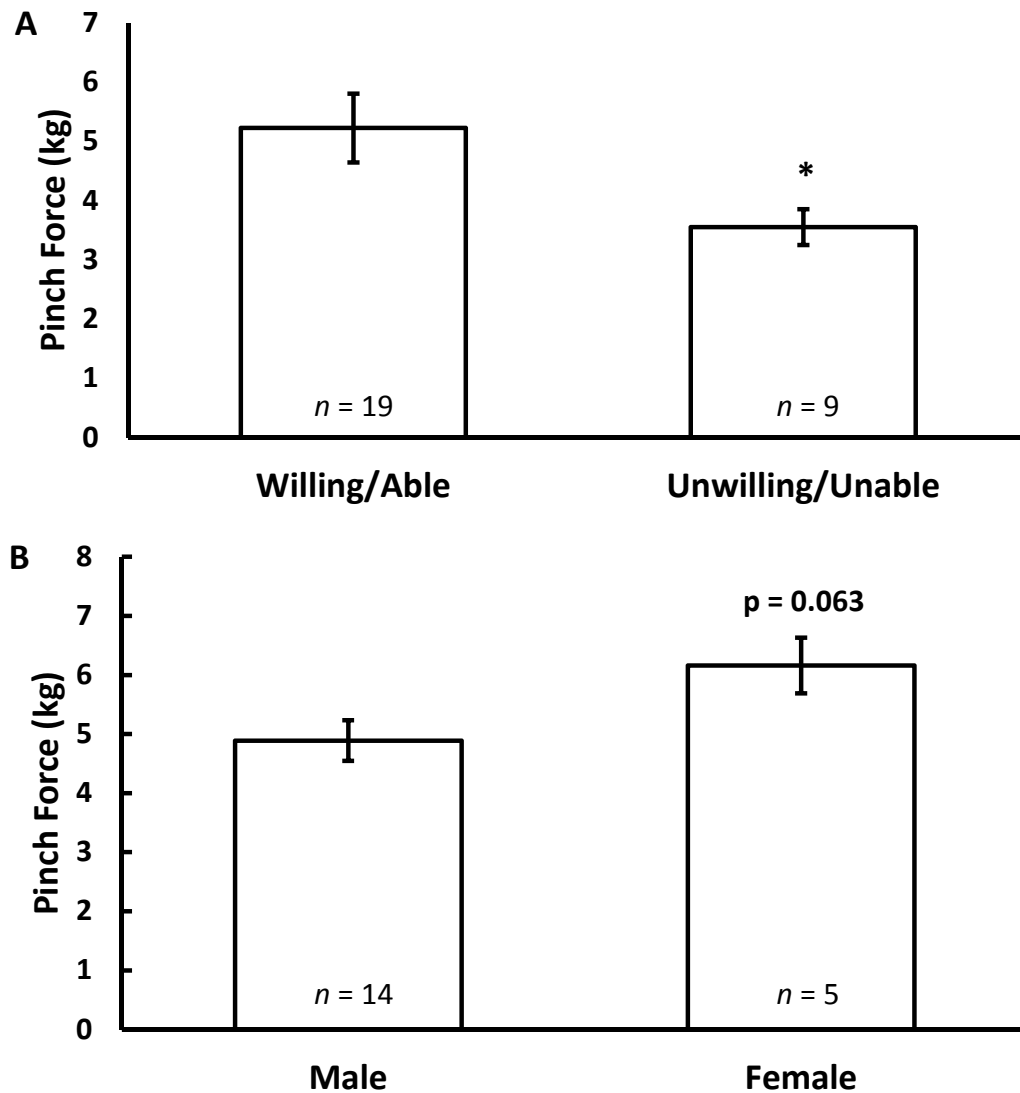


Figure 4: Turtles noted as unable or reluctant to withdraw (two 10-14 y.o. females, three 15-19 y.o. females, three 15-19 y.o. males, one 20+ male) into the shell had significantly lower average pinch force ($\pm$ SE) than those noted as quick and able to withdraw (A; $p = 0.0091$). In turtles quick and able to withdraw, however, there was no difference between male and female turtles, although females were nearly significantly greater in their average pinch force than males ($p = 0.063$; B).

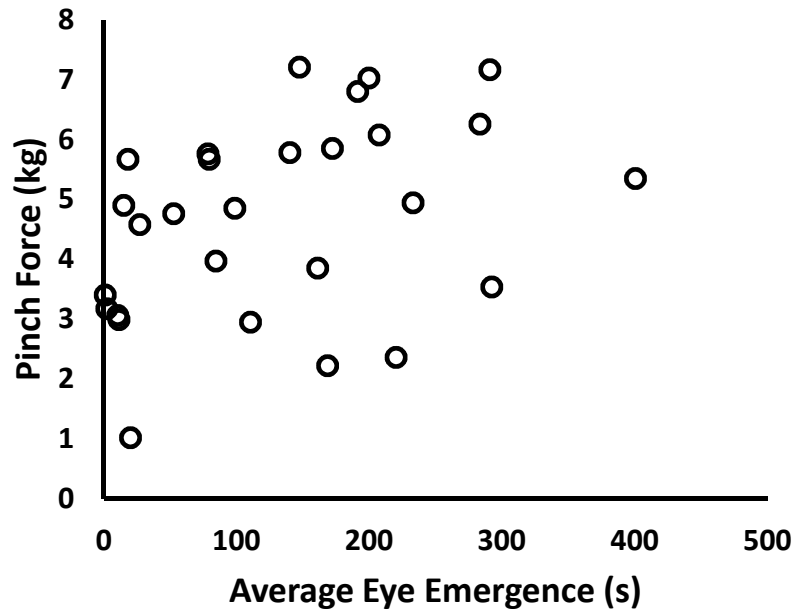


Figure 5: Each box turtle's average eye emergence latency plotted against their pinch force value. Average eye emergence was significantly correlated with pinch force ($R_p = 0.38$, $p = 0.045$).

Shell Injury Scores

Injury scores were similar across sexes ($W = 96$, $p = 0.77$) and across bolder and less bold categories when sexes were pooled ($W = 91.5$, $p = 0.78$). Correlation analysis indicated average eye emergence was not significantly related to shell injury scores ($p = 0.85$; Fig. 6). In a generalized linear regression, FTD, DTD, and vBCI were significant predictors of injury scores in a positive manner. Including DTD and vBCI in a model together was determined as the best fit model from those possible ($p < 0.0001$). However, only DTD remains significant in this best fit model, an effect that may be largely driven by 3 turtles that had daytime differentials between 4 and 5°C above the surface temperature and injury scores between 4 – 7, while all other turtles had injuries scores below 3 (Fig. 7B). In summary, this model predicts that turtles maintaining warmer

temperatures (and possibly higher body condition) will be more likely to have higher shell injury scores. Correlation analyses also indicated a trend for injury scores to be positively correlated with FTD ($R_S = 0.34$, $p = 0.075$), DTD ($R_S = 0.37$, $p = 0.054$; Fig. 7A), and vBCI ($R_S = 0.35$, $p = 0.067$; Fig. 7B), although these trends appeared to be biased by the three turtles with the highest injury scores, as the trends disappear after the removal of these outliers ($p > 0.35$).

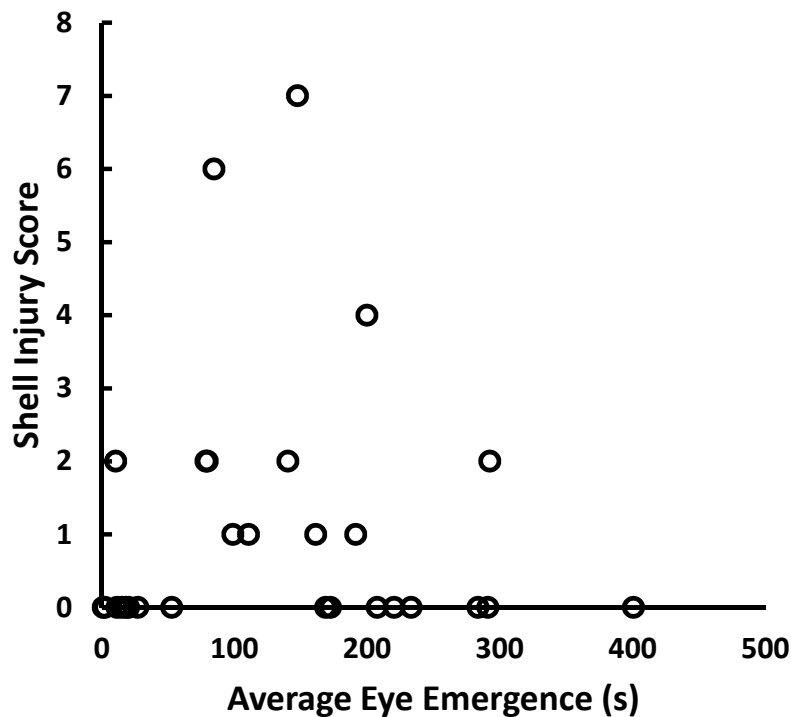


Figure 6: Each box turtle's average eye emergence latency plotted against their shell injury score. Average eye emergence was not significantly related to shell injury score ($R_S = 0.038$, $p = 0.85$).

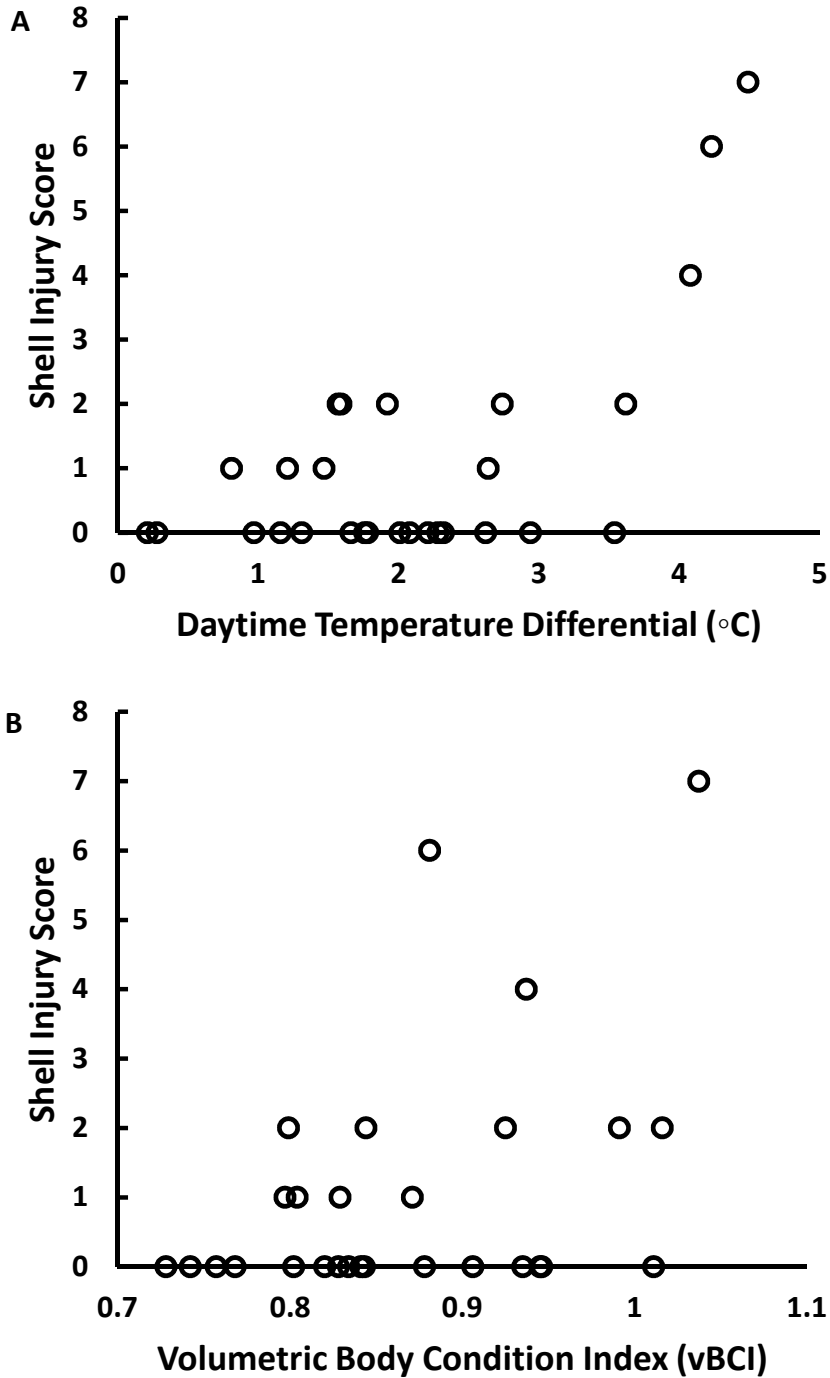


Figure 7: Each box turtle's average daytime temperature differential (A) or volumetric body condition (B) plotted against their shell injury scores. Injury scores nearly positively correlated with daytime temperature differentials ($R_s = 0.37$, $p = 0.054$) and volumetric body condition ($R_s = 0.35$, $p = 0.067$). Daytime temperature differential was also a significant predictor of injury score in the generalized linear regression ($p < 0.0001$).

Hemolysis scores

Hemolysis of sheep RBCs occurred in all plasma samples of turtles following 3 h of incubation. The mean hemolysis across all turtles was 4.0 and ranged from 2.0 to 6.0. Agglutination was not apparent in any samples for the 3 h scoring, although signs of agglutination appeared after 5 h of incubation. Male and female turtles did not differ significantly in their hemolysis scores ($t = -0.29$, $df = 26$, $p = 0.78$) and, after pooling across sexes, hemolysis scores remained similar between bolder and less bold turtles ($t = 1.72$, $df = 26$, $p = 0.098$; Fig. 8). Hemolysis scores also did not correlate with average eye emergence ($p = 0.28$), or with either vBCI ($p = 0.47$) or injury scores ($p = 0.75$) (Table 6).

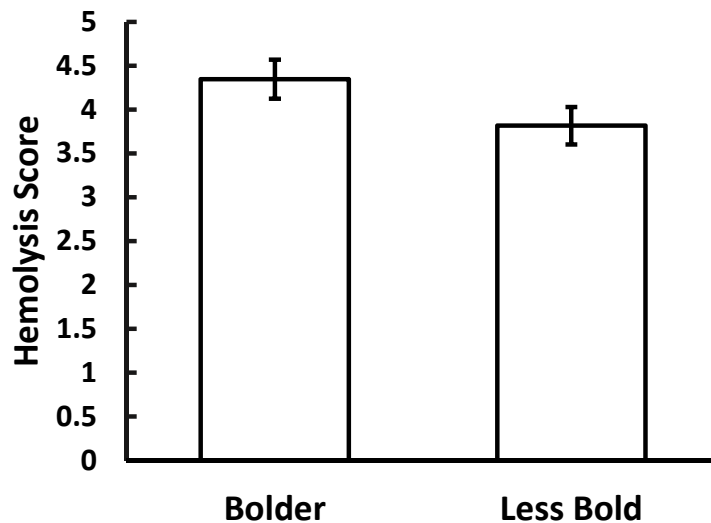


Figure 8: Average ($\pm$ SE) hemolysis scores for bolder ($n = 13$) and less bold ($n = 15$) box turtles. No significant difference was found between the categories ($t = 1.72$, $df = 26$, $p = 0.098$).

Fecal nematodes and ectoparasites

Fecal nematode counts that included and excluded microcapillarids were not correlated ($R_s = -0.126$, $p = 0.70$). Additionally, both of these counts were similar between sexes (including microcapillarids: $t = -0.49$, $df = 10$, $p = 0.64$; excluding microcapillarids: $W = 34.5$, $p = 0.20$) and boldness categories (including microcapillarids: $t = 0.49$, $df = 10$, $p = 0.64$; excluding microcapillarids: $W = 18.5$, $p\text{-value} = 0.52$). In general, microcapillarids were the most common nematode for 11 out of 12 turtles for which they were counted (see Fig. 9). Other nematode ova frequently noted were suspected to be “pinworms” or “hookworms”, such as those in the order Oxyurida or suborder Strongylida, respectively. Parasite counts did not correlate with vBCI, however there was a negative trend between parasite counts (including microcapillarids) and injury scores ($R_s = -0.53$, $p = 0.078$) (Table 6). Only a single ectoparasite was observed: a leech, believed to be a smooth turtle leech (*Placobdella parasitica*) (McCoy *et al.*, 2007) (Fig. 10), was found on a single turtle and remained attached to the shell for at least one week in the late summer, although it moved several times to different locations on the plastron and marginal scutes of the carapace.

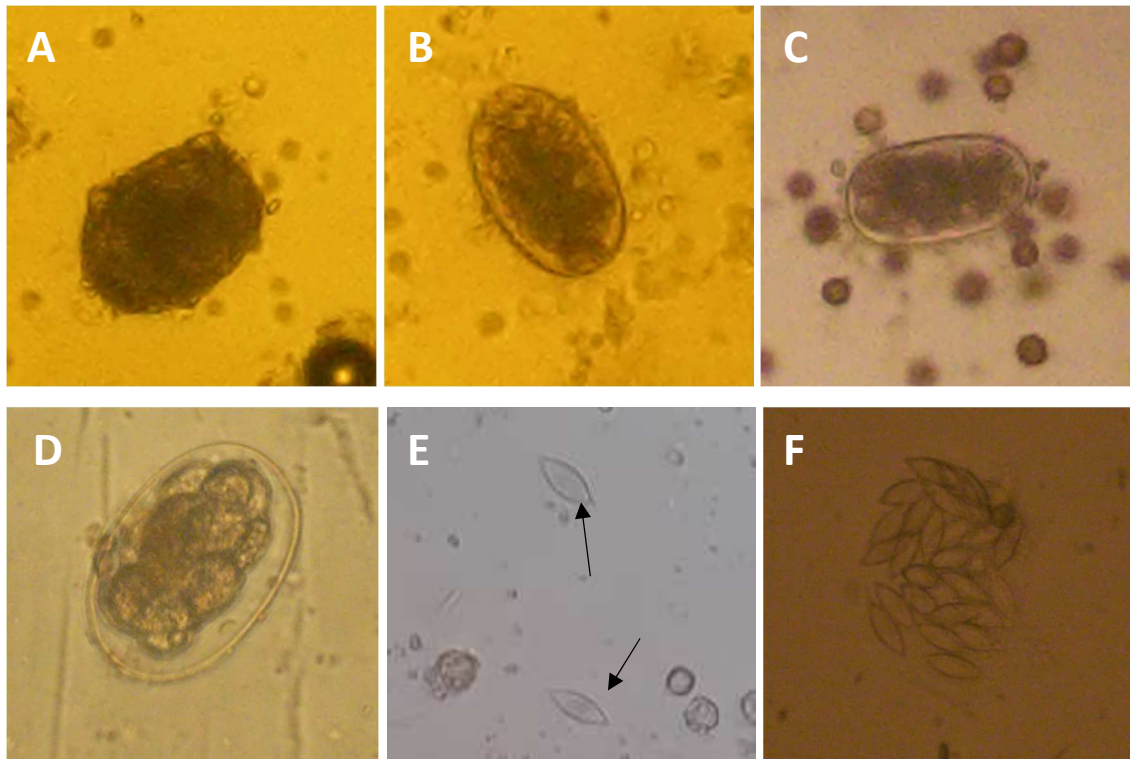


Figure 9: Photographic examples of suspected nematode ova found in the fecal matter of *T. carolina* using the Mini-FLOTAC method and light microscopy at 20-40x. (A-C) suspected pinworm ova (e.g., order Oxyurida) (D) a suspected hookworm ova (e.g., suborder Strongylida), (E) individual microcapillarids (arrows), (F) an aggregate of microcapillarids.



Figure 10: Image of a single leech attached to the marginal scutes of a turtle's carapace and near a radio transmitter (A) and after moving to the underside of the marginal scutes (B). The leech remained on the turtle for at least one week and moved between the upper and lower sides of the carapace on several occasions.

Triglycerides

Females exhibited significantly higher triglyceride levels than males (mean males = 38.23 mg/dL; mean females = 184.04 mg/dL) ($W = 168.5$, $p = 0.00018$) and were therefore tested separate from males for comparisons between bolder and less bold categories. However, there were similar triglyceride levels between bolder and less bold males ($W = 53$, $p = 0.27$) and females ($t = 0.36$, $df = 8$, $p = 0.73$) (Fig. 11). Plasma triglycerides did not correlate with average eye emergence ($R_s = -0.212$, $p = 0.28$). Triglycerides also did not correlate with hemolysis scores, injury scores, or nematode counts, but they were nearly positively correlated with vBCI ($p = 0.079$) (Table 6).

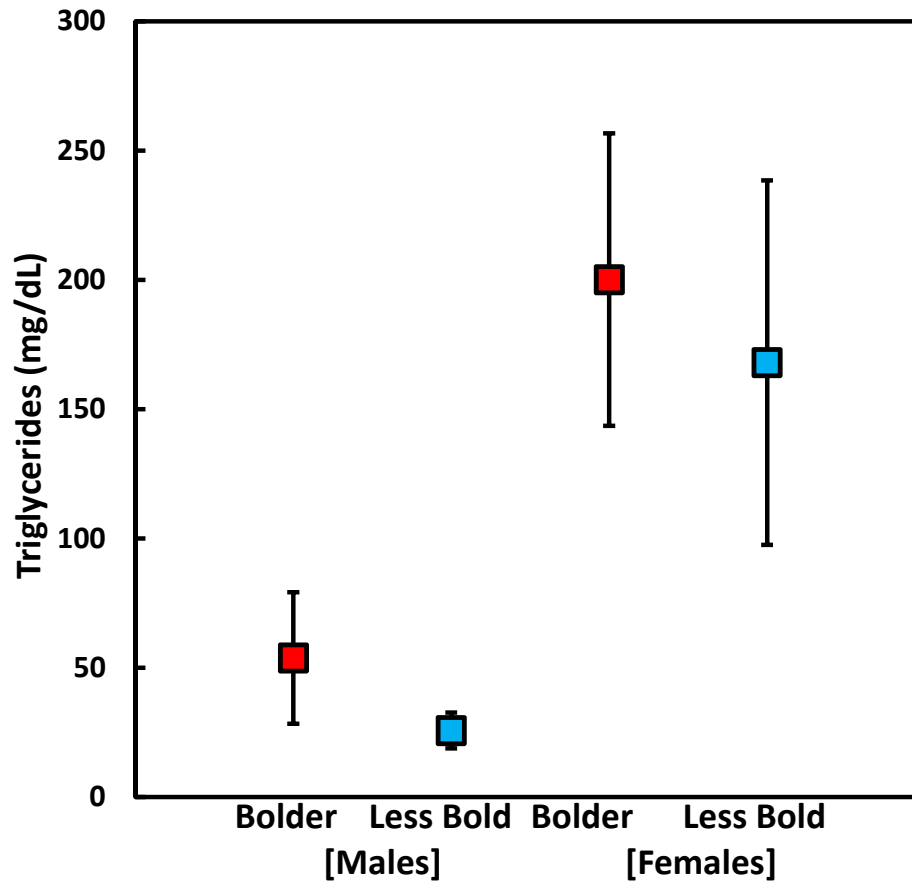


Figure 11: Average ($\pm$ SE) plasma triglycerides differences across box turtle categories of bolder males ($n = 8$), less bold males ($n = 10$), bolder females ($n = 5$), and less bold females ($n = 5$). Although the sexes differed in plasma triglycerides ($W = 168.5$, $p = 0.00018$), there was no significant difference between the boldness categories within either sex ($p > 0.05$).

Steroid hormones

Baseline plasma CORT levels were found to be similar between the sexes ($W = 108$, $p = 0.41$) as well as between bold and less bold turtles ($W = 109$, $p = 0.62$; Fig. 12). Additionally, average eye emergence and baseline CORT were not correlated ($R_s = -0.061$, $p = 0.76$) (Fig. A2).

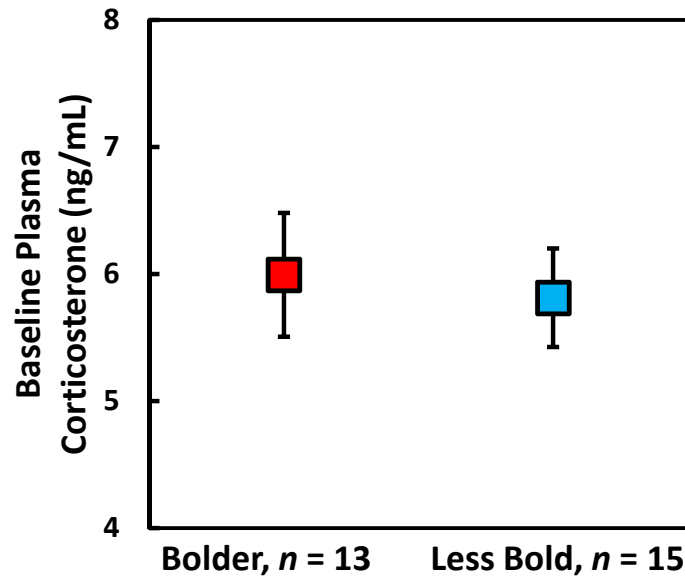


Figure 12: Average baseline plasma corticosterone concentrations ($\pm$ SE) in bolder and less bold box turtles.

Plasma CORT increased following 22-h of laboratory post-confinement (paired $t = -5.72$, $df = 8$, $p < 0.001$; Fig. 13A). Of the 9 turtles bled a second time, 6 were males and 3 were females, while 4 were categorized as bolder and 5 as less bold. The difference in CORT between baseline and post-confinement bleeds did not significantly differ between the sexes ($W = 10$, $p = 0.91$) or between boldness categories ($W = 16$, $p = 0.19$; Fig. 13B).

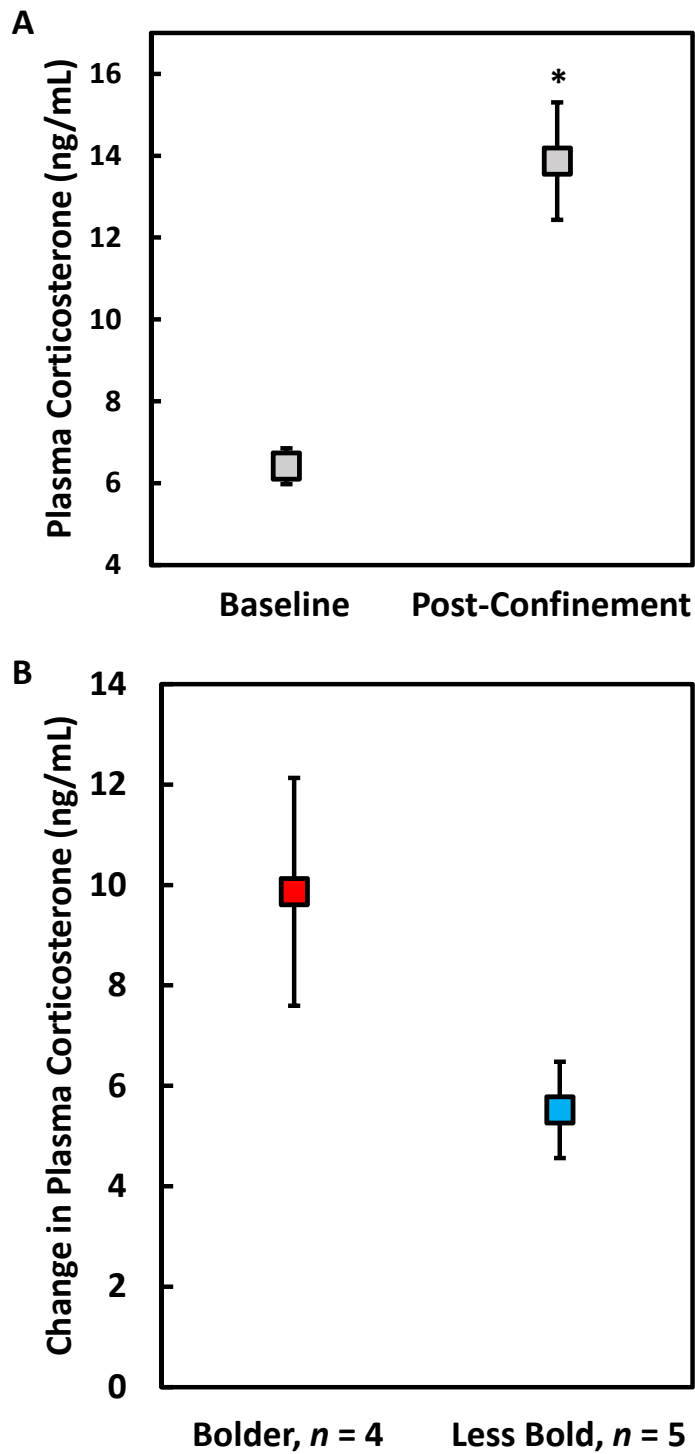


Figure 13: Plasma corticosterone levels from bleeds ($n = 9$) of post-22 h confinement was significantly higher than baseline corticosterone levels (A; paired $t = -5.721$, $p < 0.001$). However, the change in corticosterone, calculated as the difference in corticosterone between the first and second bleeds, did not significantly differ between bolder and less bold turtles (B; $W = 16$, $p = 0.19$).

Similar to CORT, no differences between bold and less bold turtles were found for testosterone in males ($W = 35$, $p = 1$; Fig 14A) or progesterone in females ($W = 17$, $p = 0.40$; Fig 14B). In addition, average eye emergence was not correlated with plasma testosterone ($p = 0.46$) or progesterone ($p = 0.85$) concentrations (Fig. A3-S4). However, testosterone of males was found to be significantly positively correlated with date bled ($R_s = 0.59$, $p = 0.015$; Fig. 15A) with individuals bled later in the season (late July- early August) having higher concentrations of testosterone than those bled earlier in the study (late May – June). This relationship was not found for date bled and the progesterone of females, although I sampled fewer females and largely lacked female blood samples from mid-June to mid-July ($R_s = 0.39$, $p = 0.27$; Fig. 15B).

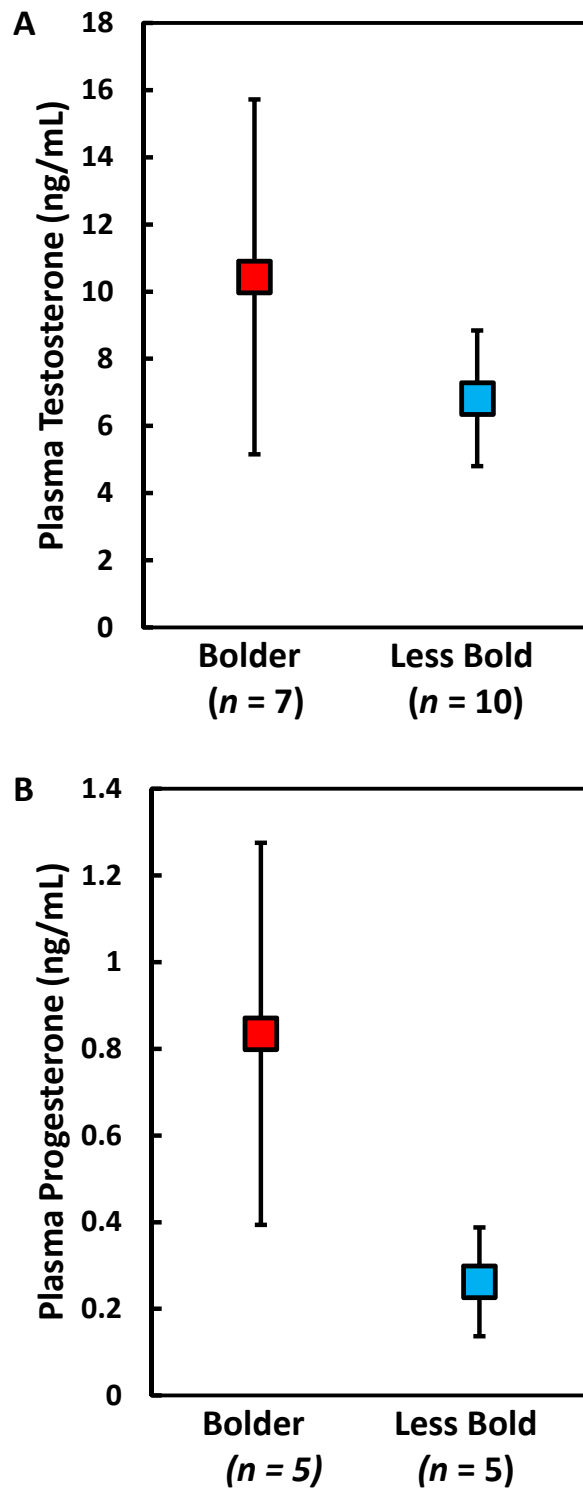


Figure 14: Average ($\pm$ SE) plasma sex steroid concentrations in bolder and less bold box turtles. No difference was indicated between categories for either testosterone in males (A; $W = 35$, $p = 1$) or progesterone in females (B; $W = 17$, $p = 0.40$).

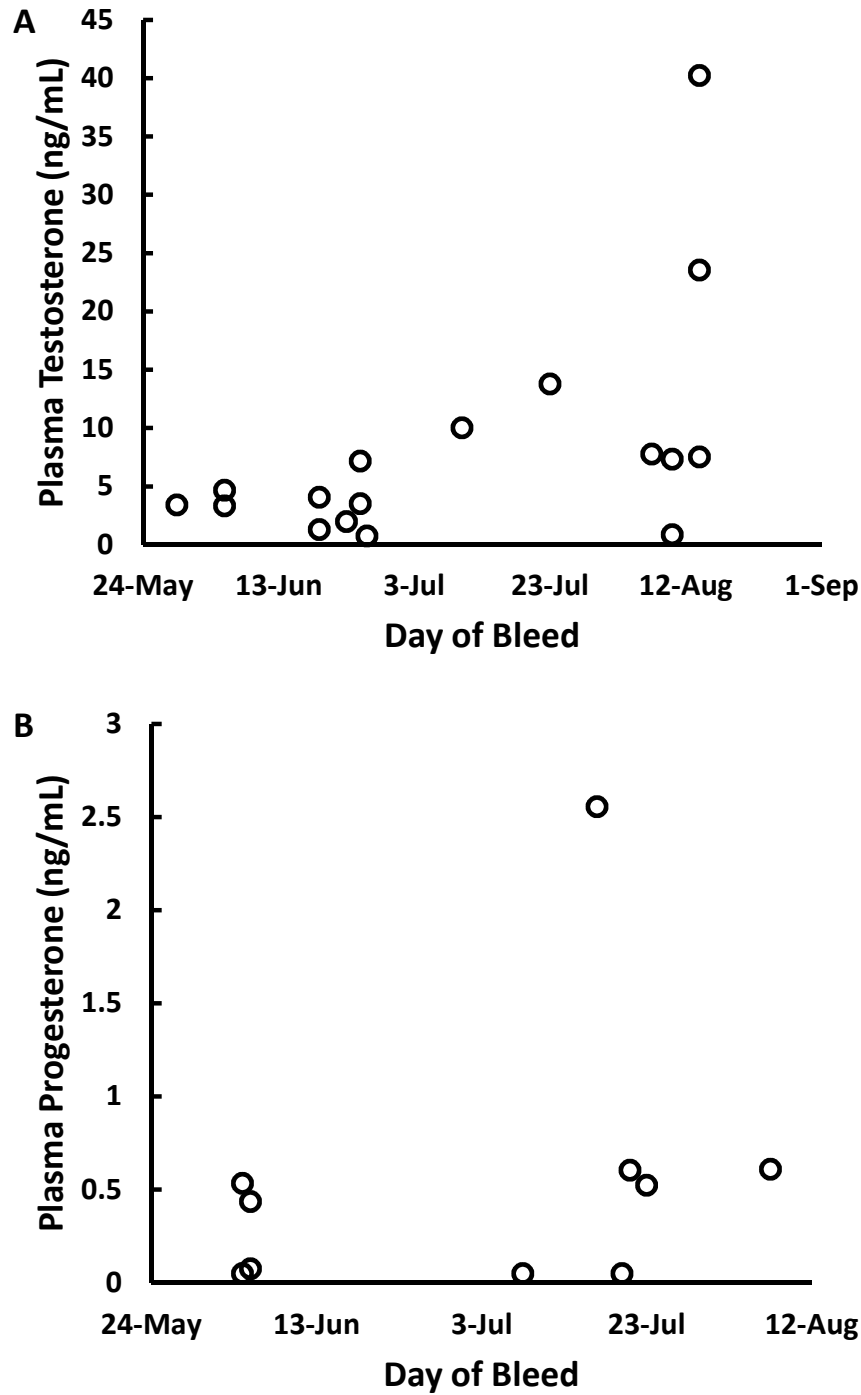


Figure 15: Relationships between the day bled for individual turtles and their plasma testosterone (A; males, $n = 17$) or progesterone (B; females, $n = 10$) concentrations. Plasma testosterone was higher in turtles bled later in the summer ($R_s = 0.59$, $p = 0.015$). Day bled had no significant relationship with progesterone ($R_s = 0.39$, $p = 0.27$).

Table 6: Results of correlation analyses between plasma triglycerides, steroid hormones, body condition, hemolysis, injury scores, and/or fecal nematode counts. No significant correlations were found. Cells shaded in light blue gray indicate nearly significant trends between the respective variables, with p-values slightly above 0.05.

Variable x Variable Relationship Tested	rho (R_p or R_s)	N	p-value
Plasma Triglycerides x Plasma CORT	0.24	28	0.226
Plasma Triglycerides x Plasma Testosterone	-0.25	17	0.326
Plasma Triglycerides x Plasma Progesterone	0.05	10	0.879
Plasma Triglycerides x vBCI	0.34	28	0.079
Plasma Triglycerides x Hemolysis Score	0.11	28	0.576
Plasma Triglycerides x Injury Score	0.03	28	0.864
Plasma Triglycerides x Nematode Counts (incl. microcaps)	-0.26	12	0.423
Plasma CORT x Plasma Testosterone	0.17	17	0.515
Plasma CORT x Plasma Progesterone	0.3	10	0.399
Plasma CORT x vBCI	0.0049	28	0.981
Plasma CORT x Hemolysis Score	0.30	28	0.118
Plasma CORT x Injury Scores	0.23	28	0.234
Plasma CORT x Nematode Counts (incl. microcaps)	-0.29	12	0.366
Plasma Testosterone x vBCI	-0.13	17	0.612
Plasma Testosterone x Hemolysis	0.17	17	0.515
Plasma Testosterone x Injury Scores	-0.22	17	0.403
Plasma Testosterone x Nematode Counts (incl. microcaps)	0.14	12	0.803
Plasma Progesterone x vBCI	0.0061	10	0.987
Plasma Progesterone x Hemolysis	0.42	10	0.222
Plasma Progesterone x Injury Scores	-0.034	10	0.924
Plasma Progesterone x Nematode Counts (incl. microcaps)	-0.051	10	0.935
Hemolysis x vBCI	0.14	28	0.467
Hemolysis x Injury Scores	0.06	28	0.749
Hemolysis x Nematode Counts (incl. microcaps)	0.25	12	0.432
Injury Scores x vBCI	0.35	28	0.067
Injury Scores x Nematode Counts (incl. microcaps)	-0.53	12	0.078

Carapace temperature

Representative environmental and carapace temperatures for bolder and less bold turtles of each sex are shown in figures 18 and 19. Simple linear regression analyses suggested average eye emergence was not a significant predictor of temperature, including FTD ($p = 0.18$), DTD ($p = 0.25$; Fig. 16), and NTD ($p = 0.22$). When comparing between sexes and boldness categories, females exhibited significantly higher FTD ($t = 2.27$, $df = 26$, $p\text{-value} = 0.032$) and DTD ($t = 2.16$, $df = 26$, $p = 0.04$; Fig 17A) values compared to males. The NTD of males and females were similar ($t = 1.32$, $df = 26$, $p = 0.20$), suggesting the difference between sexes in FTD is primarily explained by their temperature differences in the DTD. Within either sex, there was no difference between bold and less bold turtles in FTD (males: $t = 1.30$, $df = 16$, $p = 0.21$; females: $t = -0.26$, $df = 8$, $p = 0.80$) or DTD (males: $t = 1.19$, $df = 16$, $p = 0.25$; females: -0.53 , $df = 8$, $p = 0.62$). Likewise, bolder and less bold turtles did not differ in their NTD after pooling for sex ($t = 1.36$, $df = 26$, $p = 0.19$).

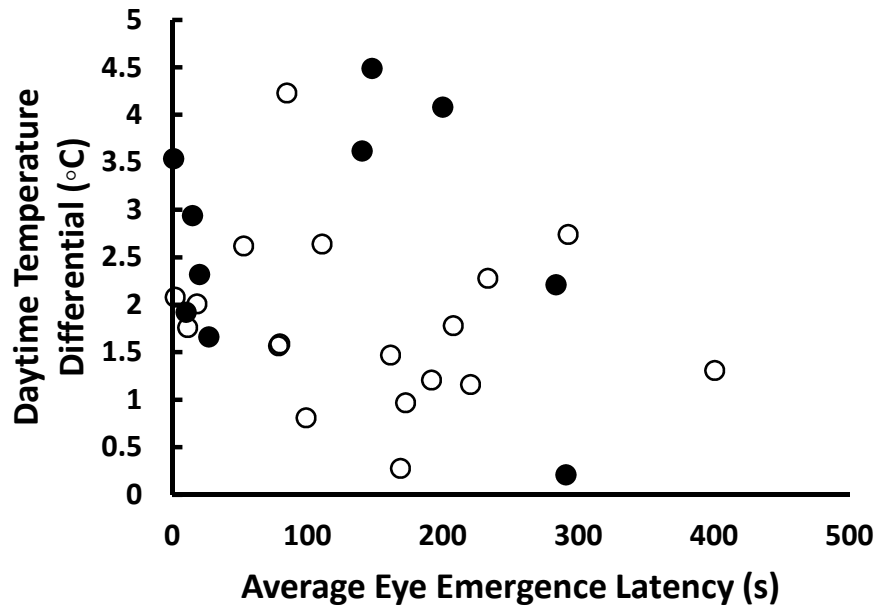


Figure 16: The average eye emergence of individual turtles plotted against their calculated daytime temperature differential from a nearby surface reference site. Male turtles are indicated by open circles and female turtles by shaded circles. There were no significant relationships between the daytime temperature differential and average eye emergence of males ($R_P = -0.20$, $p = 0.42$) or females ($R_P = -0.06$, $p = 0.87$), nor when sexes were combined ($R_P = -0.22$, $p = 0.25$).

Multiple linear regressions indicated both age (best count) and sex to be significant predictors for full study and daytime temperature differentials, with increasing age and males having a negative effect (Table 7). Similar results were found when age and sex predictor variables were tested separately, but in all cases the adjusted R^2 was higher when they were both included in the model together. Indeed, females had a higher FTD and DTD than males but the ages of each sex in this study were similar ($t = -0.554$, $df = 26$, $p = 0.584$), suggesting this to be true effects of sex and age on temperature rather than a bias of age within a sex. Conversely, neither age nor sex significantly predict nighttime temperature differentials, although age was nearly significant ($p = 0.07$ when alone or $p = 0.094$ when sex included).

Interestingly, integrating vBCI into the models slightly increases the explained portion of the percent of variation in temperature, but sex and vBCI become non-significant (albeit nearly significant for vBCI ($p = 0.087$)). Removing sex from the model then causes vBCI to become significant, suggesting these variables are likely related. Correlation analyses indicated no correlation between age and vBCI, suggesting that vBCI's effects on FTD are largely uncoupled from the effects of age. Although the t-tests revealed no significant sex differences in vBCI, females did tend to have higher vBCIs ($p = 0.09$) which may explain the similar explanatory power between sex and vBCI on temperature in the multiple linear models. Therefore, the effect of sex on FTD and DTD may be more directly explained by the effects of volumetric body condition.

Table 7: Multiple linear regression results showing the effects of age, sex (male), and volumetric body condition (vBCI) on temperature differentials calculated as the difference between box turtle carapace temperatures and nearby environmental reference sites over the entire study (FTD), daytime (DTD; 0900 – 1700), or nighttime (NTD; 2100 – 0500) periods. Younger, higher body condition, or female turtles were predicted to have higher temperature differentials (experience warmer temps) during the full study and daytime periods than their older, lower body condition, or male counterparts, respectively. This was not true of nighttime periods, although age was also nearly a significant predictor for NTD ($p = 0.094$). Similar results were found when predictor variables were tested separately, but in all cases the adjusted R^2 was higher when included together. Significance is indicated by bold values.

outcome var ~ predictor variables	$\beta_1, \beta_2, \beta_3$	Adjusted R^2	p-values: var1, 2, 3
FTD ~ Age + SexM	-0.10, -0.395	0.345	0.005, 0.033
DTD ~ Age + SexM	-0.189, -0.784	0.29	0.012, 0.046
NTD ~ Age + SexM	-0.028, -0.103	0.097	0.094, 0.252
FTD ~ Age + vBCI	-0.107, +2.332	0.355	0.003, 0.026
FTD ~ Age + SexM + vBCI	-0.101, -0.294, +1.795	0.397	0.004, 0.110, 0.087

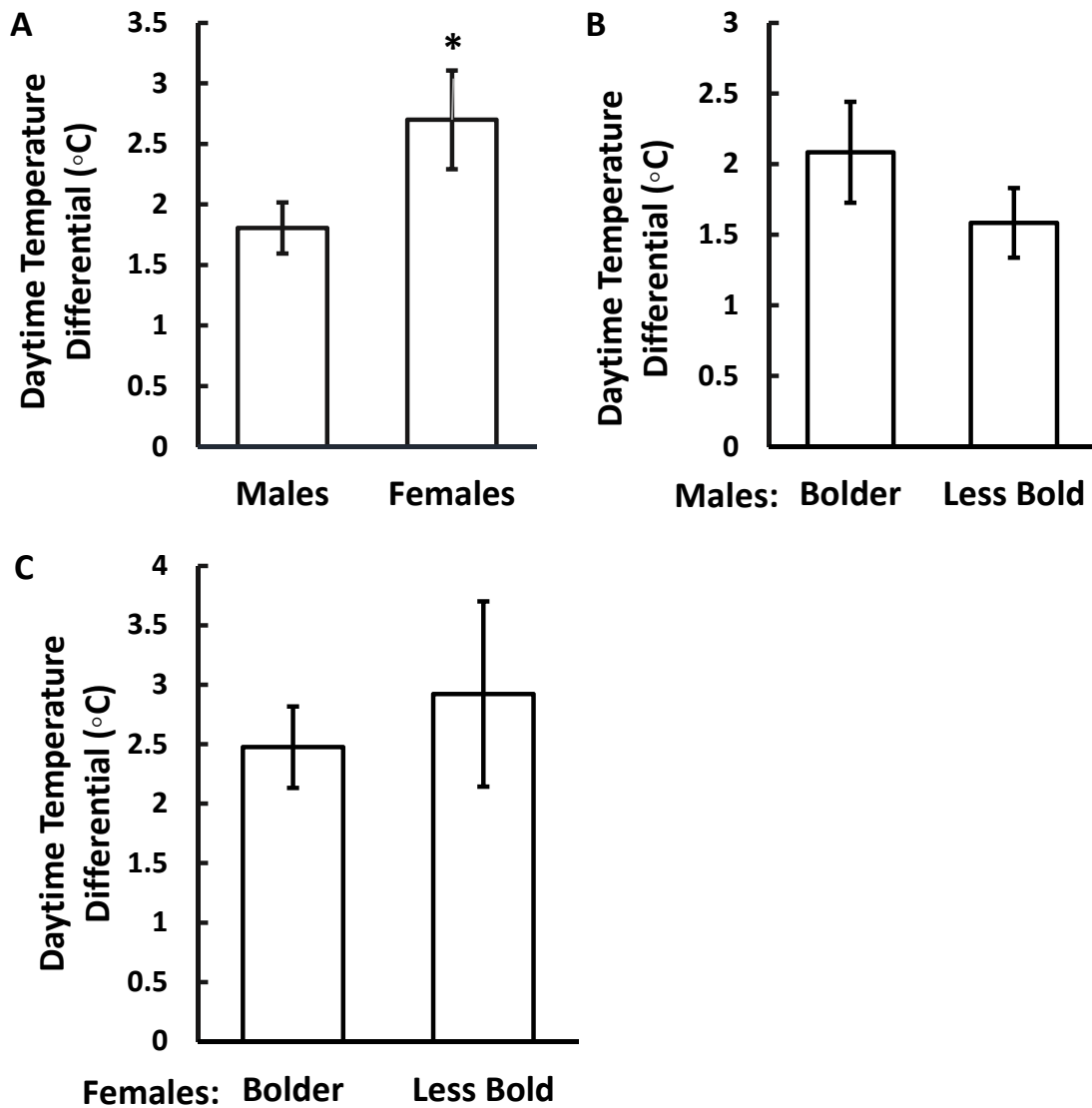


Figure 17: Comparisons between male ($n = 18$) and female ($n = 10$) box turtles (A) and bolder and less bold of either male (B) or female (C) box turtles in their mean daytime temperature differentials ($\pm$ SE) calculated as the difference between carapace temperature and the temperature of the environmental surface reference sites between 0900 and 1700 hrs. Females had significantly higher daytime temperature differentials than males ($t = 2.16$, $df = 26$, $p = 0.04$), but no differences in temperature differentials were detected between categories within either sex ($p > 0.05$ in both).

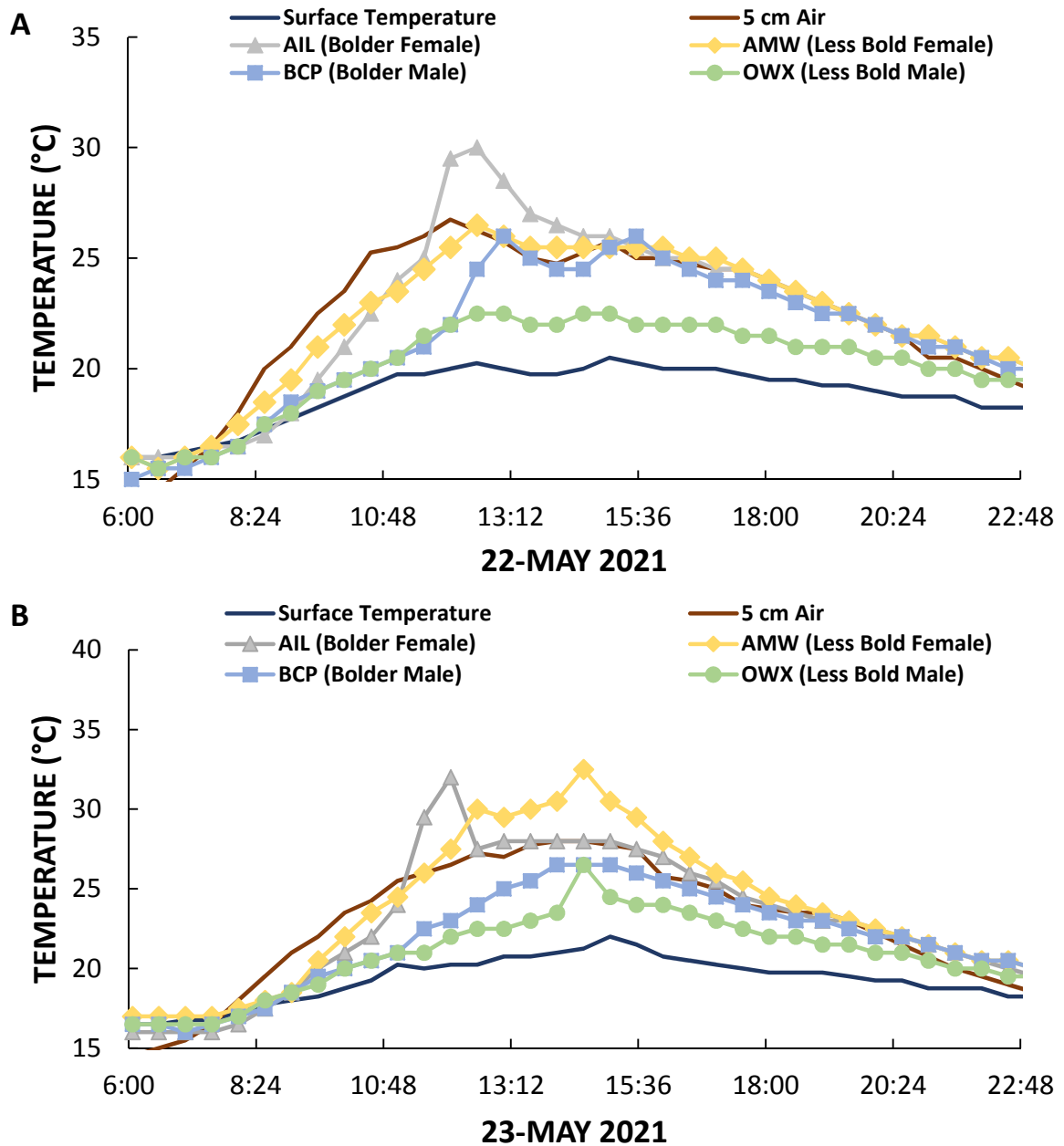


Figure 18: Temperatures of surface and 5 cm air iButtons from environmental stations plotted along with temperatures from iButtons mounted onto turtles during the 22nd (A) and 23rd (B) of May 2021 (late spring study period) from 0600 to ~23:00. These days had largely clear skies and temperature highs were 30 and 32°C, respectively. Two turtles from each boldness category are shown, one of each sex. Males tended to more closely track surface temperatures during the day whereas females readily went above surface temperatures and sometimes above air temperatures. Bolder females appeared to have sharper inclines and declines in temperature than less bold females.

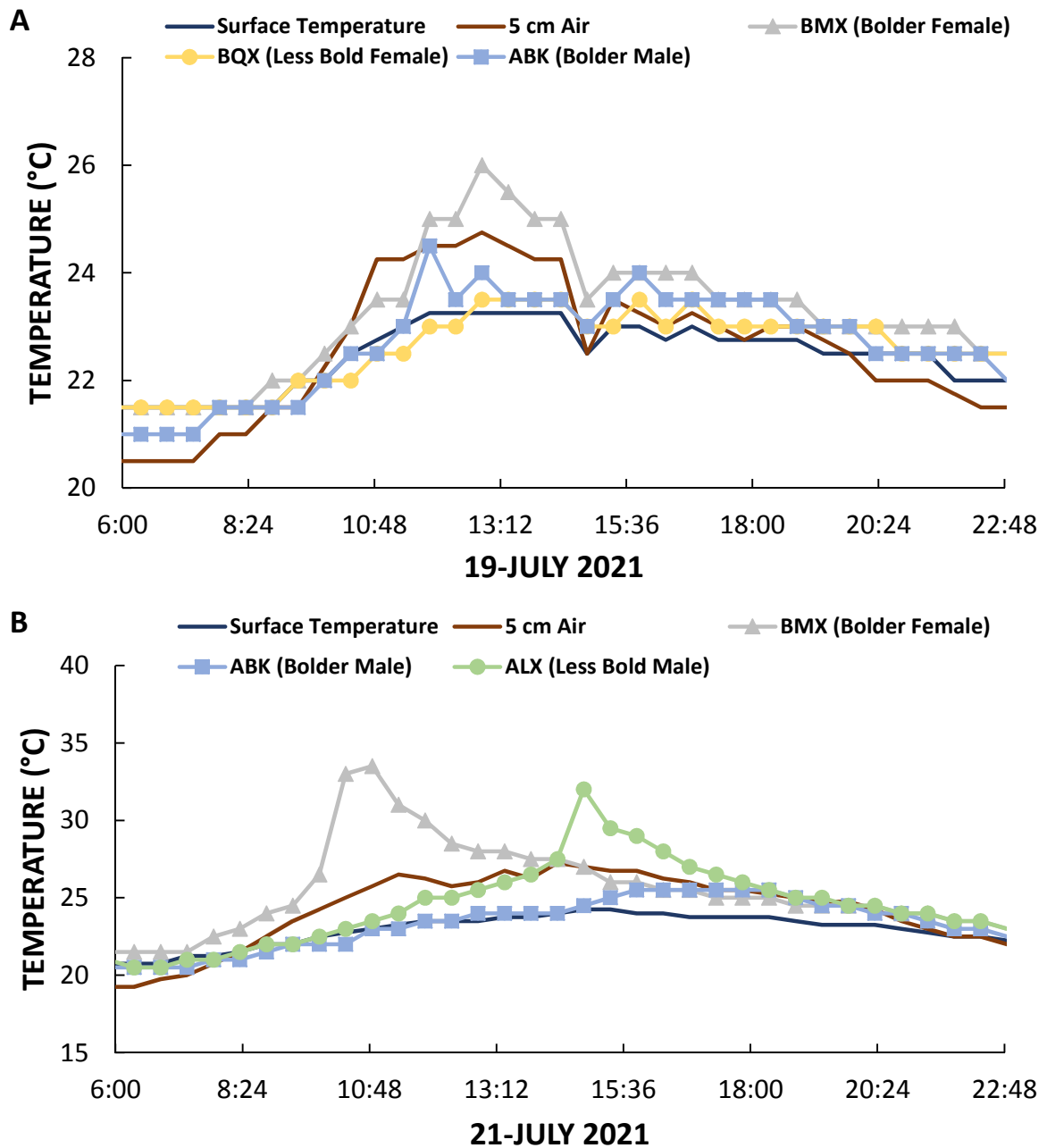


Figure 19: Temperatures of surface and 5 cm air iButtons from environmental stations plotted along with temperatures from iButtons mounted onto turtles during the 19th (A) and 21st (B) of July 2021 (summer study period) from 0600 to ~23:00. These days had largely clear skies and temperature highs were 30 and 32°C, respectively. Consistent with the graphs from May, females (particularly bold ones) readily went above surface temperatures and sometimes above air temperatures. However, males also appeared to do this on some days in July.

DISCUSSION

Sample demographics

In terms of general sample demographics regarding age and sex, the findings of this study are largely consistent with previous work on this population of turtles in 2016 (West & Klukowski, 2016), although in that study nearly 60% of adults fell in the 10-14 age group and ~37% in the 15-19 age class (with only ~3.0% in the 20+ age class). However, that study involved a much higher sample size ($n = 138$ for turtles within these age classes) and is therefore likely a more realistic representation of the population's overall demographics (West & Klukowski, 2016).

Repeatability of boldness and differences in plasticity

Results of the behavioral assays indicate that bold personality traits are present in this Tennessee population of wild adult *T. carolina*. This is indicated by the low within individual variation in boldness behaviors across the 5 assays, accompanied by relatively high variation in average behaviors across the 28 individuals. The findings agree with those of prior studies on populations of eastern box turtles of Tennessee (this population; Preston *et al.*, 2020) and Indiana, USA (Carlson & Tetzlaff, 2020; Kashon & Carlson, 2018; Pich *et al.*, 2019). Although only adults were tested in this study, Carlson & Tetzlaff (2020) have also shown this individual variation in boldness in 8-month-old juvenile eastern box turtles and demonstrated consistencies in their behavioral types by testing them a year later. Together these studies, along with this one, support boldness as a personality trait in wild eastern box turtles of central and southern USA.

Although the variability in eye emergence latency remained relatively low in the turtles of the bolder category (mean standard deviation: 20.8 sec; range: 0.0 – 60.7 sec), several turtles classified as less bold had relatively high variation in eye emergence (mean standard deviation: 102.1 sec; range: 32.5 – 207.7 sec). Therefore, it is possible that less bold turtles generally exhibit greater plasticity in this behavior than bolder individuals, as has been shown in other animal groups, including several fish (Jolles *et al.*, 2019; Kareklas *et al.*, 2016; Magnhagen & Bunnefeld, 2009) and some mammals (e.g., Dammhahn & Almeling, 2012). This also agrees with studies of “coping-styles”, which suggest that more “proactive” individuals, characterized by higher degrees of boldness and aggression, also tend to have lower flexibility in their behavioral responsiveness towards stressors than their “reactive”, less bold, and non-aggressive counterparts (Koolhaas, 2008; Koolhaas *et al.*, 2007; Øverli *et al.*, 2007). However, because there were still a few of these ‘less bold’ individuals that were consistent in their eye emergence latency (i.e., had low variation in eye emergence across assays), it may be more appropriate to view turtles in this study as either (1) consistently bolder, (2) consistently less bold, or (3) flexibly less bold. Similar personality groups have been reported in pea aphids, with some individuals consistently dropping or consistently not dropping in response to predators whereas a third group was flexible with their behavioral responses, exhibiting inconsistent drop times across assays (Schuett *et al.*, 2011, 2015). Indeed, 6 of the 15 ‘less bold’ turtles in this study had one or more of their behavioral assays with emergence times less than the 100 sec boldness cut-off (i.e., would be considered bolder for one or more of their assays), but ultimately had an average emergence that placed them into the less bold category. Likewise, 5 of the 13

bolder turtles had one or more behavioral assays with emergence times slightly above the 100 sec cut-off, although the standard deviations remained low for these individuals and hardly surpassed this cut-off. Nonetheless, by the standards of this categorization method, these 11 turtles were flexible enough to be considered bolder some days and less bold other days, with those placed in the less bold group exhibiting higher degrees of flexibility than those in the bolder group.

The difference in among-assay variance for wrist emergence and 2 body lengths was also higher in the less bold group than the bolder group, although to a lesser extent than eye emergence. This makes sense given that these behaviors were positively correlated with eye emergence both at the level of individual assays and at the level of average behaviors per turtle. However, the latency to move 1 m, which was also positively correlated with eye emergence, had relatively similar among-assay variance in the bolder group. Also, the number of total defenses employed during handling (which is negatively correlated with eye emergence latency) shows considerably higher among-assay variability in the bolder group than the less bold group, as turtles in the less bold group almost always exhibited zero defenses across all assays. Therefore, the differences in variance between bolder and less bold turtles does depend on the behavioral measure considered.

Effects of assay conditions on behavior

The GLMMs indicated both sky code and immediate shell temperature (from IR readings) to negatively predict eye emergence latency (Table 4). This suggests that with an increasing overcast or higher immediate shell temperatures during the day, turtles may

be inclined to emerge more quickly from the shell. Eastern box turtles rely largely on type III (true) orientation, using solar cues to actively choose a homeward direction and show reduced homing ability in overcast situations (DeRosa & Taylor, 1980; also reviewed in Dodd, 2001). It is possible that box turtles of this study increased their reliance for active exploratory behavior on heavily overcast days due to the effects of overcast on navigation. Studies have suggested boldness to be closely tied to exploratory behavior (e.g., Michelangeli *et al.*, 2019; Patrick *et al.*, 2017) and perhaps the more rapid emergence by box turtles during highly overcast days could reflect this. The reduced emergence latency effect of immediate shell temperature suggests that turtles may also adjust their boldness behaviors depending on their current body temperature. Together, the effects of cloud cover and immediate body temperature on emergence latency suggest at least some plasticity in boldness in response to environmental conditions.

Additionally, test number was a significant and negative predictor of eye emergence, in which turtles were predicted to have quicker emergence as their number of previous assays increased, suggesting turtles were habituating to the repeated behavioral assays involving handling by the investigator. Kashon & Carlson (2018) also found their box turtles to have similar habituation to their repeated handling assays which were similar to those conducted in this study. Because less bold turtles were noted to have more flexibility in their eye emergence latencies across behavioral assays, any habituation and reduction in eye emergence with increasing test number likely occurred primarily in these turtles as opposed to those in the bolder category. Additionally, bolder individuals repeatedly had emergence times already at or near the minimum of 1 sec and therefore could not reduce their emergence time any further.

Morphology and pinch force

Females were generally larger in weight but had similar carapace lengths as males. Because this study took place during active mating and nesting this size difference could be due to active vitellogenesis and egg production by females. A few females may have been gravid and nearing oviposition based on their relatively large movements into horse pastures and form constructions in tall grasses, however gravidity and nesting was never confirmed.

Average eye emergence had a negative, but non-significant trend with vBCI ($p = 0.076$). Therefore, bolder turtles may gain opportunities that allow for maintenance of better body condition. Alternatively, turtles may tend to behave more boldly when they exhibit higher body condition (e.g., more fat stores). Further investigation is needed given that this trend was non-significant and because the boldness categories did not significantly differ in vBCI.

Pinch force was nearly significantly higher in females compared to males ($p = 0.063$), perhaps reflecting the larger size (weight) of females. Additionally, individuals that were unable to completely close the shell (some bolder, some less bold) and individuals that were consistently unwilling to close (all bolder), when pooled together, exhibited significantly reduced pinch force values than turtles without those distinctions. Therefore, there may be similar consequences of (1) an anatomical misfit between elements or a lack of muscular strength associated with the hinge, and (2) active bold behavior during a potentially threatening interaction. Also, pinch force and reluctance/inability to close the shell may be behaviors that are linked to boldness (e.g.,

cross-traits). Roughly half of the turtles classified as bolder (~54%) either appeared incapable of closing fully or were reluctant to close, whereas this was true of only ~13% of turtles classified as less bold. Furthermore, average eye emergence was positively correlated with pinch force, suggesting turtles that took longer to emerge (less bold) also elicited higher pinch force values.

Physiological measures and parasites

Male and female turtles exhibited similar hemolysis scores, as did bolder and less bold turtles of combined sexes, suggesting there is no strong consequence of innate immunity relating to boldness in *T. carolina*. In general, the hemolysis immunoassay proved to be an effective method for testing the innate immune response to foreign RBCs by *T. carolina* plasma and may therefore be a useful health assessment in future work on box turtles, such as population studies and conservation efforts (also see West & Klukowski, 2018).

Fecal nematode counts were also similar between the sexes and boldness categories, suggesting that gastrointestinal nematode load is not a likely consequence of either sex or boldness in this species. However, this study did not investigate individual nematode species and also suffers from a small sample size of turtles for which feces was analyzed ($n = 14$). It has been noted in other studies that eastern box turtles typically exhibit a low prevalence of helminth endoparasites and tend to generally lack ectoparasites (Moraga *et al.*, 2012; Rose *et al.*, 2011). The findings of this study are largely consistent with these previous studies given that gastrointestinal nematodes counts were relatively low and only one ectoparasite (a leech) was observed on a single

turtle. Wild *T. carolina* may be particularly resistant to parasites, or our sample may have been biased towards low parasitized individuals. Regardless, other endoparasites, such as gastrointestinal trematodes and coccidia, as well as hemoparasites, should also be considered (e.g., see Doke *et al.*, 2022; Moraga *et al.*, 2012). Additionally, the most common fecal nematode noted in this study were microcapillarids which are cited in only a single source (Klingenberg, 2016) and may be of no significance to box turtles. Future studies might further investigate these so called microcapillarids to determine if they are parasitic nematodes, and if so, how pathogenic they are towards their turtle hosts. Lastly, it remains unclear if leeches frequently parasitize box turtles of wetland populations or cause any harmful effects.

Females had significantly higher plasma triglycerides than males which is consistent with prior work in this population (West & Klukowski, 2018). Plasma triglycerides may increase in females as they allocate more lipids for vitellogenesis or nesting migration, the latter of which may involve traveling far distances from a female's typical home range (West & Klukowski, 2018; Stickel, 1950). Given that active mating and nesting was likely occurring in this population during the time of this study, the high levels of plasma triglycerides in females might therefore relate to their allocation of lipids for reproductive purposes (although peak mating for this population likely occurs during early fall) (West & Klukowski, 2018).

Baseline plasma CORT was similar between the sexes and boldness categories and showed no significant correlations with any other physiological or morphological measure, including parasites and hemolysis scores. The latter was unexpected given that

prior work in this population had identified a positive relationship between plasma CORT and hemolysis (West & Klukowski, 2018). Similarly, testosterone and progesterone were not different between boldness categories and showed no significant correlations with other physiological and morphological measures, suggesting that these hormones do not serve significant roles in facilitating the trade-offs of boldness (although see discussion on the limitations of this study). However, testosterone tended to be significantly higher in males measured later in the season, which could be associated with a shift towards more active spermatogenesis in males as they approach the peak mating of autumn (West & Klukowski, 2018).

Unsurprisingly, plasma CORT did increase significantly after confining individuals in buckets within a novel lab environment for ~22 h when compared to their baseline bleeds which occurred after the final 10-min field behavioral assay. Additionally, the change of plasma CORT (post-confinement minus baseline) between bolder and less bold turtles was similar but should be further investigated given the small sample size of turtles bled a second time in this study (bolder $n = 4$; less bold $n = 5$). If turtles with varying degrees of boldness do indeed exhibit similar CORT responses, *T. carolina* may differ from the typical hypothesized pattern of “proactive” (bold, explorative, aggressive) animals which are said to have lower CORT responses to stressors than “reactive” (timid, shy, submissive) animals (Koolhaas *et al.*, 1999; Hau & Goymann, 2015). In fact, the change in plasma CORT tended to be higher (albeit non-significant) in the bolder group which suggests that bolder box turtles may actually have greater stress responses to confinement than less bold turtles. It is unknown whether turtles differed in their levels of activity during their 22 h of confinement, but because

turtles were free to move within their confinement buckets (and many did so), it is possible that their CORT response was influenced by locomotor activity during the time of confinement (Hare *et al.*, 2014) which could have been boldness dependent.

Temperature and its interactions with age, sex, body condition, and injury scores

The temperature data from iButton loggers revealed that female and younger box turtles experienced significantly higher temperature differentials for full study and daytime periods than males and generally older turtles, but this was not true of the nighttime differentials. Female turtles may therefore be selecting warmer microhabitats during the day at this time of year. Indeed, several females were repeatedly captured outside of the forested areas of the field site in horse pastures containing only limited shading by tall grasses. I suspected that some of these females may have been gravid with eggs and preparing for nesting and oviposition. Because *T. carolina* is one of many turtle species with temperature sex determination, the temperature of the nesting site chosen by ovipositing females will likely have drastic effects on offspring development and sex ratio outcomes, making nest site selection by females important to hatchling success and offspring phenotypes (e.g., Delaney & Janzen, 2019; Refsnider *et al.*, 2022). Nest site selection could also be repeatable and related to personality traits, such as boldness and exploration (e.g., Patrício *et al.*, 2018; Seltsmann *et al.*, 2014). It could be worth investigating if there are potential interactions between boldness and temperature in nesting versus non-nesting female box turtles.

In late spring (late May), males tended to more closely track the environmental surface temperatures whereas females often exceeded surface temperatures and more

closely tracked air temperatures. Additionally, several graphs indicated that bolder females tended to have sharper inclines and declines in temperature than less bold females, possibly indicating differences in thermoregulation. These patterns appeared largely true for the later summer period (late July) of the study, although on some days males were also observed to increase their temperatures above the air temperature similar to females.

Linear regression analysis suggested that turtles with higher full study and daytime temperatures (and possibly higher body conditions) were also predicted to have higher injury scores. Turtles maintaining warmer temperatures may be more likely to encounter situations that lead to injury (such as predation attempts by predators). This would support the claims of Kashon and Carlson (2018), which suggested a trade-off between thermoregulation and injury score in *T. carolina* that was possibly linked to boldness. Alternatively, turtles which have sustained higher injuries may thermoregulate to higher temperatures.

On the contrary, linear regression analyses showed that a turtle's average eye emergence was not a significant predictor of any of its temperature differential measures or injury scores. Likewise, FTD, DTD, NTD, and injury scores did not differ between the two boldness categories. Therefore, bolder and less bold turtles of this study appear to be choosing similar thermal habitats at our field site and any possible trade-off between thermoregulation and injury score may be decoupled from boldness. Nonetheless, because the GLMMs indicated that immediate shell infrared temperature was a significant predictor for the turtle's eye emergence, it is possible that temperature may

still have at least some influence on an individual's short-term processing and/or decision-making for risk-taking behaviors in response to a potential threat. This result is largely consistent with the conclusions drawn from Pich *et al.* (2019) which suggested that “the effects of temperature [on boldness behaviors] are present but not strong”. Perhaps the relationship between temperature and injury scores is better explained by other behaviors not considered in this study (e.g., exploration) that might also relate to boldness to some extent, as such a phenomenon could explain the sometimes present but weak association between temperature and boldness in studies of box turtles.

Given that immediate shell temperatures and overcast skies influence eye emergence and therefore boldness, both age and sex (and relatedly, body condition) should also be further investigated in their interactions with personality, as both variables were determined to interact significantly with a turtle's daytime temperature. Indeed, turtles that were younger, female, or had higher body condition exhibited significantly higher daytime temperatures than their respective counterparts in this study. Could it be that turtles of these distinctions are choosing different microhabitats and/or actively thermoregulating differently? If so, this could have short-term implications for their boldness behavior and therefore interactions with conspecifics or predators. This seems plausible given that daytime temperature was also positively associated with shell injury scores and that both injury scores and daytime temperature exhibited positive trends with body condition.

Limitations of present study and future considerations

The pace-of-life hypothesis is a common theory for the existence and evolutionary persistence of animal personality traits. Further investigations into the survival and reproductive consequences of boldness in eastern box turtles is needed, perhaps using more direct assessments of these life history traits than the measures used in this study. For example, it would be interesting to test if bolder and/or aggressive males or females have different copulatory (or overall reproductive) success than their timid counterparts. Such a finding was demonstrated in male zebrafish in which bolder males fertilized more eggs than shy males (Ariyomo & Watts, 2012). Additionally, turtles could be choosy with mates either directly prior to copulation or, in the case of female choice, via sperm storage post-copulation (reviewed in Pearse & Avise, 2001). Box turtles could therefore rely on personality traits to make decisions based on mate quality or compatibility, as has been demonstrated in several species of birds with assortative mating (e.g., Both *et al.*, 2005; Collins *et al.*, 2019; Pogány *et al.*, 2018). In general, the survival and reproductive consequences of boldness in box turtles needs further and more direct consideration.

It should be noted that the categorization of individuals into bold and less bold turtles using a simple method based solely on average eye emergence is likely an oversimplification of boldness in box turtles. However, the simple method did agree with the more complex, compiled behavior method of categorization (using various behaviors during and immediately following handling) for all but one individual. Nonetheless, both methods may suffer from oversimplification given that (1) all the behaviors used in the

compiled method were highly correlated and therefore may not encompass many aspects of true bold (or less bold) behavior, (2) one could easily interpret the majority of these behaviors as exploratory or activity driven behaviors rather than bold responses (although these are often not mutually exclusive and are assumed to be related here), and (3) boldness is more appropriately viewed as a continuous trait (i.e., a “shy-bold continuum”; e.g., Bubac *et al.*, 2018; Oswald *et al.*, 2012; Wilson *et al.*, 1993) rather than the division of two discrete groups such as ‘bolder’ and ‘less bold’. To the latter point, this study does attempt to test boldness as a more continuous trait when using individual and average eye emergence values in correlation and regression analyses, for which an absence of significant interactions between physiological measures and boldness remains, although some testing conditions were found to affect boldness within these tests.

Another limitation in this study is that behaviors were tested under similar contexts and therefore do not adequately test for contextual consistency. It is possible that the individual state-variables here have a more non-linear association with boldness, whereas this study primarily considered linear associations exclusively and within a single major context (summer season, presence of- and handling by- a human investigator). There could be more complex interactive effects of boldness in eastern box turtles, in which certain contexts (such as different seasons) may present different consequences for boldness. For example, Broecke *et al.* (2021) found the effects of stress-sensitivity personality in wild mice (*Mastomys natalensis*) to be present only during the “population decrease phase” of their study, in which food resources became scant and competition was high. Although Tetzlaff & Carlson (2020) have already demonstrated that differences in enrichment early in development appears to have no

effect on the repeatability of boldness in lab-reared juvenile eastern box turtles, different seasons could still create differential pressures on bold and less bold turtles in Middle Tennessee which are unrelated to food availability (e.g., temperature, shelter availability).

It is also possible that the methodology of this study fails to consider the primary mechanisms by which these intrinsic states influence behavior. For example, regarding hormones, only differences in the concentration of certain hormones among individuals were considered and not differences in receptor density, carrier molecules, or expression across tissues which could be just as, if not more, important in governing the hormonal effects of behavioral phenotypes in this species (e.g., Oswald *et al.*, 2012; Niemelä & Dingemanse, 2018). Additionally, several of the physiological traits were measured at only a single point, such as at the end of the study, and therefore do not account for repeatability in these physiological traits of individual state or the possibility of the short- and long-term differences in their responsiveness between individuals and this deserves further attention. For example, it is possible that bolder and less bold turtles differ in their stress responsiveness (non-significant in this study but small sample size).

Furthermore, there may be other physiological and/or ecological variables not considered in this study that do interact more directly with boldness and present potential explanations for its trade-offs (or fitness implications) in *T. carolina*, such as other aspects of immunity like inflammatory responses or specific immune responses. Boldness could also be linked to other behaviors within a behavioral syndrome and these other behaviors may have more explicit or direct trade-offs with intrinsic and extrinsic state variables such as those measured in this study. For example, aggressive behavior was

noted in one of the male turtles of this study which attacked a submissive male subject for nearly an hour (see Appendix) (although the seemingly aggressive behaviors may be confused with courtship behaviors; see Stickel, 1989). Perhaps aggressive behaviors, which are also often considered ‘risk-taking’, are more directly dependent on individual state than the measures of boldness considered in this study, although the behaviors might be correlated.

Conclusion

To my knowledge, this is the first study to investigate intrinsic physiological correlates of boldness (besides sex and age) in eastern box turtles. There were no differences in hemolysis, fecal nematodes, triglycerides, or body condition between bolder and less bold turtles, nor did any of these variables significantly correlate with average eye emergence. This suggest that neither impairments in innate immunity, increased loads of endoparasites, availability of fat stores, nor body condition status are likely to be strong consequences of boldness in eastern box turtles of Middle Tennessee. Therefore, as it currently stands, this study suggests boldness to be largely independent of sex, age, short-term aspects of circulating triglycerides, endocrine profiles, innate immunity, nematode loads, and general morphology, although bolder turtles may have higher body condition (non-significant trend observed in this small study) and less bold turtles may have higher pinch force values. This appears true at least in the context of adult *T. carolina* during these active months of the summer season, as was the focus of this study. It is possible that these variables might interact with boldness during other (e.g., cooler) months of the year, or in juvenile turtles. More long-term studies which

include a greater number of ecologically relevant contexts (e.g., the other seasons) and developmental periods for eastern box turtles may help to test this possibility. On the other hand, daytime temperature differed between age and sex groups, and showed positive trends with body condition and injury scores, calling attention to the possibility of thermoregulation by some turtles and potential trade-offs of thermoregulators vs thermoconformers. Specifically, this study suggests that turtles maintaining warmer daytime temperatures may be more likely to have higher body condition but also exhibit higher degrees of shell damage. One would expect that either boldness or its association with other behaviors (e.g., exploration, aggression, etc.) could interact with thermoregulation in at least some contexts (e.g., nesting or preparation for overwintering), and therefore facilitate these trade-offs.

In conclusion, this study further shows that eastern box turtles display consistent bold personalities across individuals and suggests that less bold individuals tend to display higher levels of plasticity in their bold responses than bolder turtles. Moreover, box turtles appear to have the ability to habituate to repeated handlings which may influence the outcome of their boldness responses. Intuitively, turtles that are consistently proactive in their use of active defenses during a potentially threatening encounter may be more vulnerable to predators as they appear less likely to tightly close the shell, however, turtles with the inability to fully close the shell (regardless of boldness) may also suffer similar consequences. Interestingly, boldness appears to be largely independent of short-term physiological state, although there was a negative trend between average eye emergence and body condition, suggesting turtles that emerge quicker (bolder) may have higher body conditions. On the other hand, boldness appeared

to be dependent on some short-term environmental conditions, such as cloud coverage and immediate body temperature, but not dependent on overall daily temperatures.

Finally, daytime temperatures differed between sex and age groups and exhibited positive trends with shell injury scores and body condition, suggesting that turtles may differ in their thermoregulation and that there may be differential consequences for thermoregulators and thermoconformers. Further studies are needed to better understand the implications of these interactions and their possible correlation with other personality traits.

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APPENDIX

Boldness categorization by simple and compiled methods

The simple and compiled behavior methods of boldness categorization provided similar results to one another. In the simple method, 13 out of 28 turtles were classified as ‘bolder’ and the remaining 15 as ‘less bold’ (Fig. 2), whereas in the compiled behavior method half (14) were classified as ‘bolder’ and the remaining half (14) as ‘less bold’ (Fig. A1). Both methods agreed on placement of turtle subjects into ‘bolder’ and ‘less bold’ categories for all but one individual which had an average eye emergence slightly above the simple methods cutoff for the ‘bolder’ group but exhibited several ‘bold’ qualities considered by the compiled behavior method. In other words, all 13 turtles considered as ‘bolder’ by the simple method were also considered ‘bolder’ by the compiled behavior method and only 1 turtle that was ‘less bold’ in the simple method was instead considered ‘bolder’ by the compiled behavior method. Similar to eye emergence latency alone, the individual scores assigned to turtles by the compiled behavior method were also significantly repeatable within individuals across the 5 behavioral assays ($r = 0.33$, $CI = [0.051, 0.582]$) (Fig. A1).

Table A1: Behaviors and scores for the compiled behavior method of boldness categorization. Higher scores indicate a bolder turtle.

Behavior	Scores Possible		
The time the turtle spent closed-up during the 3-min handling period	Score 0	Score 0.5	Score 1.0
	If turtle remained in shell for ≥ 120 sec	If turtle remained in shell between 61 and 119 secs	If turtle remained in the shell for ≤ 60 secs
Did the turtle deploy any air walking (kicks) with the front and/or back legs during the 3-min handling period	Score 0	Score 0.5	
	If the turtle did not deploy air walking (kicks)	If the turtle deployed air walking (kicks)	
The latency in seconds for the turtle to extend its head so the eyes surpass the anterior margins of the shell during the observation period	Score 0	Score 0.5	Score 1.0
	If eye emergence did not occur until ≥ 400 secs or at all	If eye emergence occurred between 201 and 399 secs	If eye emergence was ≤ 200 secs
The latency in seconds for the turtle to move 2-body lengths in distance during the observation period	Score 0	Score 0.5	Score 1.0
	If 2-body lengths were not reached until ≥ 400 secs had passed or at all	If the turtle reached 2-body lengths between 201 and 399 secs	If 2-body lengths were reached in ≤ 200 secs
Whether or not the turtle crawled 1 m during the observation period	Score 0	Score 0.5	
	If the turtle had not moved 1 m	If the turtle had moved at least 1 m	
Final Score 0 - 4 (<i>sum of all above scores divided by total possible = final score</i>)	If final score is less than half (< 2) then the individual is considered 'less bold'. If final score is more than or equal to half (≥ 2) then the individual is considered 'bolder'		

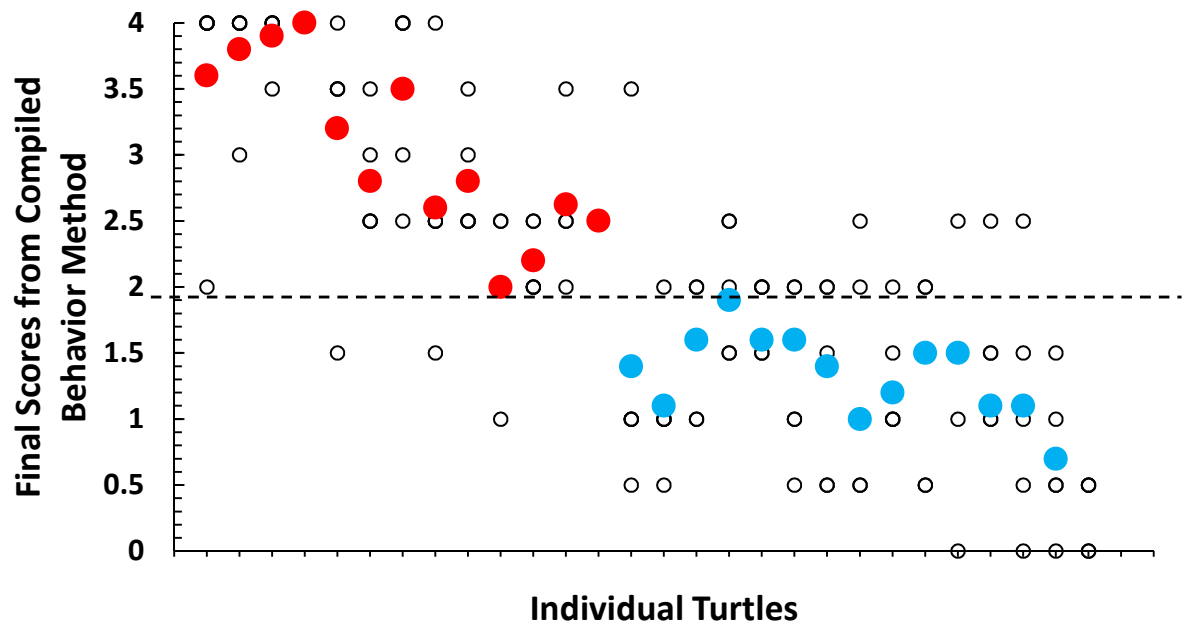


Figure A1: Final scores of individual turtles by the compiled behavior method. Scores ranged very 0 – 4, with 4 being the boldest and 0 the least bold. The threshold line at $y = 2$ represents the cut-off for boldness categorization by this method, and red dots above this line indicate bolder individuals (average final scores ≥ 2) while blue dots below the line indicate less bold individuals (average final scores < 2). Individual turtles are placed in order from lowest to highest average eye emergence to allow comparison with Fig. 2.

An observed account of aggression

While radio tracking turtles between captures, two observations of interactions between conspecifics were noted. Upon stumbling on these interactions, I retreated approximately 10 m and observed for up to an hour. Of these, two male subjects were once observed in a seemingly one-sided combative interaction (BNX attacking ABK,). During the observed portion of this encounter BNX appeared to be the aggressor while ABK remained submissive. Although both turtles were later found to be ‘bolder’, behavioral assays suggest BNX may be the slightly bolder of the two due to an average eye emergence that is quicker than that of ABKs (mean of BNX: 52.8 sec, ABK: 98.8

sec). During the observed combat that lasted nearly an hour, BNX continuously bit at the margins of ABK's carapace, occasionally climbing onto and over ABK who remained mostly closed the entire time. The aggressor (BNX) also began bleeding on his mouth/snout, presumably due to repeatedly biting at ABK's carapace. This was the only observed account of aggression that I noted during this field season and within these subjects, with the only other interaction observed between subjects being a copulation event between OWX ('bolder') and HKW ('less bold').

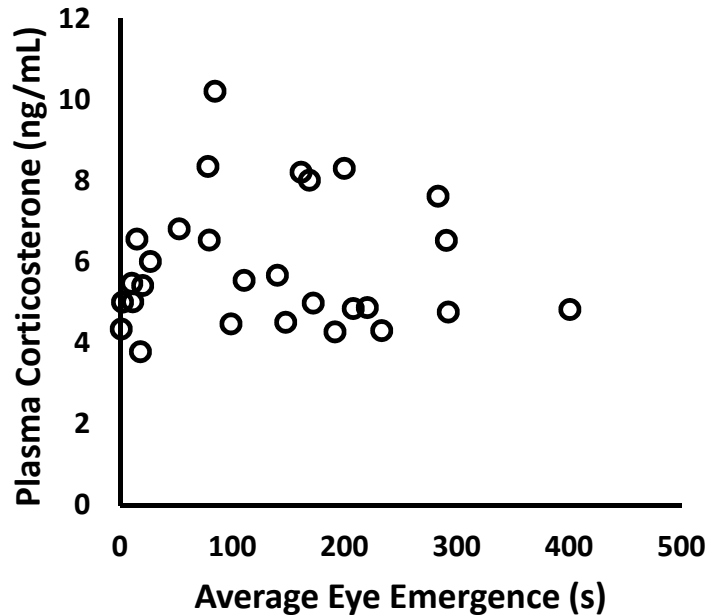


Figure A2: Relationship between the average eye emergence of individual box turtles and their baseline plasma corticosterone ($n = 28$) concentration. There was no significant correlation between the average eye emergence and plasma corticosterone ($R_s = -0.061$, $p = 0.76$).

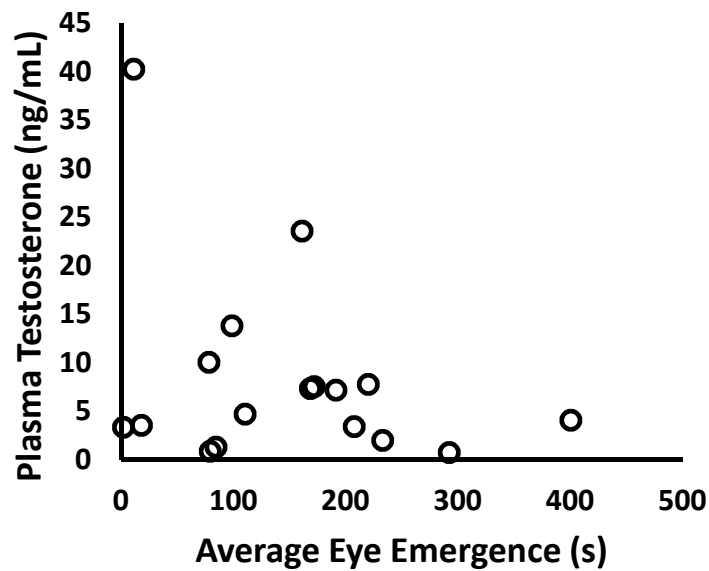


Figure A3: Relationship between the average eye emergence of individual male box turtles and their baseline plasma testosterone ($n = 17$) concentration. There was no significant correlation between the average eye emergence and plasma testosterone ($R_s = -0.19$, $p = 0.46$).

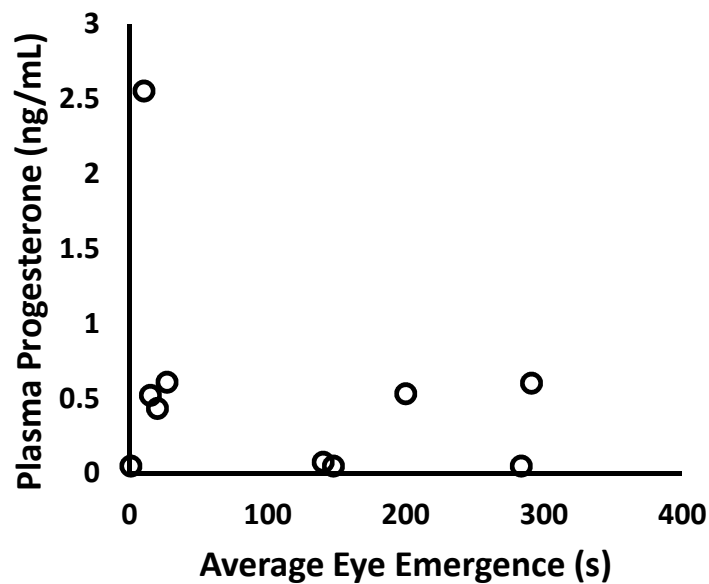


Figure A4: Relationship between the average eye emergence of individual female box turtles and their baseline plasma progesterone ($n = 10$) concentrations. There was no significant correlation between the average eye emergence and plasma progesterone ($R_s = -0.068$, $p = 0.85$).