The regulation of stress steroid release in freshwater turtles

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Honors Biology Thesis Proposal

Introduction

Reptiles serve in important roles as predators, prey, grazers, and seed dispersers within ecosystems and their presence or absence can serve as bioindicators of the health of their respective environments (Bohm et al., 2013; Lovich et al., 2018). There is however, a large discrepancy between a species' ecological importance and its lack of use in research (Bohm et al., 2013). Specifically, the International Union for the Conservation of Nature (IUCN) creates a Red List of Threatened Species in which only 45% of the 10,400 currently recognized reptiles have been assessed; and this number is small compared with the 83% of amphibians and nearly 100% of mammals and birds (Tingley et al., 2016). Additionally, Bohm et al. (2013) examined the conservation status of reptiles and found 21% were considered data deficient. These statistics illustrate the lack of attention towards these species and the need to direct more future research towards reptiles, especially turtles. Turtles are considered the most imperiled reptiles with 61% of species endangered or extinct (Lovich et al., 2018). Some of the factors implicated in the decline of turtles include: destruction and degradation of their habitat; overexploitation in the pet trade, reliance on them as nutritional staples, and climate change (Lovich et al., 2018). Despite these factors, turtles are often commonly spotted, and people do not realize they are in decline. Thus, this project aims to use turtles as the model organism to study stress.

Stress Physiology

Turtles in a trap present an interesting problem for the stress physiologist. Stress is defined as an external or internal force that threatens the homeostatic state and triggers a physiological "stress response" (Sapolsky et al., 2000). This series of physiological and behavioral changes aim to restore homeostasis, and thus, is a necessary response for surviving a stressor (Martinez-Silvereste, 2014; Tsigos et al., 2000). The stress response is pleiotropic as a singular stressor can result in a multitude of corresponding physiological changes (Martinez-Silvereste, 2014). There are different types of stressors (environmental, behavioral, and demographic) which can each provoke a stress response, but a stimulus is only considered a "stressor" when it involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1), a hormone cascade that leads to an increase in glucocorticoid concentration (Tsigos et al., 2000; Sapolsky et al., 2000; Cockrem, 2007).

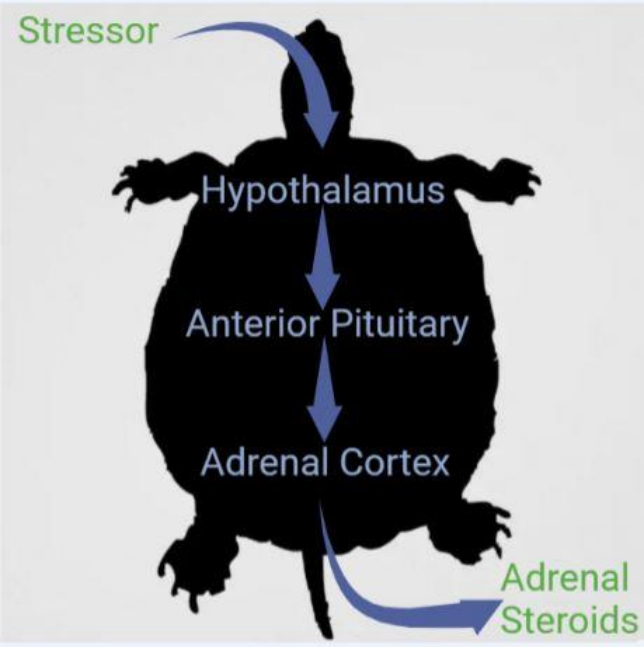

Figure 1. Hormonal Pathway for the synthesis of the adrenal steroids. This process begins by the hypothalamus perceiving a stressor and then communicating with the anterior pituitary gland, and finally the cortex of the adrenal gland. At the adrenal gland, cholesterol is converted to first pregnenolone and then multiple intermediates before synthesis and secretion of glucocorticoids (cortisol and corticosterone) and dehydroepiandrosterone (DHEA).

This HPA axis can be activated by both external and internal stimuli, as well as, by real or perceived events (Whitham, 2020). Once activated, cortical releasing hormone (CRH) is released by the parvocellular cells within the paraventricular nucleus (PVN) of the hypothalamus (Tsigos et al., 2000). These neurons deliver CRH from the hypothalamus through the hypophyseal portal system to the anterior pituitary (Sapolsky et al., 2000). In the anterior pituitary, adrenocorticotropic hormone (ACTH) is secreted in response to the CRH binding to the CRH-R1 receptors on corticotropic cells (Tsigos et al., 2000; Whitham, 2020). Then, ACTH is secreted into the blood stream to travel beyond the brain to the adrenal cortex, the location for the conversion of cholesterol to pregnenolone and the first precursor for all steroid hormones (Figure 2).

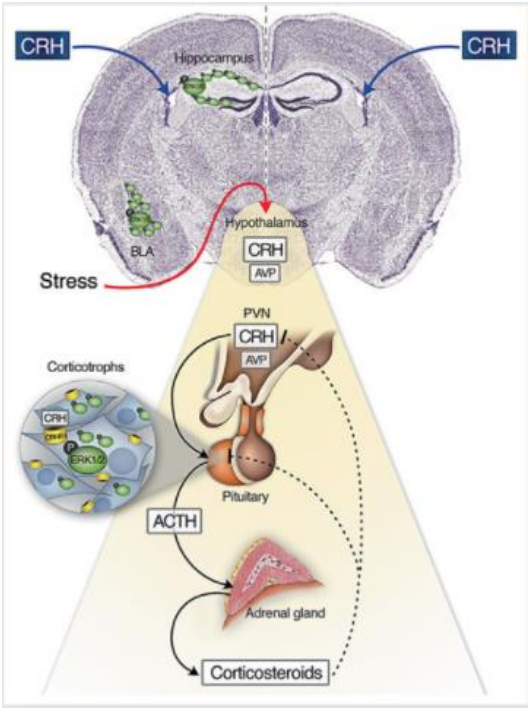

Figure 2. Pathway for the synthesis of adrenal steroids. The yellow shading shows the HPA axis system and its regulation of steroid hormones in response to stress. In the HPA axis, corticotropin releasing hormone (CRH) is produced, as released by the paraventricular nucleus (PVN). Next, CRH binds to the CRH-R1 receptors on the anterior pituitary gland which incites the secretion of adrenocorticotropic hormone (ACTH). Then, ACTH travels to its target organ, the adrenal gland which initiates the conversion of cholesterol to pregnenolone the precursor to all steroids. (Image taken from Bonfiglio et al., 2011).

Steroid hormones are cholesterol-derived compounds with four hydrocarbon rings and oxygenated side chain functional groups which makes them hydrophobic (Figure 3). Glucocorticoids secreted during stress include corticosterone and cortisol, which due to the similarity in structure both bind to glucocorticoid receptors with similar affinity. Thus, the relative quantity of each glucocorticoid determines which plays a greater role in stress adaptation (Sapolsky et al., 2000; Whitham, 2020).

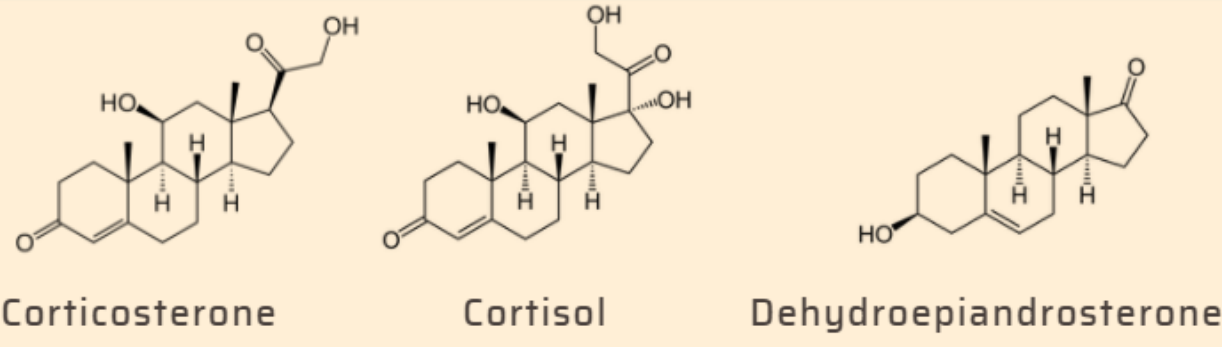

Figure 3. Molecular structures for the three end products of the HPA axis: corticosterone, cortisol, and dehydroepiandrosterone (DHEA).

After stress passes, negative feedback in the HPA axis system can occur through two mechanisms, by blocking the CRH receptors or inhibiting secretion of ACTH (Tsigos et al., 2000). In this way, the hormones secreted as output will influence the hormones secreted at the beginning of the cascade to regulate the HPA axis, and thus, the increase in glucocorticoid concentration influences the further release of other hormones (Fokidis, 2016; Tsigos et al., 2000). However, when this negative feedback is compromised, a chronic secretion of glucocorticoids can result in negative effects, such as depressed immunity and inhibited reproduction (Sapolsky et al., 2000). Thus, it is critical to study the stress steroid hormones and how they are produced.

Corticosterone

Within 1-2 minutes after experiencing a stressor, glucocorticoid concentrations increase, and in reptiles, the primary glucocorticoid is corticosterone (Cockrem, 2013). Corticosterone is produced in the adrenal cortex, and it responsible for restoring homeostasis during stress (Cockrem, 2007; Whitham, 2020). An increase in corticosterone levels alter an organism's metabolism, most notably increasing blood glucose levels, as well as alter behaviors that are adaptable to their given stressors (Cockrem, 2007). The time frame for this adaptation to stress was noted in Silverin and Wingfield (1998), who found corticosterone levels increase quickly upon capture but return to a baseline concentration after 30-60 min.

Cortisol

In contrast, cortisol is less frequently used as a stress biomarker in reptile research as it is considered a secondary glucocorticoid product in reptiles (Cockrem, 2013; Hellhammer et al., 2009). Nonetheless, several studies have measured increased plasma cortisol levels in reptiles, after exposure to restraint, predators, food deprivation, heat, close captivity, or aggressive behavioral interactions, suggesting it may also play a physiological role. It is expected that the endogenous supply of cortisol exists in a lower concentration than corticosterone. This difference means that cortisol binds less to the receptor, and overall may play a smaller role in the adaptive stress response, perhaps by inducing local effects.

Dehydroepiandrosterone (DHEA)

In addition to glucocorticoids, the adrenal gland also synthesizes DHEA in response to ACTH. Within the blood it is largely found in the sulfated form, DHEA-S (Schwartz, 2002). With steroid sulfatases, DHEA-S can later be converted to bioactive DHEA (Schwartz, 2002). It is thought that DHEA is an antagonist to glucocorticoids (i.e., an "anti-stress" hormone) and can be measured to provide a more holistic understanding of the stress response beyond glucocorticoids (Whitham, 2020).

Neuropeptide Y and Stress

Stress responses involve metabolic shifts, and so, energy deficits are considered highly stressful. Neuropeptide Y (NPY) is an extremely orexigenic neurohormone that is released when an organism is in an energy deficient state (Joly-Amado, 2014). NPY is composed of 36 amino acids that are highly conserved across both mammalian and non-mammalian species (Mercer et al., 2011). Due to its high conservation, it is believed that NPY is involved in many physiological processes, beyond energy conservation (Mercer et al., 2011). Some of the physiological processes NPY is believed to be involved in include sleep, stress, metabolism, and cardiovascular functioning (Joly-Amado, 2014). The hypothalamus, a specialized region in the brain, includes an integration site for nutrient related signals that respond to hunger. Within the hypothalamus there are several collections of nerve cells bundled together to form nuclei, one such site is labeled as the arcuate nucleus which has downstream connections to the paraventricular nucleus (PVN), ventromedial nucleus (VMN), lateral hypothalamic nucleus

(LHA), and dorsomedial nucleus (DMN) (Mercer et al., 2011). These downstream connections aid in the maintenance of energy homeostasis by regulating food intake and increasing fat storage (Joly-Amado, 2014). Despite NPYs importance in many critical processes, research is still limited. Some research has focused on the effect of NPY on increasing an organism's resiliency, defined as the ability to resist cognitive impairments during stress (Villarroel et al., 2018).

Signaling with NPY can occur through multiple receptor types (Y1 through Y5), and in the context of stress Y1, Y2, and Y5 receptor appear particularly important. Y1 is a receptor with a wide distribution in the human and rodent brain (Mercer et al., 2011). In rodents, energy homeostatic effects of NPY are at least partially dependent on the Y1 receptor. Mercer et al. (2011) noted this phenomenon as the use of Y1 agonists stimulated food intake in satiated rats (Stanley et al., 1992). Y2 is a receptor mostly centralized within the brain that can be stimulated by both NPY and the similar peptide YY (PYY), a gut hormone, both of which have anorexigenic effects when Y2 is stimulated (Mercer et al., 2011). Y5 receptors similarly have been found to be expressed within the brain, with very limited peripheral expression (Mercer et al., 2011), and studies using NPY analogs found this receptor increases food consumption (Cabere et al., 2000). Thus, this receptor is targeted as potentially important for anti-obesity treatments (Mercer et al., 2011).

The goal of this study is to characterize the HPA axis regulators of the stress response in a turtle species, the red-eared slider, *Trachemys scripta elegans* and to determine if NPY acts as a secretagogue for adrenal steroids in reptilian species, as it does in mammals. Here, I will use pharmacological injections of ACTH, dexamethasone (DEX, a synthetic steroid that blocks ACTH release and induces HPA negative feedback), NPY, and NPY receptor agonists. Each turtle will receive a single injection upon capture, and I will record whether glucocorticoid secretion is stimulated or decreased due to negative feedback (see example in Fokidis and Deviche, 2010).

I hypothesize that ACTH injection will increase glucocorticoid and DHEA production, Opposingly, the injection of DEX should block the release of ACTH as it is a pituitary inhibitor and limit the secretion of glucocorticoids and DHEA. I hypothesize that NPY injection will activate the stress response and increase steroid levels, and using different NPY receptor

agonists, I can identify the specific receptor involved. Thus, this study may help establish a novel connection between stress response and food intake.

Methods

Model Species

Red-Eared Sliders, *Trachemys scripta elegans*, are a member of the Family Emydidae (Meylan, 2006). This family of "pond turtles" comprises 15 of Florida's 30 turtle species (Meylan, 2006). Within these 15 species of 'pond turtles'' there are several subspecies of Pond Sliders (sliders). Along with Red Eared Sliders, other subspecies of Pond Sliders include yellow-bellied sliders, *Trachemys scripta scripta*, and Cumberland slider, *Trachemys scripta troosti* (Ernst and Lovich, 2009). All sliders are semiaquatic, meaning they venture onto land to lay eggs or move to a different aquatic environment (Ernst and Lovich, 2009; Gibbons, 1990). These omnivorous turtles need an aquatic environment rich with vegetation and accessible basking spots (Gibbons, 1990).

The model organism for this work is the Red-Eared Slider, which is native to the East and Central United States, but can now be found worldwide, on all continents other than Antarctica due to releases from the pet trade, and are named the only globally invasive species of turtle (Rodrigues et al., 2016; Taniguchi et al., 2017). This species was first described in 1885 from a specimen obtained near Charleston, South Carolina; it was the first reptile admitted into the United States National Museum collection (Gibbons, 1990). While originally cataloged as *Emys serrata*, it has later been referenced as *Pseudemys scripta*, and then *Chrysemys scripta*, and is now classified as *Trachemys scripta* (Gibbons, 1990). There are many subspecies of sliders, as shown in Figure 4, but the most common worldwide is the Red-Eared Slider characterized by a red post-orbital stripe on the lateral portion of its face (Thomas, 2006).

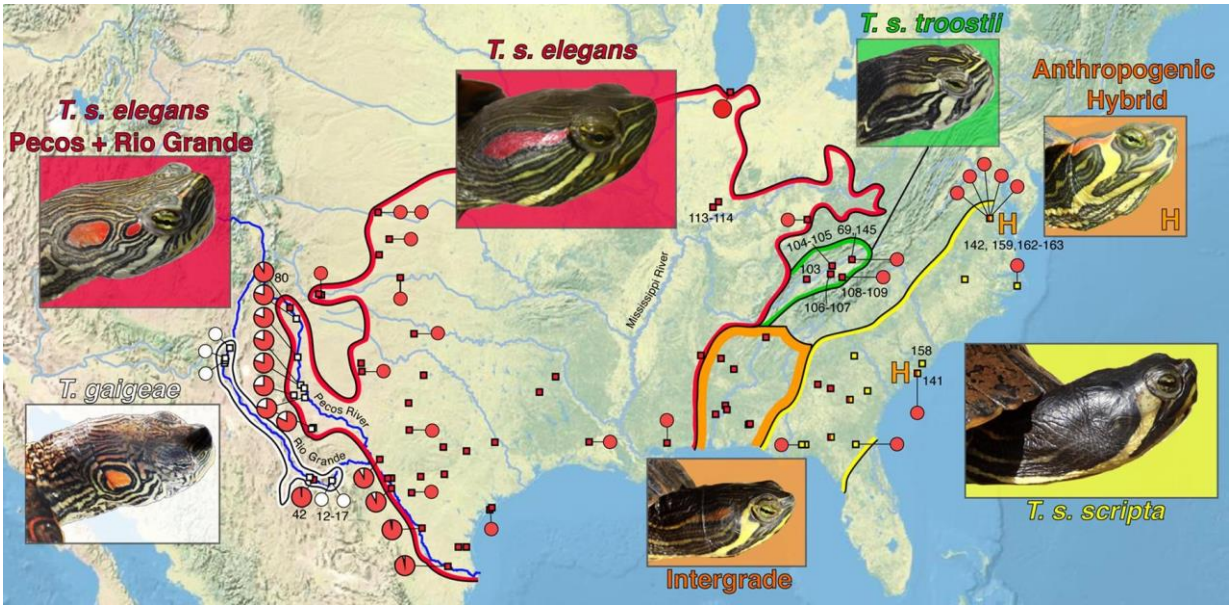

Figure 4. A distribution map to show the relationship between pond sliders in the United States. There are four distinctive species of pond sliders within the United States including *T.s. scripta*, *T.s. troostii*, *T.s. elegans*, and *T.s. gaigeae*. Additionally, there are several hybrids located at contact zones. Note in Central Florida, only the invasive *T. s. elegans* is found. (Image obtained from Parham et al. (2020).

Red-Eared Sliders are originally native to North Florida and other places within the Mississippi River drainage area and the Gulf of Mexico (Figure 5, Meylan, 2006), but they are now found throughout the US, including Central Florida, as well as the West Indies, into South America, Europe, the Middle East, Northern/Southern Africa, East Asia, and Australia (Ernest and Lovich, 2009; Meylan 2006; Seidel 2002). Its introduction into new areas has led to competition and exclusion of native turtle populations (Meylan, 2006; Thomas, 2006).

Figure 5. Native range map of the Slider turtle. However, it is expected that the current range is much greater than is illustrated because of the pet trade. Image obtained from Ernst & Lovich, (2009).

The ability of sliders to dominate aquatic ecosystems can be attributed to several factors. Firstly, they are active year-round in places where the climate permits them (Ernst and Lovich, 2009). Secondly, their high reproductive success coupled with a long mating season from April to July allows red-eared sliders to produce a large clutch size of 12.5-15.1 eggs (Tucker et al., 1998). Finally, they are a common pet with a long life span and their subsequent release into the wild means they have been introduced into many areas where they have outcompeted native turtle species, including other sliders and similar pond turtles (Meylan, 2006; Riedle et al., 2016; Thomas, 2006). The widespread invasion of the Red-Eared Slider and their disproportionately high frequency in the Southern US, threatens other species through alimental and spatial competition (Chen, 2006). Red-eared sliders also dilute the gene pool by forming hybridized populations with other species at contact zones (Figure 4), and they increase spread of disease amongst species that have not formed natural immunity towards the pathogens (Harrison, R. and Larson, E., 2014; Meyer et al., 2015; Parham et al., 2013).

While human actions are significant factors in why the Red-Eared slider turtles became invasive, humans are also there largest threat (Thomas, 2006). Ways in which humans threaten Red-Eared sliders can mainly be outlined using three categories: fishing, trade, and transport.

Fishing is detrimental as some fishermen use their eggs as bait, or they are inadvertently caught and killed sliders as they attempt to remove them from their fishing line (Thomas, 2006). Some fishermen even purposefully kill turtles because of the incorrect belief that their presence decreases the success of fish populations (Thomas, 2006). Humans also threaten sliders due to their trade (Thomas, 2006). During 1999, approximately 8,000,000 recently hatched turtles were shipped to 60 different countries for use as pets (Thomas, 2006). The increase in farm-raised turtles reduces genetic diversity and increases resource competition in wild populations (Thomas, 2006). Finally, transport and the creation of roads near their native ecosystems, combined with an increased number of drivers, has led to high vehicular mortality of turtles (Thomas, 2006). Furthermore, transport has indirect effects on slider populations as the increase in vehicular transport results in a direct increase in pollutants (Thomas, 2006), which can also hamper endocrine functions (Thomas, 2006).

Study Site and Turtle Trapping

This study was conducted in Lake Virginia in Winter Park, Florida along the shoreline of Rollins College between September 2021 to March 2022. Red-Eared Sliders are the dominant turtle found in Lake Virginia, accounting for almost all turtle captures during this period. Lake Virginia is part of the Winter Park Chain of Lakes and is connected to other lakes through the Howell Branch Watershed (Orange County Water Atlas). Lake Virginia is a 223-acre public waterbody and is surrounded by the Rollins College campus and housing developments. This ecosystem is the focus for this study due to its large population of red-eared sliders, which have been studied for about four years, including a previous honors thesis by Katie Caldwell. Three to four hoop traps were baited with cat food and chicken, and they were placed in the shallow water along the shoreline of Rollins College. Traps were set out over 2-3 nights and checked daily. Captured turtles were examined on shore and released at the site of capture afterwards.

Pharmacological Injections and Blood Sampling

For each subject, a blood sample was taken from the subcarapacial sinus on the base of the carapace. Turtles were then injected with 100 ul of one of the seven treatments (Table 1) in

the left hind leg. Turtles will be held in isolation in a cloth bag, and then a second blood sample was collected at 30 minutes. This time corresponds to a typical maximum stress response in pond turtles, and blood was stored on ice in the field, until blood cells and plasma were separated through centrifugation in the laboratory.

Table 1. Pharmacological treatments used in this study. Each subject received only a single injection of one treatment group at a time with blood samples collected before and 30 minutes after injection. All injections were administered as 5 mM doses in 100 ul volumes of 0.75% saline.

Treatments	Saline	ACTH	DEX	NPY	NPY-Y1 agonist	NPY-Y2 agonist	NPY-Y5 agonist
	0.75% NaCl				BIBO3304	BIEE0246	LUAA33810
Dose		10 IU/kg	16 ug/kg	3.54 ug/kg	3.12 ug/kg	3.73 ug/kg	1.765 ug/kg

Each turtle was uniquely marked using the 1-2-4-7 numbering system developed by Cagle (1939) and shown in Figure 6. Marks were made in the marginal scutes of the turtle shell using a small rotary tool providing each turtle a unique number for future identification. Throughout this study, any recaptures were still used as subjects provided that at least 10 days had passed since its prior use, allowing enough time for any residual effects of previous fast acting injections to wear off.

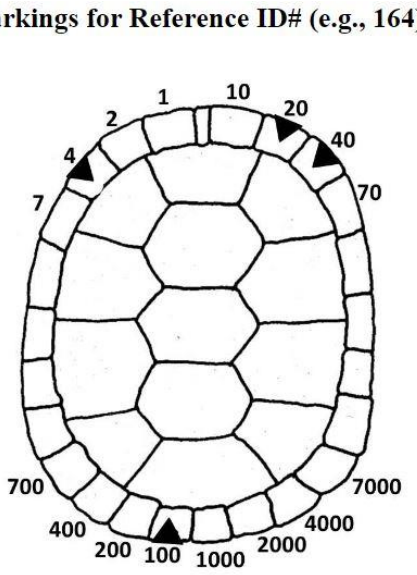

Figure 6. The system for turtle identification utilizes a series of quadrants and a 1-2-4-7 numbering system. Triangular shaped notches are then made on the corresponding scute with a rotary tool to clearly distinguish between turtles and identify recaptures. Here an example of turtle #164 is provided.

Determining Sex and Age in Pond Sliders

The sex of all subjects was identified based on several characteristics including: nail length, shape of plastron, and overall size, see Figure 7 (Moldowan, 2014). Male turtles have longer second and third nails than females which is an adaptation that facilitates the use of their claws to better grip the female turtle during copulation and for courtship displays (Gradela et al., 2017). Conversely, the most obvious secondary sex characteristic in female sliders is their domed shell which is wider with greater curvature and a greater absolute shell height (Gradela et al., 2017; Moldowan, 2014; Vega and Stayton., 2011).

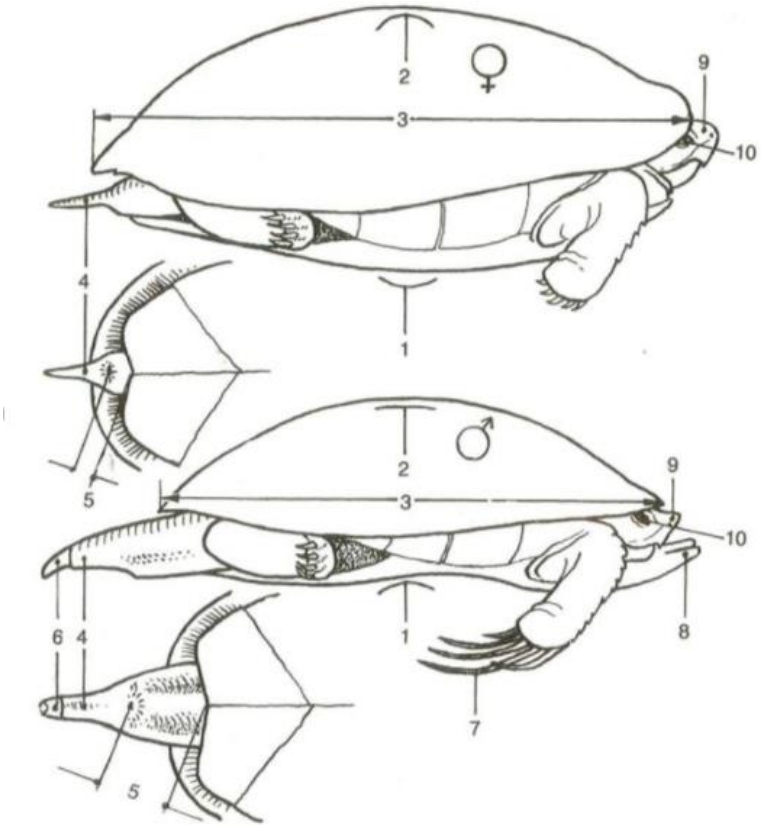

Figure 7. Labeled anatomical differences between female and male turtles. 1-3. Females have a greater curvature than males resulting in a greater absolute shell height for females. 4. Larger tail in terms of length and broadness in males. 5. Distance between cloaca and posterior edge of plastron is greater in males. 6. Keratinized tip absent/minimal on female's tails. 7. Elongated 2nd-3rd foreclaws in males. 8-9. Elongated gular scutes in males and snout in males. 10. Vibrant eye color in males. Image taken from Moldowan, (2014).

The age of turtles was crudely estimated using size of the shell and skin melanin content. Older turtles have a larger, smoother carapace than young turtles as they grow (Aresco et al., 2006). Older turtles, especially males, also accumulate melanin as they age (Aresco et al., 2006). Younger turtles typically have distinct carapace markings and melanin in discrete spots on their plastron; however, with age, the rich colors of the carapace fades which leaves the older turtles with a light brown, yellow-brown, gray, or black shell (Aresco et al., 2006).

Morphometrics

The body size of turtle was measured by obtaining the carapace and plastron lengths and widths as shown in Figure 8 and also the height of the shell and body mass. Additional notes taken include the shell temperature recorded to the nearest 0.1^{o}C using an infrared non-contact thermometer held about 2 cm distance from the top of the carapace and provides an estimate of thermometabolic state. Lastly, any abnormalities in the turtle's physical appearance were recorded, including unique physical markings, scars or other signs of injury or disease, and the presence of leech parasites. All turtles were then released at their site of capture.

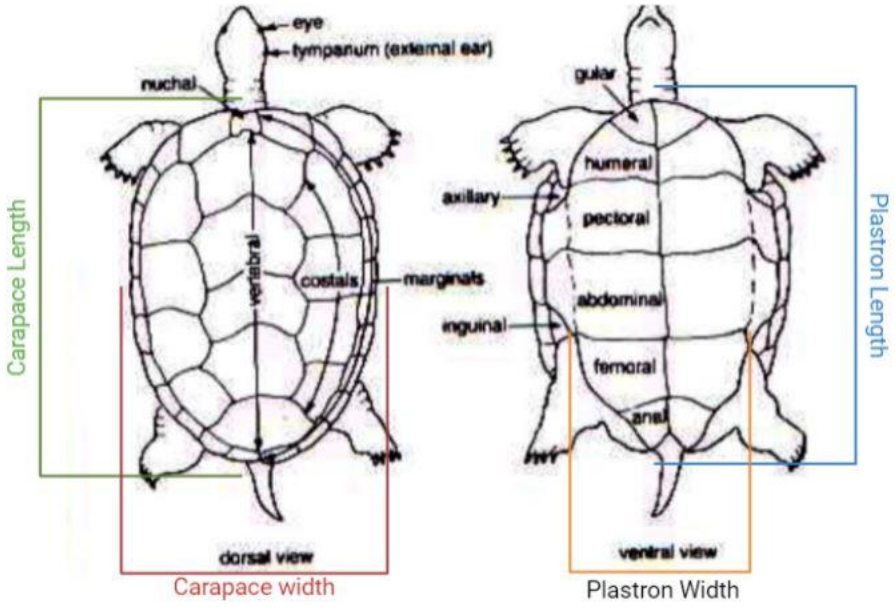

Figure 8. Morphometrics of turtle body size. Carapace length was measured along the vertebral line from the nuchal to pygal scute as indicated by the green bracket. The red bracket shows that the carapace width was measured between the sixth marginal scute of the right and left side of the carapace. Additionally, the plastron length was measured between the intergular and anal scutes as shown by the blue bracket. The plastron width was demarked by the orange bracket and is measured at the line of intersection between the abdominal and femoral scutes. Image adapted from Body Plan (http://reptilis.net/chelonia/bodyplan.html).

Enzyme-linked immunoassays (ELISA) for steroid hormones

The quantification of plasma corticosterone, cortisol and DHEA were conducted using commercial enzyme-linked immunoassays (ELISA) which has been specifically used for turtle

samples (DeVries et al., 2015; Pryor and Casto, 2015). Assays were run according to manufacturer's instructions with plasma samples first diluted 100x prior to the assay. To reduce interassay variation and to ease comparisons, samples from the same subjects were run on the same 96-well microplate. The concentration of steroids in each sample was then calculated by interpolation from the standard curves using GraphPad Prism version 4 software (La Jolla, CA, USA).

The sensitivity of the corticosterone ELISA (Arbor Assays Inc, Ann Arbor, MI, USA) ranged between 20.9 pg/mL and the mean intra-assay and inter-assay precision were 9.4% and 13.2%, respectively (N = 2 plates, 64 samples total). Similarly, the sensitivity and precision of the cortisol ELISA (Arbor Assays Inc, Ann Arbor, MI, USA), were 27.6 pg/mL and the mean intra-assay and inter-assay precision were 11.7% and 16.2%, respectively (N = 2 plates, 64 samples total). The sensitivity, intra-assay, and inter-assay precisions of the DHEA ELISA (Enzo Life Sciences, Farmingdale, NY, USA), were 2.90 pg/mL, 8.9%, and 14.1% respectively.

Statistical Analysis

All data was tested for normality prior to statistical analysis. The effects of each treatment on steroid levels before and after injection were tested with paired t-tests. Linear regression was used to determine how sampling date, body size, body mass, sex, body temperature, and whether the turtle was a recapture effected steroid levels. Significance was set at $p < 0.05$.

Results

In total, 64 turtles were sampled with 13 being recaptures. Recaptures were only tested if at least ten days had passed from previous capture and injection. To verify that recaptures did not create a compounding variable in our experiment, a linear regression showed no significant effect of recapture on cortisol, corticosterone, or DHEA concentrations (all $p > 0.284$). In total, 26 turtles were male and 38 were female, but sex also showed no significant effect for any of the three steroids measured (all $p > 0.073$). The size and weights of the turtles ranged greatly (Table

2) and these measures were highly correlated ($r > 0.893$, $p > 4.601 \times 10^{-42}$). There was no significant effect of size on cortisol, corticosterone, and DHEA levels (all $p > 0.373$).

Table 2. Morphometric Data for red-eared sliders from this study. The overall sizes were measured as demonstrated in Figure 8.

Range in:	Low	Mean	High
Carapace Length (cm)	13.5	23.3 (+/- 0.49)	28.3
Carapace Width (cm)	12.1	20.93 (+/- 20.93)	25.3
Plastron Length (cm)	11.6	19.3 (+/- 0.42)	24.1
Plastron Width (cm)	5.5	9.2 (+/- 0.21)	12.3
Shell Height (cm)	4.6	8.1 (+/- 0.21)	10.4
Shell Mass (kg)	0.3	1.5 (+/- 0.08)	2.6

Date, body mass, carapace length, and temperature were not associated with steroid levels (all $p > 0.063$). However, of note was a consistent negative and positive correlation between body temperature and cortisol concentrations, before ($r = -0.299$, $p = 0.013$) and after treatment ($r = 0.469$, $p = 0.001$) respectively.

Injection of DEX did not see any significant increases in blood steroid concentration (all $t > -1.380$, all $p > 0.102$) (Figure 9). However, injection of saline saw a significant increase in corticosterone concentration ($t = -1.959$, $p = 0.013$), but not in cortisol ($t = 0.472$, $p = 0.325$) or DHEA concentrations ($t = 0.677$, $p = 0.259$) (Figure 9). Similarly, a significant increase in corticosterone concentration ($t = -2.237$, $p = 0.0279$) was seen after injection of ACTH but not in cortisol ($t = -0.252$, $p = 0.404$) nor DHEA concentration ($t = -0.294$, $p = 0.388$) after the same injection.

On the converse, the injection of NPY saw a significant increase in DHEA ($t = -0.931$, $p = 0.035$) and cortisol concentrations ($t = -1.469$, $p = 0.0900$) but not in corticosterone concentration ($t = -0.144$, $p = 0.445$). When considering the receptors of NPY specifically, only

one receptor saw a significant increase in blood steroid concentration. Specifically, the injection of NPY-Y1 and NPY-Y5 agonists did not see any significant changes in blood steroid concentration (all $t > -0.816$, $p > 0.118$). However, the injection of the NPY-Y2 agonist saw a significant increase in DHEA ($t = -1.165$, $p = 0.015$) and cortisol concentrations ($t = -1.165$, $p = 0.015$) but not in corticosterone concentration ($t = -0.211$, $p = 0.419$). This increase in only DHEA and cortisol concentration matched the increases seen with the injection of NPY.

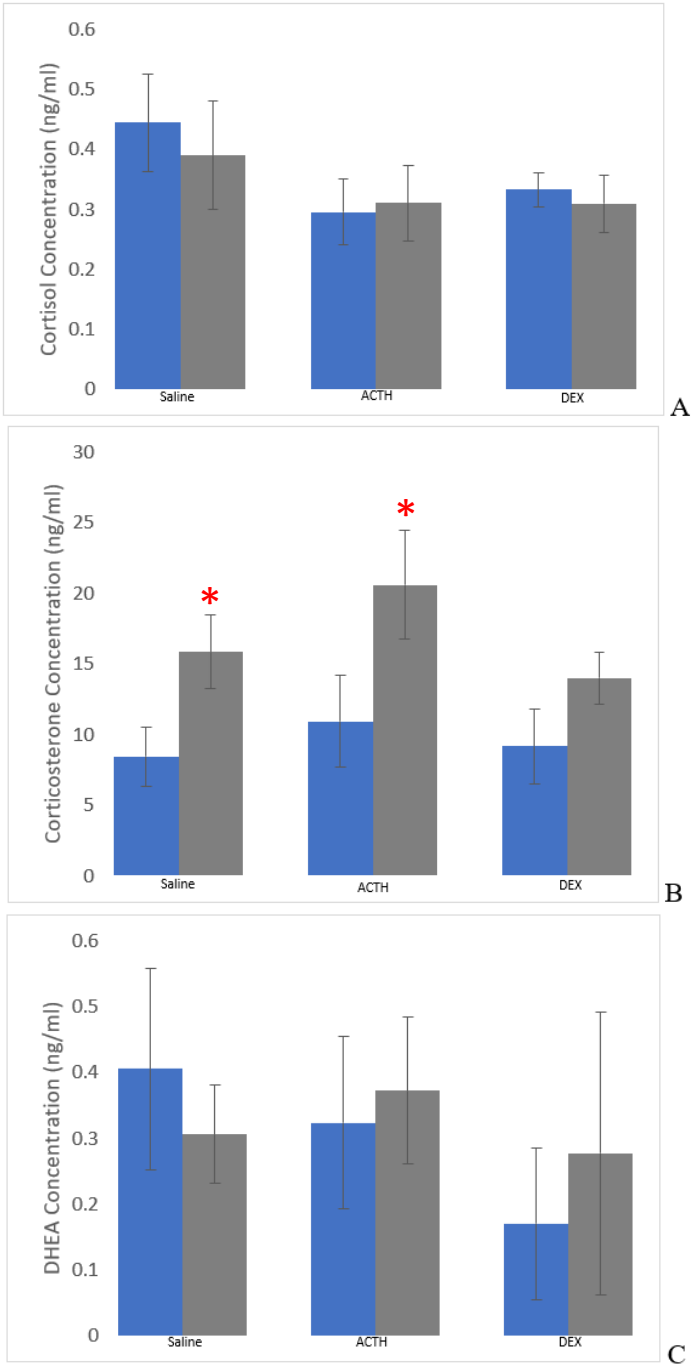

Figure 9. Concentration of steroid hormones after ACTH, DEX, and Saline Injection. Concentrations of cortisol, corticosterone and DHEA before and after treatment with saline, ACTH, or DEX. The blue columns represent the concentration of these steroids prior to treatment while the grey columns represent steroid levels after treatment. The asterisks indicate statistically significant increases in hormone levels after injection ($p < 0.05$).

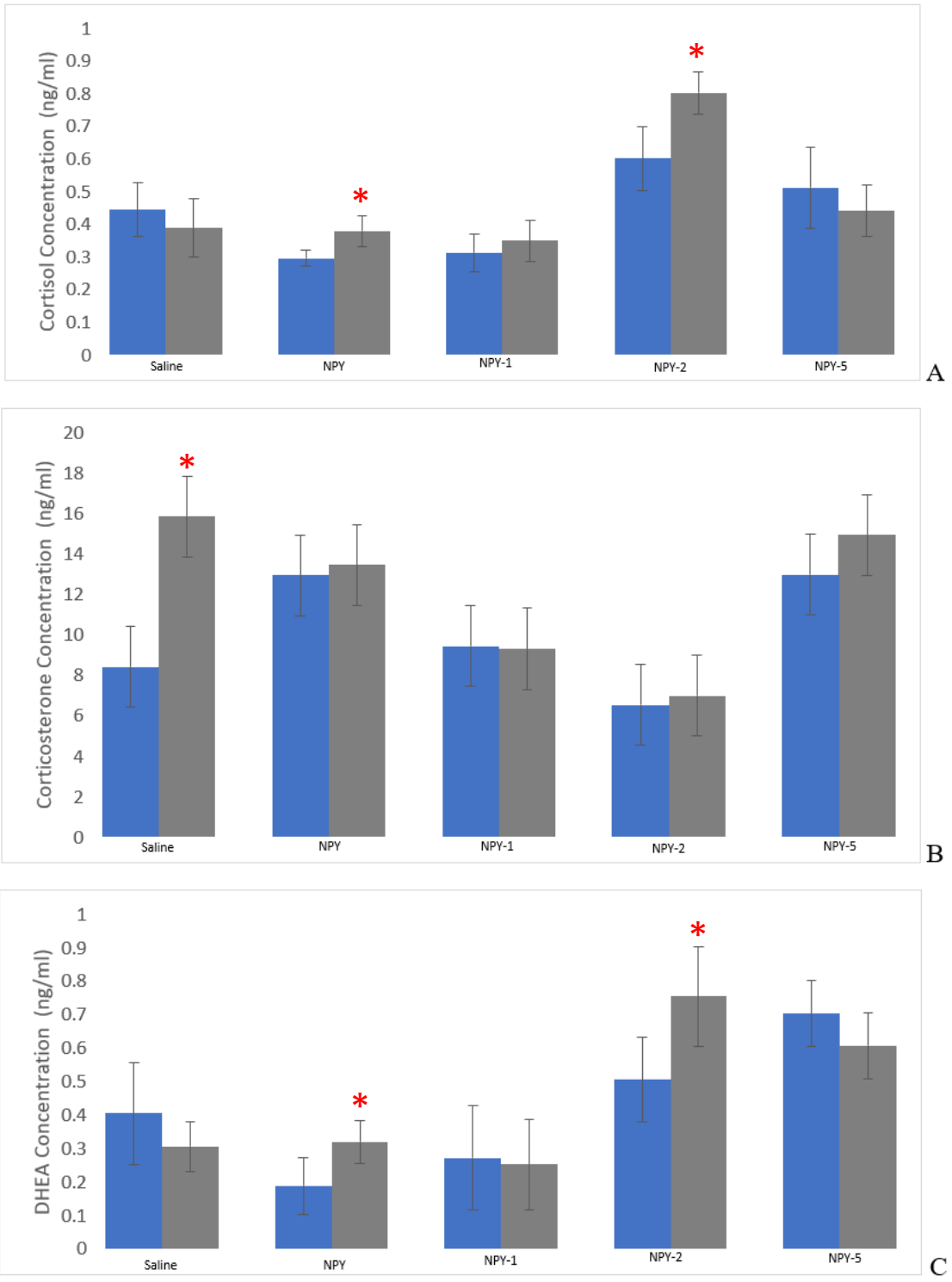

Figure 10. Concentration of steroid hormones after injection of NPY or three different receptor agonists. Concentrations of cortisol, corticosterone and DHEA before and after treatment with saline, NPY, or NPY-Y1, NPY-Y2, and NPY-Y5 receptor agonists. The blue columns represent the concentration of these steroids prior to treatment while the grey columns represent steroid levels after treatment. The asterisks indicate statistically significant increases in hormone levels after injection ($p < 0.05$).

Discussion

This study aimed to understand the functioning of the HPA Axis in Red-Eared Sliders and determine if NPY acts as a secretagogue in reptiles as it may in mammals. The data reveal that ACTH only increased the concentration of corticosterone, and not cortisol or DHEA. Interestingly, NPY only increased the concentration of cortisol and DHEA and an agonist for the NPY-2 receptor also produced this effect. Thus, corticosterone levels are regulated by ACTH, but NPY may be responsible for regulating cortisol and DHEA secretion in turtles, potentially through the Y2 receptor for NPY.

Independent Pathways for Steroid Hormone Release

The hormone ACTH was used to maximize the secretion of both glucocorticoids and DHEA. Surprisingly, ACTH injection only led to an increase in corticosterone concentrations, but not cortisol or DHEA in our study. In contrast, the injection of NPY only led to increases in cortisol and DHEA concentrations. This polarization suggests a pathway for corticosterone release independent of DHEA and cortisol secretion. The results indicate that ACTH could simply be responsible for increases in corticosterone, the major stress marker in reptiles and, that in addition to regulating energy homeostasis, NPY could be the regulator of these secondary steroids, cortisol, and DHEA (Mercer et al., 2011). Additionally, the data presented a correlation between cortisol and DHEA levels; however, this relationship was not present with corticosterone, as seen by the p value in the correlation test. These results reaffirm the idea that DHEA and cortisol may be produced in the same way, independently of corticosterone. This connection between NPY and steroid synthesis is novel, as NPY is currently recognized to be secreted during starvation, which can be clearly perceived as a stressful event (Richardson et al., 1995).

However, these findings do not lessen the importance of the adrenal gland in hormone release. While ACTH only increased the concentration of corticosterone, the injection of DEX was able to shut down the formation of cortisol, corticosterone, and DHEA. Considering DEX is functions by initially blocking ACTH release to induce negative feedback in the HPA Axis, these results suggest that the adrenal gland's production of ACTH is foundational in forming all steroid hormones. This phenomenon is called the permissive effect because ACTH is necessary

to produce basal levels of steroid hormones (Sapolsky et al., 2000). However, considering the effect of NPY injection on DHEA and Cortisol concentration specifically, it is likely that NPY is still required for a significant increase in the concentration of these steroids. Future research could further investigate the independent pathways for corticosterone versus cortisol/DHEA secretion.

Independent Pathways for Stress: Energy vs Capture and Handling

Interestingly, our saline injected group provided mixed data. As a control, saline injections expectedly saw small steroid increases due to the turtle's exposure to the experiment being a stress inducing environment (i.e. capturing turtles and completing injections). The secretion of corticosterone following saline injection matched the original expectation with an increase in concentration that was much less compared to the ACTH injection group (a 9.647 ng/ml increase compared to a 7.422 ng/ml for saline). However, no significant changes in either cortisol or DHEA occurred in the saline group. This suggests that acute stress, unaffiliated with energy balance, does not alter these steroids. Future research could try to separate energy stress from the stress of capture and handling.

Relationship Between Temperature and Steroid Release

Within this research, there was also a surprising relationship between temperature and steroid release. A statistically significant correlation was found between temperature and steroid release. Both before and after injection cortisol levels suggest that the warmer you are, the less cortisol is circulating in the blood stream. While these results initially appeared spurious, the relationship between these two factors (temperature and cortisol concentration) should be further explored. Jaxion-Harm and Ladich (2014) gradually changed the temperature of common carp and found the opposing relationship between cortisol and temperature, stating that cortisol levels decrease as internal temperatures decrease. Our findings were based on an even more gradual temperature change as the organism's body adapted to environmental changes. Future research could further test the effect of temperature by using water baths to quickly change the

temperature of the turtle during the 30-minute isolation period between before/after blood samples.

Novel Connection between NPY and Stress

Quintana et al. (2016) reports that 83-85% of the primary structure of NPY is conserved across birds and teleost fish. Additionally, research by Blomqvist et al. (1992) shows that the amino acid sequence encoding NPY only varies by 1-5 positions in chicken, goldfish, and the ray *Torpedo marmorata*. Thus, it is likely that NPY is also conserved between mice and turtles, meaning our injections will work in both species. Similarly, the NPY receptor system is evolutionarily conserved, and the receptor agonists used in this study have been used in a variety of mammal and bird species, according to the manufacturer (Quintana et al., 2016). However, there is concern that the agonists used match the receptors known to exist in mice and not turtles, and perhaps turtle receptors are structurally too different to be affected by the same agonists. No previous work has been focused on turtle NPY receptors; however, this study saw noticeable significant effects in the agonist for the NPY-2 receptor. The data suggests that the NPY-2 receptor is involved in DHEA and cortisol secretion but did not alter corticosterone levels. The effects were similar to the NPY injection results suggesting that NPY may be exerting its effects through the NPY-2 receptor. More research should attempt to identify whether the NPY-2 receptor is actually present on the adrenal gland of turtles and whether it is structurally similar to those in mammals.

NPY uses a family of G-proteins, including the NPY-2 receptor, localized within the brain and sympathetic neurons of the central and peripheral nervous system; its extensive reach is essential for signaling (Ammar et al., 1996). Current research on NPY-2 receptors shows that it is localized in the brain predominantly due to its role in presynaptic inhibition of neurotransmitter release (Ammar et al., 1996). Specifically, NYP-2 receptors accumulate in the amygdala, hypothalamus, hippocampus, and frontal lobe, but they can also be found in areas that sympathetic neurons innervate including the vas deferens, blood vessels, kidney, and intestinal mucosa. It is possible that the reduced cortisol and DHEA levels could be attributed to the constriction of blood vessels that NPY-2 controls, preventing as much blood from exiting the pituitary gland and in turn hampering the HPA axis system. Alternatively, this study may have

found a novel connection between NPY-2 and the adrenal gland. This connection could be further investigated by injecting the model organism with radioactively labeled NPY-2 agonists and then completing scans of the adrenal gland post-mortem.

This research is important because it provided evidence for a novel connection between NPY and the stress response system. Previously, NPY was believed to only be relevant in energy utilization; however, the data suggests its role in producing cortisol and DHEA. The formation of these steroids appears to occur independent of corticosterone secretion. While these findings were produced in turtles, the high conservation of NPY, specifically that the gene sequence is identical in humans and nine other species, allows one to apply this knowledge to humans (Mercer et al., 2011; Quintana et al., 2016). Thus, for our health, it is critical to know how these steroid hormones are produced because chronically high levels are devastating (Chuanaxin et al., 2020; Jaxion-Harm and Ladich, 2014). Specifically, an excess of cortisol in the blood stream can induce Cushing's syndromes, abdominal obesity, hypertension, and/or osteoporosis (Chuanaxin et al., 2020). Generally, chronically high steroid hormone levels create negative consequences because they challenge the body's negative feedback system and in turn, inhibits the proper functioning of the HPA Axis system (Sapolsky et al., 2000).

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Abbreviations for reference

Abbreviation	Full Name	Function
HPA	Hypothalamic-Pituitary-Adrenal axis	Hormone cascade that leads to an increase in glucocorticoid concentration
CRH	Cortical Releasing Hormone	Released by the hypothalamus at the start of the HPA-Axis after one perceives a stressor
ACTH	Adrenocorticotropic Hormone	Secreted in response to the CRH binding to CRH-R1 receptors and travels to the adrenal cortex to further the HPA Axis hormone cascade
DEX	Dexamethasone	A synthetic steroid that blocks ACTH release and induces HPA negative feedback
PVN	Paraventricular Nucleus	The cells of the hypothalamus that secrete CRH. Also, a downstream connection for NPY.
DHEA	Dehydroepiandrosterone	Steroid hormone produced in the HPA Axis System. Also, the bioactive form of DHEA-S
DHEA-S	Dehydroepiandrosterone Sulfate	The sulfated form of DHEA which is how it is normally found in the blood
NPY	Neuropeptide Y	Extremely orexigenic neurohormone that is released when an organism is in an energy deficient state
Y1	NPY-Y1 Receptor	One of five G-protein receptors. Energy homeostatic effects of NPY are at least partially dependent on the Y1 receptor
Y2	NPY-Y2 Receptor	One of five G-protein receptors. Centralized to the brain and activated by NPY and a gut hormone (PYY) to induce anorexigenic effects
Y5	NPY-Y5 Receptor	One of five G-protein receptors. Primarily localized to the brain and known to increase food consumption