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The regulation of stress steroid release in freshwater turtles

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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 minor 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). Since there are so many different ways stress can be exhibited behaviorally and physiologically within a turtle, it is important to select a

specific way in which stress is measured. In this study, I used the HPA axis system to characterize the stress response and understand the role of specific hormones/intermediates.

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, CRH binds to the CRH-R1 receptors on corticotropic cells and then adrenocorticotropic hormone (ACTH) is secreted in response (Tsigos et al., 2000; Whitham, 2020). This step is important because, prior the production of ACTH, the HPA axis is localized to the brain. However, since ACTH is blood soluble it can be secreted into the blood stream to travel beyond the brain to the target organ. The target organ is the adrenal cortex which is the location for the conversion of cholesterol to pregnenolone and the first precursor for all steroid hormones (Figure 1).

Steroid hormones are cholesterol-derived compounds with four hydrocarbon rings and oxygenated side chain functional groups which makes them hydrophobic. 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). In addition to glucocorticoids, within the group of steroid hormones is dehydroepiandrosterone (DHEA), a possible antagonist to glucocorticoids.

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 in birds (Pied Flycatchers, Ficedula hypoleuca) 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.

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).

Negative Feedback

While steroid hormones appear to be the end of the HPA axis system, the cascade is more complex. Thus, the concentration of these steroid hormones is relevant in controlling the earlier parts of the HPA axis, known as negative feedback. 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). This second mechanism (inhibition of ACTH secretion) will be artificially induced by injection of DEX.

Negative feedback works to regulate the HPA axis by having the hormones secreted as output influencing the hormones secreted at the beginning of the cascade, 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.

NPY and Stress

Stress responses involve metabolic shifts, and so, energy deficits occurring in starvation periods are considered highly stressful. Neuropeptide Y (NPY) is an extremely orexigenic neurohormone that is released from the hypothalamus when an organism is in an energy deficient state (Joly-Amado, 2014). In other words, it is a neurohormone released when an organism is in a starvation state. 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 degree of conservation across organisms, it is believed that NPY is involved in many physiological processes, beyond energy conservation, including sleep, stress, metabolism, and cardiovascular functioning (Joly-Amado, 2014; Mercer et al., 2011).

NPYs largest role is during a starvation state which makes sense as it is released from the hypothalamus, a specialized region in the brain that 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 known 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. Thus far, most research focuses on NPYs effect on the brain and cardiovascular system. Specifically, 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).

To further study NPY, it is also important to consider its structure. NPY has multiple receptor types (Y1 through Y5) through which signaling can occur. To target one of these specific receptors an agonist can be used. An agonist can be either an exogenous (injected/ingested) or endogenous (produced by the body) chemical that binds to a specific receptor to fully activate it. For example, in order to activate the Y1 receptor, ones body must have access to the Y1 receptor agonist. This agonist is specific, and thus, it will bind to only the Y1 receptor, not Y2, Y3, Y4, or Y5.

While little research has been conducted on NPY Y1, Y2, and Y5 receptors, some research has indicated that these receptors appear particularly important in the context of stress.

Specifically, 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 receptor 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 with an agonist (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). Considering the roles of these agonists and the fact that NPY is important in starvation, it seems plausible that one of these receptors is particularly important in the stress response. Thus, NPY and its receptors should be studied to find this connection between stress and starvation.

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 caught turtles using baited hoop traps. Then, I used pharmacological injections of ACTH (intermediate in HPA axis), dexamethasone (DEX, a synthetic steroid that blocks ACTH release and induces HPA negative feedback), NPY (starvation hormone), and NPY receptor agonists. Each turtle received a single injection upon capture, and I recorded whether adrenal steroid secretion is stimulated or decreased due to negative feedback (see example in Fokidis and Deviche, 2010).

I hypothesized that ACTH injection would increase glucocorticoid and DHEA production. This hypothesis is supported by the evidence that suggests that ACTH is an intermediate in the HPA axis system. ACTH is a blood soluble molecule produced by the anterior pituitary gland, and it allows for the stress response system to extend beyond the brain and form adrenal steroid hormones. 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. Specifically, DEX will prevent ACTH from entering the blood stream, confining the stress response system to the brain. Lastly, I hypothesized that NPY injection will activate the stress response and increase steroid levels, because the literature indicates NPY is involved in starvation, a stressful state for the body. Additionally, I expect one specific NPY receptor will be

particularly important in the stress system, and so, I predict an increase in steroid hormone concentration after injection of an NPY receptor agonist. Thus, this study may help establish a novel connection between stress response and food intake.

Methods

Model Species

The model organism for this work is the Red-Eared Slider, *Trachemys scripta elegans.* The Red-Eared Slider was the first reptile admitted into the United States National Museum Collection, after being first described in 1885. Red-Eared Sliders, 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 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 Red-Eared Slider is native to the East and Central United States, but due to releases from the pet trade, they can now be found worldwide, on all continents other than Antarctica, and are named the only globally invasive species of turtle (Rodrigues et al., 2016; Taniguchi et al., 2017). The invasion of sliders into new areas has led to competition and exclusion of native turtle populations (Meylan, 2006; Thomas, 2006). So, while a specimen of this species was first obtained near Charleston, South Carolina, it is now found throughout the world.

The ability of sliders to dominate aquatic ecosystems can be attributed to several factors. Firstly, sliders 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 sliders to produce a large clutch size of 12.5-15.1 eggs (Tucker et al., 1998). Finally, sliders 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 limitations on food and space as they are territorial creatures (Chen, 2006). Red-Eared sliders also dilute the gene pool by forming hybridized populations with other species at contact zones, 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 sliders became invasive, humans are also there largest threat (Thomas, 2006). Ways in which humans threaten sliders include fishing, trade, and transport. Fishing is detrimental as some fishermen use slider eggs as bait, or sliders are inadvertently caught and killed as fishermen 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 turtles 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 vehicles, has led to high vehicular mortality of turtles (Thomas, 2006).

Field Methods:

Study Site and Turtle Trapping

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 study was conducted in Lake Virginia in Winter Park, Florida along the shoreline of Rollins College between September 2021 to March 2022. This ecosystem is the focus for this study due to its large population of Red-Eared sliders. Red-Eared sliders within Lake Virginia have been studied by Fokidis and his students for about four years.. During this project, baited hoop traps were set out every week from September 2021 to March 2022 (except for December) to catch turtles. 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 every week, usually

over three nights and checked daily. Red-Eared Sliders are the dominant turtle found in Lake Virginia, accounting for almost all turtle captures during this period. Captured turtles were examined on shore and released at the site of capture immediately after the examination was completed.

Pharmacological Injections and Blood Sampling

After successfully capturing a turtle, the first step was to obtain a blood sample immediately upon removal from the trap to measure blood steroid concentration prior to injection. For each captured turtle, a blood sample was taken from the subcaparial sinus on the base of the carapace. A sinus is a large pool of blood, and this sinus is specifically located just below the carapace of the turtle. Then, turtles were = 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. Thirty minutes 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 captured turtle received only a single injection of one treatment group 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.

Treatment Group	Saline	ACTH	DEX	NPY	NPY-Y1 agonist	NPY-Y2 agonist	NPY-Y5 agonist
Chemical Name	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

While in the field, each turtle was uniquely marked using the 1-2-4-7 numbering system developed by Cagle (1939), providing each turtle a unique number for future identification (Figure 2). Marks were made in the marginal scutes of the turtle shell using a small rotary tool. 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 injections to wear off. Ten days is considered beyond the typical span these injections work within the body as they are fast-acting compounds (Fokidis, 2016).

Determining Sex and Age in Red-Eared Sliders

The sex of all subjects was identified based on several characteristics including: nail length, shape of plastron, and overall size. (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).

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 (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. The height of the shell and body mass were also measured using calipers and a spring scale with drawstring bag, respectively. 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 as it 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.

Laboratory Methods:

Enzyme-linked immunoassays (ELISA) for steroid hormones

Enzyme-linked immunoassays (ELISA) can be used to quantify peptide, hormone, or protein concentrations. Previous research by DeVries et al. (2015) and Pryor and Casto (2015) have been successful in measuring adrenal steroid levels in turtle blood samples. So, the

quantification of plasma corticosterone, cortisol and DHEA were conducted using a commercial ELISA kit. 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. The precision of the assays was important because two plates were necessary to check the concentration of each steroid due to there being 128 samples (n = 64). The precision indicated that there were minimal differences between wells on the same or different plates.

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, 51 unique turtles and 13 recaptures were tested. This totaled to 128 turtles blood samples being collected at two time points (n = 64). 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).

Table 2. Morphometric Data for Red-Eared sliders from this study. The overall sizes were measured as demonstrated in Figure 4.

Ranges 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 result in any significant increases in blood steroid concentration (all $t > -1.380$, all $p > 0.102$) (Figure 3). However, injection of saline resulted in 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 3). Similarly, a significant increase in corticosterone concentration ($t = -2.237$, $p = 0.0279$) was measured 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 resulted in 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 resulted in a significant increase in blood steroid concentration. Specifically, no significant changes in blood steroid concentration (all $t > -0.816$, $p > 0.118$) were measured after the injection of NPY-Y1 and NPY-Y5 agonists. However, after injection of the NPY-Y2 agonist 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 ($t = -0.211$, $p = 0.419$) were measured. This increase in only DHEA and cortisol concentration matched the increases seen with the injection of NPY.

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 increased the concentration of corticosterone, but not the concentration of cortisol or DHEA. However, 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 and Handling Stress

While the injection of ACTH was expected to maximize the secretion of both glucocorticoids and DHEA, ACTH injection only led to an increase in corticosterone concentrations. Cortisol and DHEA concentration levels did not increase in response to ACTH injection. 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 my suggestion that DHEA and cortisol may be produced in the same way, independently of corticosterone. This connection between NPY and

steroid synthesis is novel, but it fits with the current knowledge on NPY and its role in starvation (Richardson et al., 1995).

Specifically, I suggest that the formation of these hormones may occur using two independent pathways: 1) ACTH → Corticosterone 2) NPY-Y2 → Cortisol/DHEA. This polarization of the pathways aligns with the levels of these hormones in the blood and the role of their precursor molecules. Corticosterone is the major steroid hormone in reptiles while cortisol and DHEA are the minor steroid hormones. This polarization of pathways suggests that acute stress, unaffiliated with energy balance, does not alter the levels of the secondary steroids. Specifically, NPY is a hormone important in starvation and energy balance, and energy balance is a vital component of handling stress. So, it seems that NPY, and specifically the Y2 receptor, could be required for the secretion of DHEA and Cortisol. Similarly, since ACTH injections induced a statistically significant increase in Corticosterone levels, ACTH seems particularly important for the production of corticosterone.

Despite the particular importance of ACTH and NPY in the release of these steroid hormones, these findings do not lessen the importance of the adrenal gland in overall 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 functions by blocking ACTH release to induce negative feedback in the HPA Axis, 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, and the NPY-Y2 receptor specifically, is still required for a significant increase in the concentration of these steroids. Future research could more thoroughly investigate the independent pathways for corticosterone versus cortisol/DHEA secretion. Then, it can further clarify the separation of energy stress (ACTH → corticosterone secretion) from the stress of capture and handling (NPY-Y2 → Cortisol/DHEA secretion).

Stress Inducing Methodology

Turtles in a trap represents an interesting problem for a stress biologist because the methodology required in assessing their stress can be stress inducing. To measure how stressful

our methodology was on the turtles, an injection of saline was used as a control. However, our saline injected group provided mixed data. As a control, saline injections expectedly resulted in small steroid increases due to the methodology of the experiment (i.e. capturing turtles and completing injections is stressful for the captured turtles). There was a slight increase in cortisol and DHEA levels after injection with saline; however, these changes were not statistically significant. Contrastingly, the secretion of corticosterone following saline injection had a statistically significant increase. However, this increase in concentration of corticosterone was much less compared to the ACTH injection group (a 9.647 ng/ml increase compared to a 7.422 ng/ml for saline). Thus, it can still be stated that the injection of ACTH increases levels of corticosterone and that ACTH is important for the production of high corticosterone levels.

Additionally, this data is further evidence for the independent pathways used for increasing hormone concentrations. Specifically, it is possible that corticosterone concentration increased specifically because it is the primary hormone secreted during a stressful event. So, when a turtle is being captured and is experiencing a fight-or-flight response, its response can be physiologically measured by examining the corticosterone level.

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, Blomqvist et al. (1992) show 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 there is enough similarity in the NPY genomic structure to use the injections for both turtles and mice. 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 focused on turtle NPY receptors; however, this study measured significant effects in steroid hormone concentration after the injection of the agonist for the NPY-2 receptor. Thus, the results suggest 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 and currently known to be localized within the brain and sympathetic neurons of the central and peripheral nervous system; the extensive reach of NPY 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.

Application

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 elevated levels are detrimental to one's health (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). Thus, 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

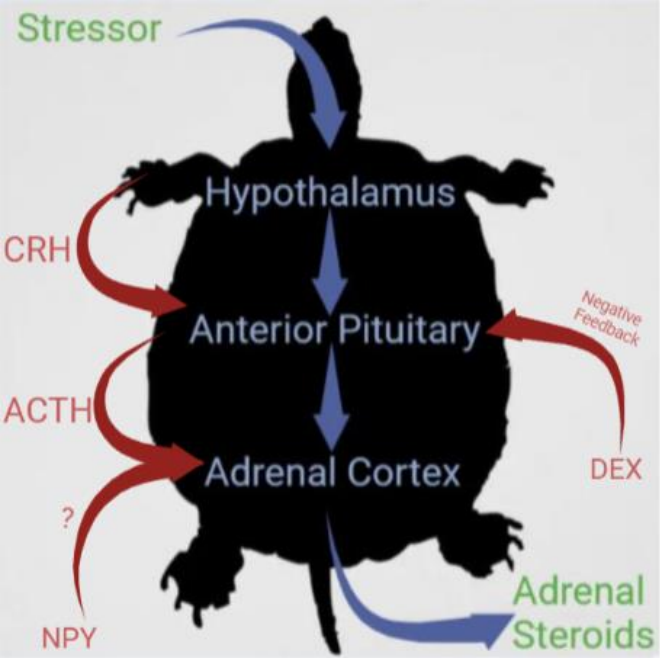


Figure 1. HPA axis system for the synthesis of the adrenal steroids. This process begins by the hypothalamus perceiving a stressor and then communicating with the anterior pituitary gland, and finally the cortex of the adrenal gland. In the HPA axis, corticotropin releasing hormone (CRH) is produced, 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.

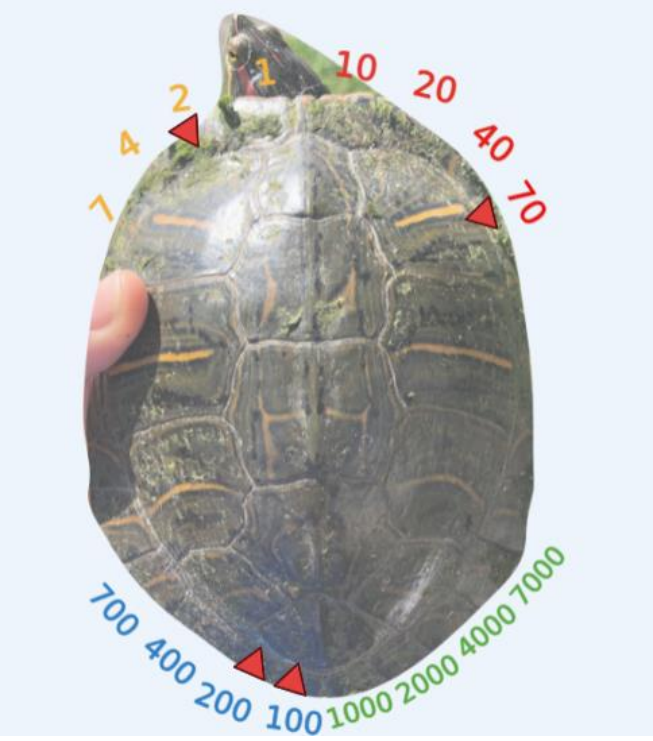
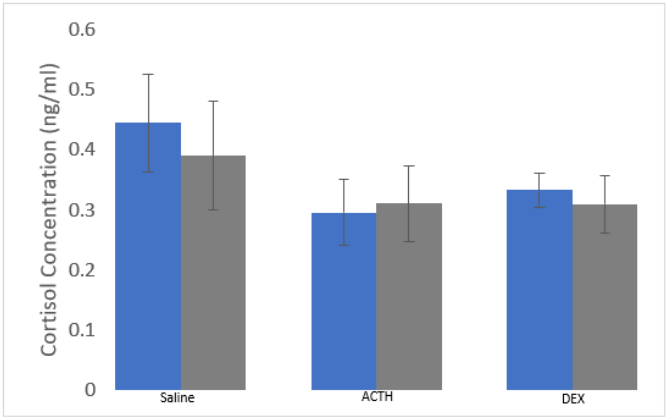
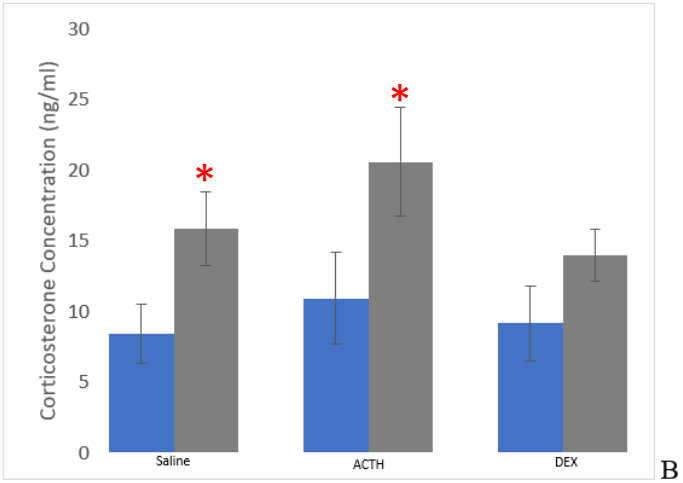


Figure 2. The system for turtle identification utilizes a series of quadrants and a 1-2-4-7 numbering system. Triangular shaped notches are then made on the corresponding scute with a rotary tool to clearly distinguish between turtles and identify recaptures. Here an example of turtle #372 is provided.



A



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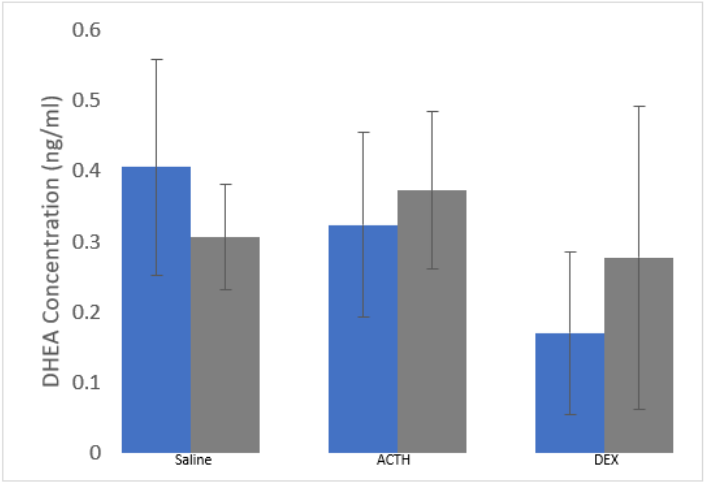

C

Figure 3. Concentration of steroid hormones after ACTH, DEX, and Saline Injection. Concentrations of cortisol (A), corticosterone (B) and DHEA (C) 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 ($p < 0.05$).

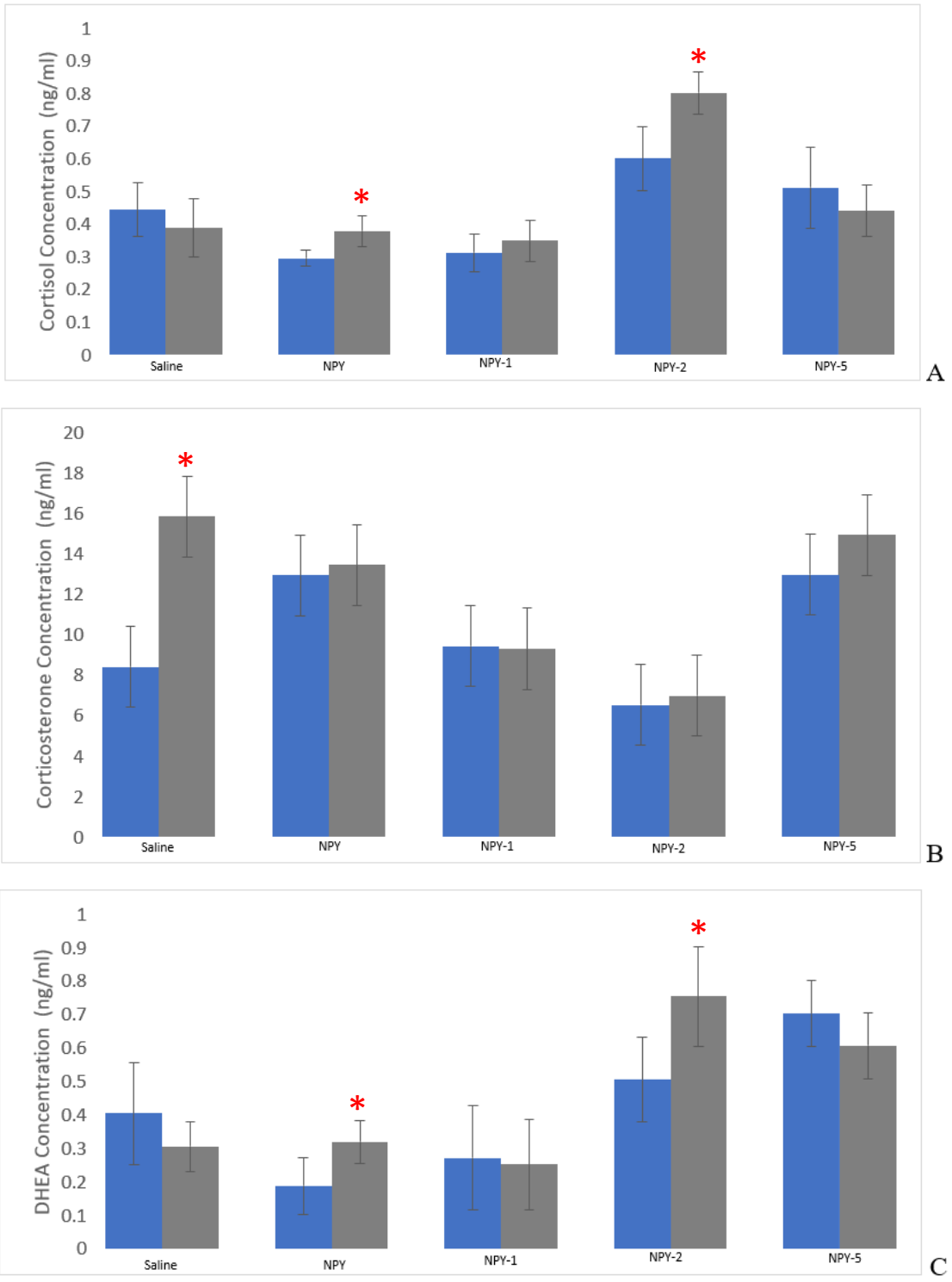

Figure 4. Concentration of steroid hormones after injection of NPY or three different receptor agonists. Concentrations of cortisol (A), corticosterone (B) and DHEA (C) before and after treatment with saline, NPY, or NPY-Y1, NPY-Y2, and NPY-Y5 receptor agonists. The blue columns represent the concentration of these steroids prior to injection while the grey columns represent steroid levels after treatment. The asterisks indicate statistically significant increases in hormone levels after injection ($p < 0.05$).