

Behavioral and Physiological Responses to Simulated Predator Induced Stress in the
Eastern Box Turtle, *Terrapene carolina carolina*

by
Victoria Lay

A thesis presented to the Honors College of Middle Tennessee State University in partial
fulfillment of the requirements for graduation from the University Honors College

Fall 2016

Behavioral and Physiological Responses to Simulated Predator Induced Stress in the
Eastern Box Turtle, *Terrapene carolina carolina*

by
Victoria Lay

APPROVED:

Dr. Matthew Klukowski
Biology Department

Dr. Lynn Boyd
Biology Department Chair

Dr. David Nelson
Biology
Second Reader

Dr. John Vile
Dean, University Honors College

OR

Dr. Philip E. Phillips, Associate Dean
University Honors College

Abstract

The behavioral and physiological responses of eastern box turtles, *Terrapene carolina carolina*, to a simulated predatory encounter were measured. Wild box turtles were randomly assigned to a stimulus intensity group, which consisted of observation (control), a visual stimulus, a poke on the leg, or a pinch on the leg before being flipped. Behavior in response to the stimulus was observed from a distance for 5 minutes, except for the control which was observed for 15 minutes. Immediately after the observation period, turtles were captured and had a small blood sample taken, after which a pinch gauge was used to measure the shell's closing force. The results indicate that the stronger the simulated predatory stimulus, the stronger the behavioral responses, with the turtles taking more advantage of their fully closable shell at the more intense stimulus groups. However, stimulus intensity did not affect the plasma corticosterone or plasma lactate response. This may be due to too short or weak of a stimulus, increased parasympathetic activity, or seasonal effects. Upon capture and during handling almost all turtles withdrew into their shells and closed their anterior plastron, although fewer actually closed their posterior plastron, and a few engaged in other behaviors such as biting, urinating, and air walking. There appeared to be a correlation between box turtle size and pinch strength; however, the relatively large size of the pinch gauge may have put smaller turtles at a disadvantage compared to larger turtles because of excessive stretching of their closing muscles. The results of this study indicate that in spite of their fully closable shell, box turtles exhibit a complex, graded response to perceived threats. This strongly suggests that full closure of the shell is likely to be costly and occurs only in response to the most intense stimuli.

Table of Contents

	Page
Abstract	iii
List of Tables	v
List of Figures	vii
List of Terms	ix
Introduction	1
Methodology	6
Treatment and Observations	6
Blood Sampling	8
Corticosterone.....	8
Lactate.....	8
Pinch Gauge.....	9
Other Measurements	9
Statistical Tests	10
Results	10
Discussion	13
References	17
Tables	20
Figures	23

List of Tables

	Page
Table 1. Descriptive statistics for the different stimulus treatment groups of eastern box turtles. The average \pm SD is shown for carapace length (CL), body mass, volumetric body condition (vBCI), and estimated age. The four groups did not differ significantly in any of these variables	20
Table 2. Behaviors observed in eastern box turtles before the stimulus was applied. *One turtle was observed walking to the creek and then swimming. Also, the posterior plastron was not always clearly visible, and turtles which were found swimming were not considered to have a posture, thus n may be less than 40.....	20
Table 3. Anterior plastron response of eastern box turtles immediately after stimulus application. See text for a description of the different plastron positions.....	21
Table 4. Head response of eastern box turtles immediately after stimulus application. See figure 5 for depiction of different head positions	21

Table 5. Behaviors observed during capture and handling in eastern box turtles after stimulus application. Air walked is defined as the turtle moving its legs in the air while being held in a fashion similar to as if it was walking or swimming. Only turtles which were fully closed for the full duration of capture and handling are included under plastron positions22

List of Figures

	Page
Figure 1.	Range of the eastern box turtle, <i>Terrapene carolina</i> . Map from Dodd (2001)23
Figure 2.	The hinged plastron of the box turtle allows it to withdraw its limbs and close its shell. Photo by Victoria Lay23
Figure 3.	A diagram from Bramble (1974) illustrating how the shell closes in <i>T. c. carolina</i> . The shell is shown open in A, closed in B, and an open shell with an overlay of a closed shell (dotted lines) is seen in C. Shell closure is possible via a hinged plastron and specialized musculo-skeletal anatomy of the pectoral and pelvic girdle joints24
Figure 4.	A diagram from Bramble (1974) illustrating the <i>scapular suspensorium</i> locking mechanism. “A” displays the <i>suspensorium</i> in the “locked” position, which is when the shell is open. “B” shows the <i>suspensorium</i> beginning to fold outward as the shell starts to close. “C” shows the <i>suspensorium</i> completely unfolded as the shell is fully closed25
Figure 5.	A series of illustrations depicting the head positions seen in box turtles besides completely closed and head extended far26

Figure 6.	A histogram displaying the number of eastern box turtles per stimulus intensity group that fell into each behavioral rank score. Each group contained 10 turtles	26
Figure 7.	Average (\pm SE) time for the head to reach mostly out (A), and time for the anterior plastron to be at least mostly open after the stimulus was applied (B).....	27
Figure 8.	Average (\pm SD) plasma corticosterone (A) and lactate concentrations (B) in eastern box turtles belonging to each stimulus intensity group.....	28
Figure 9.	A scatter plot showing the association between maximum pinch strength of an eastern box turtle and its age.....	29

List of Terms

Analysis of Variance (ANOVA) – Statistical models used to determine variation among multiple groups

Carapace – The top portion of the shell of a turtle

Chelonian – A reptile in the order *Testudines*, formerly *Chelonia*; all turtles

Corticosterone – A steroidal stress hormone released from the adrenal gland in reptiles

Enzyme-Linked Immunosorbent Assay (ELISA) – A lab technique utilizing antibodies and hormones conjugated with an enzyme used to measure the concentration of a substance in a solution where a reaction leads to a color change in the sample solution, with stronger color changes indicating a lower concentration of the substance

Lactate – A chemical produced by the body during vigorous physical activity in which some anaerobic metabolism occurs; the ionized version of lactic acid

Limb Girdles – The bones which support and attach the limbs to the trunk of the body

Mesic – A habitat containing a moderate amount of moisture

Nonparametric Statistics – Statistical methods where the data does not have to fit a normal distribution; based on ranked scores

Plastron – The bottom portion of the shell of a turtle

Spearman Rank Correlation – A nonparametric statistical test that measures the strength of correlation between two variables

Spectrophotometric Enzyme Assay - A lab technique used to measure the concentration of a substance in a solution by determining the strength of an absorption line at a certain wavelength using a spectrophotometer, with a stronger absorption line indicating a higher concentration of the substance

Spectrophotometer – A machine that measures the amount of light that a substance absorbs at specific wavelengths

Subcarapacial Sinus – A large vein found on the back of the neck of a turtle

Introduction

Animals have evolved a diverse assortment of dramatic antipredator defense behaviors and mechanisms. For example, disturbed bombardier beetles will spray a jet of exploding acid at would-be attackers (Emlen, 2014). Rather than exhibiting defensive aggression, hognose snakes puff up and writhe about onto their back, mouth open and tongue hanging out with no obvious signs of breathing in order to feign death (Burghardt & Greene, 1988). Taking a subtler approach, leaf-mimicking katydids blend into the surrounding foliage by using their incredible camouflage and swaying gait to look like a leaf fluttering in the wind (Emlen, 2014). While not as dramatic as the previous examples, turtles themselves are equipped with a dependable and effective defense system: their shells. The most protective shells are fully closable, such as those of the box turtles.

The eastern box turtle, *Terrapene carolina carolina*, is an inhabitant of mesic forests across the eastern United States from the coastal plains of Georgia up to the southern part of Maine and west to the Mississippi River (Fig. 1). It lives on the forest floor eating slugs, insects, berries, and whatever else it may catch to satisfy its omnivorous diet (Dodd, 2001). During hot, dry weather the turtle will burrow under vegetation, but will emerge during summer rain storms (Conant & Collins, 1998). Turtles are well known to be long-lived animals (Gibbons, 1987), and eastern box turtles have a lifespan of 40 years or more (Henry, 2003). Box turtles may be preyed upon by small to medium sized carnivores such as coyotes, raccoons, skunks, and rodents, as well as predatory birds such as vultures and barn owls (Dodd, 2001). Juveniles are more vulnerable than adults to predators because of their small size and weaker shell (Herrel &

O'Reilly, 2005). The box turtle has the benefit of a strong carapace (upper shell) and a hinged plastron (lower shell), which enables it to withdraw into and tightly close its shell to form an effective barrier from an outside threat (Dodd, 2001).

Several Chelonian families have independently evolved plastrons that close at one or both ends, with the family *Emydidae* (which includes the genus *Terrapene*) having the most genera with these closable, hinged plastrons (Bramble, 1974). In order for turtles to have a closable shell, four characteristics are necessary: a plastron with a movable joint, mechanisms for displacing the limb girdles in order to pull the limbs within the shell, the musculature to close the shell, and extra space in the shell to accommodate the limbs. These features are highly developed in box turtles. In *Terrapene* the hinged plastron is movable on both the anterior and posterior portions (Bramble, 1974; Fig. 2 and 3). The hinge is located on a suture between the hyoplastron and phyoplastron bones, as it is in other turtles in the family Emydidae. The rod-shaped scapula extends dorsally in the body and, in *Terrapene*, is attached to two additional bones by synovial joints: the suprascapula, which is attached distally to the scapula, and the episcapula, which is attached distally to the suprascapula. Collectively, these two bones are called the *scapular suspensorium*, which can fold inward and act as a locking mechanism to keep the front plastron of the shell open (Fig. 4). It can provide girdle stability while the turtle is walking, and helps accommodate the girdle during closing. To close the anterior hinge the *scapular suspensorium* is unlocked and unfolded by the Muscularis (M.) testoscapularis attached to the posterior of the suprascapula. The front plastron is drawn up by the cirvico-plastral ligament, which in the genus *Terrapene* is attached directly to the M. retrahens capitis collique muscle allowing it to more directly pull on the anterior

plastron as compared to other emydine turtles. To keep the posterior plastron open in a “locked” position the ilium sits in a recess in the pleural bone, and is supported by bony processes and the *M. retrahens pelvium* muscles. The posterior plastron may be raised by the *M. testoiliacus* muscle pulling the pelvis up and forward, as the ilia slides forward over the ilial process, and the plastron is pulled up by muscles that are attached between the girdle and xiphiplastron (Bramble, 1974).

While the anatomical basis of shell closure in box turtles has been studied in detail, there is a lack of literature on the behavior of shell closure itself. For example, it is not known under what circumstances shell closure occurs as opposed to other antipredator behaviors, what level of perceived threat is necessary to elicit shell closure, how much variation in closing behavior exists among individuals, and for how long the turtle may keep the shell closed. These questions are important to study because shell closure is a behavior with both direct (e.g., muscular/metabolic) and indirect costs (e.g. opportunity cost). A turtle closed within its shell cannot forage, shift locations, or search for mates. Shell closure may also impede oxygen uptake because of either impaired inspiration or organ compression due to the method by which turtles breathe, the movement of the limb girdles (Johnson & Creighton, 2005).

Turtles may respond to a simulated predator in a variety of ways. Gatten (1975) observed that it was common for ornate box turtles, *Terrapene ornata*, to withdraw into their shells in response to electric shock. It was noted by Smith and De Carvalho (1985) that captured ornate box turtles would react to a visual stimulus by either walking away or by withdrawing into their shells. Upon being touched on the head and carapace, all turtles withdrew their head and legs into their shells. It was also noted that wild turtles

responded to capture in a variety of ways. All of the ornate box turtles urinated, some of the turtles withdrew into their shells and remained motionless, and others attempted to crawl away while trying to bite at the researchers until a more intense stimulus was administered in the form of a tap on the head or a pinch on the foot in which case they would withdraw into their shells (Smith & De Carvalho, 1985). Dodd (2001) observed similar responses in wild box turtles (*Terrapene* spp.), with turtles defending themselves by withdrawing into their shell, concealing themselves, biting, or urinating and/or defecating. Based on the literature it would appear that the known behavioral responses of threatened box turtles include urination, defecation, biting, attempting to flee, immobilization, and withdrawal into their shells. It is possible that the responses of individual turtles may vary based on personality traits, which are commonly defined as persistent differences in behavior between individuals. For example, in a study on Namibian rock agama lizards Carter *et al.* (2012) observed consistent differences in risk taking behavior (i.e., how close the lizards would let a perceived predator get to them before fleeing) between individuals. In eastern box turtles boldness may be reflected in the intensity of a stimulus required to elicit limb and head withdrawal and shell closure, as well as in the degree and duration of shell closure.

The physiological responses of box turtles to a predatory encounter may include increased plasma corticosterone levels, the major steroidal stress hormone released from the adrenal gland in reptiles (Thaker *et al.*, 2009), and increased plasma lactate levels due to enhanced use of anaerobic metabolism (Bennett & Licht, 1972). Cash *et al.* (1997) reported that plasma corticosterone increased in response to capture at both 30 and 60 minutes after initial capture and handling in wild red-eared sliders, *Pseudemys scripta*.

Elevated plasma corticosterone concentrations are thought to help the animal meet the energetic demands of activity (Cash & Holberton, 1999; Cash *et al.*, 1997). In our case, increased activity may be spurred by the simulated predator and lead to an increase in plasma corticosterone concentrations. This is supported by a study by Thaker *et al.* (2009) where elevated corticosterone levels were seen to enhance antipredator behavior in male tree lizards during a predatory encounter. Cash *et al.* (1997) also showed that corticosterone levels were not correlated with the time needed to collect a blood sample within the first 10 minutes of capture. This would suggest that quickly obtaining a blood sample from each box turtle after capture will provide us with the plasma corticosterone concentrations in response to the applied stimulus, rather than in response to capture and handling itself. As to why lactate levels would be increased, Gatten (1975) noted that increased activity in response to an electric shock produced increases in blood lactate concentrations in both *T. ornata* and *P. scripta*. Similarly, Bennett & Licht (1972) stated that lactate levels increase during periods of activity in reptiles due to increased reliance on anaerobic respiration. Also, since lung ventilation may be restricted in box turtles during shell closure (Kardong, 2015) and since shell closure requires some muscular effort (Bramble, 1974), it is expected that their lactate levels will be elevated by relatively long periods of shell closure.

The use of the hinged shell, including under what circumstances it is closed, the force and duration of shell closure, as well as other antipredator behavioral and physiological responses need to be studied in order to understand how box turtles may react to predatory threats (and human disturbance). The purpose of this study is to determine the effect of stimulus intensity on the behavior and physiology of wild eastern

box turtles. We predict that the greater the intensity of the applied stimulus, the stronger the antipredator behavioral and physiological response of the box turtles. Furthermore, we also predict that the reactions of turtles will vary between individuals, perhaps based on individual temperament. Expected physiological responses include elevated plasma corticosterone and lactate concentrations.

Methodology

Treatment and Observations

Turtles from the Nickajack Trace Wetlands in Murfreesboro, TN were observed due to the high density of box turtles that live there. Discovered box turtles were randomly divided into different stimulus intensity groups ranging from 0-3. At intensity level 0, the control or minimal disturbance group, VL backed away at least 5 meters from the turtle and recorded the turtle's behavior for 15 minutes in order to record baseline behavior. At intensity level 1, turtles were presented with a visual stimulus, a hand waved rapidly (5 back and forth motions in about 5 seconds) in front of the turtle's face at a distance of about 30 cm. At intensity level 2, VL approached the turtle and firmly poked it on the leg without picking the turtle up. At intensity level 3 one leg was pinched and the turtle was turned over for 5 seconds before being placed upright. All stimuli and observations were conducted in the wetlands.

Each stimulus intensity group contained 10 turtles and each individual in groups 1-3 was observed for 5 minutes after the stimulus was applied. In order to minimize the potential for observer interference after the stimulus was administered, the observer recorded behavior, typically with binoculars, from a distance of at least 5 meters while

remaining as motionless as possible. All individuals within the groups had their behavior ranked with a number from 1-4 (described below). Observations during stimulus application were recorded with a video camera (GoPro® Hero, San Mateo, CA, USA) to serve as an objective record of the response and thus rule out observational bias, which is possible in this study since we were not able to conduct the observations blind to the treatment of each subject.

The behavioral responses of the turtles were scored as follows: individuals who were unresponsive to the stimulus for 30 seconds or more received a score of 1. Individuals who withdrew their heads to at most barely out (where the nostrils were just beyond the perimeter of the carapace but the eyes were not) but did not withdraw their legs received a score of 2 (Fig. 5). Individuals who withdrew their head and legs at least mostly into their shell (legs withdrawn at least to barely out, where only the toes were beyond the perimeter of the shell, and head withdrawn to at least barely out) with no noticeable closing of the shell received a score of 3. Individuals who withdrew their head and legs completely within the perimeter of their shell but did not completely close and seal their shell (had at least a 5 mm gap for barely open or at least a 15 mm gap for mostly open) received a score of 3.5. Individuals who completely withdrew their head and legs and completely or almost completely (≤ 5 mm gap) closed their shells received a score of 4. Initially we had additional behavioral scores (e.g. 1.5, 5) but these were omitted because no subjects fell into those categories. For all individuals in stimulus groups 1-3, behavior was monitored for the 5-minute post-stimulus observation period. The behaviors recorded included shell closure and reopening time, to what degree the head and legs were withdrawn, movements, and other activities (e.g. urination, biting).

Blood Sampling

After the observation period, a small (<150 µl) blood sample was taken from the subcarapacial sinus in the back of the neck using a 25-gauge needle. Samples were temporarily stored in heparinized microcapillary tubes until transported that afternoon to the lab where they were centrifuged for 10 minutes at approximately 1000 g. After hematocrit was determined, plasma was divided into two 0.5 ml Eppendorf tubes (one for corticosterone determination, the other for lactate) and stored in a freezer at -80°C.

Lactate

Plasma concentrations of lactate were measured via a spectrophotometric enzyme assay using a lactate reagent set (Catalog no. L7596, Pointe Scientific, Inc., Canton, MI, USA) to estimate the extent of anaerobic metabolism. After the first 20 plasma samples were thawed and vortexed, 12 µl of plasma (or water or standards) was added to a cuvette, after which reagent 1 (peroxidase) and reagent 2 (lactate oxidase) were added, and cuvettes were again vortexed thoroughly. Samples were left at room temperature for 10 minutes, and then absorbance was read at 550 nm using a spectrophotometer (Thermo Scientific™ Spectronic Genesys 5, Model 336008). This protocol was then repeated for the remaining 20 samples. Our intra-assay coefficient of variation (CV) was 1.6% and our inter-assay CV was 5%.

Corticosterone

Plasma corticosterone levels were measured via an enzyme-linked immunosorbent assay (ELISA) test (K014-H1, Arbor Assays, Ann Arbor, MI, USA). After plasma samples were thawed, 20 µl of plasma was mixed with 20 µl of dissociation reagent and allowed to sit for 10 minutes (30 minutes max), after which 560 µl of assay

buffer was added to each sample to bring the dilution to 30 fold. The standards and samples were then treated as described in the ELISA kit insert and read on a plate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA, USA). Two ELISA kits were used in order to run all 40 samples in duplicate, as each plate could only hold 36 samples in duplicate. The intra-assay coefficient of variation (CV) was 5.6% and the inter-assay CV was 17%. While our inter-assay CV is relatively high, it should not bias our results since an equal number of turtles from each treatment group were run on each of the two plates.

Pinch Gauge

Immediately after blood collection, a pinch gauge dynamometer (Baseline® 30 lb Pinch Gauge, Catalog no. 12-0200, Fabrication Enterprises Inc. White Plains, NY) was inserted in between the plastron and carapace (between the second and third most cephalad marginal scutes). Each turtle was gently probed in an attempt to elicit its maximum closing ability. We tested for an effect of stimulus intensity group, sex, age, body size, body condition (see below), and behavioral response scores on closing force or “pinch strength”.

Other Measurements

All captured turtles were marked on the edge of their shell with a triangular file for identification purposes, and had their sex, weight, carapace length (CL), width (CW), and height (SH) recorded. Sex was determined via the morphology of the plastron as well as eye color. Weight was measured using a Pesola spring scale (± 5 g). Carapace length, CW, and SH were measured using 200 mm digital calipers. The volumetric body condition index (vBCI) was calculated using the formula $vBCI = \text{Weight} / (\pi * (CL) * (SH) * ((CW)/6000))$ as described in Ashton, *et al.* (2015). Because ranavirus has been

detected in box turtles from our field site (Vannatta et al., 2016), we were careful to handle each turtle with new gloves and to disinfect our equipment between turtles (Bryan et al., 2009).

Statistical Tests

The effect of stimulus intensity group on behavioral scores, plasma corticosterone levels, plasma lactate levels, and pinch strength was tested using a one-way Kruskal-Wallis ANOVA on ranks. The Student-Newmann-Keuls Method, Tukey Test, and Dunn's Method were used for post-hoc pairwise comparisons. Independent samples t-tests were used to test for sex differences in body size, body condition, pinch strength, corticosterone, lactate, and hematocrit. Because box turtles are known to be sexually dimorphic (Vannatta et al., 2016), a two-way analysis of variance was used to test for effects of sex and treatment on vBCI. Finally, the Spearman rank correlation test was used to test for associations between the dependent variables. SigmaStat program (Version 3.1, Systat Software, San Jose, CA) was used to calculate statistics.

Results

There was not a significant difference between the sexes in behavioral scores ($T=281$, $P=0.88$), plasma corticosterone levels ($T=268.5$, $P=0.61$), or plasma lactate levels ($T=282.5$, $P=0.91$), and so the results for both sexes were pooled for subsequent analyses. Similarly, treatment groups did not differ in body size (CL: $F_{3,40}=0.18$, $P=0.91$; Mass: $H_{3,40}=1.68$, $P=0.64$), vBCI ($F_{3,40}=0.05$, $P=0.98$), or age ($F_{3,40}=1.21$, $P=0.32$; Table 1). Also behavioral scores did not vary with turtle size (CL: $r=0.04$, $n=40$,

$P = 0.79$; Mass: $r = 0.11$, $n = 40$, $P = 0.5$), vBCI ($r = 0.02$, $n = 40$, $P = 0.90$), or age ($r = -0.04$, $n = 40$, $P = 0.81$).

There was a significant effect of stimulus intensity group on behavioral scores ($H_{3,40} = 33.51$, $P < 0.001$; Fig. 6), and a significant difference between all pairwise comparisons (Treatment group 3 vs 0: $q = 7.60$, $P < 0.05$; 3 vs 1: $q = 5.93$, $P < 0.05$; 3 vs 2: $q = 4.17$, $P < 0.05$; 2 vs 0: $q = 7.29$, $P < 0.05$; 2 vs 1: $q = 4.65$, $P < 0.05$; and 1 vs 0: $q = 6.20$, $P < 0.05$). All 10 of the control (undisturbed) box turtles were unresponsive to our presence (behavioral score = 1), whereas the majority of turtles exposed to the visual stimulus at least partially withdrew their heads but not their limbs (score = 2). Most of the turtles that were poked fully withdrew and had some shell closing (score = 3.5), and most of the turtles that were pinched and flipped completely withdrew and closed their shell (score = 4; Fig. 6).

In all but a single turtle, turtles were seen to have had their anterior plastron fully open before the stimulus was applied (the exception was a turtle in group 3 who was found slightly open; Table 2). Stimulus intensity was shown to influence how open the anterior plastron was immediately after the stimulus was applied ($H_{3,40} = 27.31$, $P < 0.001$; Table 3), and also had an effect on head position immediately after stimulus application ($H_{3,40} = 30.09$, $P < 0.001$; Table 4). The pairwise comparisons indicate significant differences for how open the anterior plastron was between treatment group 0 vs 3 ($q = 6.1$, $P < 0.05$) and 1 vs 3 ($q = 5.3$, $P < 0.05$). For head position, the pairwise comparisons indicate significant differences between treatment group 0 vs 3 ($q = 6.90$, $P < 0.05$), 0 vs 2 ($q = 5.28$, $P < 0.05$), and 0 vs 1 ($q = 4.06$, $P < 0.05$).

Stimulus intensity group had a significant effect on the time it took after the stimulus was applied for the turtle's head to reach at least the mostly out state ($H_{3,39} = 22.67, P < 0.001$; Figure 7A). The pairwise comparisons indicate significant differences between treatment group 3 vs 0 ($q = 4.01, P < 0.05$), 2 vs 0 ($q = 3.70, P < 0.05$), and 1 vs 0 ($q = 3.66, P < 0.05$). There was also a significant effect of stimulus intensity group on time for the anterior plastron to reach the mostly open state ($H_{3,39} = 19.50, P < 0.001$; Figure 7B). Pairwise comparisons showed a significant difference between treatment group 3 vs 0 ($q = 3.65, P < 0.05$) and 3 vs 1 ($q = 2.97, P < 0.05$). Only two turtles attempted to flee during the 5-minute post-stimulus observation period, both were in treatment group 3.

While stimulus intensity group had a pronounced effect on behavior, there was not a significant effect on plasma corticosterone levels ($H_{3,40} = 3.8, P = 0.28$; Fig. 8A) or plasma lactate levels ($H_{3,40} = 3.62, P = 0.31$; Fig. 8B).

Regardless of stimulus intensity group, there were no noticeable differences in behavior once the turtles had been captured and handled (Table 5). During capture and handling, out of 40 turtles, 8 urinated, 1 defecated, 7 attempted to bite, and 5 air walked. Surprisingly, only 14 of the 40 turtles had their posterior plastron closed for the full duration of capture, while 28 had their anterior plastrons fully closed. After release 29 turtles remained immobilized during the entire 10 minute observation period and 11 turtles fled.

Pinch strength was positively correlated with body size (Mass: $r = 0.44, n = 36, P = 0.01$; CL: $r = 0.34, n = 36, P = 0.04$), vBCI ($r = 0.33, n = 36, P = 0.05$), and age ($r = 0.52, n = 36, P = 0.001$; Fig. 9). There was not an effect of sex ($T = 217.50, P = 0.46$) or

stimulus intensity group on pinch strength ($F_{3,40} = 1.29$, $P = 0.30$), but there was almost a correlation between pinch strength and behavioral score ($r = 0.32$, $n = 36$, $P = 0.06$).

There was a positive correlation between corticosterone and pinch strength ($r = 0.41$, $n = 36$, $P = 0.01$). Finally, lactate concentrations were not correlated with pinch strength ($r = -0.27$, $n = 36$, $P = 0.12$).

Discussion

The results indicate that a more intense simulated predator stimulus, e.g., involving greater contact between the box turtle and the simulated predator, produces a more intense behavioral response, likely due to a higher perceived threat. This is shown by the effect of stimulus group on behavioral rank scores and the latency to open the shell and extend the head (Figs. 6, 7). Once turtles had been captured and handled, there was no longer obvious behavioral differences between the stimulus groups. This is most likely because all turtles were exposed to the same post-stimulus capture and handling protocol, and the stress of capture and handling greatly exceeded that of even our most intense applied stimulus (pinch flip). There was noticeable individual variation between turtles, most evidently seen in turtles that were poked (stimulus treatment = 2; Table 3) and during the capture and handling procedures, in behaviors such as urination, defecation, biting, opening their shell, and air walking. It is unclear why many of the turtles did not close their posterior plastron during capture and handling. This finding may be related to the observation that turtles that fully closed their anterior plastron would often slightly open their posterior plastron, and when the posterior plastron was pushed to fully closed the anterior plastron would slightly open. One possibility is that these individuals did not

have enough space within their shells to accommodate the full withdrawal of both sets of limbs. Alternatively, failure to close the posterior plastron may have been observed in turtles with a high rate of breathing, perhaps elicited by capture and handling, since movement of the limb girdles is known to help turtles respire (Kardong, 2015).

The lack of effect of stimulus intensity on plasma corticosterone concentrations suggests either that box turtles did not perceive even our most intense predator stimulus (pinch flip) as that of a real predator, or they have a slow, weak corticosterone response to predator encounters. Weak corticosterone responses have been seen in male fence lizards, *Sceloporus undulatus*, but only during the breeding season (Klukowski, 2011), and so it is possible that if the corticosterone responses of the turtles were measured in a different season there would be a more pronounced response. As mentioned earlier, Cash *et al.* (1997) note that corticosterone levels are not affected significantly by capture and handling if the blood sample is taken in 10 minutes or less. None of the collected turtles were bled past 10 minutes after capture, suggesting that the results obtained were indeed from the stimulus alone. It is possible that our stimulus was too short in duration for plasma corticosterone to appreciably increase. Cash *et al.* (1997) also mention that the greatest increase in corticosterone in response to capture and handling occurred within the first 30 minutes, and for vertebrates it is generally seen that the release of corticosterone in response to acute stress begins with low initial concentrations and increasing over time before declining. Our observation period amounted to 5 minutes per individual, capture was almost immediate, and blood sampling time (time between capture and successful collection of a blood sample) ranged from about 2 minutes to 7 minutes. Thus, the total time we potentially disturbed each subject was perhaps too short

(7-12 minutes) to elicit an increase in plasma corticosterone. Khan *et al.* (2007) reported that glucocorticoid levels were unaffected by short-term captivity and handling and mildly invasive procedures like blood sampling in gopher tortoises, *Gopherus Polyphemus*. Similarly, Hunt *et al.* (2015) observed that during transportation of juvenile Kemp's ridley sea turtles, *Lepidochelys kempii*, the turtles only showed a significant increase in corticosterone during the longer 26-hour transport duration, not the shorter 13-hour duration. This all suggests that a longer stimulus application time and perhaps more extreme stimuli may have produced increased corticosterone levels. A possible example is if we had sampled the turtle's blood after handling, for it was during the capture and handling duration that we saw more extreme behavioral responses, such as urination and biting.

The lack of effect of stimulus intensity on plasma lactate concentrations may be due to, as noted by Smith & De Carvalho (1985), an increase in parasympathetic activity in turtles who immobilize or hide under cover (which for box turtles may include their shell), leading to a decrease in metabolism and ventilation. While this would potentially mean a decreased oxygen intake from the reduced ventilation, the decreased energy demands could explain the lack of buildup of lactate.

The results seem to indicate that there is a correlation between the size of a box turtle and its pinch strength. However, due to the relatively large size of the pinch gauge (insertion width 16.8 mm) it is likely that the closing muscles of older, larger turtles were more likely to be operating in the optimal initial muscle length range as compared to smaller turtles whose closing muscles were likely overstretched. This is due to the fact that the less stretched out a muscle is, the more cross bridges that may form between the

actin and myosin in the muscles, producing more tension. This means the large turtles were able to form more cross bridges and thus create more muscle tension (Eckert *et al.*, 1997). Further study with a smaller, custom built pinch gauge would give more accurate results.

Eastern box turtles exhibited stronger and more prolonged behavioral responses the more intense the predatory stimulus, with box turtles taking advantage of their fully closable shell at the most intense stimulus groups. The box turtles did not exhibit a significantly strong corticosterone or lactate response, which may be due to seasonal effects, too short or weak of a stimulus, or increased parasympathetic activity. Upon capture almost all turtles withdrew into their shells and closed their hinges, with a few engaging in other behaviors such as biting, urinating, and air walking. Although there appears to be a correlation between box turtle size and pinch strength, the relatively large size of the pinch gauge means that older, larger turtles would have an unfair muscle length-tension advantage. Future studies will examine if longer and/or more intense stimuli have an effect on box turtle antipredator behavior, plasma corticosterone, and plasma lactate concentrations, or if there is seasonal variation in any of these variables.

References

- Ashton, D. T., Bettaso, J. B., & Welsh, H. H., Jr. (2015). Changes across a decade in size, growth, and body condition of western pond turtle (*Actinemys marmorata*) populations on free-flowing and regulated forks of the trinity river in northwest California. *Copeia*, 103(3), 621-633.
- Bennett, A. F. & Licht, P. (1972). Anaerobic metabolism during activity in lizards. *Journal of Comparative Physiology*, 81(3), 277-288.
- Bramble D. M. (1974). Emydid shell kinesis: biomechanics and evolution. *Copeia*, 1974(3), 707-727.
- Bryan, L.K., Baldwin, C.A., Gray, M.J., & Miller, D.L. (2009). Efficacy of select disinfectants at inactivating *Ranavirus*. *Diseases of Aquatic Organisms*, 84, 89-94.
- Burghardt, G. M. & Greene, H.W. (1988). Predator simulation and duration of death feigning in neonate hognose snakes. *Animal Behavior*, 36(6), 1842-1844.
- Carter, A., Goldizen A., & Heinsohn, R. (2012). Personality and plasticity: temporal behavioral reaction norms in a lizard, the Namibian rock agama. *Animal Behavior*, 84, 471-477.
- Cash, W. B. & Holberton, R. L. (1999). Effects of exogenous corticosterone on locomoter activity in the red-eared slider turtle, *Trachemys scripta elegans*. *Journal of Experimental Zoology*, 284, 637-644.
- Cash, W. B. & Holberton, R. L., & Knight, S.S. (1997). Corticosterone secretion in response to capture and handling in free-living red-eared slider turtles. *General and Comparative Endocrinology*, 108, 427-433.

- Conant, R., & Collins, J. T. (1998). *Peterson Field Guides: Reptiles and Amphibians, Eastern / Central North America* (3rd ed., pp. 158–166). New York, NY: Houghton Mifflin Company.
- Dodd Jr., C. K. (2001). *North American Box Turtles: a Natural History* (pp. 39-183). Norman, OK: University of Oklahoma Press.
- Eckert, R., Randall, D., Burggren, W., & French, K. (1997). *Eckert Animal Physiology: Mechanisms and Adaptations* (4th ed., pp. 357). New York: W.H. Freeman and Company.
- Emlen, D. J. (2014). *Animal Weapons: the Evolution of Battle* (pp.13-23). New York, NY: Henry Holt and Company, LLC.
- Gatten, R. E., Jr. (1975). Effects of activity on blood oxygen saturation, lactate, and pH in the turtles *Pseudemys scripta* and *Terrapene ornata*. *Physiological Zoology*, 48(1), 24-35.
- Gibbons, J. W. (1987). Why do turtles live so long? *Bioscience*, 37(4), 262-269.
- Henry, P. F. P. (2003). The eastern box turtle at the Patuxent Wildlife Research Center 1940s to the present: another view. *Experimental Gerontology*, 38(7), 773-776.
- Herrel, A. & O'Reilly, J.C. (2005). Ontogenetic scaling of biting force in lizards and turtles. *Physiological and Biomechanical Zoology*, 79(1), 31-42.
- Hunt, K. E., Innis, C. J., Kennedy, A. E., McNally, K.L., Davis, D.G., Burgess, E.A., & Merigo, C. (2015). Assessment of ground transportation stress in juvenile Kemp's ridley sea turtles (*Lepidochelys kempii*). *Conservation Physiology*, 4(1), 1-13.

- Johnson, S.M., & Creighton, R.J. (2005). Spinal cord injury-induced changes in breathing are not due to supraspinal plasticity in turtles (*Pseudemys scripta*). *American Journal of Physiology*, 289, 1550 – 1559.
- Kahn, P. F., Guyer, C., & Mendonca, M.T. (2007). Handling, blood sampling, and temporary captivity do not affect plasma corticosterone or movement patterns of Gopher Tortoises (*Gopherus polyphemus*). *Copeia*, 2007(3), 614-621.
- Kardong, K. V. (2015). *Vertebrates Comparative Anatomy, Function, Evolution* (7th ed., pp. 437-437). New York, NY: McGraw-Hill Education.
- Klukowski, M. (2011). Effects of breeding season, testosterone, and ACTH on the corticosterone response of free-ranging male fence lizards (*Sceloporus undulatus*). *General and Comparative Endocrinology*, 173 (2), 295-302.
- Smith, E. N. & De Carvalho, M. C. (1985). Heart rate response to threat and diving in the ornate box turtle, *Terrapene ornata*. *Physiological Zoology*, 58(2), 236–241.
- Thaker, M., Lima, S. L., & Hews, D. K. (2009). Acute corticosterone elevation enhances antipredator behaviors in male tree lizard morphs. *Hormones and Behavior*, 56(1), 51-57.
- Vannata, J. M. (2016). Demographic characteristics, incidence of ranavirus infection, and seasonal corticosterone levels in the eastern box turtle, *Terrapene carolina carolina*, in a suburban wetlands habitat of middle Tennessee (Master's thesis). Retrieved from James E. Walker Library Digital Repository. (Accession No. 4543)

Tables

Stimulus Treatment		n	Female	Male	CL (mm)	Mass (g)	vBCI	Age
0	Control	10	6	4	125.0 ± 13.3	376.9 ± 131.9	0.926 ± 0.1	15.5 ± 3.6
1	Visual	10	6	4	125.9 ± 14.8	405.5 ± 124.8	0.943 ± 0.1	17.6 ± 3.3
2	Poke	10	8	2	127.4 ± 10.1	410.9 ± 67.3	0.961 ± 0.1	16.9 ± 2.0
3	Pinch Flip	10	6	4	128.5 ± 6.6	406.6 ± 49.4	0.947 ± 0.1	15.9 ± 1.5

Table 1. Descriptive statistics for the different stimulus treatment groups of eastern box turtles. The average ± SD is shown for carapace length (CL), body mass, volumetric body condition (vBCI), and estimated age. The four groups did not differ significantly in any of these variables (see text for details).

Characteristic	n	Behaviors Observed		
Anterior Plastron	40	39 Fully Open	1 Slightly Open	
Posterior Plastron	37	35 Full Open	2 Slightly Open	
Posture	39	28 Lying Down	8 Standing	2 Standing Tall
Movement	40	37 Immobile	2 Brief Walk	2 Swimming*

Table 2. Behaviors observed in eastern box turtles before the stimulus was applied. *One turtle was observed walking to the creek and then swimming. Also, the posterior plastron was not always clearly visible, and turtles which were found swimming were not considered to have a posture, thus n may be less than 40.

Treatment		n	Fully Open	Mostly Open	Slightly Open	Fully Closed
0	Control	10	10	0	0	0
1	Visual	10	8	1	1	0
2	Poke	10	3	2	3	2
3	Pinch Flip	10	0	1	1	8

Table 3. Anterior plastron response of eastern box turtles immediately after stimulus application. See text for a description of the different plastron positions.

Treatment		n	Fully In	Barely Out	Mostly Out	Out Normal	Extended Far
0	Control	10	0	0	1	8	1
1	Visual	10	3	7	0	0	0
2	Poke	10	6	4	0	0	0
3	Pinch Flip	10	10	0	0	0	0

Table 4. Head response of eastern box turtles immediately after stimulus application. See figure 5 for depiction of different head positions.

Treatment	n	Urinated	Defecated	Attempted to Bite	Air Walked	Ant. Plastron Fully Closed	Post. Plastron Fully Closed
0 Control	10	3	0	2	1	8	3
1 Visual	10	0	0	0	2	6	3
2 Poke	10	2	0	2	1	8	7
3 Pinch Flip	10	3	1	3	1	6	1
Sum	40	8	1	7	5	28	14

Table 5. Behaviors observed during capture and handling in eastern box turtles after stimulus application. Air walked is defined as the turtle moving its legs in the air while being held in a fashion similar to as if it was walking or swimming. Only turtles which were fully closed for the full duration of capture and handling are included under plastron positions.

Figures

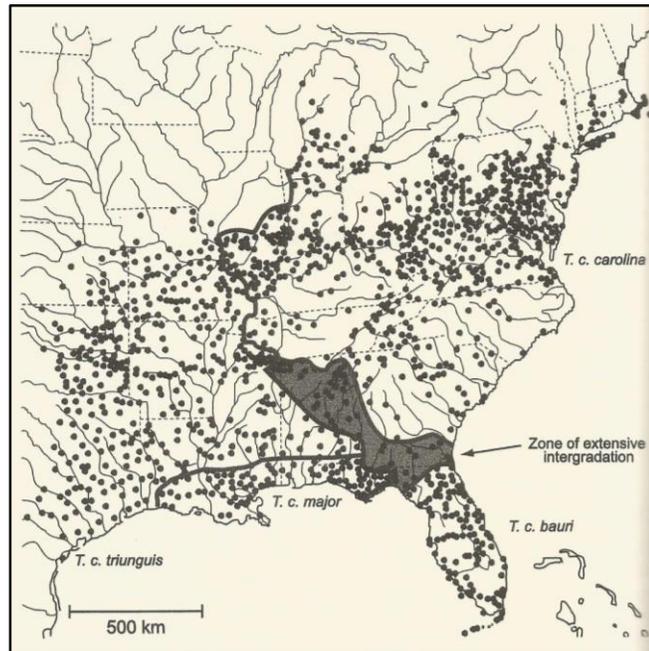


Figure 1. Range of the eastern box turtle, *Terrapene carolina*. Map from Dodd (2001).



Figure 2. The hinged plastron of the box turtle allows it to withdraw its limbs and close its shell. Photo by Victoria Lay.

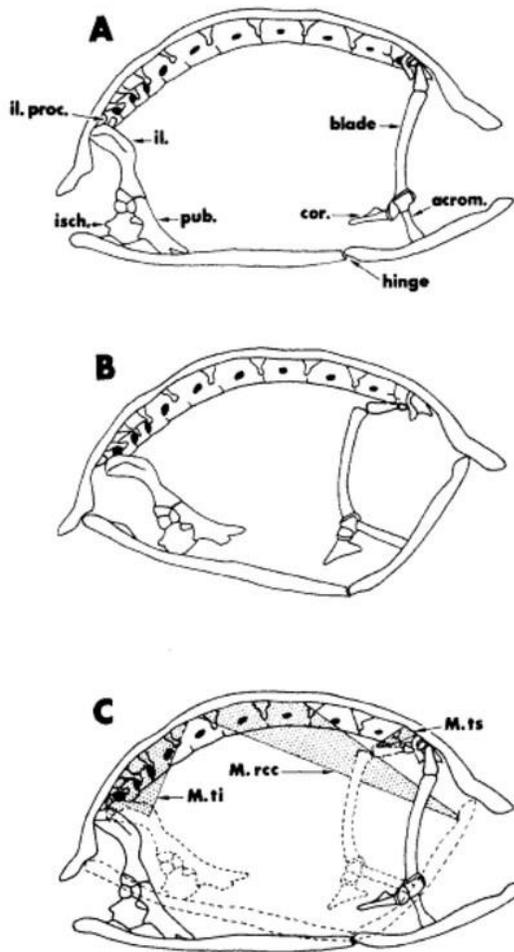


Figure 3. A diagram from Bramble (1974) illustrating how the shell closes in *T. c. carolina*. The shell is shown open in A, closed in B, and an open shell with an overlay of a closed shell (dotted lines) is seen in C. Shell closure is possible via a hinged plastron and specialized musculo-skeletal anatomy of the pectoral and pelvic girdle joints. See introduction for description of system. Abbreviations: il. Proc., iliac process; il, ilium; isch, ischium; pub, pubis; blade, scapular blade; cor, coracoid; acrom, acromian process; M. ti, testoiliacus muscle; M. rec, retrahans capitis collique muscle; M. ts, testoscapularis muscle.

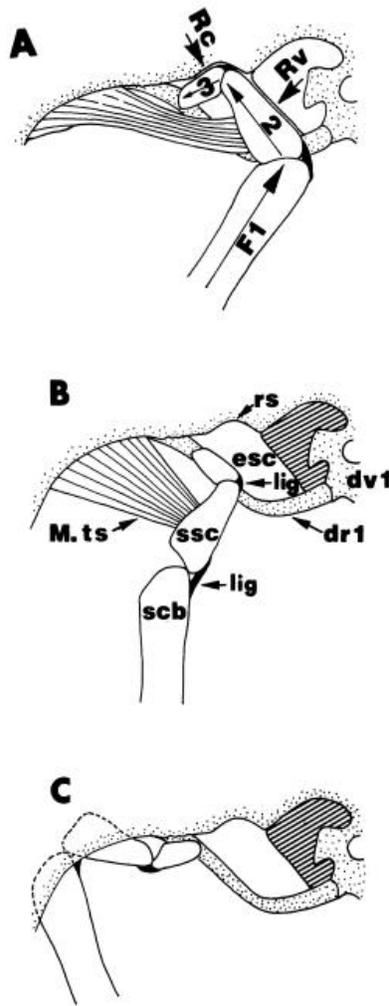


Figure 4. A diagram from Bramble (1974) illustrating the *scapular suspensorium* locking mechanism. “A” displays the *suspensorium* in the “locked” position, which is when the shell is open. “B” shows the *suspensorium* beginning to fold outward as the shell starts to close. “C” shows the *suspensorium* completely unfolded as the shell is fully closed. Abbreviations: dr 1, dorsal rib 1; dv 1, dorsal vertebra 1; lig, ligament; M. ts, testoscapularis muscle; rs, scapular recess; esc, episcapula; ssc, suprascapular; scb, scapular blade.

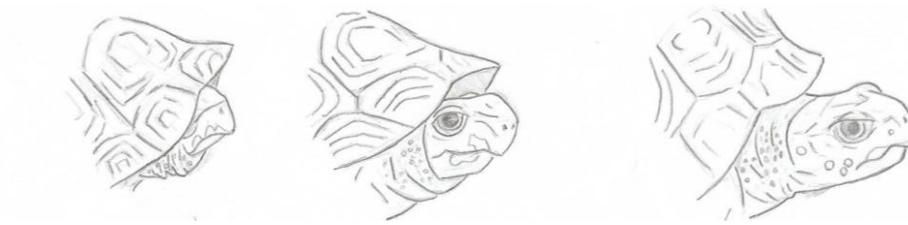


Figure 5. A series of illustrations depicting the head positions seen in box turtles besides completely closed and head extended far. The order goes (from left to right) barely out, mostly out, and out normal. Illustrations by V. Lay.

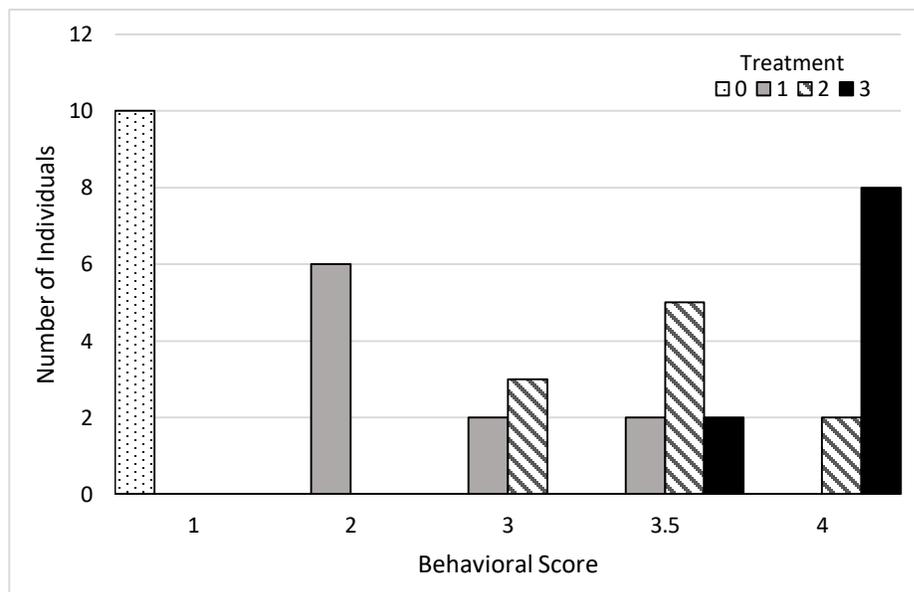


Figure 6. A histogram displaying the number of eastern box turtles per stimulus intensity group that fell into each behavioral score. Each group contained 10 turtles. Control turtles were observed from a distance (Stimulus treatment = 0), other turtles were presented with a visual stimulus but not touched (Treatment = 1), were poked (Treatment = 2), or pinched on the leg and flipped over for 5 sec (Treatment = 3). There was a significant difference between all pairwise comparisons ($H_{3,40} = 33.51, P < 0.001$). See methods for descriptions of behavioral scores.

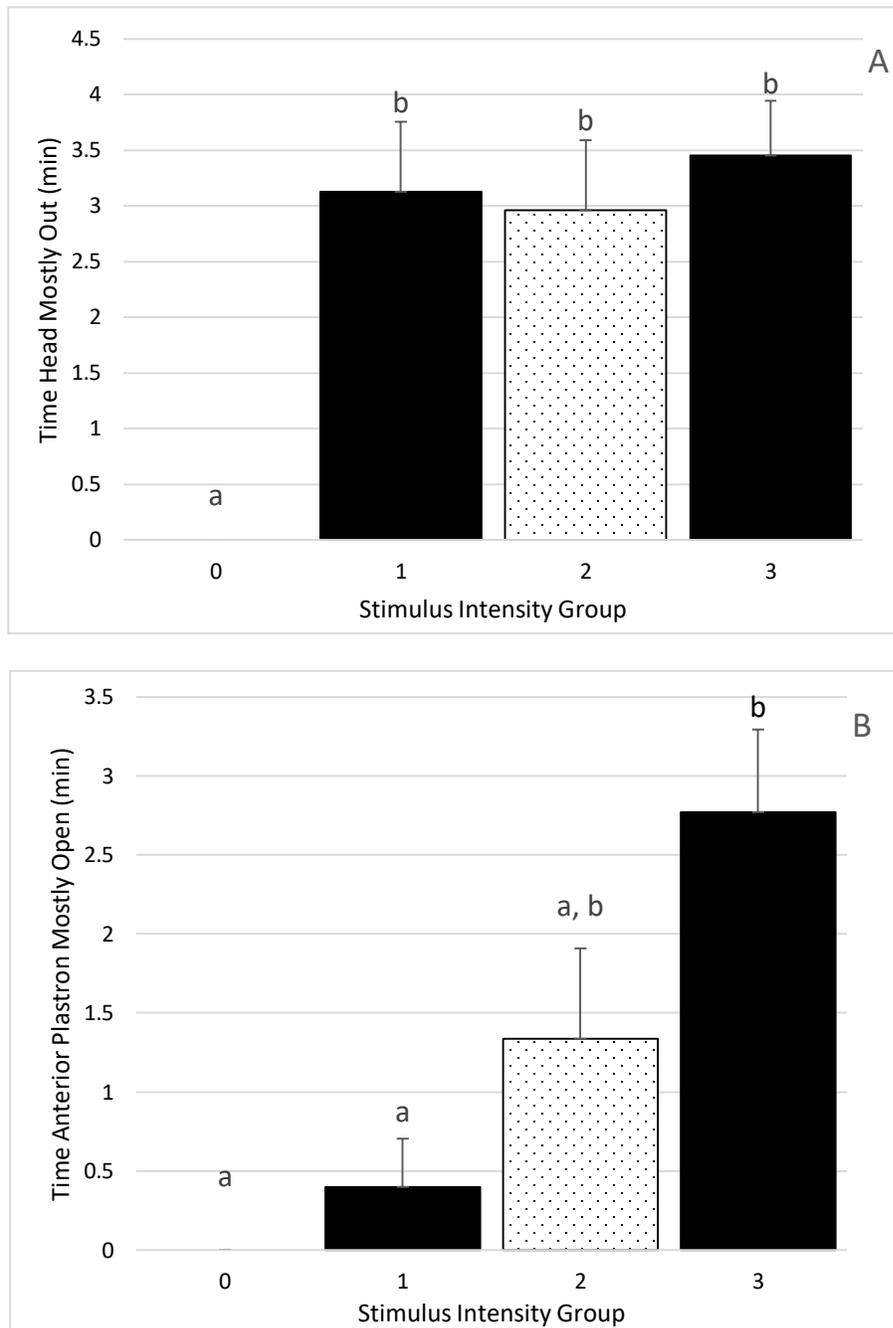


Figure 7. Average (\pm SE) time for the head to reach mostly out ($H_{3,39} = 22.67$, $P < 0.001$; A), and time for the anterior plastron to be at least mostly open in eastern box turtles after the stimulus was applied, ($H_{3,39} = 19.50$, $P < 0.001$; B). Different letters represent statistical differences between groups indicated by post hoc tests.

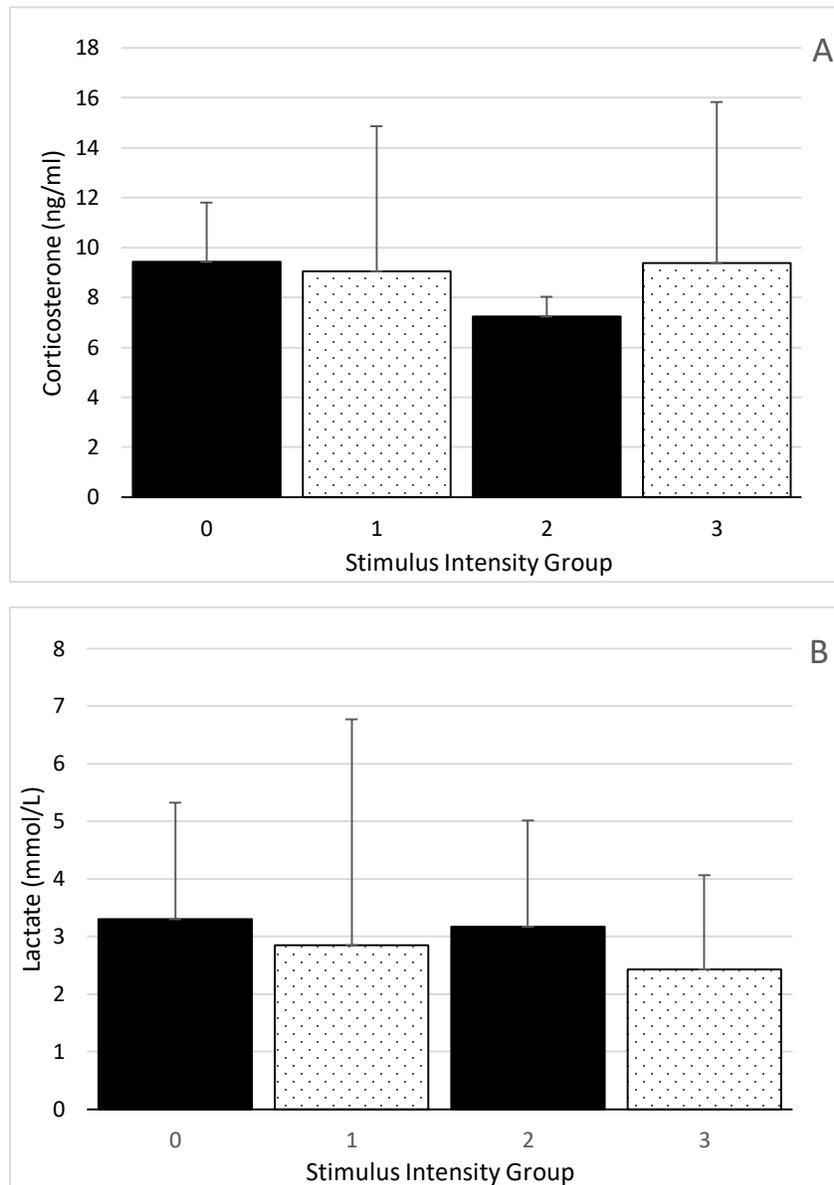


Figure 8. Average (\pm SD) plasma corticosterone ($H_{3,40} = 3.8$, $P = 0.28$; A) and lactate ($H_{3,40} = 3.62$, $P = 0.31$; B) concentrations in eastern box turtles belonging to each stimulus intensity group. Each group contained 10 turtles. Blood was sampled after the approximate 30 second stimulus application and the 5 minute post stimulus period (in control group, after 15 minute observation from a distance). There were no significant differences between the groups.

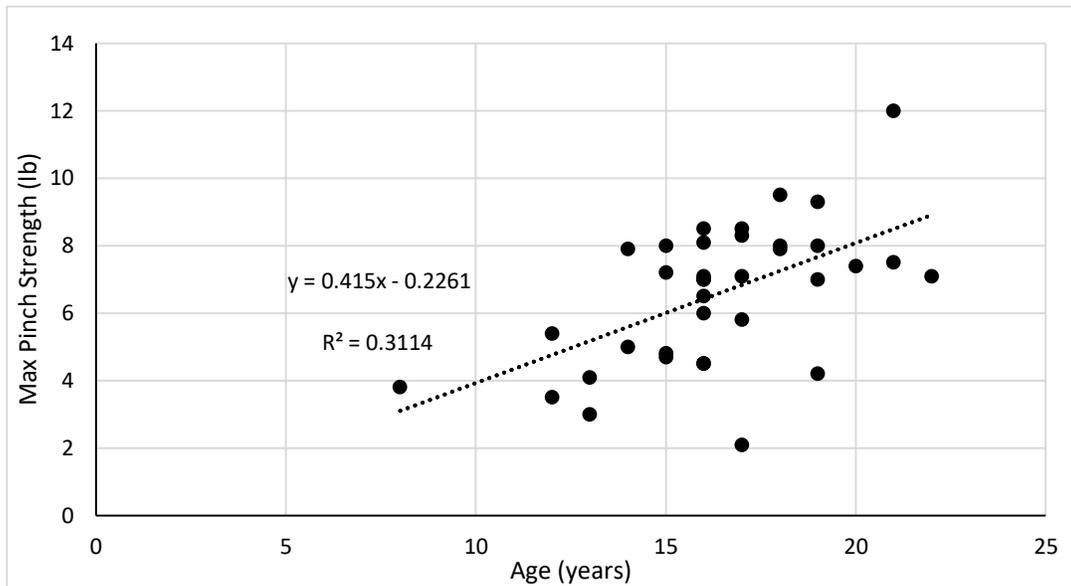


Figure 9. A scatter plot showing the association between maximum pinch strength of an eastern box turtle and its age, ($r = 0.52$, $n = 36$, $P = 0.001$).

