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Exploring the Glucocorticoid Actions of 1α -Hydroxycorticosterone (1α -OH-B) in the Elasmobranch Fishes

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The University of Southern Mississippi

EXPLORING THE GLUCOCORTICOID ACTIONS OF

1α -HYDROXYCORTICOSTERONE (1α -OH-B) IN THE ELASMOBRANCH FISHES

by

Faith Nichole Lambert

A Thesis
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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December 2014

ABSTRACT

EXPLORING THE GLUCOCORTICOID ACTIONS OF 1 α -HYDROXYCORTICOSTERONE (1 α -OH-B) IN THE ELASMOBRANCH FISHES

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The corticosteroid 1 α -hydroxycorticosterone (1 α -OH-B) is unique to the elasmobranch fishes. It is thought that 1 α -OH-B acts as both the primary glucocorticoid (GC) and mineralocorticoid (MC) in these fishes, a dual role analogous to that of cortisol in the teleost fishes. The MC characteristics of 1 α -OH-B are well supported, but data supporting its GC functions are lacking. In this study, the putative GC actions of 1 α -OH-B were examined. The first experiment characterized the physiological stress response of the Atlantic stingray (*Dasyatis sabina*) to air exposure, with particular regards to the roles of corticosteroids and metabolic fuels. Results demonstrate that corticosteroids increase in a corresponding manner to glucose, supporting a GC role for 1 α -OH-B. Also, to determine the ability of 1 α -OH-B to regulate the transcription of genes classically regulated by GCs, I isolated and sequenced the mRNA encoding serum- and glucocorticoid- inducible kinase 1 (SGK1). *SGK1* mRNA abundance was upregulated in red blood cells incubated with 1 α -OH-B. The results of these studies support the putative GC actions of 1 α -OH-B, including the correlation of 1 α -OH-B with basal glucose *in vivo* and the first report of direct mRNA regulation by this unique corticosteroid. Future studies should focus on the characterization of transcriptional regulation by 1 α -OH-B, including the identification of GC response elements in the promoter of *SGK1* and other genes, and also the development of a specific antibody for the quantification of 1 α -OH-B.

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CHAPTER I

INTRODUCTION

Steroid hormones are a class of biological chemicals produced from cholesterol precursors in the adrenal/interrenal and gonadal tissues of vertebrates. Corticosteroids are a type of steroid hormone that are further divided into two groups: the mineralocorticoids (MC) and glucocorticoids (GC). MCs play a central role in the control of hydromineral balance, regulating ion balances through varying mechanisms including the control of membranous ion transporters. GCs regulate multiple metabolic processes, including glucose metabolism, and are especially important in mobilizing energy stores during the stress response. Both of these molecules play a significant role in many physiological functions.

MC and GC molecules are lipophilic and therefore cannot be readily stored by cells. They are produced as they are needed and immediately released into circulation. These steroid hormones readily pass through cellular membranes, where they bind to intracellular mineralocorticoid receptors (MR) or glucocorticoid receptors (GR), respectively. MRs and GRs form dimers that then activate processes downstream, including transcriptional regulation of genes containing hormone response elements (HRE) in their promoter regions.

In mammalian, amphibious, reptilian, and avian species mineralocorticoid and glucocorticoid functions are carried out by separate molecules. Either cortisol or corticosterone acts as the main glucocorticoid while aldosterone mainly performs mineralocorticoid functions. In teleosts, however, cortisol is the dominant corticosteroid hormone, and aldosterone is absent. Cortisol functions as both a mineralocorticoid and

glucocorticoid in these fish by binding to both the MR and GR with high and low affinity, respectively (Mommsen et al., 1999). If bound to the MR, cortisol elicits MC functions. If bound to the GR, cortisol elicits GC functions. In some respects, this can be considered a more simplified system.

A unique situation is present in the elasmobranch fishes, where 1α -hydroxycorticosterone (1α -OH-B) is the predominant corticosteroid. 1α -OH-B is only found in these taxa, where it is the major product of the interrenal tissue (Idler et al., 1967a; Klesch and Sage, 1975; Nunez and Trant, 1998). Previous publications have indicated that it likely possesses a dual role as a mineralocorticoid and glucocorticoid, similar to cortisol in the teleost fishes (for review, see Anderson, 2012). The MC properties of 1α -OH-B have been well documented, but evidence supporting the GC functions of this molecule are almost entirely lacking. Elucidating the potential GC functions of this ancient steroid is vital to our understanding of the elasmobranch stress response, which has important implications for the conservation of these species. Traditionally, cortisol is utilized as a stress indicator in teleost fishes. Understanding the role of 1α -OH-B would provide a similar tool for the elasmobranch fishes, allowing researchers to more readily evaluate the elasmobranch stress response. Additionally, elucidating the role of this corticosteroid will help aid in understanding the evolution of the HPA/HPI axis among different taxa.

Until recently, the lack of synthetic 1α -OH-B availability and the need for accurate measurement techniques significantly limited progress in this field. There is no specific antibody for 1α -OH-B, which has made developing a specific assay for this corticosteroid complicated. However, the validation of a corticosterone enzyme linked

immunosorbant assay (ELISA) for the quantification of 1α -OH-B (Evans et al., 2010) and availability of synthetic 1α -OH-B (University of Mississippi) are now allowing for further advances in its study.

The Physiological Stress Response and Corticosteroids

The stress response is an organism's reaction to a perceived threat and is composed of three parts (primary, secondary, and tertiary responses) (Barton, 2002). During the primary response, the endocrine system is activated. Catecholamines (epinephrine and norepinephrine) are released from storage almost immediately and facilitate the "fight or flight" response (Reid et al., 1998). This consists of energy store releases, improved cardiovascular tone, enhanced cognition, and the suppression of processes not necessary for immediate survival (Romero and Butler, 2007). These factors allow the animal to respond quickly by either fleeing the threat or fighting it off.

The primary stress response also stimulates the hypothalamic-pituitary-interrenal (HPI) axis (Figure 1), which initiates a cascade of hormones culminating in glucocorticoid release (Barton, 2002; Sapolsky et al., 2000). Initially, the hypothalamus releases corticotrophin releasing hormone (CRH), which stimulates the pituitary to release adrenocorticotrophic hormone (ACTH) (Romero and Butler, 2007). ACTH then activates production of glucocorticoids in the adrenal or interrenal tissue (Romero and Butler, 2007). The course of these actions takes a significantly longer time than the release of catecholamines. Initial effects of glucocorticoids may not be detected until approximately 3-5 minutes or more after the initiation of a stressor (Sapolsky et al., 2000). These effects also last for an extended period of time (from hours to days).

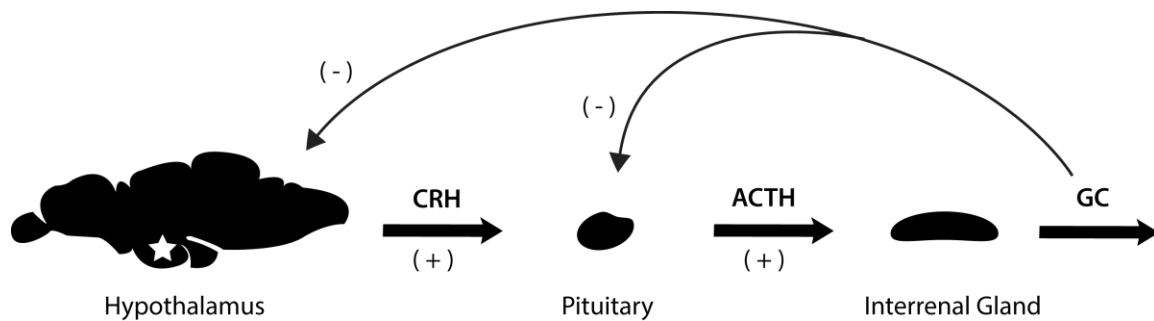


Figure 1. Hypothalamic-Pituitary-Interrenal Axis. Corticotropin releasing hormone (CRH) is released from the hypothalamus after perception of a stressor. CRH stimulates release of adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn stimulates glucocorticoid (GC) release from the interrenal gland. GCs then act in a negative feedback loop on the hypothalamus and pituitary to shut-down the hormonal cascade.

The secondary stress response consists of changes caused by catecholamines and glucocorticoids. These include changes in blood constituents and occur in order to maximally aid in survival (Barton, 2002). The affected organism will experience the release of energy stores such as glucose, accumulation of lactate from anaerobic glycolysis, respiratory acidosis (increased $p\text{CO}_2$), blood acidosis (decreased pH), increases in plasma electrolytes, changes in hematocrit levels, and upregulation of genes involved in combating cell-level stress including transcription factors and other regulatory proteins, e.g., the serum- and glucocorticoid-inducible kinases (SGK) (Barton, 2002; Romero and Butler, 2007; Sapolsky et al., 2000; Webster et al., 1993a, 1993b).

Persistent stressors may cause secondary response effects to become chronic, resulting in a tertiary response (Barton, 2002). Short-term allocation of energy stores towards combating stress can provide the necessary means for immediate survival. However, extended states of disturbed homeostasis result in long-term deprivation of metabolic fuels in processes that include growth, immune function, and reproduction. Insufficient maintenance of these systems results in the degradation of the organism's

overall health and fitness (Romero and Butler, 2007). Persistent issues can result in a population level crisis (Barton, 2002).

1 α -hydroxycorticosterone (1 α -OH-B)

Unlike its putative role as a glucocorticoid, the role of 1 α -OH-B as a mineralocorticoid has been well defined. Mineralocorticoid actions have been demonstrated by sodium retention as the result of 1 α -OH-B injections into rats (Idler et al., 1967b) and sodium transport across an isolated toad bladder (Grimm et al., 1969). 1 α -OH-B increases in dogfish (*Squalus acanthias*) following stepwise transfers to 50% salinity, which is further evidence for a role of 1 α -OH-B in ion retention (Hazon and Henderson, 1984). However, there is a lack of direct evidence supporting its role as a glucocorticoid. Injections of mammalian CRH and ACTH into dogfish (*Scyliorhinus canicula*) increased levels of circulating 1 α -OH-B, suggesting its importance in the stress response (Hazon and Henderson, 1985). 1 α -OH-B has also been shown to bind the ligand-binding domain of the putative glucocorticoid receptor of several elasmobranch species *in vitro* (Carroll et al., 2011). Previous studies have reported low levels of circulating corticosterone, a possible precursor to 1 α -OH-B, in the blood of several elasmobranch species (Rasmussen and Crow, 1993; Snelson et al., 1997; Truscott and Idler, 1972). However, the elasmobranch interrenal gland has only been shown to generate 1 α -OH-B in response to stimulation by ACTH (Klesch and Sage, 1975; Nunez and Trant, 1998). Furthermore, reported elevations in plasma corticosterone have been acknowledged as likely being caused by a low cross-reactivity of corticosterone antibodies with 1 α -OH-B rather than the presence of corticosterone itself (Manire et al., 2007). To date there have been no published measurements of circulating 1 α -OH-B in

elasmobranchs during exposure to stress. Furthermore, while injection of ACTH results in increased plasma glucose in *S. acanthias* (DeRoos and DeRoos, 1973), there are no records of direct effects of 1α -OH-B on glucose metabolism, a primary action of glucocorticoids and hallmark of the secondary stress response. However, this may be complicated by the unusual energy metabolism of the elasmobranch fishes, as discussed below.

Elasmobranch Ketone Body Metabolism

The elasmobranch fishes seem to have a unique dependence on ketone bodies. Ketone bodies are metabolic fuels produced by the liver and include acetoacetate, β -hydroxybutyrate (β HB), and acetone. These molecules generally circulate in low concentrations and are used as metabolic fuels when glucose (the primary oxidative fuel) is not readily available (Laffel, 1999; Nordlie et al., 1999). However, multiple studies have indicated that the energy metabolism of elasmobranchs has a heavy reliance on ketone bodies (primarily β HB; for review, see Speers-Roesch and Treberg, 2010). β HB appears to increase the efficiency of multiple physiological functions in these fishes including osmoregulation (Walsh et al., 2006) and muscle recovery (Richards et al., 2003). In addition, the comparatively high activity of D- β -hydroxybutyrate dehydrogenase (β HBDH, which is utilized in ketone body metabolism) in elasmobranch tissues, as opposed to that of teleosts, suggests that these compounds are of primary importance as fuel in elasmobranchs (Zammit and Newsholme, 1979).

The liver, which is a primary organ for producing oxidative fuels, has been shown on multiple occasions to preferentially produce ketone bodies in the elasmobranch fishes. The livers of multiple shark species demonstrated high capacities for ketogenesis in a

study conducted by Watson and Dickson (2001). Furthermore, hepatocytes from the little skate, *Raja erinacea*, produce ketone bodies *in vitro* (Mommsen and Moon, 1987). Therefore, whereas the glucocorticoids of most taxa stimulate gluconeogenesis in the liver, the primary “glucocorticoid” function of 1α -OH-B may instead be to induce ketogenesis.

Experimental Design and Hypotheses

This study provides deeper insight to the physiological stress response of elasmobranchs, with emphasis on the role of 1α -OH-B during these changes. The project is divided into two main parts that encompass both *in vivo* and *in vitro* experiments. The first focuses on the role of 1α -OH-B in the endocrine stress response and evaluates changes in circulating levels of 1α -OH-B, glucose, β HB, and other relevant blood constituents following induced stress. The second set of experiments focuses on a likely gene target for direct regulation by 1α -OH-B, serum- and glucocorticoid-inducible kinase 1 (*SGK1*). The mRNA encoding SGK1 was isolated and characterized. Its involvement during the stress response and regulation at the transcriptional level, after exposure to 1α -OH-B *in vitro*, was also evaluated.

In the following chapters, experiments will address four main hypotheses:

1. Circulating concentrations of 1α -OH-B in elasmobranchs increase as a part of the physiological stress response.
2. β HB increases as circulating levels of 1α -OH-B increase *in vivo*.
3. *SGK1* mRNA increases in red blood cells *in vivo* during the stress response of elasmobranchs.
4. When elasmobranch tissues are exposed to 1α -OH-B *in vitro*, *SGK1* mRNA transcript abundances increase.

Animal Model

Dasyatis sabina, the Atlantic stingray, was the model species for this study. *D. sabina* are accompanied by a wealth of published knowledge regarding their biology, physiology, and molecular endocrinology. This species has previously been used to study the *in vitro* regulation of 1 α -OH-B synthesis by stress and osmoregulatory hormones (Evans et al., 2010). Additionally, mRNA encoding multiple enzymes involved in 1 α -OH-B steroidogenesis have been isolated and sequenced from this species (Evans and Nunez, 2010).

D. sabina are abundant inhabitants of the Mississippi sound, where they are easily attainable by trawling. Captured animals were maintained at The University of Southern Mississippi Gulf Coast Research Laboratory and fed a diet of shrimp every other day until satiated. All procedures involving *D. sabina* were approved by The University of Southern Mississippi Animal Care and Use Committee (IACUC protocol #13031403, see Appendix).

CHAPTER II

THE PHYSIOLOGICAL RESPONSE OF THE ATLANTIC STINGRAY, *DASYATIS SABINA*, TO AN ACUTE STRESSOR

Background

It is well known that the classical endocrine stress response leads to glucocorticoid production (Barton, 2002; Sapolsky et al., 2000). Glucocorticoids aid in an organism's fight or flight response through the mobilization of energy stores including stimulation of gluconeogenesis (Sapolsky et al., 2000). Although it is presumed that 1α -OH-B functions as the main glucocorticoid hormone of elasmobranchs, there has been no published data directly indicating the effects of stress on its production or direct effects of 1α -OH-B on glucose metabolism (Anderson, 2012).

The elasmobranch stress response is composed of physiological disturbances that closely reflect those of stressed teleost species. The secondary stress response has been well documented for a variety of elasmobranchs (Brooks et al., 2011; Hoffmayer et al., 2012; Hoffmayer and Parsons, 2001; Kneebone et al., 2013). Stress levels in these species can be assessed by characteristic changes in blood chemistry, such as osmolytes imbalance, blood acidosis, and increased metabolic fuel mobilization, as described previously.

Ketogenesis may play a unique and essential role in the elasmobranch stress response. The relatively high concentrations of ketone bodies (especially β HB) in elasmobranch species may indicate their importance as an energy source for these organisms. To date, no measurements of β HB concentration during exposure to stressors have been documented in elasmobranchs. The findings of this study therefore provide

novel information regarding the role of ketone bodies in elasmobranch energy metabolism.

Air exposure is a common stressor experienced by elasmobranchs caught as bycatch in both commercial and recreational fishing (Cicia et al., 2012; Renshaw et al., 2012) and results in significant departures from physiological homeostasis. Air exposure causes collapse of the gill lamellae, thereby inhibiting gas exchange and severely reducing oxygen availability (Cicia et al., 2012; Ferguson and Tufts, 1992). Decreased oxygen availability for even brief intervals can cause severe disruptions in metabolism and blood chemistry (Ferguson and Tufts, 1992; Milston et al., 2006). Air exposure has successfully been used as a consistent and repeatable stressor in many experiments on both teleosts and elasmobranchs (Acerete et al., 2004; Cicia et al., 2012; Davis and Schreck, 2005; Ferguson and Tufts, 1992; Suski et al., 2007).

In this experiment, *D. sabina* were assessed for changes in physiology during a 30 minute air exposure stressor and also 48 hours after the stressor (to examine recovery). Measurements of secondary stress response indicators (pH, lactate, $p\text{CO}_2$) were used to assess the severity of the stressor over time. Additionally, a large suite of plasma osmolytes was quantified to determine any ionic imbalance associated with stress. Corticosteroids were assessed during the stressor alongside changes in metabolic fuels (glucose and βHB) to determine any potential glucocorticoid effects. Additionally, alanine, an intermediate in glucose metabolism, was evaluated to provide potential insight about observed changes in plasma glucose concentrations. The combination of these measurements provides a complete picture of the physiological changes occurring during the stress response of elasmobranchs.

Methods

Animal collection and handling

In this study, *D. sabina* ($n = 11$) were captured by trawl off the coasts of Mississippi and Alabama. Only mature male animals were used in order to minimize differences in circulating hormones due to sex or age. Animals were maintained in four recirculating 500-gallon tanks at 21 ppt seawater that were monitored regularly for nutrient concentrations and dissolved oxygen. Stingrays resided in this facility for no less than two weeks prior to experimentation. Tanks were housed in a covered wet lab facility with screened-in windows; therefore, stingrays experienced natural temperatures and photoperiods. Water temperature on the morning of the stress experiment was 22°C while air temperatures were only slightly warmer at 25°C. All stingrays appeared healthy and were feeding regularly, on shrimp, before the study was conducted. They were fasted for three days prior to experimentation to avoid changes in circulating glucose as a result of food intake.

Stressor and sample collection

All stingrays from a single tank ($n = 2-3$) were rapidly captured by two individuals such that all animals from the tank were sampled within an average of 3 minutes. Following removal from the water, stingrays were placed on a table atop dampened cloths. Air exposure lasted 30 minutes, with blood draws at 0 minutes (immediately upon removal from the tank), 15 minutes, and 30 minutes. After the last blood draw, stingrays were replaced in their tanks and allowed to reacclimate. Following an additional 48 hours, animals were once again briefly removed from the water for a

final blood draw (representing recovery). Following the 48 hour blood draw, stingrays were weighed, and disc width was measured.

Blood was drawn from the dorsal wing of each animal using a 1 mL syringe and 22 gauge needles. At each time point, approximately 0.5 mL of blood was taken and immediately transferred to a Vacutainer[®] tube containing lithium heparin.¹ Whole blood that was not used for immediate analysis (iSTAT VetScan measurements and hematocrit) was transferred to 1.5 mL tubes and centrifuged at 4100 x g for 5 minutes. Plasma and red blood cells were separated by pipetting into separate tubes and immediately frozen on dry ice, followed by long-term storage at -80°C.

iSTAT VetScan and Hematocrit Measurements

Approximately 95 µL of whole blood was used to measure blood lactate, pH, *p*CO₂, and hematocrit. Lactate, pH, and *p*CO₂ analysis was carried out using the Abaxis iSTAT VetScan equipped with CG4+ cartridges following the manufacturer's instructions. Hematocrit was measured by filling capillary tubes approximately ¾ full and centrifuging for 5 minutes in a microcapillary centrifuge. Measurements were then made from tubes using the hematocrit reader rotor.

Osmolality and osmolytes

Plasma osmolality was measured using a Vapro 5520 osmometer following the manufacturer's instructions with 10 µL of each plasma sample. Plasma chloride was measured using a Labconco chloridometer on the low setting with 10 µL of each plasma sample. A suite of osmolytes (bromine, sodium, calcium, potassium, ammonium, nitrate, sulfate, and trimethylamine oxide (TMAO)) was measured from plasma using ion-exchange chromatography (Metrohm-Peak, Herisau, Switzerland) at the University of

¹ Vacutainer[®] is a registered trademark of Becton, Dickinson and Company.

Manitoba (Winnipeg, Manitoba, Canada) using methods previously outlined for measurement of cations and anions (Anderson et al., 2012). Plasma used for urea measurements was diluted 1:25 in dH₂O followed by analysis conducted using an end-point quantitative colorimetric assay (QuantiChrom urea assay kit, DIUR-500, BioAssay Systems, CA, USA), read at 520 nm on a SpectraMax M2 (Molecular Devices) plate reader after a room temperature incubation period of 20 minutes.

β-hydroxybutyrate, glucose, and alanine

β-hydroxybutyrate (βHB), glucose, and alanine were measured with enzymatic assays using resazurin and measured on a FLUOstar Omega (BMG Labtech) 96-well plate reader. To measure βHB, plasma samples were diluted 1:20 in dH₂O and combined with 200 μL of reaction buffer (250 μL NAD⁺ (15 mM), 250 μL diaphorase (40 IU/mL), 500 μL resazurin (1mM), 25 mL Tris buffer (150 mM)) and 10 μL of β-hydroxybutyrate dehydrogenase (βHBDH, 10 IU/mL). The reaction was allowed to run for 30 minutes at room temperature during which time fluorescence intensity was measured with an excitation wavelength of 544 nm, emission wavelength of 590 nm, and a gain of 1450. Samples were evaluated against a βHB standard curve.

To measure glucose, plasma samples were diluted 1:10 in dH₂O and combined with 200 μL of reaction buffer (250 μL NADP⁺ (15 mM), 250 μL diaphorase (40 IU/mL), 500 μL resazurin (1mM), 500 μL ATP (25 mM), 23.5 mL Tris/MgCl₂ buffer (150 mM/2 mM), followed by addition of 0.2 IU/mL glucose-6-phosphate dehydrogenase (G6PDH) and 10 μL of hexokinase (HK, 10 IU/mL). The reaction was allowed to run for 30 minutes at room temperature during which time fluorescence intensity was measured with an excitation wavelength of 544 nm, emission wavelength at 590 nm, and a gain of

800. Samples were evaluated against a glucose standard curve ranging from 10 μM to 100 μM .

To measure alanine, plasma samples were diluted either 1:40 or 1:20 (depending on the volume of plasma remaining for analysis) and combined with 200 μL of reaction buffer (250 μL NAD^+ (15 mM), 250 μL diaphorase (40 IU/mL), 500 μL resazurin (1mM), 25 mL Tris buffer (150 mM)) and 20 μL of alanine dehydrogenase (AldH, 25 IU/mL). The reaction was allowed to run for 30 minutes at room temperature during which time fluorescence intensity was measured with an excitation wavelength of 544 nm, emission wavelength at 590 nm, and a gain of 1200. Samples were evaluated against a glucose standard curve ranging from 2.5 μM to 25 μM .

1-alpha-hydroxycorticosterone

There is no specific antibody for $1\alpha\text{-OH-B}$; therefore, measurements of this corticosteroid must be conducted by other means. Initial attempts to quantify $1\alpha\text{-OH-B}$ followed the methods of Evans et al. (2010), which were developed for the quantification of $1\alpha\text{-OH-B}$ in *ex vivo* interrenal incubation media using a validated corticosterone assay. Steroids were extracted from 100 μL of plasma by vortexing with a 10:1 addition of dichloromethane. Samples were then centrifuged at 1000 x g to separate aqueous and organic layers. The lower organic layer was transferred to a new vial, and the aqueous layer was discarded. Samples were dried down using a constant stream of nitrogen gas, and then reconstituted in 175 μL of methanol.

HPLC was performed with columns and solvents as outlined in Evans et al. (2010). Standards of 25 μM $1\alpha\text{-OH-B}$ (provided by Dr. John Rimoldi, University of Mississippi) and B (corticosterone; Sigma-Aldrich) were used to determine the

appropriate window to collect specific corticosteroid fractions. Although B has never been observed as a corticosteroid in the Atlantic stingray using HPLC, TLC, and GC/MS methods (Nunez and Trant, 1999; B. Nunez personal communication), HPLC isolation of the 1α -OH-B fraction from plasma samples is necessary to ensure specific quantification. Using standards as described above, 1α -OH-B was eluted from 5.5-8 minutes, and B was eluted from 9-12 minutes. 150 μ L of each 175 μ L extracted steroid sample was injected into the machine, and the 1α -OH-B fraction was collected at its respective time. 1α -OH-B mobile phase samples were dried down at 42°C with a constant stream of nitrogen and reconstituted in 230 μ L of assay buffer from a commercial corticosterone assay kit (ADI-900-097, Enzo Life Sciences, NY, USA). The assay was conducted following the manufacturer's protocol and measured against a 1α -OH-B standard curve ranging from 0.042 nM to 1000 nM. Cross reactivity of the corticosterone antibody for 1α -OH-B was calculated from 5 standard curves each of B and synthetic 1α -OH-B, with 50% B_0 selected for comparing values.

To determine percent recovery during extraction, four samples were spiked with synthetic 1α -OH-B to a concentration of 100 μ M and measured against their non-spiked counterparts. Spiked and non-spiked samples were handled throughout all steps (steroid extraction, HPLC, assay measurement, etc.) in the same manner as experimental samples.

Total corticosteroids

Due to difficulties with the method described above (outlined in Results), a second method was utilized to measure total corticosteroids using the previously validated corticosterone EIA kit (500655, Cayman Chemical, MI, USA; Evans et al., 2010). For this method, plasma samples were directly diluted 1:10 in EIA buffer and

assayed in duplicate against a corticosterone standard curve ranging from 8.2 pg/mL to 5000 pg/mL following the manufacturer's protocol. To validate the direct use of plasma in the assay, 6 additional plasma samples from *D. sabina* were spiked with corticosterone to a concentration of 2000 pg/mL prior to dilution and measured against their non-spiked counterparts. Only 6 of the 11 stingrays used in the stress study were analyzed for total corticosteroids; these individuals were chosen based upon the quantity of plasma remaining after all other analyses.

Plasma Metabolomics

In a separate effort, 100 uL of each plasma sample was sent to St. Boniface Hospital in Manitoba, Winnipeg, Canada, for metabolomics analysis using gas chromatography / mass spectrometry (GC/MS).

Data analysis

Blood and plasma parameters were analyzed using a repeated measures one-way analysis of variance (ANOVA) over the time series (factors: 0 minutes, 15 minutes, 30 minutes, and 48 hours). Variables that violated the assumptions of homogeneity and normality of variance were transformed by the most appropriate power function. Significant trends were followed by a post-hoc paired t-test to separate the significant mean values. Data was considered statistically significant at an α level of <0.05 .

Results

Whole blood components

Lactate changed significantly over the course of the experiment ($p < 0.001$, Figure 2a). Concentrations increased from 0.406 ± 0.08 mmol/L at 0 minutes to 1.542 ± 0.216 mmol/L at 15 minutes and 2.654 ± 0.304 mmol/L at 30 minutes. Recovery measurements

of lactate (0.348 ± 0.048 mmol/L) were not significantly different from basal concentrations. CO_2 also changed significantly during the experiment ($p < 0.001$, Fig. 2b). $p\text{CO}_2$ increased from 10.333 ± 0.485 mmHg at 0 minutes to 12.178 ± 0.427 mmHg at 15 minutes and 13.790 ± 0.677 mmHg at 30 minutes. Recovery measurements of CO_2 (9.190 ± 0.398 mmHg) were not significantly different from basal measurements. Changes in whole blood pH were also significant ($p < 0.001$, Fig. 2c). Blood pH decreased from 7.095 ± 0.028 at 0 minutes to 6.922 ± 0.024 at 15 minutes and 6.827 ± 0.030 at 30 minutes (Fig. 2c). Recovery pH (7.203 ± 0.025), although significantly different from basal measurements (t-test, $p = 0.008$), was no longer acidic relative to time 0. Hematocrit did not change significantly during the course of the experiment, with values ranging from 16.286 ± 1.625 to $18.889 \pm 1.401\%$ (Fig. 1d).

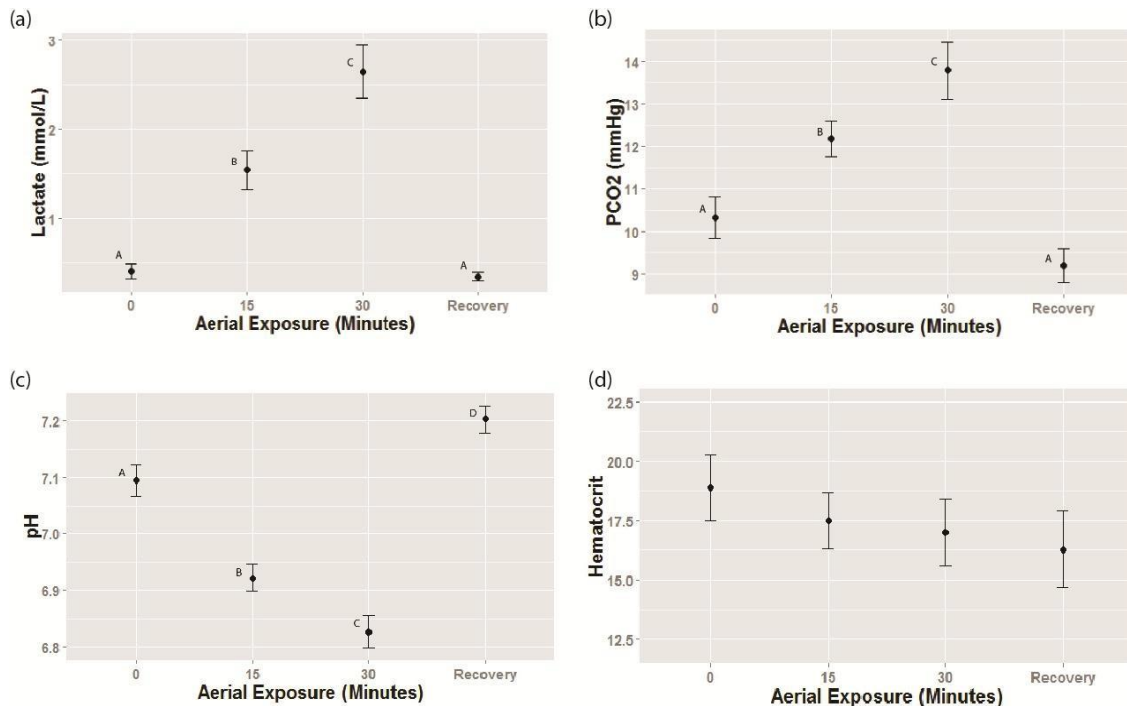


Figure 2. Mean (\pm SEM) (a) blood lactate, (b) blood $p\text{CO}_2$, (c) blood pH, and (d) hematocrit in the Atlantic stingray during 30-minute air exposure stressor and recovery (48 hours). Letters indicate values that are significantly different ($p < 0.05$) from each other.

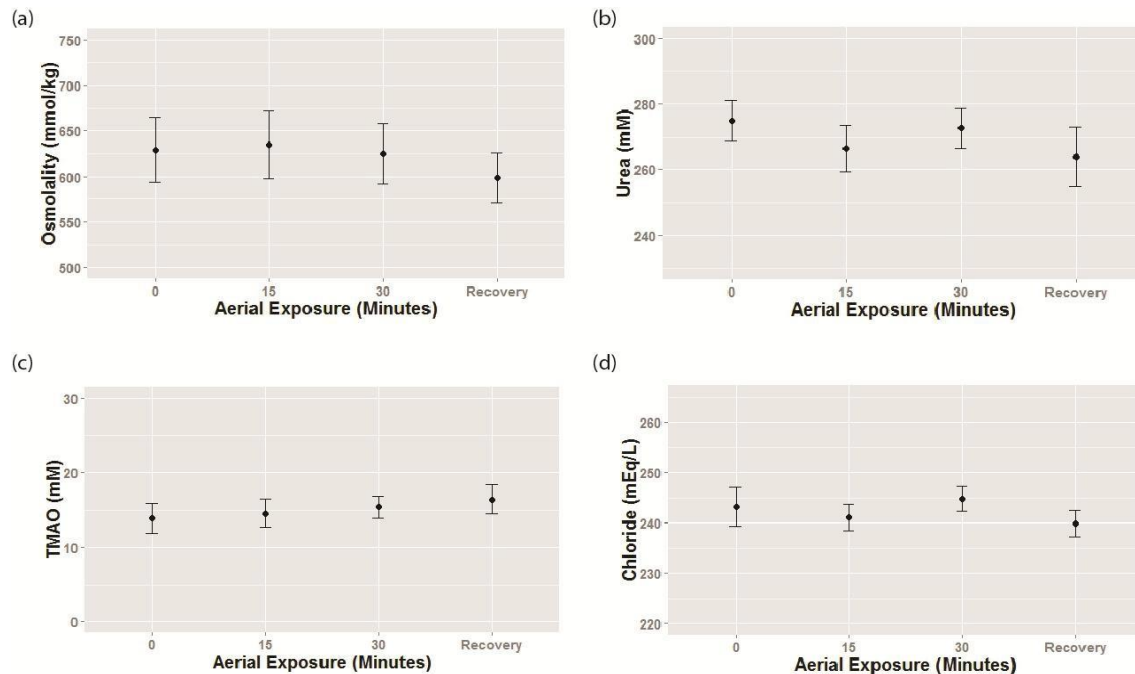


Figure 3. Mean (\pm SEM) (a) plasma osmolality, (b) plasma urea, (c) plasma TMAO, and (d) plasma chloride in the Atlantic stingray during a 30-minute air exposure stressor and recovery (48 hours). Letters indicate values that are significantly different ($p < 0.05$) from each other.

Plasma osmolality and osmolytes

Plasma osmolality did not change significantly during the experiment (Fig. 3a). Out of all the plasma osmolytes measured (Fig. 3b-d, Fig. 4, Fig. 5), only potassium ($p < 0.01$, Fig. 5b) and sulfate ($p < 0.01$, Fig. 5c) changed significantly. Plasma potassium concentrations increased from 3.339 ± 0.303 mM to 4.398 ± 0.213 mM over the 30-minute air exposure. At 48 hours, potassium concentrations had decreased to 3.102 ± 0.377 mM and were not significant from basal measurement. Sulfate increased significantly during the experiment ($p < 0.01$). Sulfate increased significantly from 0.264 ± 0.027 mM at 0 minutes to 0.300 ± 0.039 at 15 minutes. Sulfate continued to increase at 30 minutes (0.353 ± 0.050 mM) but was not significantly different from measurements at 15 minutes or 48 hours. The 48 hour increase (0.921 ± 0.322 mM) was significantly different from both 0- and 15-minute measurements. Concentrations of plasma chloride,

urea, TMAO, ammonium, calcium, sodium, bromine, and nitrate did not change significantly during the course of the experiment (Fig. 3b-d, Fig. 4, Fig. 5a).

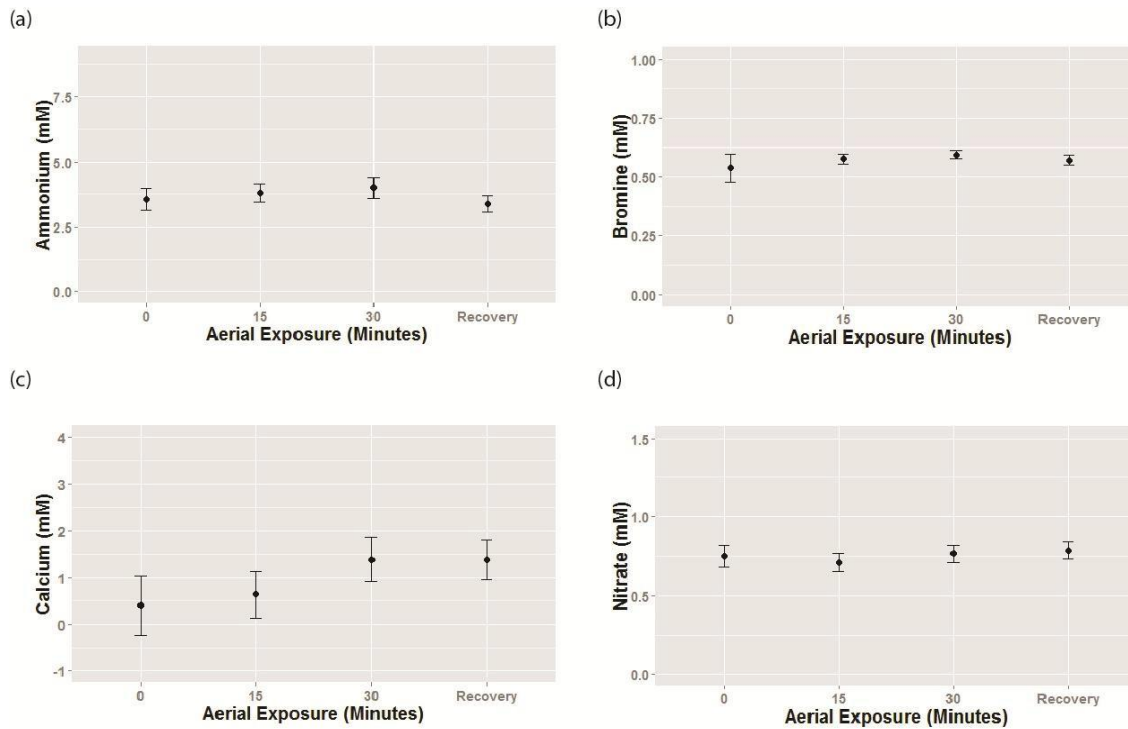


Figure 4. Mean (\pm SEM) (a) plasma ammonium, (b) plasma bromine, (c) plasma calcium, and (d) plasma nitrate in the Atlantic stingray during a 30-minute air exposure stressor and recovery (48 hours). Letters indicate values that are significantly different ($p < 0.05$) from each other.

Metabolic fuels

Plasma glucose changed significantly during the course of the experiment ($p < 0.01$, Figure 6a). Glucose concentrations decreased significantly from 0.713 ± 0.029 mM at 0 minutes to 0.634 ± 0.02 mM at 15 minutes. After 15 minutes, plasma glucose increased through 30 minutes and reached a concentration of 0.925 ± 0.057 mM at 48 hours. Neither plasma alanine nor β HB changed significantly over the course of the experiment (Figure 6b-c).

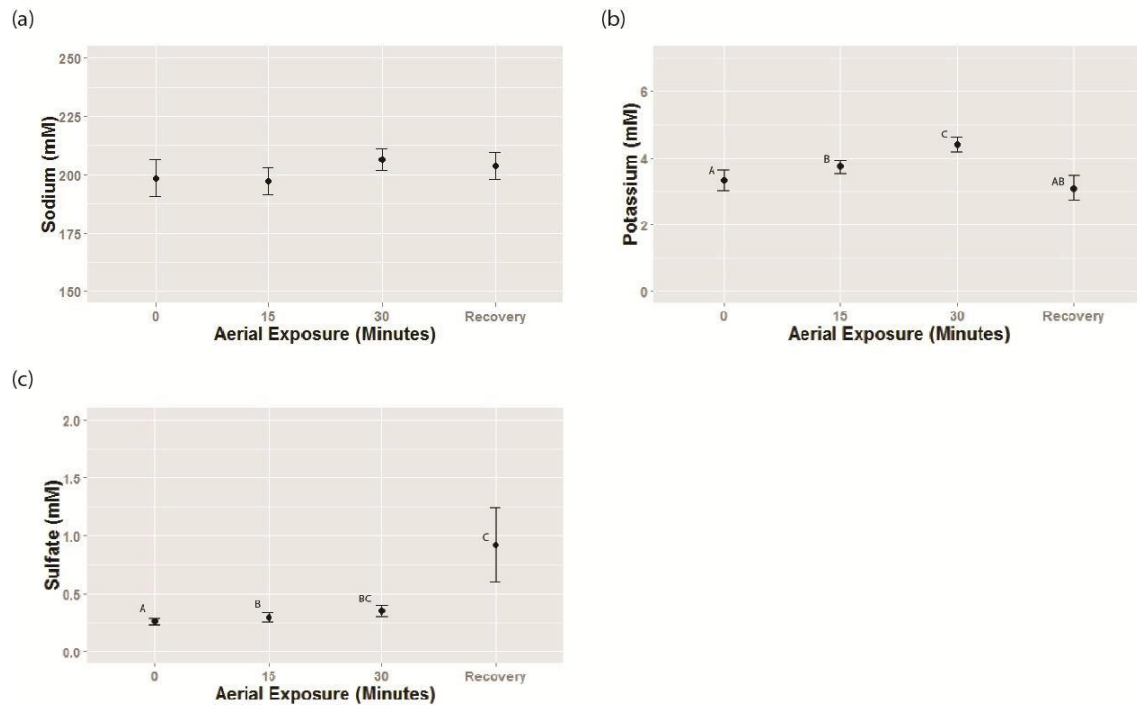


Figure 5. Mean (\pm SEM) (a) plasma sodium, (b) plasma potassium, and (c) plasma sulfate in the Atlantic stingray during a 30-minute air exposure stressor and recovery (48 hours). Letters indicate values that are significantly different ($p < 0.05$) from each other.

Corticosteroids

Direct measurements of 1α -OH-B were unsuccessful due to a leak in the HPLC apparatus leading to significant sample loss. Also, while initial tests indicated that cross reactivity of the Enzo ELISA for 1α -OH-B was 8.3% (improving upon the Cayman Chemical ELISA used by Evans et al., 2010; 5.5%), it was determined via HPLC that the 1α -OH-B standard used for this calculation was degraded. A new test for cross reactivity using fresh standard revealed a value of only 2.3%, which likely compounded the effects of sample loss during HPLC.

However, measurements of total corticosteroids using the 1:10 direct dilution method were successful. Total corticosteroids changed significantly across experimental time points ($p = 0.004$, Fig. 6d). Plasma corticosteroids did not change significantly

during the 30-minute air exposure but were significantly elevated at 48 hours (452.22 ± 172.74 pg/mL) vs. all other time points (0 minutes: 179.07 ± 38.26 pg/mL, 15 minutes: 152.40 ± 24.07 pg/mL, 30 minutes: 164.70 ± 21.94 pg/mL).

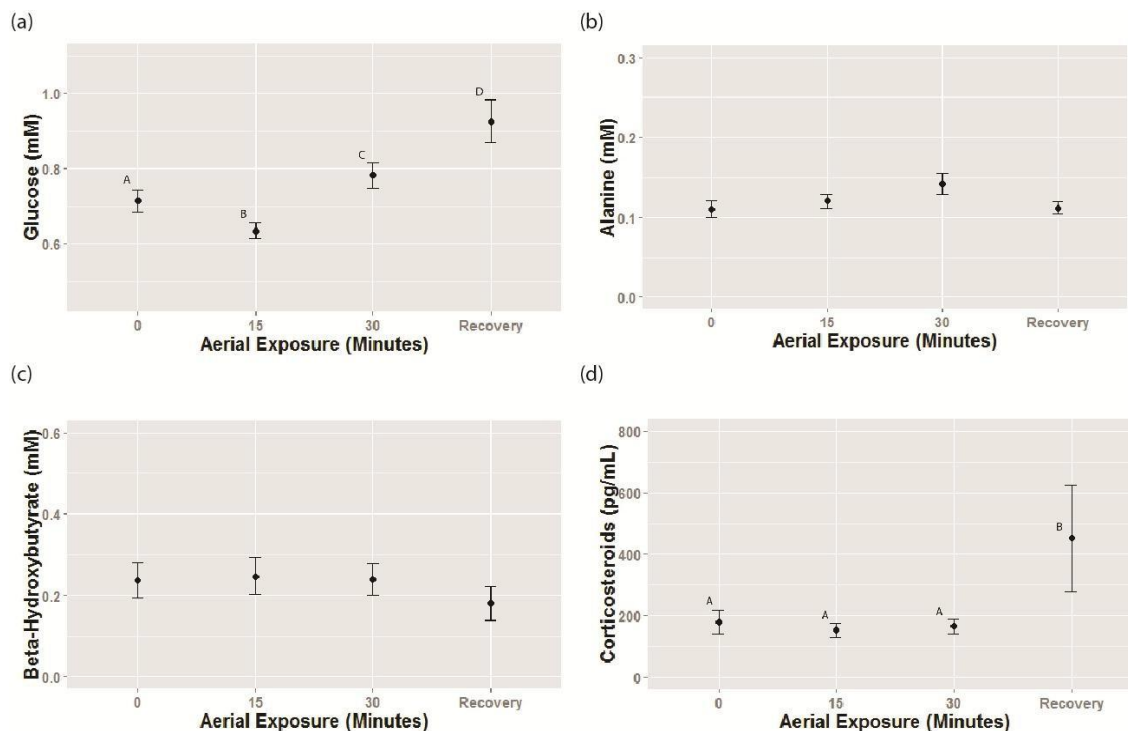


Figure 6. Mean (\pm SEM) (a) plasma glucose, (b) plasma alanine, (c) plasma β HB, and (d) plasma total corticosteroids in the Atlantic stingray during a 30-minute air exposure stressor and recovery (48 hours). Letters indicate values that are significantly different ($p < 0.05$) from each other.

Plasma samples spiked with corticosterone standard had an average concentration of 1480.45 ± 47.15 pg/mL ($\pm 3.2\%$). The equivalent standard in ELISA buffer alone was 1823.3 pg/mL; therefore spiked samples averaged 81% of the expected corticosterone value with excellent repeatability ($\pm 3.2\%$). The reported corticosteroid values are therefore considered to be highly accurate relative to each other using this method. Additionally, the relationship between basal measurements of plasma glucose

and plasma total corticosteroids were analyzed using a linear regression and were significantly different ($p < 0.014$, Figure 7).

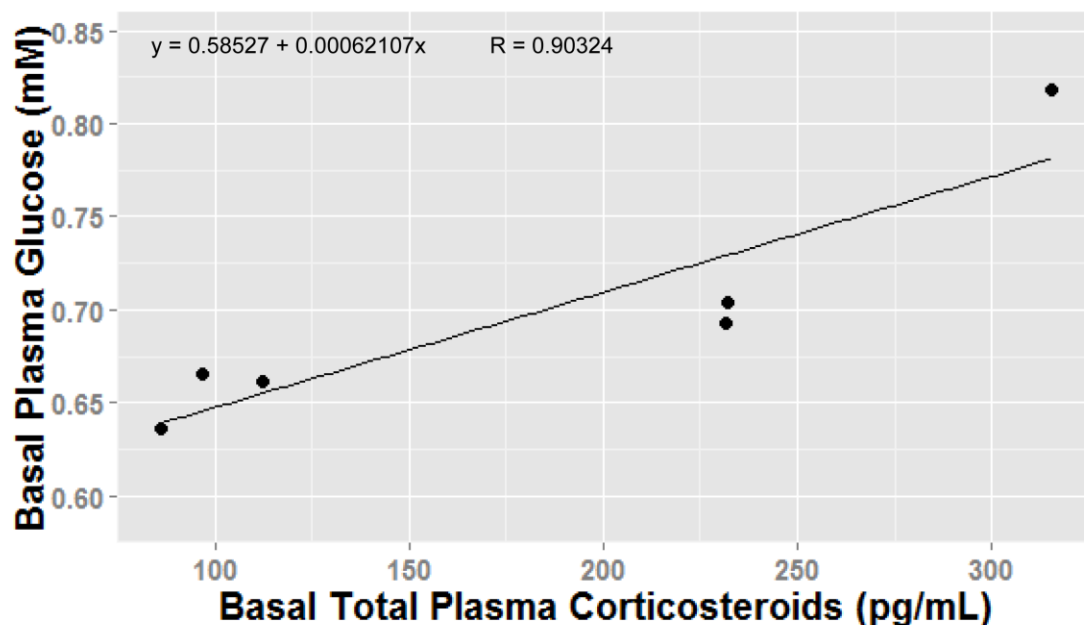


Figure 7. Linear regression of basal plasma total corticosteroids and basal plasma glucose in *D. sabina*, displaying a significant correlation.

Plasma Metabolomics

Over 2400 metabolites were detected in each plasma sample, 92 of which changed significantly over the course of stress and recovery (Figure 8). This data is preliminary. More extensive research will need to be conducted in order to identify the names of these 92 compounds. However, this information provides insight to the dissimilarity between stingray metabolism during homeostasis and an acute stressor.

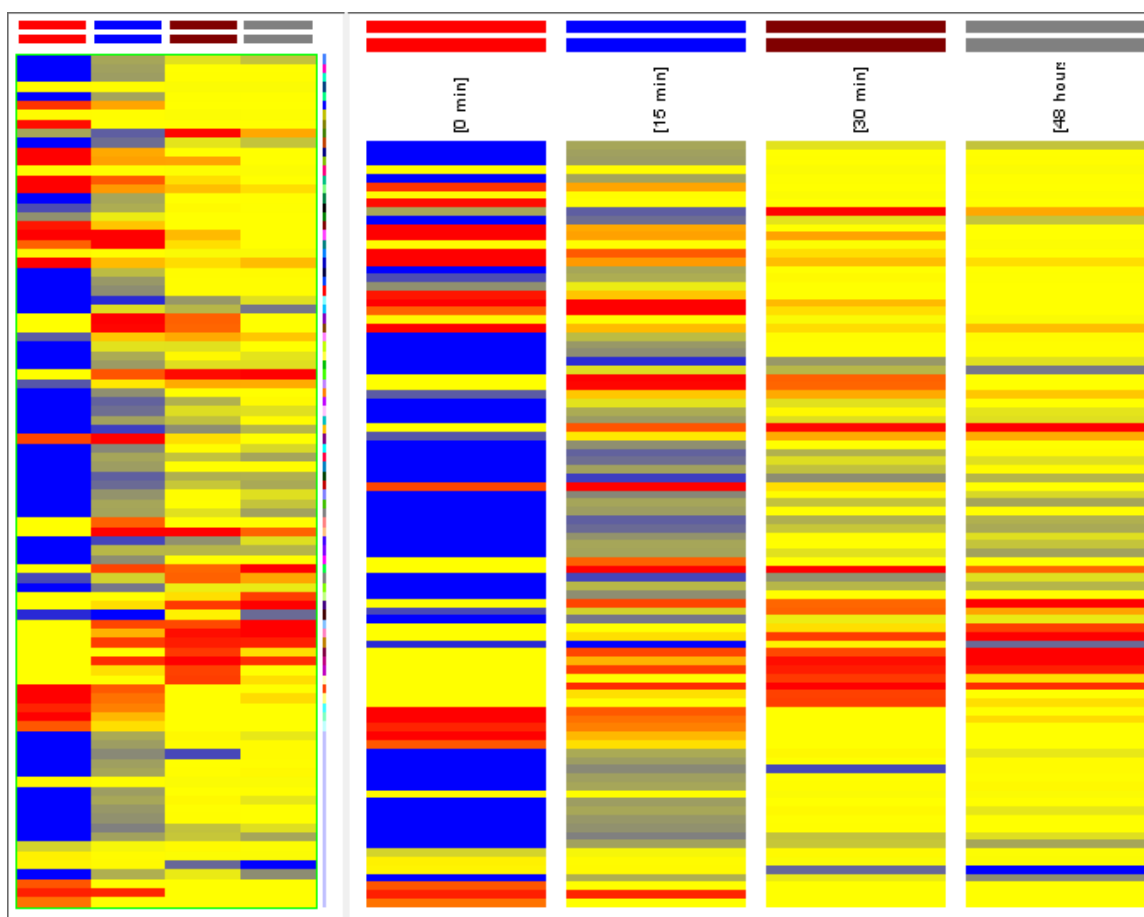


Figure 8. Metabolites from Atlantic stingray plasma exhibiting significant changes ($p < 0.05$) over the course of a 30-minute air exposure and recovery (48 hours). Cool colors (blues) indicate low relative levels of metabolites. Warm colors (reds > yellow) indicate high relative concentrations of metabolites. Each row indicates a single metabolite.

Discussion

The primary purposes of this study were to assess changes in 1α -OH-B, β HB, and glucose over the course of an acute stressor. While direct measurements of 1α -OH-B were not attainable, this study presents the first measurements of corticosteroids in an elasmobranch in response to a controlled stressor. Corticosteroids did not increase during the 30-minute air exposure, contrary to expectations. However, a significant increase at 48 hours was observed that corresponded with significant increases in glucose. This may

indicate that air exposure has more long-term damaging effects. Plasma concentrations of β HB did not significantly change at any point during the experiment, indicating that β HB may not play a prominent role in the stress response, at least for aerial exposure.

D. sabina exposed to air for an extended period of time exhibit a characteristic secondary stress response as observed in other vertebrates with regards to changes in blood lactate, $p\text{CO}_2$, and pH. These changes are commonly associated with increased anaerobic metabolism as a result of strenuous physical activity associated with the fight or flight response (Skomal and Bernal, 2010). Stingrays in this study were most active during the brief period of net capture. They minimized their movements shortly after the first blood draw and remained relatively still for the remainder of the 30-minute stressor. However, lactate, blood acidosis, and $p\text{CO}_2$ continued to climb despite decreased physical activity. The collapse of gill lamellae and a resulting decrease in gas exchange efficiency are the most likely causes of these departures from homeostasis. The inability to shed excess CO_2 causes this gas to build-up, resulting in decreased blood pH. This is further compounded by the increased lactate produced by anaerobic metabolism resulting from reduced O_2 uptake. Similar findings have been demonstrated in other studies with elasmobranchs and teleosts (Cicia et al., 2012; Ferguson and Tufts, 1992; Milston et al., 2006). In either case, departures from basal levels in these blood parameters have been used in a wide variety of taxa, including elasmobranchs, as clear indicators of an acute stress response (Barton, 2002; Skomal, 2007). The continued increases in $p\text{CO}_2$, lactate, and blood acidosis over the entirety of the 30-minute experimental period indicate that longer bouts of aerial exposure produce a more substantial stress response.

Despite the severe departures from homeostasis experienced by these animals, when whole blood parameters were measured again at 48 hours, they had all returned to basal levels, indicating that the acute stress response had subsided. There were no mortalities within the 48-hour recovery period, and there was only one mortality within the week after experimentation. This high survival rate following such a severe stressor supports the use of *D. sabina* as a model organism for further *in vivo* research regarding the stress response of elasmobranchs. Additionally, the novel blood sampling method, i.e., dorsal wing draws, conducted with these animals provides a straightforward way to assess an individual animal over time in a relatively non-intrusive manner versus traditional phlebotomy techniques requiring extensive handling.

Hematocrit measurements did not change during the experiment. Multiple studies using elasmobranchs have also shown consistency of hematocrit measurements during stress (Cicia et al., 2012; Hoffmayer et al., 2012; Hoffmayer and Parsons, 2001). However, this is in contrast to what is observed in many teleosts (Suski et al., 2007). As previously suggested by Hoffmayer et al. (2012), this may be a unique feature of elasmobranch species.

There were no changes in plasma osmolality or the majority of plasma osmolytes (with the exception of K^+ and SO_4^{-2}) during the course of this experiment. This is in contrast to what is commonly seen in other stress studies. Sharks sampled over the course of a 60-minute hook-and-line stressor experienced significant increases in osmolality (Hoffmayer et al., 2012; Hoffmayer and Parsons, 2001) and departures from basal levels of Na^+ , Cl^- , and Ca^+ (Hoffmayer et al., 2012). Departures from osmolality and osmolyte homeostasis have also been reported in *S. acanthias* as a result of capture, transport, and

captivity stress (Mandelman and Farrington, 2007). The aforementioned studies were all conducted in conditions where fish were maintained in the water for the majority of experimental time. The ability to exchange osmolytes with the surrounding water may have allowed for changes in these parameters during these experiments. This exchange would be unable to occur in animals exposed to air and accounts for the consistent osmolytes levels of *D. sabina* in this study. Cicia et al. (2012), who evaluated the physiological stress response to air exposure in the little skate (*Raja erinacea*), also observed no changes in plasma osmolytes in winter, when water and air temperatures did not differ significantly. However, changes in plasma osmolytes were observed during the summer months, when air temperatures far exceeded holding tank values. It was hypothesized that these changes reflected differences in water and ion compartmentalization between plasma and cells; however, comparison of the winter and summer studies was also confounded by differences in basal levels of osmolytes (Cicia et al., 2012). Regardless, the results of Cicia et al. (2012) have interesting implications regarding the added effects of temperature differences during the stress of air exposure. In this study, air and water temperatures only differed by 3°C.

Plasma K^+ concentrations most likely increased due to stress-induced damage at the cellular level due to acidosis and have previously been observed in elasmobranchs exposed to air (Cicia et al., 2012; Frick et al., 2010). Also, the opening of K^+ channels during cardiac cellular stress has been described and hypothesized to protect mitochondria when conditions become anoxic (Ozcan et al., 2002). As the ability of stingrays to obtain O_2 during aerial exposure is reduced, this seems a likely mechanism for the resultant rise in K^+ concentrations. Significant increases in plasma SO_4^{-2} were also

observed throughout the entire 48-hour experiment. However, little research has been conducted on changes in divalent ions during stress; therefore, the role of SO_4^{2-} in the stress response warrants further research.

The surprising lack of change in βHB indicates that this metabolic fuel may not be as important during the stress response as hypothesized. βHB circulates at higher concentrations in elasmobranch species (~0.2-4.9 mM) as opposed to teleosts where concentrations are practically undetectable (Speers-Roesch and Treberg, 2010). Despite this apparent importance, the results of the current study suggest that glucose is the predominant metabolic fuel mobilized during stress, potentially by corticosteroid actions.

Patterns of glucose correlated to both physical activity and changes in corticosteroids over the entirety of the experiment. The significant decrease in plasma glucose at 15 minutes is likely due to usage of this metabolic fuel during activity immediately following capture. The subsequent lack of movement after placement on the exposure table and hence the decreased need for glucose, along with stable plasma corticosteroid concentrations, are both likely contributors to this observed decrease. The significant increase in glucose at 30 minutes may then result from continued exposure to air, causing extreme physiological changes and the need for energy to maintain enantiostasis. The most significant increases in both glucose and corticosteroids, however, were observed at the 48-hour mark. These corresponding trends give reason to believe that circulating corticosteroids are acting as glucocorticoids. It is most likely that the major or only corticosteroid measured in this study is $1\text{-}\alpha\text{-OH-B}$. Previous studies have shown that $1\alpha\text{-OH-B}$ is the dominant corticosteroid in elasmobranchs (Anderson, 2012; Table 1) and the only product of interrenal tissue exposed to mammalian ACTH

(Klesch and Sage, 1975; Nunez and Trant, 1998). For these reasons, it is likely that the observed changes in glucose are mediated by 1α -OH-B. However, because of the difficulties in specifically measuring this steroid, future studies should examine this possibility when a specific assay for 1α -OH-B is available. While current suggested methods have potential, e.g., Evans et al. (2010), the most desirable scenario is the development of an antibody and assay specific to this corticosteroid. This would provide the accuracy necessary to establish a thorough understanding of the role 1α -OH-B plays in the functioning of the endocrine stress response.

Alongside corresponding trends during the experiment, a linear regression of basal plasma glucose with basal corticosteroids exhibits a significant correlation (Fig. 6). Basal level measurements were chosen to evaluate this relationship because stingrays at this time were considered to be in the most similar, and hence most comparable, physiologic state of homeostasis. The significant relationship between the amount of circulating corticosteroids and amount of circulating glucose strongly supports the role of 1α -OH-B as a glucocorticoid and is also indicative of the natural range in physiological function amongst animals. These differences in basal level glucose and total corticosteroids suggest that animals do not all function the same way and may have variable responses to external stimuli, such as stressors. These individual variations are even more apparent in the total corticosteroid trends seen amongst animals, an observation that has also been documented in other species (Figure 9; Cockrem, 2013).

The lack of significant changes in corticosteroids during the 30-minute aerial exposure, followed by an increase within the subsequent 48-hour period may be related to the type of stressor. When *R. erinacea* were exposed to air, a similar lack of change in

glucose throughout the 50-minute exposure was observed (Cicia et al., 2012). Given the lack of physical activity during the course of aerial exposure, it may be that this stressor does not require large inputs of metabolic energy. Increases in corticosteroids and glucose at a point long delayed after the 30 minute air exposure period may therefore indicate that this stressor has more long-term damaging effects than short-term ones. Support for this hypothesis is provided by the only fatality observed during the week following air exposure in the current study. The individual with the largest corticosteroid increase (Stingray 5, Figure 9) died two days following the end of the experiment (96 hours post-exposure). Further evidence that *D. sabina* were not fully recovered from potential tissue damage and inflammation resulting from air exposure is provided by preliminary metabolomics results. While efforts to identify specific metabolites are ongoing, 92 entities were identified as significantly affected by stress. The majority of these metabolites increased throughout the course of air exposure and either remained elevated or continued to increase up to 48 hours later (Fig. 8). This result was unexpected and strongly supports the hypothesis that 30-minute air exposure results in long-term deleterious effects on elasmobranch physiology.

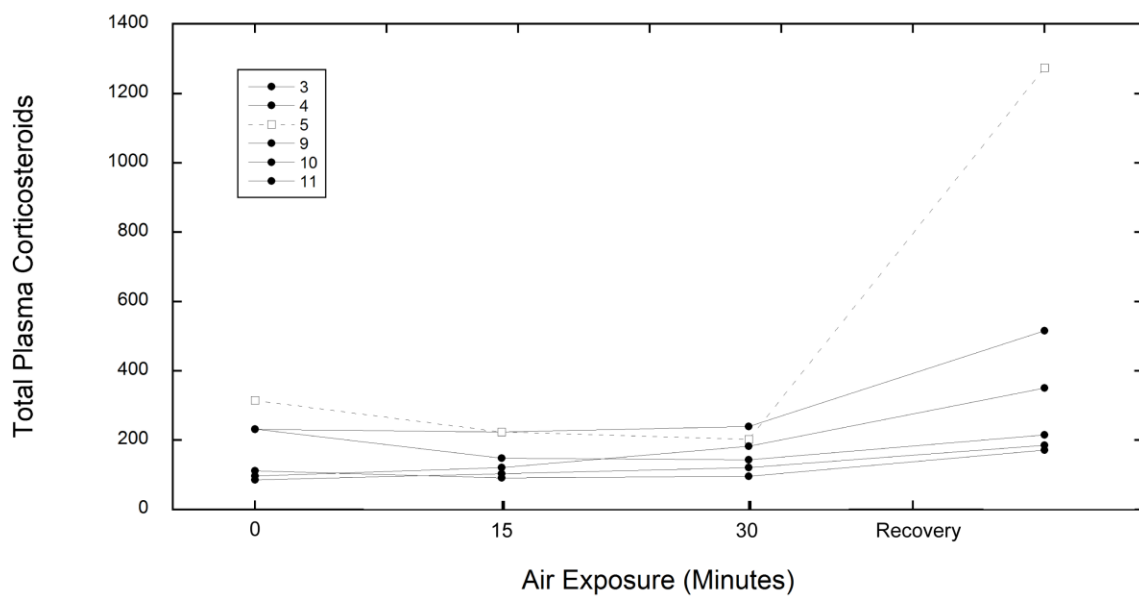


Figure 9. Total plasma corticosteroids in individual Atlantic stingrays during a 30-minute air exposure stressor and recovery (48 hours).

CHAPTER III
REGULATION OF SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE 1
mRNA BY 1 α -OH-B

Background

The steroid hormones elicit most of their effects by regulating gene transcription. With this being the case, a highly conserved GC regulated gene was chosen to evaluate the potential role of 1 α -OH-B in gene regulation. Serum- and glucocorticoid-inducible kinase 1 (SGK1) is a member of the family of serine/threonine kinases and plays an important role in many cellular response systems (Lang and Cohen, 2001). *SGK1* transcription is upregulated by an integrated system of hormonal and nonhormonal signals that include osmotic shock, peptide hormones, glucocorticoids, and mineralocorticoids (Lang et al., 2006; Waldegger et al., 1998; Webster et al., 1993a, 1993b). This kinase plays a role in many cellular response systems, including ion regulation and transport mechanisms, cell growth, and apoptosis.

Since its discovery in rats (Webster et al., 1993a), *SGK1* has been cloned in other organisms, including amphibians (Chen et al., 1999), humans (Waldegger et al., 1997), and sharks (Waldegger et al., 1998). Similar to other protein kinases, it has been highly conserved throughout evolutionary history (Lang and Cohen, 2001). This is evidenced by the significant homology between the amino acid sequences of species from different taxa.

Research in mammals has shown that *SGK1* is present in practically all tissues (Lang et al., 2006; Webster et al., 1993a). However, transcript levels between these tissues vary greatly. These differences appear to reflect the varying need for SGK1 in

tissue specific processes. This is supported by findings observed in tissue suites of the spiny dogfish (*S. acanthias*). While nearly all tissues showed some *SGK1* transcripts, the highest expression was observed in the liver, heart, kidney, and intestine (Waldegger et al., 1998). It is hypothesized that a similar distribution will be present in *D. sabina*.

SGK1 transcription is sensitive to increases in circulating levels of glucocorticoids as a result of the glucocorticoid response element (GRE) located within its 5'-flanking sequence (Webster et al., 1993b). Ligand-bound, dimerized GR-GR complexes bind to GREs and act as a transcription factor to promote the production of mRNA (Romero and Butler, 2007). Similar to other protein kinases, SGK1 has been highly conserved throughout evolutionary history (Lang and Cohen, 2001), and it is therefore hypothesized that the *D. sabina* *SGK1* gene sequence also contains a GRE in its promoter region.

SGK1 is classified as an immediate-early response gene because of the rapid increase in its mRNA levels following exposure to glucocorticoids or serum (Webster et al., 1993b). Significant changes in its expression have been shown to occur as rapidly as 30 minutes after stimulation in mammalian and teleost subjects, making it an excellent candidate for evaluation during acute stress studies such as air exposure (Baskin and Sayeski, 2012; Brennan and Fuller, 2000; Johnstone, 2011; Webster et al., 1993b).

Unlike mammalian taxa, mature red blood cells in fish retain a nucleus. Since steroid hormones travel through blood to reach target tissues, nucleated red blood cells may be a prime candidate for first response to stressors at the transcriptional level (Boutilier and Ferguson, 1989). Previous studies have investigated the correlation between stressors and the transcription of heat shock proteins in teleost red blood cells, demonstrating that induced stress increases levels of mRNAs encoding these protective

proteins (Currie et al., 1999; Currie and Tufts, 1997; Delaney and Klesius, 2004; Lund et al., 2003). *SGK1* in red blood cells may therefore exhibit a similar pattern of transcriptional upregulation in response to stress. Glucocorticoids produced after the detection of a threat would bind to the GRE in the *SGK1* promoter, if present in *D. sabina*, inducing transcription of this mRNA.

It is important to note that SGK1 is also involved in osmoregulatory processes. It is particularly important in the control of epithelial ion transport and is known to affect the activity of a vast array of membrane transport proteins (e.g., ENaC, ROMK, NKA) (for review, see Loffing et al., 2006). The role of this protein in osmoregulation is also evidenced by the moderators of its activity. Aldosterone, the main mineralocorticoid of many vertebrate species, has been shown to increase *SGK1* expression in the nephron of the rat kidney (McCormick et al., 2005). Angiotensin II (Ang II), a prominent player in the renin-angiotensin system (RAS), has previously been shown to increase transcription of *SGK1* in fibrosarcoma-derived cell line (Baskin and Sayeski, 2012). The actions of the RAS are counteracted by the natriuretic peptides, which may therefore have opposing effects on *SGK1* transcription.

The rectal gland is important to hydromineral balance in the elasmobranchs (Forrest 1996) and is thus an appropriate model for addressing the function of SGK1 in these fishes. Furthermore, there is precedence for regulation of this gene in the elasmobranch rectal gland, as tissue of *S. acanthias* exposed to a hypertonic solution resulted in a significant increase of *SGK1* transcripts (Waldegger et al., 1998). Exposure of this tissue to the primary elasmobranch corticosteroid, 1α -OH-B, may therefore elicit

the same result. This is an excellent model for examining the capacity of 1α -OH-B as a transcription factor.

In this experiment, all but a short (~ 75 bp) portion of the *D. sabina SGK1* coding region was cloned and sequenced. *SGK1* mRNA tissue distribution was characterized, including the relative prominence of transcripts between tissues. To analyze the regulation of *SGK1* during the stress response, mRNA was quantified in red blood cells collected from the stress series. Two *in vitro* experiments were also conducted to determine if 1α -OH-B was capable of regulating *SGK1* transcription. Rectal gland tissue was incubated with synthetic 1α -OH-B, Ang II, and C-natriuretic peptide (CNP), and fresh whole blood was also incubated with synthetic 1α -OH-B.

Methods

Isolating the SGK1 sequence

5' and 3' RACE and Genome Walker reactions

The sequence of a 483 nt fragment of the *D. sabina SGK1* coding region (CDS), isolated using degenerate PCR, was kindly provided by Dr. Scott Nunez of Wake Tech Community College, Raleigh, NC. To isolate the remainder of the CDS and 5' and 3' untranslated regions (UTRs), total RNA was prepared from freshly isolated *D. sabina* interrenal glands using the Directzol RNA Miniprep kit (Zymo Research, USA) following the kit protocol. 10 μ g of total RNA was then prepared for 5' and 3' RACE PCR using the FirstChoice RLM-RACE kit (AM1700, Ambion Inc., USA) following the manufacturer's instructions. 5' and 3' rapid amplification of cDNA ends (RACE) reactions were each conducted using two gene-specific primers (Table 1) in addition to the primers provided by the RLM-RACE kit. The first round of 5' RACE reactions

amplified a partial sequence that does not extend fully to the upstream start codon relative to dogfish *SGK1* (CAA11527.1). Additional RACE reactions attempting to extend 5' sequence were unsuccessful; therefore, upstream genomic DNA sequence was isolated using a commercial kit (Universal Genome Walker 2.0, Clontech Laboratories Inc., CA, USA). Four libraries of genomic DNA from *D. sabina* interrenal gland were prepared by enzyme digests (DraI, EcoRV, PvuII, and StuI) as outlined in the kit's protocol. Both inner and outer reactions for each digest were carried out following the manufacturer's instructions and gene specific primers (Table 1). Exonic regions of DNA sequences obtained using genome walking methods were identified via alignment to the dogfish *SGK1* nucleotide sequence (AJ223715.1).

cDNA cloning and sequencing

5' and 3' RACE PCR products, as well as Genome Walker PCR products, were ligated into the PGEM T-vector following the PGEM T-Vector System I kit protocol with T4 DNA ligase (A3600, Promega Corporation, WI, USA). Ligation reactions were then used to transform premade Z-competent *Escherichia coli* cells (Zymo Research, USA), which were subsequently spread onto isopropylthio- β -galactoside (IPTG) and x-galactosidase (x-gal) coated Luria Burtani (LB) medium plates with ampicillin. Plates were incubated overnight at 37°C, and a subset of white colonies were selected for PCR screening for insert size using vector-specific primers. Colonies containing the appropriate sized inserts were then isolated and incubated overnight in 2 mL of LB broth with ampicillin at 37°C with shaking at 250 rpm. Plasmids were isolated the following day using the Zyppy plasmid Miniprep kit (R2052, Zymo Research, USA). Plasmids were then sent out for sequencing (Genewiz, Inc.).

Sequence alignments and molecular phylogenetic analysis

NCBI BLAST was used to align protein sequences to the one translated from the isolated nucleotide sequence. To determine the conservation of critical amino acid residues within the sequence, *D. sabina* SGK1 was aligned using the Clustal W algorithm in CLC workbench with protein sequences of diverse taxa representing all major vertebrate groups. These included one elasmobranch (*S. acanthias* CAA11527.1), one holocephalan (*Callorhinchus milii* XP_007901699.1), one teleost (*Danio rerio* NP_954682.1), one amphibian (*Xenopus laevis* NP_001083809.1), one avian (*Gallus gallus* NP_989807.1), and two mammals (*Mus musculus* NP_035491.1, *Homo sapiens* NP_005618.2). These protein sequences, combined with an additional two teleost species (*Oryzias latipes* XP_004083751.1, *Fundulus heteroclitus* Q5Q0U5.1), were also used in a phylogenetic analysis to determine the evolutionary relationship of *D. sabina* SGK1 to that of other taxa. Amino acid sequences were aligned using the Clustal W algorithm (Thompson et al., 1994), and phylogenetic relationships were inferred using the Neighbor-Joining method in Mega version 6 (Tamura et al., 2007) and 2000 iterations to generate a bootstrap consensus tree.

Regulation of SGK1 by 1 α -OH-B and tissue specific distribution of SGK1

Tissues for distribution analysis

Tissues for analysis of SGK1 distribution were collected from two male and one female *D. sabina* as part of a previous experiment in which animals were sacrificed by cervical dislocation and tissues rapidly collected and stored in RNALater until RNA isolation. Tissues collected included heart, gill lamellae, liver, spiral valve, rectal gland, interrenal gland, red blood cells, muscle, testes, and gonad.

In vitro rectal gland incubations

To determine if 1α -OH-B plays a role in the regulation of *SGK1*, *D. sabina* rectal gland tissue was incubated in the presence of multiple concentrations of this corticosteroid. Additional incubations were conducted with homologous angiotensin II and C-natriuretic peptide (custom synthesis by Biomatik), two key components of the osmoregulatory system in elasmobranchs. Rectal glands were collected from 5 mature male *D. sabina* and rapidly divided into 6 equal pieces. Each piece of tissue was briefly transferred to the laboratory and then incubated for 6 hours with gentle shaking at room temperature in 1 mL of elasmobranch Ringer's solution (240 mM NaCl, 7 mM KCl, 10 mM CaCl₂, 4.9 mM MgCl₂, 8 mM NaHCO₃, 0.5 mM Na₂HPO₄•(2H₂O), 0.5 mM Na₂SO₄, 360 mM urea, 60 mM trimethylamine oxide (TMAO) and 1% glucose) with one of the following treatments: control, 100 nM 1α -OH-B, 250 nM 1α -OH-B, 500 nM 1α -OH-B, 100 nM AngII, or 100 nM CNP. At the end of the incubation, tissues were removed from media and stored at -80°C until RNA extraction.

In vitro blood incubations

1 mL of blood was collected from 5 mature male stingrays and immediately transferred to a heparinized Vacutainer[®] tube. Blood was transferred to the laboratory and then incubated for 2 hours with gentle shaking at room temperature under one of the following treatments: control, 100 nM 1α -OH-B, or 500 nM 1α -OH-B. At the end of the incubation period, blood was transferred to microcentrifuge tubes and spun at 4100 x g for 5 minutes. The plasma was discarded, and red blood cells were stored at -80°C until RNA extraction.

Blood from stress series

Red blood cells from the stress series experiment were also used for analyzing changes in *SGKI* over the course of stress.

RNA isolation and cDNA synthesis

RNA was isolated using the Directzol RNA Miniprep kit (Zymo Research, USA) following the manufacturer's protocol including on-column DNase treatment. cDNA was then generated in a 20 µl reaction using random primers and the GoScript reverse transcription system (A5000, Promega Corp.) with 1 µg of total RNA per sample.

qPCR

Quantitative real-time PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green as a fluorescent marker. 2 µl of each cDNA sample was used in 20 µl reactions with SYBR Select Master Mix (4473369, Applied Biosystems) as outlined in the manufacturer's protocol. Reactions were run in duplicate on MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems) covered with adhesive film for PCR plates (VWR). Samples were run through one 2 minute cycle at 50°C, one 2 minute cycle at 95°C, and then 40 consecutive cycles of 15 seconds at 95°C and 1 minute at 60°C. A dissociation curve was conducted at the end of all cycles to assess the purity of products.

The forward and reverse primers used in qPCR for the gene of interest (*SGKI*) and a housekeeping gene (*18S*) are listed in Table 1. Primer efficiencies for *SGKI* primers were estimated using five serial dilutions (1:10) of rectal gland cDNA and the qPCR protocol described above. The *SGKI* primers' efficiency was 99.73%. *18S* primer

efficiencies for this species and instrument have been previously determined in our laboratory. The *18S* primers' efficiency was 100.21%.

Table 1

RACE, Genome Walker, and qPCR Primers

Gene	Primer	Oligonucleotide Sequence
SGK1	3' RACE Outer Reaction	5'-ATGTGAGTGGACCTTCTGACCTGC-3'
SGK1	3' RACE Inner Reaction	5'-AGCACTTTGACCCCGAGTTCACAG-3'
SGK1	5' RACE Outer Reaction	5'-GTGCACGTTGTGTCATTTGATTCA-3'
SGK1	5' RACE Inner Reaction	5'-CACAATATGGCCCTGGCGGTCAA-3'
SGK1	5' Genome Walker Outer	5'-CGAAGCTTCCTTTGCCGATGACTTTG-3'
SGK1	5' Genome Walker Inner	5'-GTTGAAGTCACTTGGTTTGGCTTGAG-3'
SGK1	qPCR Forward	5'-AGCAGGAACACGGCGGAA-3'
SGK1	qPCR Reverse	5'-ATCCGCTTTGTTCTGTCCTTT-3'
18S	qPCR Forward	5'-GTTAATTCCGATAACGAACGAGACTC-3'
18S	qPCR Reverse	5'-ACAGACCTGTTATTGCTCAATCTCGTG-3'

Data Analysis

Real-time data for tissue incubations and the stress experiment was analyzed using a *t*-test on ΔC_t values as outlined by Yuan et al. (2006) after being assessed for normality and equality of variances. For significant changes in *SGK1*, fold changes of

transcripts were calculated by calculating $2^{-\Delta\Delta C_t}$. Data for tissue distribution was analyzed using the equation described by Fink et al. (1998), which provides relative expression values without the use of a standard curve.

Results

RACE and Genome Walker sequence

While genome walking successfully extended the 5' CDS sequence that was obtained using RACE, the final sequence lacks approximately 26 amino acids of the 5' CDS based on alignment to dogfish *SGK1* (CAA11527.1). A total of 2187 nucleotides were obtained for *SGK1* in this study, including 1100 nt of 3' untranslated region (UTR) and the polyadenylated tail. Translation of the isolated, 5'-partial coding sequence results in a 726 amino acid protein sequence. Aligning the protein sequence from *D. sabina* with SGK1 proteins of other taxa using NCBI BLAST shows very high homology (Fig. 10). *D. sabina* SGK1 shares 91% identity with *S. acanthias* and 83% identity to *H. sapiens*. Residues important for SGK1 function are conserved. These include residues corresponding to Thr256 and Ser422 phosphorylation sites known in mammals (Lang and Cohen, 2001). Phylogenetic analysis groups *D. sabina* SGK1 with that of another elasmobranch species and also separates it from other taxonomic groups (Figure 11).

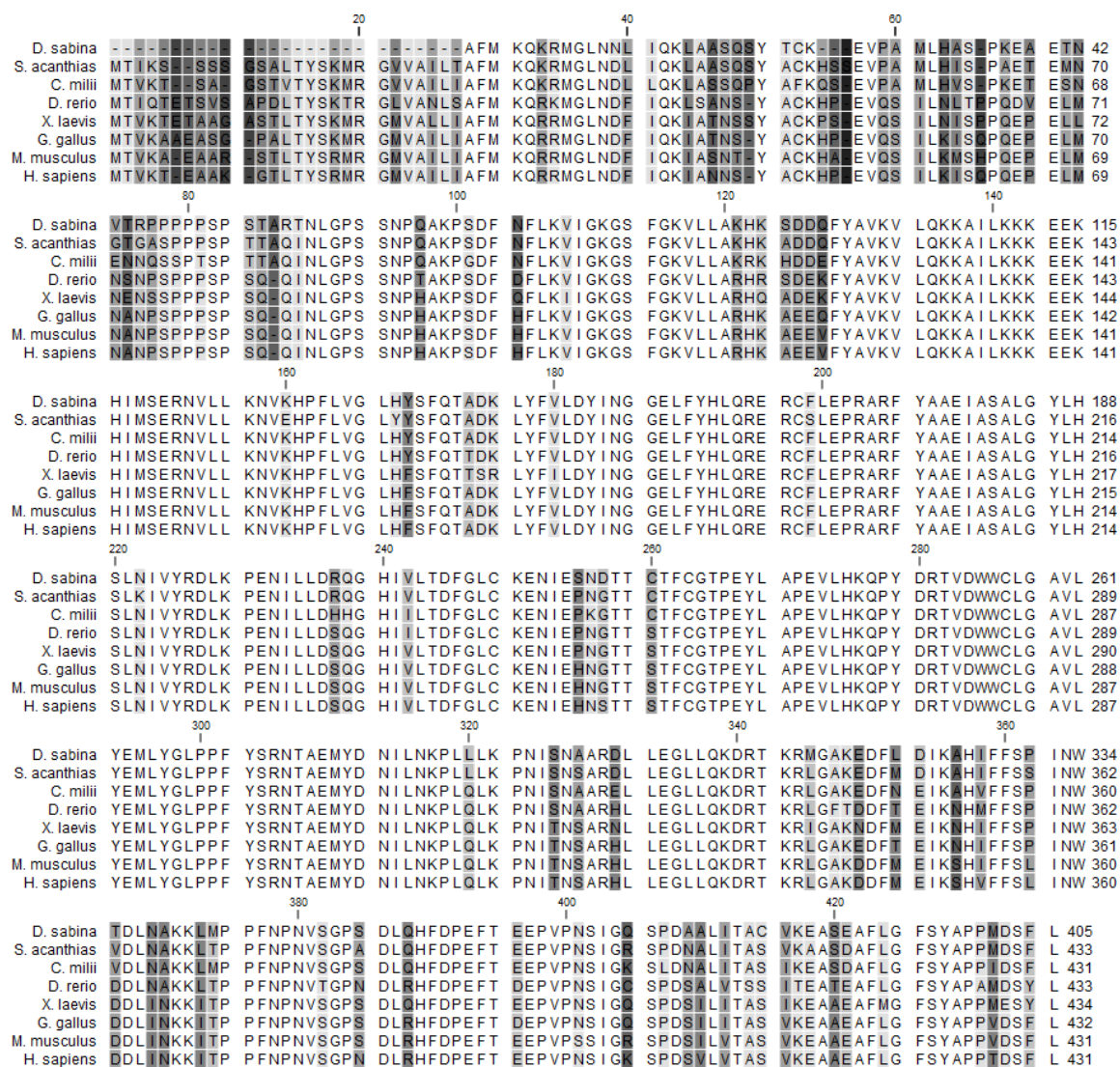


Figure 10. Amino acid alignment of *D. sabina* SGK1 with that of *S. acanthias* (CAA11527.1), *C. milii* (XP_007901699.1), *D. rerio* (NP_954682.1), *X. laevis* (NP_001083809.1), *G. gallus* (NP_989807.1), *M. musculus* (NP_035491.1), and *H. sapiens* (NP_005618.2).

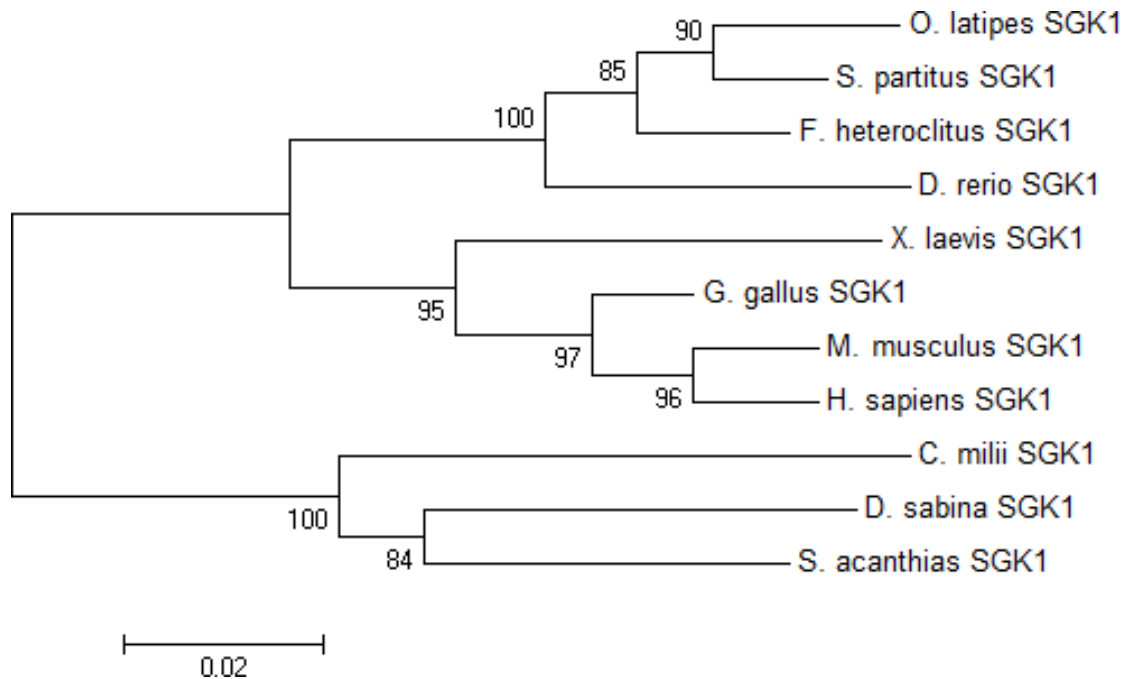


Figure 11. Phylogenetic analysis of *D. sabina* SGK1 with that of *S. acanthias* (CAA11527.1), *C. milii* (XP_007901699.1), *D. rerio* (NP_954682.1), *O. latipes* (XP_004083751.1), *F. heteroclitus* (Q5Q0U5.1), *X. laevis* (NP_001083809.1), *G. gallus* (NP_989807.1), *M. musculus* (NP_035491.1), and *H. sapiens* (NP_005618.2).

Tissue distribution of SGK1

SGK1 was present in all tissues tested. Although graphing the relative concentrations of *SGK* shows apparent differences in transcript concentrations between tissue types (Figure 12), there were no significant differences based upon the Wilcoxon analysis. Keeping this in mind, the tissues with highest transcript concentrations were blood, gill lamellae, and spiral valve. These concentrations appeared two-fold higher than those of muscle, heart ventricle, liver, and rectal gland tissues. The interrenal gland, gonad, and blood showed relatively low concentrations of transcripts, and kidney tissue had the lowest concentration.

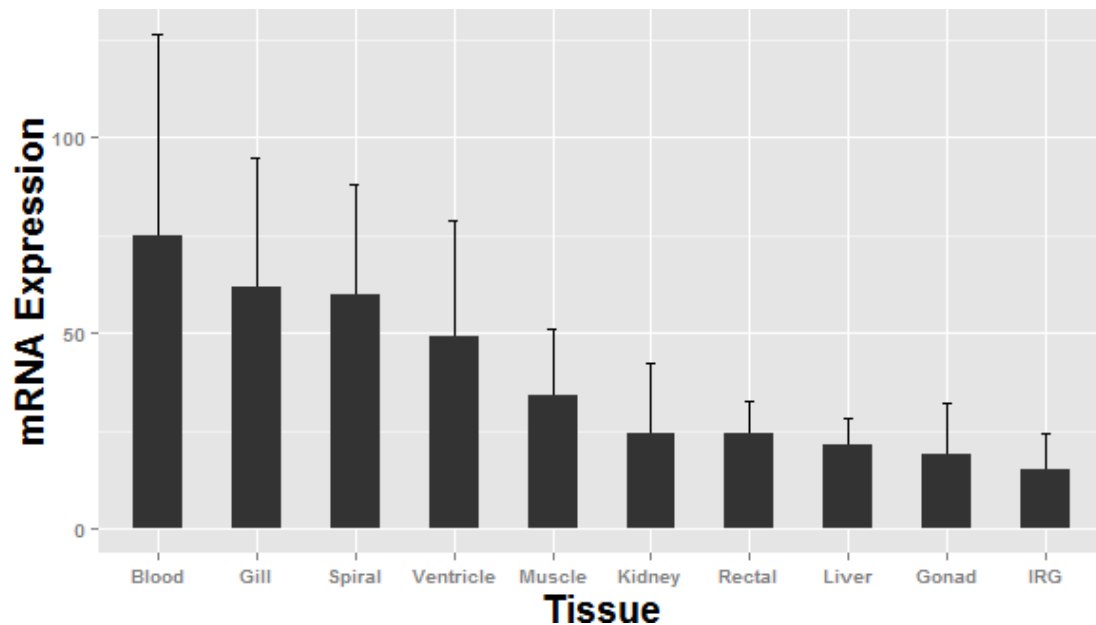


Figure 12. Mean (\pm SEM) relative abundances of *SGK1* mRNA in *D. sabina* tissues.

SGK1 transcript measurements from *in vitro* tissue incubations and stress series RBCs

Incubation of whole blood with 100 nM 1α -OH-B resulted in significant increases in *SGK1* transcripts ($p < 0.05$) (Figure 13). The two-hour incubation resulted in approximately a two-fold increase of *SGK1* transcripts. Whole blood incubated with a concentration of 500 nM 1α -OH-B did not show any significant changes in *SGK1*. None of the treatments used in the rectal gland incubations resulted in significantly different levels of *SGK1* transcripts from the control treatment (Figure 14). Additionally, there were no significant changes in *SGK1* mRNA levels in red blood cells at any point during the stress series (Figure 15).

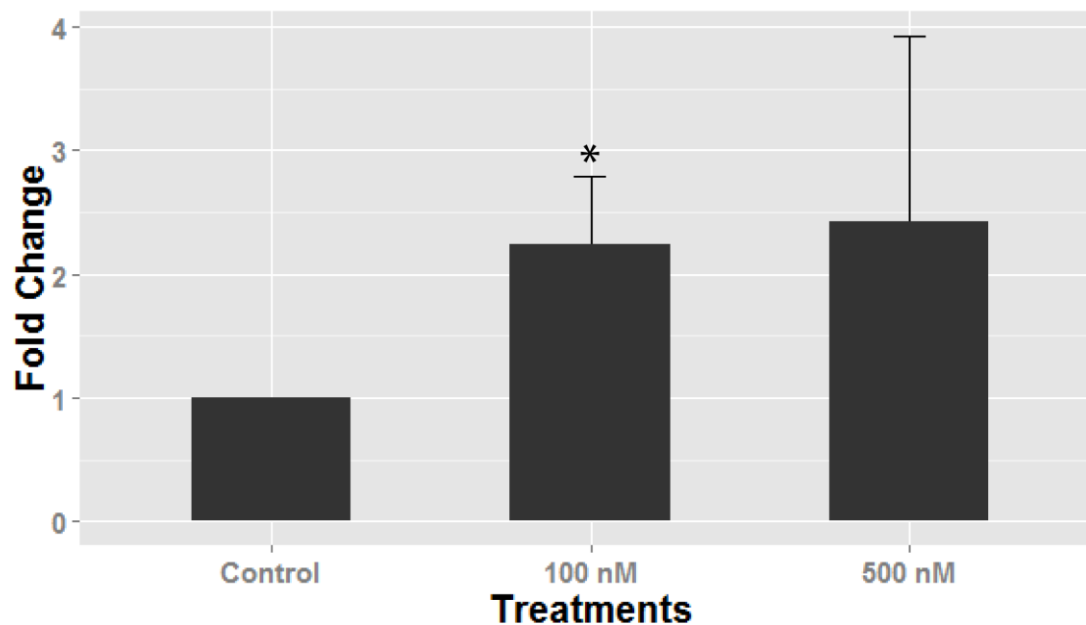


Figure 13. Fold-changes in *SGK1* mRNA abundances in *D. sabina* red blood cells exposed to 100 nM and 500 nM 1α -OH-B *in vitro*. Asterisk indicates a significant difference from control conditions ($p < 0.05$).

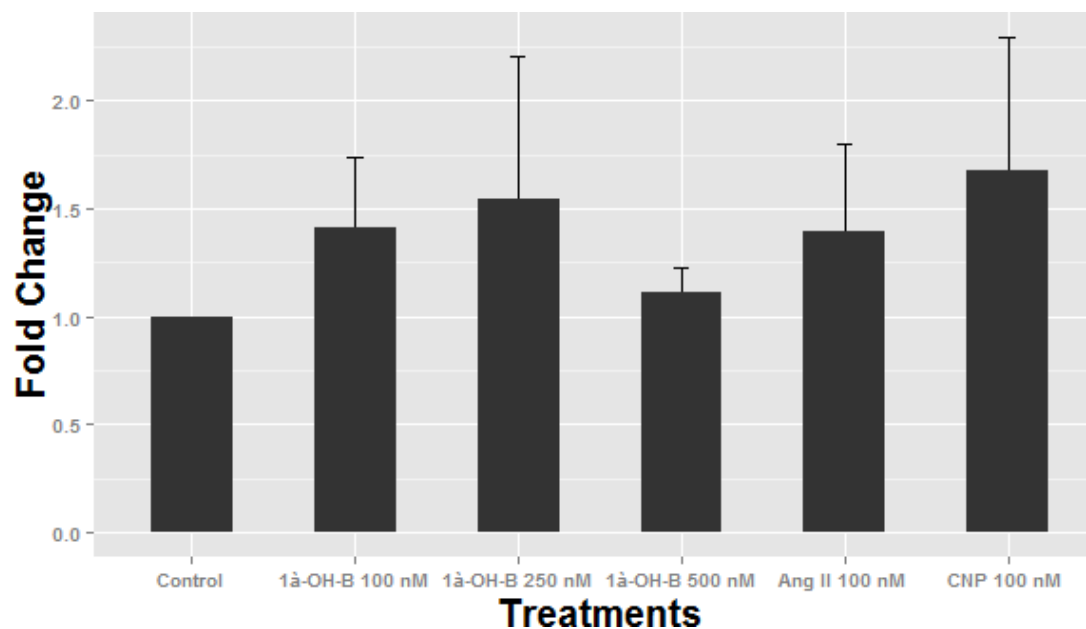


Figure 14. Fold-changes in *SGK1* mRNA abundances in *D. sabina* rectal gland tissue exposed to 100 nM 1α -OH-B, 250 nM 1α -OH-B, 500 nM 1α -OH-B, 100 nM Ang II, and 100 nM CNP *in vitro*.

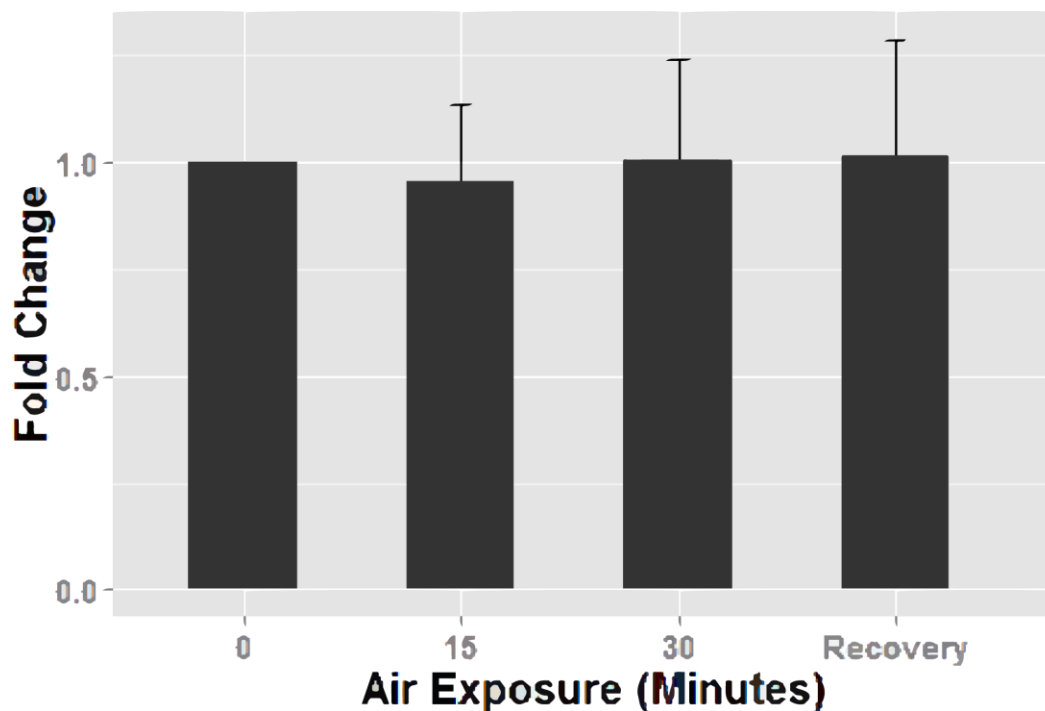


Figure 15. Fold-changes in *SGK1* mRNA abundances in *D. sabina* red blood cells over the course of a 30-minute air exposure stressor and recovery (48 hours).

Discussion

This study reports, for the first time, direct regulation of gene expression by the unique elasmobranch corticosteroid 1α -OH-B. The selected target gene, *SGK1*, was chosen due to the significant body of literature describing the regulation of this gene by corticosteroids as well as the critical function of the encoded protein in the cellular stress response. Multiple lines of evidence support the identification and characterization of *D. sabina SGK1* in the current study. Alignment of the *D. sabina* SGK1 protein sequence with those of other vertebrate taxa demonstrates the prominent homology and conservation of residues important for function. The high degree of sequence identity across diverse taxa supports a conserved function for SGK1 in vertebrate physiology and also the hypothesis that mechanisms regulating *SGK1* gene expression will be conserved in elasmobranchs. Additional support for positive identification of SGK1 is provided by

phylogenetic analysis, which groups the *D. sabina* protein with the only other available elasmobranch sequence (*S. acanthias*) and separates it from more distant taxonomic groups.

SGK1 is ubiquitously expressed in the tissues of *D. sabina*, as in other vertebrates. There is a lack of significant differences in *SGK1* transcript concentrations between tissue types, but this is likely an artifact of small sample size as large differences seem to be apparent based on average values. A previous study in *S. acanthias* demonstrated significantly higher concentrations of *SGK1* in tissues involved in glucose metabolism (liver) and some ion transport (intestine and kidney) versus other tissues (Waldegger et al., 1998). Although not significantly different from other tissue types in this study, average concentrations of *SGK1* in gill lamellae and spiral valve, which are actively involved in ion transport, were much higher than in other tissues. With this being the case, it is curious that the salt secreting rectal gland does not show higher concentrations. However, low concentrations of *SGK1* in rectal gland were also found in *S. acanthias* (Waldegger et al., 1998) using Northern blot.

Contrary to my hypothesis, none of the treatments in rectal gland incubations with 1α -OH-B elicited significant changes in *SGK1* mRNA expression. This could be caused by several factors. As discussed above, *SGK1* mRNA expression in tissue suites showed a relatively low amount of this gene in rectal gland. These low levels could indicate that *SGK1* does not play a significant role in the functioning of the rectal gland. It is also possible that this *SGK1* function is regulated at the protein level rather than the transcriptional level. This occurs by phosphorylation of Ser422 and Thr256, rather than binding to a GRE element in the *SGK1* promoter by corticosteroids (Lang and Cohen,

2001). In the rectal gland of *S. acanthias*, *SGKI* mRNA was upregulated when tissue was incubated *in vitro* with hypertonic solutions or the osmoregulatory hormone vasoactive intestinal polypeptide, the latter of which acts to regulate blood pressure (Waldegger et al., 1998). Therefore, the activity of the rectal gland may be more responsive to changes in extracellular tonicity or altered blood flow rather than stimulation by corticosteroids.

Given the significant increase in *SGKI* mRNA in red blood cells exposed to 100 nM 1α -OH-B for 2 hours, it is clear that this corticosteroid does regulate cellular processes at the gene level. This is the first instance in which 1α -OH-B has been shown to regulate gene transcription and supports the hypothesis that 1α -OH-B functions in a manner similar to glucocorticoids in other taxa. Although not yet identified in *D. sabina* *SGKI*, it is likely that regulation by 1α -OH-B is mediated through binding to a GRE in its promoter, as seen in other taxa. Lack of significant change in *SGKI* mRNA after exposure to 500 nM 1α -OH-B may have been due to the biologically irrelevant high dosage.

SGKI mRNA in *D. sabina* RBCs from the stress experiment did not exhibit any significant changes. The experiment may have not provided ample time to exhibit significant changes. Although *SGKI* has been described as an early-response gene and has been shown to change significantly within 30 minutes, this has been in the presence of elevated glucocorticoids (Baskin and Sayeski, 2012; Johnstone, 2011), a situation similar to the red blood cell *in vitro* incubations described above. Elevated concentrations of plasma corticosteroids in this experiment were not observed until 48 hours after air exposure. It is possible that steroid concentrations at 48 hours were not elevated enough

or for a long-enough duration to produce a detectable change in *SGK1* mRNA at this time point.

In conclusion, this study is the first to demonstrate a role for 1α -OH-B in gene regulation. Regulation of *SGK1* in red blood cells indicates these cells respond to changes in circulating corticosteroids, and future studies should further examine the role of 1α -OH-B in the cellular stress response of red blood cells and other elasmobranch tissues. To further elucidate the mechanisms of gene regulation as well as the glucocorticoid versus mineralocorticoid actions of 1α -OH-B, studies should also pursue the remainder of the 5' *SGK1* mRNA sequence and promoter region of *D. sabina SGK1* to identify transcription factor binding sites and response elements including the GRE. This will help to both better understand the functioning of SGK1 in *D. sabina*, as well as provide additional evidence for or against the role of 1α -OH-B as a glucocorticoid.

CHAPTER IV

SUMMARY

This study provides several lines of evidence for the role of 1α -OH-B as a GC, and demonstrates, for the first time, that 1α -OH-B has the ability to regulate genes at the transcriptional level. Trends of total plasma corticosteroids (which are presumed to be 1α -OH-B) match changes in glucose during the stress response. Additionally, individual measurements of basal total plasma corticosteroids and glucose are significantly correlated. This supports a role for 1α -OH-B as a regulator of glucose metabolism, which is a key component of GC function observed for both cortisol and corticosterone.

Whole blood incubated with 1α -OH-B *in vitro* exhibits increases in transcript abundances of a highly conserved glucocorticoid-regulated gene, *SGK1*. Given that a GRE is present in the promoter region of *SGK1* in other vertebrates, it is likely that *SGK1* regulation in the blood of elasmobranchs is controlled by the same mechanism.

Although evidence from this study supports the role of 1α -OH-B as a glucocorticoid, further research is still warranted in this area. For example, an understanding of the mechanism by which 1α -OH-B regulates the expression of genes such as *SGK1* transcripts is needed. Isolating the promoter region of this gene will indicate whether or not a GRE is present and therefore whether 1α -OH-B regulates transcription in a manner similar to other GCs. Additionally, methods for precisely measuring circulating levels of 1α -OH-B (e.g., assays using specific antibodies) still need to be developed in order to definitively understand its role in the physiological stress response and regulation of glucose metabolism.

APPENDIX

UNIVERSITY OF SOUTHERN MISSISSIPPI
Office of the Vice President for Research
Institutional Animal Care and Use Committee
118 College Dr. #5116
Hattiesburg, MS 39406-0001

APPROVED PROTOCOL MODIFICATION FORM

NOTES:

If changes requested in this amendment cannot be satisfactorily justified as fitting within the original objectives of the protocol noted, a new protocol should be submitted.

Amendments adding to or modifying Radiological and/or Hazardous Agents or Surgical Procedures should attach the appropriate form from the ACUP form. Be sure to note the protocol number **AND** the date of your amendment at the top of the attached pages.

Investigators should conduct literature searches to assure that amendments fit with the same guidelines as those required for the original protocol.

Protocol #: **13031403**

Original Approval Date: **4/11/13**

Title of Protocol: **Collection, maintenance, breeding and studies on the endocrinology and physiology of elasmobranch fishes**

Principal Investigator: **Andrew N. Evans**

Department & College: **Coastal Sciences, Coll. of Sci/Tech**

Office Address: **703 East Beach Dr., Ocean Springs, MS 39564**

Office Phone #: **(228) 872-4298**

Office Fax #:

E-Mail Address: **andrew.n.evans@usm.edu**

Nature of change in Protocol:

Addition/change in Personnel: ☐

Change in animal housing: ☐

☐ Other:

Addition/deletion of procedure: ☒

Change in animal species: ☐

Change in animal numbers: ☐

Change in title: ☐

Explain:

As described in the approved protocol, our laboratory studies the actions and regulation of a corticosteroid hormone entirely unique to the elasmobranch fishes, 1alpha-hydroxycorticosterone (1alpha-B), which is thought to play a central role in both osmoregulation and the stress response. With regards to this modification, we are proposing new experiments designed to examine the primary stress response of Atlantic stingrays and specifically the role that 1alpha-B plays in this response. The specific role of 1alpha-B in the stress response has never before been examined despite the fact that this steroid is the major, and possibly only, corticosteroid produced in elasmobranchs (corticosteroids facilitate the stress response in other vertebrates). Previous studies have attempted to examine this, but did not run controlled stress experiments (instead examining other samples e.g. from longline captured sharks; Manire et al., 2007) and were not able to specifically quantify 1alpha-B as historically there have not been methods to do so. We are working with a colleague at the University of Mississippi that has developed the first methods for synthesizing 1alpha-B and have published the development of a specific assay for this steroid as cited in the approved protocol.

The tissue sampling methods will not differ from the currently approved protocol, as we will only conduct blood draws for this experiment. The experimental procedure is new: we are proposing to use aerial exposure as a stressor, consistent with both anthropogenic stress experienced by captured stingrays (either via hook-and-line angling or in the shrimp trawl fishery) and recently published work detailing the secondary stress response (i.e. NOT the corticosteroid response) in skates (Cicia et al. 2012; University of New England protocol UNE03-2010). Aerial exposure is an excellent method for examining stress in batoids (i.e. skates and stingrays) because it mimics typical fishing stress as described--particularly lengthy on-deck exposure in the shrimp trawl fishery--and also provides a consistent but not overwhelming or exhausting stimulus.

For our experiments we would use the same general procedure as used for skates but with less exposure time: stingrays would be netted and placed on wet tables, exposed to air in this manner for 30 minutes with serial blood draws taken at 0, 15 and 30 minutes (0.5 mL per draw) and then placed back into their tanks. 1 α -B, blood gases, ions and other physiological indicators would be quantified. A follow-up blood sample would be taken 2-5 days later to assess recovery of 1 α -B and physiological parameters. The number of animals used for this experiment would be <20 (likely 12-16) and would be part of the total already proposed in the initial protocol. This new experiment does not include euthanizing the animals except in cases where deemed necessary (e.g. poor condition following aerial exposure, which is expected to be rare).

The total amount of blood drawn per animal within the 30 minute stress period (1.5 mL) is less than the maximum suggested volume for fish. A veterinarian colleague recommends limiting a blood draw event to 1% total body weight for stingrays/sharks; this is also stated by other veterinarians regarding fish in general, e.g.: (<http://veterinarymedicine.dvm360.com/vetmed/article/articleDetail.jsp?id=256178&sk=&date=&pageID=2>). For our animals, which will be in the 500-1000g range, 1% body weight would allow for a maximum 7.5-10 mL of blood. The proposed total of 1.5 mL of blood within 30 minutes (three samples of 0.5 mL) would only equal 0.3 to 0.15% total body weight. Additional references are provided by the following documents and websites:

1) UC Davis (and others, using the same text) IACUC guidelines for blood volumes: state that as a general rule 1% of an animal's weight in grams can be collected in a 24-hr period: (safetyservices.ucdavis.edu/ps/a/IACUC/po/bloodVolumes)

Similar to most fish species, elasmobranchs have approximately 5% blood volume (i.e. ~5 mL / 100g body weight; Good et al, 2008); in that light:

2) LSU IACUC, policy #2, point 2.1: Using a calculation that arrives at a maximum blood draw equal to 1.5% of animal weight for mammals, which is based on % of the total blood volume, the equivalent in elasmobranch fishes would be [22% of the total blood volume (5%)] = 1.1% of a fish's weight can be drawn in any 2-week period.

3) University of Pittsburg IACUC: maximum survival bleed volume without fluid replacement = 10% CBV (circulating blood volume). Elasmobranchs: 10% of 5 mL/100g = 0.5 mL per 100g body weight (2.5 – 5 mL for stingrays 500 to 1000g).

Additional literature searches were conducted specific to this protocol modification, again using Web of Knowledge and PubMed (1995-2013). New search strings were: '1 α -hydroxycorticosterone & stress & primary', 'elasmobranch & stress & primary' & 'elasmobranch & aerial'. Relevant results for the first and third search strings were limited to the Cicia et al. (2012) and Manire et al. (2007) papers described above and cited at the end of this document. The second string ('elasmobranch & stress & primary') returned 7 (WofK) and 6 (PubMed) hits, with only a few being relevant to the proposed experiment including the Cicia/Manire papers and others describing secondary stress parameters and not corticosteroids, with the exception of 1 paper authored by the PI (Evans) describing the ex vivo regulation of 1 α -hydroxycorticosterone synthesis.

Date amendment is needed by: **9/5/13 if possible**

ASSURANCE BY INVESTIGATOR:

I assure that these activities do not unnecessarily duplicate previous experiments conducted here or elsewhere. I agree to conduct this project in accordance with the protocol originally submitted and approved by the IACUC, and to obtain prior approval from the committee before modifying the protocol.

Investigator Signature: _____

Date: 9/5/13

References

Cicia, A.M., Schlenker, L.S., Sulikowski, J.A. and Mandelman, J.W. (2012). Seasonal variations in the physiological stress response to discrete bouts of aerial exposure in the little skate, *Leucoraja erinacea*. *Comp. Biochem. Physiol. A* 162: 130-138.

Good, J.P., Wells, A. and Hazon, N. (2008). Measurement of blood volume in the elasmobranch fish *Scyliorhinus canicula* following acute and long-term salinity transfers. *J. Fish Biol.* 73: 1301-1313.

LSU IACUC Policies: http://www.vetmed.lsu.edu/dlam/IACUC%20policies_introd.htm

Manire, C.A., Rasmussen, L.E., Maruska, K.P. and Tricas, T.C. (2007). Sex, seasonal, and stress-related variations in elasmobranch corticosterone concentrations. *Comp. Biochem. Physiol. A* 148: 926-935.

University of Pittsburgh IACUC: www.iacuc.pitt.edu/sites/default/files/documents/Blood%20Volume%20Withdrawal%20Policy.pdf



**THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.**

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001

Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

AMENDMENT NOTIFICATION

The *proposal amendment* noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **13031403**
 PROJECT TITLE: **Collection, Maintenance, Breeding & Studies on the Endocrinology & Physiology of Elasmobranch Fishes**
 PROPOSED PROJECT DATES: **03/2013 – 9/2015**
 AMENDMENT NUMBER: **1**
 PRINCIPAL INVESTIGATOR(S): **Andrew Evans**
 DEPARTMENT: **Coastal Sciences/USM GCRL**
 FUNDING AGENCY/SPONSOR:
 IACUC COMMITTEE ACTION: **Full Committee Approval**
 PROTOCOL EXPIRATION DATE: **9/30/2015**

Frank Moore, Ph.D.
IACUC Chair

Date 29/06/2013

REFERENCES

- Acerete, L., Balasch, J.C., Espinosa, E., Josa, A., and Tort, L., 2004. Physiological responses of Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. *Aquaculture*. 237, 167-178.
- Anderson, W.G., 2012. The endocrinology of 1 α -hydroxycorticosterone in elasmobranch fish: a review. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 162(2), 73-80.
- Anderson, W.G., Nawata, C. M., Wood, C. M., Piercey-Normore, M. D., and Weihrauch, D., 2012. Body fluid osmolytes and urea and ammonia flux in the colon of two chondrichthyan fishes, the ratfish, *Hydrolagus colliei*, and spiny dogfish, *Squalus acanthias*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 161, 27-35.
- Barton, B.A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* 42, 517-525.
- Baskin, R. and Sayeski, P., 2012. Angiotensin II mediates cell survival through upregulation and activation of the serum and glucocorticoid inducible kinase 1. *Cell Signal*. 24(2), 435-442.
- Boutilier, R.G. and Ferguson, R.A., 1989. Nucleated red cell function: metabolism and pH regulation. *Can. J. Zool.* 67, 2986-2993.
- Brennan, F.E. and Fuller, P.J., 2000. Rapid upregulation of serum and glucocorticoid-regulated kinase (sgk) gene expression by corticosteroids *in vivo*. *Mol. Cell. - Endocrinol.* 166(2), 129-136.
- Brooks, E.J., Sloman, K.A., Liss, S., Hassan-Hassanein, L., Danylchuk, A.J., Cooke, S.J., Mandelman, J.W., Skomal, G.B., Sims, D.W., Suski, C.D., 2011. The stress

- physiology of extended duration tonic immobility in the juvenile lemon shark, *Negaprion brevirostris* (Poey 1868). J. Exp. Mar. Biol. 409, 351-360.
- Carroll, S.M., Ortlund, E.A., and Thorton, J.W., 2011. Mechanisms for the evolution of a derived function in the ancestral glucocorticoid receptor. PLoS Genet. 7(6), e1002117.
- Cicia, A.M., Schlenker, L.S., Sulikowski, J.A., and Mandelman, J.W., 2012. Seasonal variations in the physiological stress response to discrete bouts of aerial exposure in the little skate, *Leucoraja erinacea*. Comp. Biochem. Physiol. 162, 130-138.
- Chen, S., Bhargava, A., Mastroberardino, L., Meijer, O.C., Wang, J., Buse, P., Firestone, G.L., Verrey, F., and Pearce, D., 1999. Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proc. Natl. Acad. Sci. 96, 2514-2519.
- Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181, 45-58.
- Currie, S. and Tufts, B.L., 1997. Synthesis of stress protein 70 (Hsp70) in rainbow trout (*Oncorhynchus mykiss*) red blood cells. J. Exp. Biol. 200, 607-614.
- Currie, S., Tufts, B.L., and Moyes, C.D., 1999. Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. Am. Physiol. Soc. 276, R990-R996.
- Davis, M.W. and Schreck, C.B., 2005. Responses by Pacific halibut to air exposure: lack of correspondence among plasma constituents and mortality. Trans. Am. Fish. Soc. 134(4), 991-998.

- Delaney, M.A. and Klesius, P.H., 2004. Hypoxic conditions induce Hsp70 production in blood, brain, and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture*. 236, 633-644.
- DeRoos, R. and DeRoos, C.C., 1973. Elevation of plasma glucose levels by mammalian ACTH in the spiny dogfish shark (*Squalus acanthias*). *Comp. Biochem. Physiol.* 52(1), 145-146.
- Evans, A.N. and Nunez, B.S., 2010. Regulation of mRNAs encoding the steroidogenic acute regulatory protein and cholesterol side-chain cleavage enzyme in the elasmobranch interrenal gland. *Gen. Comp. Endocrinol.* 168, 121-132.
- Evans, A.N., Rimoldi, J.M., Gadepalli, R.S.V. and Nunez, B.S., 2010. Adaptation of a corticosterone ELISA to demonstrate sequence-specific effects of angiotensin II peptides and C-type natriuretic peptide on 1α -hydroxycorticosterone synthesis and steroidogenic mRNAs in the elasmobranch interrenal gland. *J. Steroid Biochem. Mol. Biol.* 120, 149-154.
- Ferguson, R.A. and Tufts, B.L., 1992. Physiological effects of brief air exposure in exhaustively exercised rainbow trout (*Oncorhynchus mykiss*): implications for “catch and release” fisheries. *Can. J. Fish. Aquat. Sci.* 49, 1157-1162.
- Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., Bohle, R.M., 1998. Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat. Med.* 4(11), 1329-1333.
- Frick, L.H., Reina, R.D., and Walker, T.I., 2010. Stress related physiological changes and post-release survival of Port Jackson sharks (*Heterodontus portusjacksoni*) and

- gummy sharks (*Mustelus antarcticus*) following gill-net and longline capture in captivity. J. Exp. Mar. Biol. Ecol. 385, 29-37.
- Grimm, A.S., O'Halloran, M., Idler, D.R., 1969. Stimulation of sodium transport across isolated toad bladder by 1α -hydroxycorticosterone from an elasmobranch. J. Fish. Res. Board Can. 26, 1823-183.
- Hazon, N. and Henderson, I.W. 1984. Secretory dynamics of 1α -hydroxycorticosterone in the elasmobranch fish *Scyliorhinus canicula*. J. Endocrinol. 103(2), 205-211.
- Hazon, N. and Henderson, I.W., 1985. Factors affecting the secretory dynamics of 1α -hydroxycorticosterone in the dogfish, *Scyliorhinus canicula*. Gen. Comp. Endocrinol. 59, 50-55.
- Hoffmayer, E.R. & Hendon, J.M. & Parsons, G.R., 2012. Seasonal modulation in the secondary stress response of a carcharhinid shark, *Rhizoprionodon terraenovae*. Comp. Biochem. Physiol. A. 162(2), 81-87.
- Hoffmayer, E.R. and Parsons, G.R., 2001. The physiological response to capture and handling stress in the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. Fish Physiol. Biochem. 25, 277-285.
- Idler, D.R., Freeman, H.C., and Truscott, B.J., 1967a. 1α -hydroxycorticosterone: synthesis *in vitro* and properties of an interrenal steroid in the blood of cartilaginous fish (genus *Raja*). Fish. Res. Board Can. 9(5), 297-317.
- Idler, D.R., Freeman, H.C., and Truscott, B., 1967b. Biological activity and protein binding of 1α -hydroxycorticosterone: An interrenal steroid in elasmobranch fish. Gen. Comp. Endocrinol. 9(2), 207-213.

- Johnstone III, W.M., 2011. Characterization of Serum and Glucocorticoid Kinase Isoforms and a Membrane Glucocorticoid Receptor in the Euryhaline Tilapia (*Oreochromis mossambicus*): Implications in Salinity Adaptation. PhD. Dissertation. North Carolina State University: USA.
- Klesch, W. and Sage, M., 1975. The stimulation of corticosteroidogenesis in the interrenal of the elasmobranch *Daysides sabina* by mammalian ACTH. *Comp. Biochem. Physiol.* 52(1), 145-146.
- Kneebone, J., Chisholm, J., Bernal, D., and Skomal, G., 2013. The physiological effects of capture stress, recovery, and post-release survivorship of juvenile sand tigers (*Carcharias taurus*) caught on rod and reel. *Fish. Res.* 147, 103-114.
- Laffel, L., 1999. Ketone bodies: a review of physiology, pathophysiology, and application of monitoring to diabetes. *Diabetes Metab. Res.* 15, 412-426.
- Lang, F., and Cohen, P., 2001. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci. STKE.* (108), re17. [DOI: 10.1126/scisignal.1082001re17].
- Lang, F., Böhmer, C., Palmada, M., Seebohm, G., Strutz-Seebohm, N., and Vallon, V., 2006. Pathophysiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol. Rev.* 86, 1151-1178.
- Loffing, J., Flores, S.Y., and Staub, O., 2006. Sgk kinases and their role in epithelial transport. *Annu. Rev. Physiol.* 68, 16.1-16.30.
- Lund, S.G., Lund, M.E.A., and Tufts, B.L., 2003. Red blood cell Hsp70 mRNA and protein as bio-indicators of temperature stress in brook trout (*Salvelinus fontinalis*). *Can. J. Fish. Aquat. Sci.* 67, 460-470.

- Mandelman, J.W. and Farrington, M.A., 2007. The physiological status and mortality associated with otter-trawl capture, transport, and captivity of an exploited elasmobranch, *Squalus acanthias*. ICES J. Mar. Sci. 64, 122-130.
- Manire, C.A., Rasmussen, L.E.L., Maruska, K.P., and Tricas, T.C., 2007. Sex, seasonal, and stress-related variations in elasmobranch corticosterone concentrations. Comp. Biochem. Physiol. 148, 926-935.
- McCormick, J.A., Bhalla, V., Pao, A.C., and Pearce, D., 2005. SGK1: a rapid aldosterone-induced regulator of renal sodium reabsorption. Physiol. 20, 134-139.
- Milston, R.H., Davis, M.W., Parker, S.J., Olla, B.L., Clements, S., Schreck, C.B., 2006. Characterization of the physiological stress response in Lingcod. Trans. Am. Fish. Soc. 135(5), 1165-1174.
- Mommsen, T.P. and Moon, T.W., 1987. The metabolic potential of hepatocytes and kidney tissue in the little skate, *Raja erinacea*. J. Exp. Zool. 244, 1-8.
- Mommsen, T.P., Vijayan, M.M., and Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish. Biol. Fisher. 9, 211-268.
- Nordlie, R.C., Foster, J.D., and Lange, A.J., 1999. Regulation of glucose production by the liver. Annu. Rev. Nutr. 19, 379-406.
- Nunez, S. and Trant, J.M., 1999. Regulation of interrenal gland steroidogenesis in the Atlantic stingray (*Dasyatis sabina*). J. Exp. Zool. 284(5), 517-25.
- Nunez, S. and Trant, J.M., 1998. Molecular biology and enzymology of elasmobranch 3β -hydroxysteroid dehydrogenase. Fish Physiol. Biochem. 19, 293-304.

- Ozcan, C., Bienengraeber, M., Dzeja, P.P., and Terzic, A., 2002. Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. *Amer. J. Physiol. Heart Circ. Physiol.* 282(2), H531-H539.
- Rasmussen, L.E.L. and Crow, G.L., 1993. Serum corticosteroid concentrations in immature captive white-tip reef sharks, *Triaenodon obesus*. *J. Exp. Zool.* 267, 283-287.
- Reid S.G., Bernier, N.J., and Perry, S.F., 1998. The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol.* 120, 1-27.
- Renshaw, G.M.C., Kutek, A.K., and Grant, G.D., Anoopkumar-Dukie, S., 2012. Forecasting elasmobranch survival following exposure to severe stressors. *Comp. Biochem. Physiol.* 162, 101-112.
- Richards, J.G., Heigenhauser, G.J.F., and Wood, C.M., 2003. Exercise and recovery metabolism in the Pacific spiny dogfish (*Squalus acanthias*). *J. Comp. Physiol. B.* 173, 463-474.
- Romero, L.M. and Butler, L.K., 2007. Endocrinology of stress. *Int. J. Comp. Psych.* 20, 89-95.
- Sapolsky, R.M., Romero, L.M., and Munck, A.U., 2000. How do glucocorticoids influence stress response? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* 21(1), 55-89.
- Skomal, G. and Bernal, D., 2010. Physiological responses to stress in sharks. In: Carrier, J.C., Musick, J.A., Reithaus, M.R. (Eds.), *Biodiversity, Adaptive Psychology and Conservation*. CRC press, pp. 459-490.

- Snelson, F.F. Jr., Rasmussen, L.E.L., Johnson, M.R., and Hess, D.L., 1997. Serum concentrations of steroid hormones during reproduction in the Atlantic stingray *Dasyatis sabina*. *Gen. Comp. Endocrinol.* 108, 67-69.
- Speers-Roesh, B. and Treberg, J.R., 2010. The unusual energy metabolism of elasmobranch fishes. *Comp. Biochem. Physiol.* 155, 417-434.
- Suski, C.D., Cooke, S.J., Danylchuk, A.j., O'Connor, C.M., Gravel, M., Redpath, T., Hanson, K.C., Gingerich, A.J., Murchie, K.J., Danylchuk, S.E., Koppelman, J.B., Goldberg, T.L., 2007. Physiological disturbance and recovery dynamics of bonefish (*Albula vulpes*), a tropical marine fish, in response to variable exercise and exposure to air. *Comp. Biochem. Physiol. A.* 148, 664-673.
- Truscott, B and Idler, D.R., 1972. Corticosteroids in plasma of elasmobranchs. *Comp. Biochem. Physiol. A.* 42, 41-50.
- Waldegger, S., Barth, P., Forrest, J.N. Jr., Greger, R., and Lang, F., 1998. Cloning of sgk serine-threonine protein kinase from shark rectal gland – a gene induced by hypertonicity and secretagogues. *Eur. J. Physiol.* 436, 575-580.
- Walsh, P.J. Kajimura, M., Mommsen, T.P., and Wood, C.M., 2006. Metabolic organization and effects of feeding on enzyme activities of the dogfish shark (*Squalus acanthias*) rectal gland. *J. Exp. Biol.* 209, 2929-2938.
- Watson, R.R. and Dickson, K.A., 2001. Enzyme activities support the use of liver lipid-derived ketone bodies as aerobic fuels in muscle tissues of active sharks. *Physiol. Biochem. Zool.* 74(2), 273-282.
- Webster, M.K., Goya, L., Ge, Y., Maiyer, A.C., Firestone, G.L., 1993a. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which

is transcriptionally induced by glucocorticoids and serum. *Mol. Cell. Biol.* 13(4), 2031-2040.

Webster, M.K., Goya, L., and Firestone, G.L., 1993b. Immediate-early transcriptional regulation and rapid mRNA turnover of a putative serine/threonine protein kinase. *J. Biol. Chem.* 268(16), 11482-11485.

Zammit, V.A. and Newsholme, E.A., 1979. Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. *Biochem. J.* 184, 313-322.