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Comparing interrelated stress measures to differentiate acute and chronic stress in urban freshwater turtles

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Comparing interrelated stress measures to differentiate acute and chronic stress in urban freshwater turtles

Katie Caldwell

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Abstract

As urban areas grow in size and number, there are increasing impacts on the wildlife. While some species adapt, others cannot thrive in these conditions. Stress is the biological response to a stimulus that threatens homeostasis, which can be used to understand the condition of an individual. While acute stress is beneficial for survival, chronic stress can lead to deleterious impacts. A majority of turtles can be classified as declining in number or endangered, yet there is little understanding of their physiological stress response. This study examined how various stress measures—including corticosterone (CORT) in plasma, nails and leeches, metabolic measures, H:L ratio, and parasite prevalence—are related. We sampled freshwater turtles from two locations in the city of Winter Park, Florida, over a period of seven months. While there were no correlations between CORT measures, we were able to find various associations between metabolism, size, and immune measure. Additionally, we have identified further research needed to have a more complete understanding of stress in freshwater turtles.

Introduction

Urbanization has been increasing dramatically on a global scale. The United Nations (UN) stated in 2020, approximately 56% of the world's populations live in an urban area compared to 1950, when less than 30% of the population did (United Nations: Department of Economic and Social Affairs, 2019). As urban centers grow in number and size, wildlife is potentially at risk. Some of the known impacts of urbanization include habitat loss and fragmentation, introduction of non-native species, increased air temperature, altered atmospheric chemistry, altered hydrology in watersheds, and altered environmental conditions (Goddard et al., 2010). As urban landscapes encroach on native environments, it becomes increasingly necessary to understand the impacts on wildlife. The environmental pressures in urban landscapes differ considerably from the pressures that species' have faced throughout their evolutionary history. While some species may be able to persist and thrive in urban areas, others will either adapt, face deleterious physiological impacts, or immigrate out of cities.

Orange County, Florida, is an appropriate example of a growing urban area. In 1980, the population in the county was only 470,865 people (Florida Legislature, 2020). Today, the population is predicted to be more than 1,400,000 (Florida Legislature, 2020), with more than 89% of Florida residents living in urban areas (Smith, 2005). To accommodate this influx in population, additional infrastructure has continuously been built. Unfortunately, this has increased the land use for urban areas, that ultimately encroaches on native Florida habitats (Sim & Mesev, 2011). Bodies of water are often conserved for both practical, recreational and scenic purposes; however, the presence of urban development on the shoreline periphery alters the aquatic ecosystem.

Freshwater turtles have demonstrated a variety of responses to urbanization. Some species are remarkably persistent, surviving in unexpected water bodies such as golf courses ponds and ditches **(**Conner et al., 2005; Mitchell, 1988; Elston et al., 2016); however, the majority of turtles are now either declining in number, endangered or already extinct (Lovich et al., 2018). The novel challenges of urban environments could be a potential source of stress, and therefore, a variety of physiological consequences. There currently is little research on such impacts on turtles in urban environments, but first an accurate understanding of the stress response in this group is necessary.

Stress Response

Stress is defined as the biological response of an individual to stimuli (stressor) that threatens homeostasis (Martinez-Silvestre, 2014). The purpose of this response is to increase the chances of survival during potentially life-threatening events. However, this has trade-offs, because resources allocated to maintenance, growth, and reproduction—which accounts for an individual's fitness—are instead directed towards short-term survival (Angelier & Wingfield, 2012). Species have evolved to optimize fitness by finding a balance between allocating resources to the stress response and to general maintenance and reproduction; therefore, the level of response to a particular stressor varies by species, based on differences in their life history (Angelier & Wingfield, 2012). As environments are changing rapidly due to urbanization, species are more likely now to face stressors in ways that differ from those encountered during their evolutionary history (Angelier & Wingfield, 2012).

The stress response is a physiological response that integrates the endocrine, nervous, and immune systems to shift priority to immediate survival. In vertebrates, the response is initiated by the hypothalamo-pituitary-adrenal (HPA) axis as illustrated in Figure 1 (Smith and Vale, 2006). During stress, hypophysiotropic neurons found in the paraventricular nucleus (PVN) of the hypothalamus secretes corticotrophin releasing factor (CRF), which is released to the anterior pituitary gland (Smith and Vale, 2006). This signals for the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Smith and Vale, 2006). Following this is the release of glucocorticoids (GCs) from the adrenal cortex. In reptiles, corticosterone (CORT) is the predominant GC, although cortisol may also be present (Smith and Vale, 2006). GCs regulate physiological responses throughout the body by ubiquitously distributed intracellular receptors (Smith and Vale, 2006).

The mechanisms of stress response are pleiotropic with one stressor leading to multiple physiological changes (Angelier & Wingfield, 2012). The responses increase likelihood of survival, while suppressing others that distract from survival (Angelier & Wingfield, 2012). The short-term effects of CORT include suppressing reproductive behavior, inhibiting territoriality, increasing gluconeogenesis, promoting foraging and escape behaviors, and raising metabolic rates, which increases oxidative stress (Wingfield et al., 1998; Pickering et al., 2012). If elevated CORT levels are maintained for extended periods of time, the outcomes are deleterious. When the stress is chronic, the reproductive system is inhibited, the immune system is repressed, neuronal cell death increases, protein loss due to oxidative stress and growth is suppressed (Sapolsky et al., 2000; Pickering et al., 2012). These differences between acute and chronic stress are outlined in Table 1. CORT is also regulated by negative feedback mechanisms that represses the stress response long-term; however, these mechanisms are imperfect, and chronic stress occurs due to its failure (Gjerstad et al., 2018; Smith & Vale, 2006).

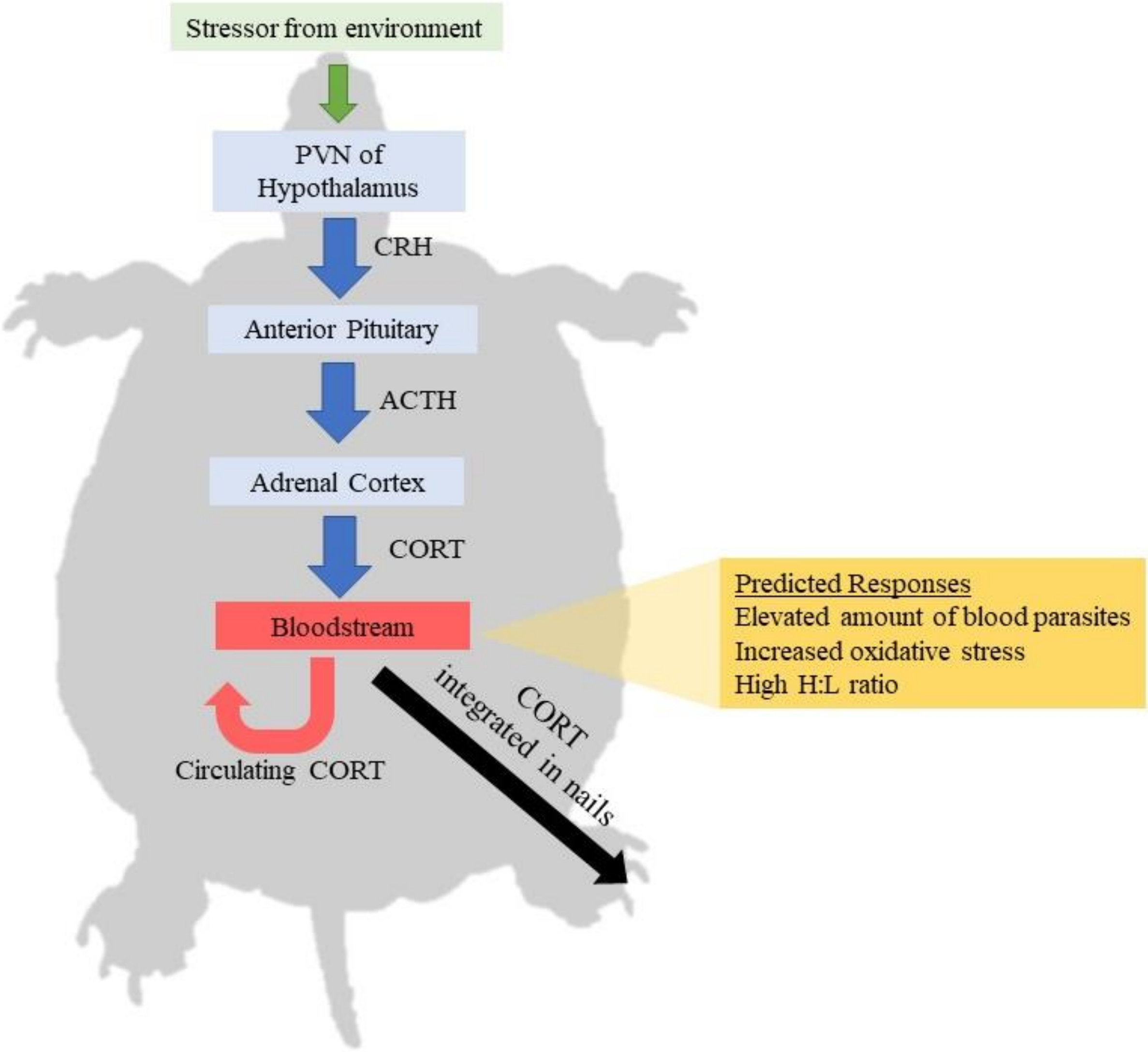

Figure 1. HPA axis regulates the physiological stress response in vertebrates. The HPA axis is represented by the blue pathway. The paraventricular nucleus (PVN) of the hypothalamus secretes corticotrophin releasing factor (CRH), which is released to the anterior pituitary gland. This signals for the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. This initiates the release of corticosterone (CORT). Some CORT will circulate in the bloodstream (shown in red) while some will be integrated into keratinized tissue (shown in black). The predicted outcome for animals facing chronic stress—indicated by high concentrations of nail CORT—are noted in yellow.

Table 1. Effects of the stress response in the short term (acute) and long term (chronic). Adapted from Wingfield et al. 1997 and Wingfield et al. 1998.

Acute Stress	**Chronic Stress**
Suppress reproductive behavior	Inhibit reproductive system
Suppress territorial behavior	Suppress immune system
Increase glucogenesis	Promote severe protein loss due to oxidative stress
Increase foraging behavior	Disrupt second messenger systems
Increase metabolic rate	Neuronal cell death
Promote recovery on return to normal life history stage	Suppress growth

Although it is important to note the physiological responses of increased CORT in times of stress, the concentration of the hormone is not entirely dependent on the intensity of the stressor. For example, while it has been shown that short-term increase in CORT does suppress reproductive behaviors in reptiles, yet the levels of plasma CORT increases during the reproductive season in some reptiles (Guillette et al., 1995; Moore & Jessop, 2003). This increase in the hormone has no impact on reproductive behavior in this scenario; instead, it is hypothesized to increase energy stores due to the high energetic requirements of reproduction (Moore & Jessop, 2003). Similarly, levels of plasma CORT have shown to vary based on body size, sex, body condition and age (Moore & Jessop, 2003; Dunlap & Wingfield, 1995).

Stress and Urbanization

Animals living in urban environments are facing new pressures that may impact their life histories. These negative impacts can include: habitat loss and fragmentation, introduction of non-native species, increased air temperature, changes in atmospheric chemistry, changes in hydrology in watersheds, and changes in environmental conditions (Goddard et al., 2010). These conditions may lead to chronic stress and therefore, its deleterious outcomes in some species; however, other species may take advantage of the benefits of urban living such as access to food and water (Anderies et al., 2007) and access to shade (Braman et al., 2000). Because certain species can utilize these resources, not all animals suffer from urbanization and instead, thrive. The research supports that stress response in urban animals is highly variable likely due to how different animals adapt to these major changes.

Avian models, along with mammals, have been frequently used to investigate the impacts of urbanization on stress response. This research has shown stress response is specific to each species. A study in European Blackbirds (*Turdus merula*) showed the hatchlings born in urban environments had a lower stress response than the hatchlings that were born in a nearby forest—likely to cope in the higher stress urban environment (Partecke et al., 2006). Another study in songbirds in desert cities, showed that three of the four species examined has the same baseline plasma CORT levels between urban and rural populations and that the stress response was not suppressed in urban birds (Fokidis et al., 2009). This variety in responses in avian species supports the notion that the effects of urbanization on stress response is species specific.

While most research is biased towards urban birds and mammals, there has been increasing research looking into the glucocorticoid stress response in urban reptiles. The baseline plasma CORT concentrations in copperhead snakes (*Agkistrodon contortrix*) did not vary between those found in the forest interior compared to those found near roads, but the latter had a lesser stress response to being restrained (Owen et al., 2014). Additionally, there was also a negative correlation between increased traffic and stress response (Owen et al., 2014). In a study of tree lizards (*Urosaurus ornatus*), the baseline and stress-induced plasma CORT levels were lower in urban lizards compared to their rural desert counterparts; however, unlike copperheads, the magnitude of the stress response did not differ (French et al., 2008). In common side-blotched lizards (*Uta stansburia),* those in urban areas had a greater CORT response to a stressor than those in rural areas (Lucas & French, 2012). The differences in responses in urban reptiles furthers the idea that adaptations in stress response are highly specific to each species.

Threats Facing Florida's Freshwater Turtles

Turtle populations are becoming increasingly threatened with 61% of modern turtles endangered or already extinct (Lovich et al., 2018). In addition to the dangers of urbanization, Central Florida turtles face two notable threats: disease and competition with invasive species. The Florida Fish and Wildlife Conservation Commission is monitoring a turtle mortality event in the Saint John's watershed caused by a novel virus (FWC, 2020). Additionally, invasive species, particularly red-eared sliders, have been widely introduced in peninsular Florida. The sliders, which were introduced through the pet trade, have been predicted to outcompete native species in the Central Florida region (Riedle et al., 2016).

Research surrounding the novel virus affecting turtles in Florida is limited, especially in areas around Winter Park, Florida. The first reported mortalities occurred in the St. Johns River watershed in 2018, but as of 2020, mortalities from the virus have spread to the Kissimmee Chain of Lakes (FWC, 2019; FWC, 2020). So far, the virus has been found to infect three species of turtles including the softshell (*Apalone ferox*), the peninsula cooter (*Pseudemys peninsularis*), and the Florida red-bellied cooter (*Pseudemys nelsoni*); however, softshell turtles are most commonly affected with 300 found dead (FWC, 2019). It is unknown if this virus is zoonotic and is capable of infecting humans and the degree to which it effects other turtle species (FWC, 2019; Ariel, 2011).

Native turtles are increasingly faced with the threat of invasive species. When invasive species like the red-eared slider are introduced, competition for food and basking spots increase (Chen, 2006). In addition, interbreeding of red-eared sliders with native slider species can cause hybridized populations and diluted gene pools (Parham et al., 2013; Harrison & Larson, 2014). Other non-turtle invasive species have also been identified to have negative impacts of turtles. For example, at Blue Springs in Orange County, Florida, the introduction of non-native fish and plant species has put stress on the ecosystem overall along with decreasing native turtle's food sources (Riedle et al., 2016).

Research Aims and Hypotheses

Research on the stress response of urban-living turtles is largely understudied with only two recent studies having been done both in one species of turtle, the painted turtle (*Chrysemys picta*). Turtles living in a habitat near highways were compared with those in a control site, and no significant differences were found in the CORT concentrations from nail tissue (Baxter-Gilbert et al., 2014). This study was a pilot for the novel technique of measuring CORT levels from nail samples in turtles; however, the results were supported by a later study measuring plasma CORT in the same species under similar conditions (Polich, 2016). As previously mentioned, the stress response in urban animals is highly specific to the species, and it would not be reasonable to extrapolate these findings to other freshwater turtle species.

This study aims to further the understanding of the stress response in turtles, by comparing and determining effective ways to quantify the stress response using various stress measures that differ in their time-scale and the evaluating the relationships between them.

Our study will focus on four freshwater turtle species (Figure 2) found in two urban water bodies in Winter Park, Florida. We will measure CORT in blood plasma to determine short term stress (i.e., minutess to hours) and in nails to determine chronic stress (i.e., weeks). Chronic stress can be inferred by measuring samples of keratinized tissue, such as nails, as CORT deposited within keratin does not display the same fluctuations as plasma CORT and is presumably integrated over a longer period of time as the nail grows (Figure 1; Berkvens et al., 2013; Mack & Fokidis, 2016). We also pilot the measurement of CORT from blood collected in leeches that adhere to the surface of turtle skin as a potential alternative, less invasive measure of stress, that would be intermediate between plasma and nail CORT. Blood smears will be used to assess immune response by identifying the presence of blood parasites and by determining the ratio of heterophils to lymphocytes (H:L ratio). The H:L ratio can also be used as a measure of chronic stress which suppresses immune function causing an increase in heterophil numbers, while suppressing lymphocytes (Vleck et al., 2000; Bonier et al. 2007; Harmon 1998). Being trapped is an inherently stressful event for turtles; therefore plasma CORT is likely to be elevated, but this single experience probably will not have a significant effect on leech or nail CORT. In turtles that do have elevated CORT in leeches or in their nails, we can expect the individual to be in a chronic stress condition. Therefore we can predict increased metabolic stress (more glycerol, less triglycerides), high H:L ratio, and high amounts of blood parasites. Turtles with lower nail CORT likely will exhibit the opposite; however, it is important to note that different life history characteristics may influence certain measures.

Methods

Species Descriptions

Figure 2 outlines the evolutionary relationships between common freshwater turtles in Central Florida: common snapping turtles, red-eared sliders, peninsular cooters, and mud turtles. Red-eared sliders and peninsula cooters are both in the family Emydidae, mud turtles are Kinosternidae and snapping turtles are Chelydridae.

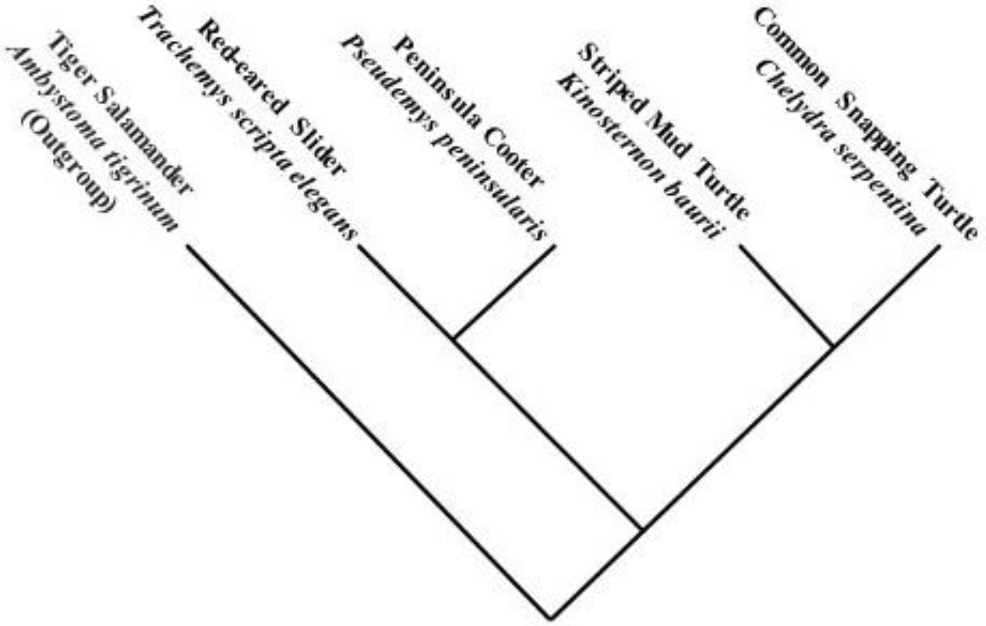

Figure 2. The phylogenetic relationship between common turtles in Central Florida. Generated using phyloT based on data from the National Center for Biotechnology Information Taxonomy Database and Ernst & Lovich (2009).

Common Snapping Turtles: Common snapping turtles, *Chelydra serpentina*, are found in the eastern and central United States, southern Canada, and Northern Mexico (Figure 3; Ernst &

Lovich, 2009). In the United States, there are supposedly two subspecies—*C. serpentina osceola*, found only in peninsular Florida, and *C. serpentina serpentina*, found throughout the rest of the United States. The morphological characteristics that separate the two include shape of tubercles, size of third vertebral scute compared to pleural scute, and maximum carapace length (Ernst & Lovich, 2009); however, this distinction is highly debated, and genetic analysis shows no difference between the two subspecies (Walker et al., 1998).

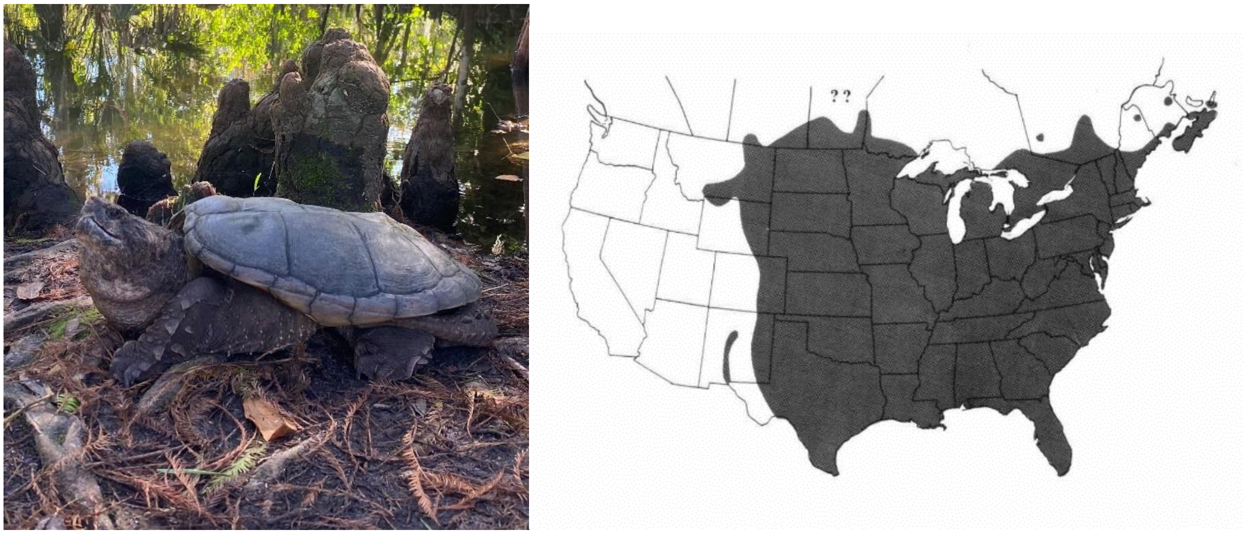

Figure 3. Adult common snapping turtle and native range map. Image by KC. Map from Ernst & Lovich 2009.

Common snapping turtles are large turtles—weighing between 4.5 and 16 kilograms on average but have been documented as heavy as 34 kilograms—with large powerful jaws (Ernst & Lovich, 2009). They are mainly aquatic and spend most of the time lying at the bottom of bodies of water or buried in mud (Ernst & Lovich, 2009). Although they can be found in almost any type of freshwater habitat, they prefer slow-moving waterways with soft substrate and abundant vegetation (Ernst & Lovich, 2009). Snapping turtles have also been found in brackish coastal waters (Kinneary, 1992). Most snapping turtles become dormant in winter, but in southern areas, particularly in peninsular Florida, they will remain active year-round (Ernst & Lovich, 2009). Mating typically occurs between the months of March and November; although, it has been reported year-round in Florida (Aresco, 2016; Ernst & Lovich, 2009). They are omnivores and will eat practically anything they can fit in their strong jaws (Ernst & Lovich, 2009).

Common snapping turtle eggs have become useful in the research of the presence of pollutants in water bodies. Throughout the Great Lakes, eggs were found to contain elevated levels of PCBs, organochlorine pesticides, PCDDs, PCDFs, and mercury; although, amounts of each varied by location. The presence of these toxins decrease survivorship in eggs and increase developmental abnormalities (Bishop et al., 1998**;** Ashpole et al., 2004; De Solla & Fernie, 2004). While this research is useful for tracking the temporal and geographic trends of polluted water bodies, the increase in fetal mortality and abnormality is not ideal for the populations of snapping turtles in these environments.

Red-eared Sliders: Red-eared sliders, *Trachemys scripta elegans,* are a subspecies of the pond slider. Their native range is around the Mississippi River and Gulf of Mexico, including Northern Florida; however, due to its popularity in the pet trade, red-eared sliders has become a prevalent invasive species (Figure 4; Ernst & Lovich, 2009). The other two subspecies of pond sliders include yellow-bellied slider, *Trachemys scripta scripta*, and Cumberland slider, *Trachemys scripta troosti* (Ernst & Lovich, 2009). Red-eared sliders are found in most freshwater habitats but prefer quiet water with soft substrate and aquatic plants (Ernst & Lovich, 2009). They need access to a basking spot as well (Ernst & Lovich, 2009). In the southern part of their range, they remain active year-round (Ernst & Lovich, 2009). Slider mating seasons lasts from April through July. They are omnivores; however, due to their weak jaw, they are limited to small, soft prey (Ernst & Lovich, 2009).

Figure 4. Juvenile Red-eared slider and native range map. Image by KC. Map from Ernst & Lovich (2009).

Red-eared sliders have been reported far from its native range including Asia, Europe, Australia, and Africa (Chen, 2006). Their success is likely due their great reproductive output (Ernst & Lovich, 2009). The presence of red-eared sliders outside its native range places other species at risk. For example, parasites only found in American turtles was found in European pond turtle populations due to the introduction of a pet red-eared slider (Meyer et al., 2015). They also increase competition for food and basking spots (Chen, 2006). Because of these impacts, the presence of invasive *T. s. elegans* could be a source of stress for native species. Furthermore, red-eared sliders have interbred with native slider species leading to hybridized populations (Parham et al., 2013). This causes genetic introgression, which means alleles from red-eared sliders are incorporated to the other sliders' gene pool, which may dilute the pool of the native species (Parham et al., 2013; Harrison & Larson, 2014).

Striped Mud Turtles: The striped mud turtles, *Kinosternon baurii*, are endemic to the southeast United States—found only in Florida, Georgia, North Carolina, South Carolina and Virginia (Figure 5; Ernst & Lovich, 2009). There are no known subspecies. Another mud turtle found in Florida is the eastern mud turtle (*Kinosternon subrubrum)*; however, the striped mud turtle is thought to be more common (Ernst & Lovich, 2009). Striped mud turtles are small, omnivorous turtles that inhabit quiet freshwater environments (Ernst & Lovich, 2009). They are very docile turtles and will rarely bite when threatened; instead, they withdraw into their shell for protection (Ernst & Lovich, 2009). They are active year-round in Florida and most breeding occurs in late summer or fall, which coincides with the rainy season (Wilson et al., 1999; Ernst & Lovich, 2009). They are semi-aquatic, and terrestrial movement is positively correlated with precipitation (Wilson et al., 1999).

Figure 5. Adult striped mud turtle and native range map. Image by FWC. Map from Ernst & Lovich, 2009.

Peninsular Cooter: The peninsular cooter, *Pseudemys peninsularis,* is a species of turtle found only in peninsular Florida (Figure 6; Ernst & Lovich, 2009). This species was formerly considered a subspecies of the river cooter, *Pseudemys concinna,* or the coastal cooter, *Pseudemys floridana* (Ernst & Lovich, 2009). Peninsular cooters are large, semi-aquatic turtles found in almost any slow-moving waterways (Ernst & Lovich, 2009). They are active both day and night with most foraging behavior seen in the morning (Ernst & Lovich, 2009). They are mainly herbivorous (Ernst & Lovich, 2009). They tend to spend the daylight hours basking and are active all year (Ernst & Lovich, 2009). Breeding occurs all year with most occurring in fall and winter months (Ernst & Lovich, 2009).

Figure 6. Adult peninsular cooter and native range map. Image by KC. Map from Ernst & Lovich 2009.

Turtle Trapping and Sample Collection

The study was conducted in Winter Park, Florida, from September 2020 to March 2021 at two study sites, Mead Botanical Gardens and Lake Virginia. Mead Botanical Gardens is a public park with wetland areas. Lake Virginia is an urban lake surrounded by Rollins College and residential areas. Howell Creek runs through Mead Gardens and flows into Lake Virginia allowing movement between locations. Baited hoop traps were placed Monday morning and checked daily until Thursday. Each specimen was uniquely marked along the marginal scutes of the shell using a small rotary tool to create notches in a pattern corresponding to a unique number. Each turtle was weighed using a spring scale and the length, width of the plastron and carapace and height of the shell was measured using calipers or measuring tape. To assess the metabolic state of the turtles we recorded the temperature on the surface of their shell and their skin using a infrared thermometer, and their internal temperature using a cloacal thermometer. Any unique physical markings and signs of injury or disease were also noted.

Nail samples were obtained using nail clippers, with only the tips collected to avoid cutting the blood vessel that runs through the center of the claw. All nail samples were stored at 4° C until processing. Blood collection followed the methods outlined in Jenkins-Perez (2012). Samples were collected using a 10 cc syringe with a 20-gauge heparinized needle. For most turtles, a blood sample were collected from the subcarapacial sinus, at the base of the carapace behind the head and neck. However, in snapping turtles, blood was collected from the dorsal coccygeal vein, located along the midline of the tail. Blood was stored on ice until brought to the laboratory for processing. A blood smear was made immediately following blood collection in the field. An approximately 50 µl drop of blood was placed on a clean glass microscope slide and using a glass coverslip, dragged to be create a one cell thick layer of blood (Bennett, 1970). These were dried under a vacuum until fixation.

Blood Sample Preparation

At the laboratory, blood samples were centrifuged to separate plasma from blood cells and both were stored in -20° C. Blood smears were fixed for 10 minutes in 100% methanol, then rinsed for 10 minutes in distilled water and then stained with Giemsa for one hour and finally rinsed in water for five minutes (Bennett, 1970). Slides were then air dried for 24 hours and cleared by xylene and coverslipped and sealed with Permount for long term storage.

Nail and Leech Sample Preparation

Solid phase extraction was used to extract CORT from both nail and leech samples. Each nail sample was first cleaned in methanol and ground into a coarse powder using a hand attrition mill. The samples were then stored at 4° C until further extraction. Nail samples and leech samples were then suspended in 84% methanol (MeOH) and homogenized using a tissue homogenizer (Precellys 24, Bertin) and stored 48 hours at 4° C until extraction. Homogenized samples were then prediluted with 10 ml of water. Carbon-bonded silica C18 cartridges on a vacuum manifold were first primed with 3 mL of 100% ethanol (EtOH) and then equilibrated with 10 mL of water. Next, 10 mL of each diluted sample was added, followed by 40% MeOH to remove fats from the column, that could interfere with the assay and columns were then allowed to dry completely. Finally, purified CORT was eluted with 5 mL of 90% MeOH and the samples were dried completely in a speed vacuum concentrator at 60° C in 3 hour increments. Dried extracts were stored 4° C.

CORT Assay

CORT concentration was quantified using an enzyme linked immunosorbent assay (ELISA) kit (Arbor Assays Inc). Dried leech and nail extracts were reconstituted with 1 μL of 100% EtOH and diluted with 119 μL of the assay buffer. Plasma was diluted with 240 μL of assay buffer. Assay was conducted according to manufacturer's protocol with each sample ran in duplicate. The concentration of CORT was determined using raw absorbance data at 495nm wavelength and interpolated from the standard curve.

Free Glycerol and Triglyceride Assay

Free glycerol and triglyceride concentrations were determined using an enzymatic determination assay kit (Sigma-Aldrich). The glycerol standard and plasma samples were added in duplicates to wells on a 96-well plate. Pre-warmed Sigma glycerol reagent was added to each well. Plates were incubated for 10 minutes at 37° C. The concentration of free glycerol was determined using raw absorbance data interpolated from the standard curve read at 540 and 750 nm. Pre-warmed triglyceride reagent was then added, and plates were incubated for 10 minutes at 37° C. Triglyceride concentrations were determined using the same wavelengths.

H:L ratio and Blood Parasite Presence

Blood smears were visually examined for parasites and the ratio of heterophils to lymphocytes (H:L) was determined. Each slide was scanned to identify if parasites were present. The amount of heterophils and lymphocytes were counted for 100 frames at 40x magnification and the results were expressed as a ratio.

Statistical Analysis

Since our sample size is limited for most species, we only analyzed red-eared sliders and snapping turtles. Pearson’s correlations between all measured variables were conducted for each species to determine interelatedness. If variables were related and highly correlated, we used principal components analysis (PCA) to reduce these variables to a single representative value. Finally, multiple linear regression analysis was used to determine if whether any of the CORT measurements were influenced by body condition, sex, date, or location. Analyses was conducted using SigmaPlot software and significance was anything with $p < 0.05$

Results

Population Demographics

A total of 84 turtles were captured with the most common turtle being the red-eared slider (53 captures), followed by snapping turtles (13); eastern mud turtles (8); three-striped mud turtles (3); common musk turtles (3); peninsula cooters (3); and red-bellied cooter (1, see Table 2). Also 15 of 84 turtles were recaptures with twelve being red-eared sliders and three being snapping turtles. One snapping turtle was recaptured twice, while the rest were single recaptures. Recaptures occurred at the same location as initial capture; however, four of the red-eared sliders were marked prior to this study, so their initial capture location is unknown. There was a generally equal sex ratio with 39 females to 36 males. The additional 9 captures were juveniles, which could not be sexed through morphological characters. Snapping turtles were the only species that had more males (8) compared to females (5). All specimens were collected via hoop trap besides one red-eared slider caught in the basking trap and one common snapping turtle caught by hand.

Table 2. Distribution of species by location.

	Mead Gardens	**Lake Virginia**	**Total**
Red-eared sliders	16	37	53
Common snapping	12	1	13
Eastern mud	6	2	8
Three-striped mud	3	0	3
Common musk	2	1	3
Peninsular cooter	2	1	3
Red-bellied cooter	0	1	1
Total	41	43	84

There was an approximately equal representation between locations with 41 captures at Mead Gardens and 43 at Lake Virginia (Table 2). Both locations had equal species richness of

S=5. The species evenness, measured by the Simpson-Dominance Index, was greater in Mead Gardens (0.75) than in Lake Virginia (D=0.26).

Table 3. Correlation matrix of metabolic data. Significance in indicated by asterisks (*)

	Skin Temp	**Shell Temp**	**Cloacal Temp**
Skin Temp	1.000		
Shell Temp	0.978*	1.000	
Cloacal Temp	0.969*	0.962*	1.000

Metabolic Stress

Skin, shell, and cloacal temperatures were highly correlated (Table 3). Using principal components analysis (PCA), a single metabolic temperature was determined for each specimen. Overall, metabolic temperature was not significantly correlated with triglycerides (r= 0.38, p = 0.121), but had a slight positive relationship with glycerol (r= 0.00012, $p < 0.001$). In red-eared sliders, there were no correlations with metabolic temperature. In snapping turtles, metabolic temperature was positively correlated with triglyceride concentration in plasma (r=0.99, P=0.012).

In snapping turtle plasma, the average concentration of glycerol was 0.11 ± 0.4 mM, and the average concentration of triglycerides was 1.91 ± 2.25 mM. In red-eared slider plasma, the average concentration of glycerol was 0.15 ± 0.10 mM, and the average concentration of triglycerides was 1.00 ± 1.35 mM.

Table 4. Correlation matrix of body size. Significance in indicated by asterisks (*)

	Carapace Length (mm)	**Carapace Width (mm)**	**Plastron Length (mm)**	**Plastron Width (mm)**	**Shell Height (mm)**
Carapace Length (mm)	1.000				
Carapace Width (mm)	0.976*	1.000			
Plastron Length (mm)	0.984*	0.988*	1.000		
Plastron Width (mm)	0.927*	0.933*	0.954*	1.000	
Shell Height (mm)	0.338	0.241	0.291	0.320	1.00

Body Size and Condition

The length and width of the carapace and plastron were also highly correlated (Table 4). As before we produced a single “body size” variable using PCA. Shell height was not significantly correlated and was excluded from this analysis. Body size and weight were strongly related (F=5.11, P=0.030). A linear regression of body size against body mass was used to develop a freshwater turtle body mass index (BMI), with the following equation: Weight (kg) = 1.172 + (0.111 * Body Size). The position of each specimen in relation to the linear regression determines the body condition, and this was based on the residuals from that linear regression.

In both species, body size was positively correlated with plasma glycerol (Red-eared sliders: r=0.63 P=0.003; snapping turtles: r=0.94, P=0.0006). In snapping turtles, body size was positively correlated with metabolic temperature (r=0.021, P=0.021) and negatively correlated with body condition (r=-0.89, P=0.003). Also, body condition was negatively associated with plasma glycerol concentrations (r=-0.80, P=0.018), metabolic temperature (r=-0.99, P=0.006), and body size (r=-0.89, P=0.003). In red-eared sliders, body size was positively correlated with weight (r=0.891 P<0.001) and with plasma glycerol (r=0.63, P=0.0032).

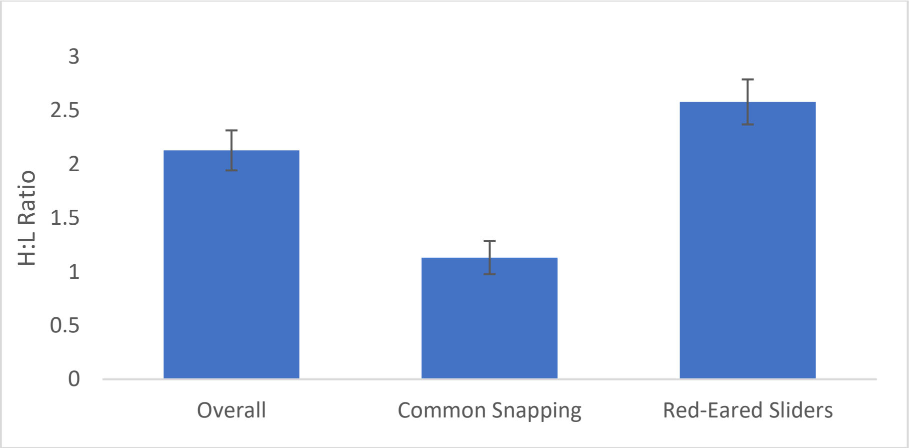

Figure 7. The H:L ratio is significant lower in common snapping turtles than in red-eared sliders.

Immune Response and Parasites

Snapping turtles had a significantly lower H:L ratio compared to red-eared sliders (Figure 7). Additionally, the H:L ratio of snapping turtles is positively correlated with body condition (r=0.83, P=0.042). In all captures, 66% had ectoparasites present and 92% hadblood parasites. Snapping turtles had the highest parasite prevalence with 85% having ectoparasites and 100% having blood parasites. All individual without blood parasites were red-eared sliders. Presence of blood parasites did not significantly impact H:L ratios (P=0.39). All ectoparasites appear to be from the genus *Placobdella*.

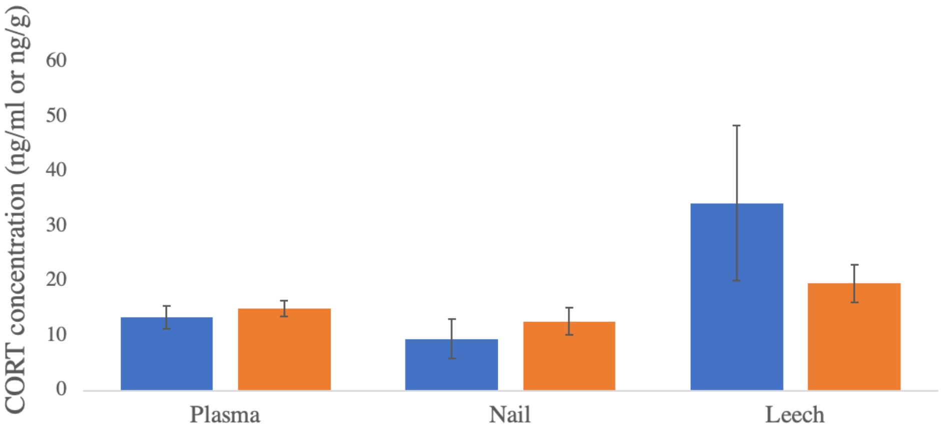

Figure 8. CORT concentrations in plasma, nails, and leeches do not vary significantly between snapping turtles (blue) or red-eared sliders (orange). All CORT concentrations were determined using an enzyme linked immunosorbent assay (ELISA) kit (Arbor Assays Inc).

CORT Measures

On average, the concentration of CORT in plasma was 14.34 ± 8.04 ng/mL, in nails was 11.83 ± 11.43 ng/g, and in leeches was 22.13 ± 21.21 ng/g. One outlier in the leech sample group was not included in the average. Neither snapping turtles or red-eared sliders differed significantly from these averages (Figure 8). There were no significant correlations between nail, plasma, and leech CORT in either species (Figure 9). In snapping turtles, CORT in general does not vary with sex, body condition, season or between locations. Plasma CORT in snapping turtles was positively associated with triglycerides (r=0.76, P=0.018). In red-eared sliders, plasma and nail CORT did not vary with sex, body condition, season, or locations; however, leech CORT concentration was significantly lower in sliders with a better body condition (F=4.135, P=0.039).

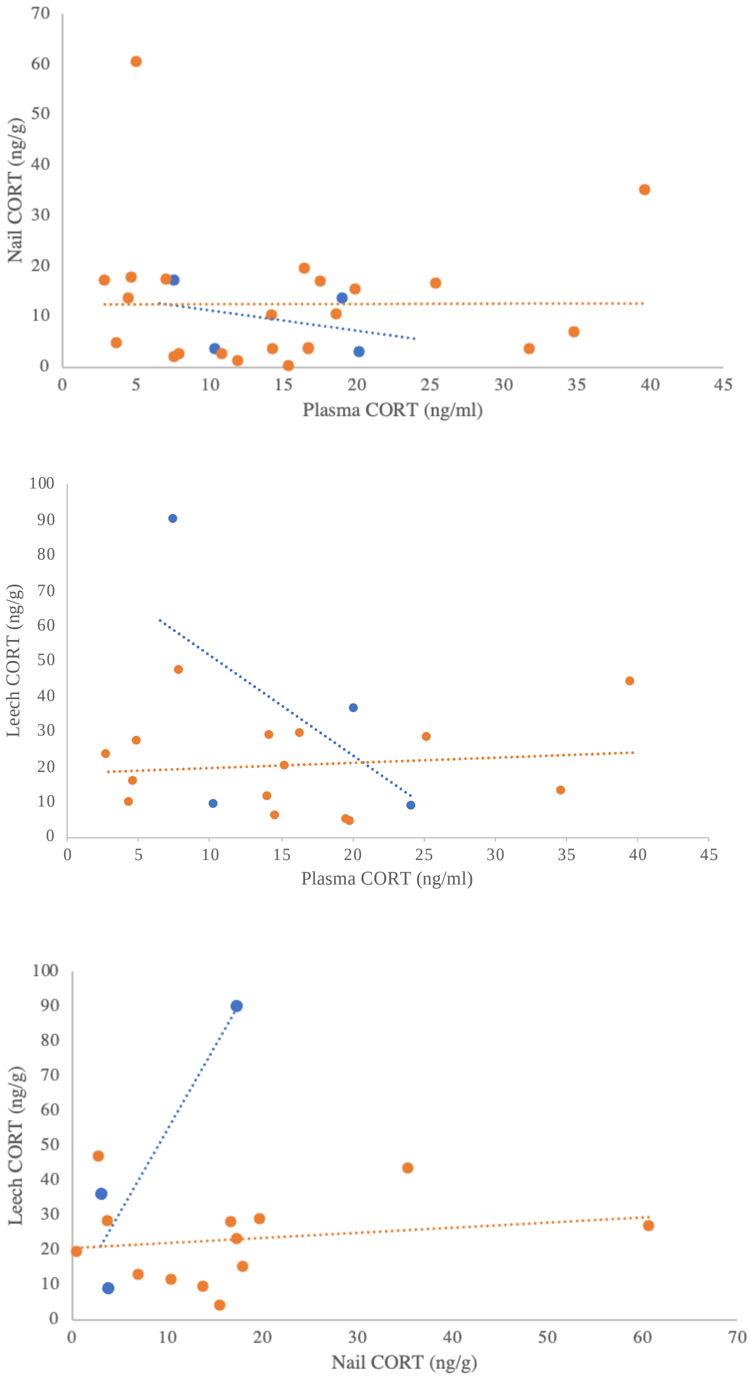

Figure 9. Plasma, nail and leech CORT concentrations were not significantly correlated in snapping turtles (blue) or red-eared sliders (orange). All CORT concentrations were determined using an enzyme linked immunosorbent assay (ELISA) kit (Arbor Assays Inc). Analysis revealed no significant correlations between nail CORT*plasma CORT, leech CORT*plasma CORT or nail CORT*leech CORT.

Discussion

Quantifying turtle stress can be a difficult task, because acute measures are often influenced by trapping methods, and chronic measures are understudied. This novel study integrated data on hormone concentrations, immune response, body size and condition, and metabolism to further investigate the stress response in freshwater turtles, as well as collecting demographic data. Because stress is associated with multiple physiological impacts, researchers proposed various variables to quantify stress. The study determined that these variables are unrelated in our two models: snapping turtles and red-eared sliders. Not only does this suggest that multiple variables should be used to quantify stress, but also that more research is needed to understand the stress response in reptiles. In addition to this, we found various relationships between stress measures, immune response, body size and condition, and metabolic measures.

While Mead Gardens and Lake Virginia had equivalent species richness, Mead Gardens had a much more even distribution of species. Of the 43 turtles caught in Lake Virginia, 37 were red-eared sliders. While it is unsurprising that red-eared sliders were the dominant species due their status as a successful invasive, the extent of invasion in Lake Virginia is concerning—particularly for species with similar life histories like the peninsula cooter. The introduction of red-eared sliders wiped out entire populations of European pond turtles (*Mauremys leprosa*) in Spain (Semenov, 2010). Mead Garden's relatively even species distribution may be a result of proper park management or simple due to species' habitat preferences. Although locations are connected by Howell Creek, none of the recaptures were known to be transient between locations. Both species have exhibited territorial behavior, which may explain recaptures remaining in initial location (Ernst and Lovich, 2009).

Metabolic Measures

Because red-eared sliders' and common snapping turtles' diets are protein-dense, the presence of glycerol and triglycerides is generally be attributed to gluconeogenesis (Ernst and Lovich, 2009; Price, 2017). In both species, there was a positive correlation with size and plasma glycerol. This is consistent with a study in loggerhead sea turtles (C*aretta caretta),* which found carapace length to be positively correlated with plasma triglycerides (Stacy et al., 2018). One possible explanation is that larger turtles cannot maintain the increased metabolic rate that occurs during a stressor and therefore, resort to gluconeogenesis to supply glucose to the blood. Indeed, snapping turtles with higher plasma CORT also had higher triglycerides present. Furthermore, higher triglycerides were associated with a higher metabolic temperature. While metabolism is influenced by a number of factors, the correlation between CORT and triglycerides implies that metabolism is influenced by the stress response in snapping turtles. An alternative possibility is that the elevation of CORT is due territory-defending or breeding behavior—particularly because snapping turtles breed year-round in Florida. A study in the green turtles (C*heloniamydas*) found that breeding season was associated with increased plasma CORT and increased metabolism (Jessop et al., 1999)While plasma glycerol in snapping turtles increased with body size, it decreased with body condition. Individuals may rely on gluconeogenesis for maintaining metabolism, which potentially can lead to a depletion of fat stores—leading to lower body condition. Another possible explanation is that turtles with less fat stores are consuming enough to maintain metabolism, which prevents further depletion.

Immune Measures

Snapping turtles were found to have a lower H:L ratio than red-eared sliders; suggesting their heterophils are lowered. While lymphocytes are associated with the adaptive immune system, heterophiles aid in innate immune response. Interestingly, the plasma of snapping turtles has particularly high antibacterial activity, which indicates a strong innate immune response (Baker, 2018) While common snapping turtles occupy almost all types of freshwater bodies, red-eared sliders have a much larger range globally due to the pet trade. The higher innate immune response from red-eared sliders could be advantageous in new environments, since there are likely different diseases present. Relying on innate response mechanisms may increase chances of survival in new habitats with new pathogens (Vilcinskas, 2015).

Blood parasites were found in the majority of turtles with an infection rate of 92%. This did not have any impact on H:L ratios, which suggests immune response is not impacted by the presence of the parasites. This is consistent with the findings of (Davis and Sterrett, 2011), which suggest that blood parasites have little impact on the health of turtles. Additionally, they found that larger turtles had more blood parasites due to increased area for leech attachment. This is consistent with the high parasite prevalence in snapping turtles, because snapping turtles were on average the largest species in this study.

CORT Measures

None of the CORT measures were correlated with each other, which validates the need for taking both acute and chronic measures when assessing stress in a population. Plasma CORT, which is a typical measure of acute stress in reptiles, was generally higher than nail CORT, which is consistent with other freshwater turtles. The novel study analyzing nail CORT in painted turtles revealed nail CORT concentrations that were lower than known plasma CORT concentrations (Polich 2016, Baxter-Gilbert et al., 2014). Since we lack nail growth rate data, we are unable to determine if this is due to external stressors increasing plasma CORT or simply the rate that CORT is integrated; however, we predict that the increased plasma CORT may be due to trapping. An additional study, similar to the study of snake shed by Berkvens et al., (2013), looking at nail growth rates could help further understanding of how plasma and nail CORT correlate. Due to these limitations, plasma CORT is likely not indicative of average life stress, which makes it a less useful measure when compared to nail CORT at assessing general stress. Using keratinized tissue provides a wholistic view of CORT levels since it is integrated over a long period of time. One limitation is that nail CORT cannot measure changes in CORT over time. Also, short-term increases in CORT may not affect long term diffusion into the nail, due to negative feedback mechanisms. Testing the HPA's negative feedback mechanism through an ACTH and dexamethasone suppression study, such as the one outlined in Fokidis and Deviche (2011) would provide greater insight to the negative feedback mechanism. Determining CORT concentration from plasma and nail samples both have benefits and limitations; therefore, using both for stress analysis provides the most complete picture of the population of study.

Because the CORT from leeches was not correlated with plasma or nail CORT, the novel part of the study reveals that leeches should not be used in place of plasma or nail CORT for acute or chronic analysis. The CORT in the leeches was found to be quite variable—possibly due to amount of blood consumed relative to body size or potentially presence of blood from another organisms. While *Placobdella* mainly feed on turtles, they have also been found to feed on alligators, amphibians, fish and aquatic invertebrates (Readel et al., 2008) Feeding on a different organism immediately prior to the turtle may impact CORT concentrations—especially because freshwater turtles have lower CORT concentrations compared to other reptiles (Selman et al., 2012). In red-eared sliders, body condition was correlated with lower leech CORT. One possible explanation is that because red-eared sliders with greater energy stores are more likely to attract leeches compared to other species, meaning that CORT does not necessarily increase when they are present.

Conclusion

An important aspect of conserving compromised groups, such as turtles, is understanding the physiological impacts on populations. This novel study validated the need for taking multiple measures of stress. Additionally, we've identified relationships between various variables relating to stress. Further studies that would increase knowledge of stress in freshwater turtles include nail growth rate study, and an ACTH and dexamethasone suppression study. In order to protect species facing peril, we need to understand their physiological conditions. Quantifying stress is becoming increasingly useful as anthropogenic activities continue to impact wildlife populations.

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