MEASURING STRESS IN CAPTIVE BONOBOS: A LOOK TO THE PAST AND FUTURE TO IMPROVE METHODS

by

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A DISSERTATION

Presented to the Departments of Anthropology and Human Physiology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

June 2016

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Doctor of Philosophy

Departments of Anthropology and Human Physiology

June 2016

Title: Measuring Stress in Captive Bonobos: A Look to the Past and Future to Improve Methods

Understanding stress in primates has wide ranging implications. It impacts how we understand human stress from an evolutionary perspective and how captive and laboratory primates are kept to best impact their health and well-being. Stress studies in non-human primates often focus on measuring cortisol. Cortisol can be measured in blood, urine, feces, saliva, or hair in primates. Quantification of cortisol is typically achieved by enzyme or radio immunoassay, high performance liquid chromatography, or mass spectroscopy.

Once cortisol is quantified, it is traditionally related to stress in primates by determining associations to variables classically seen as potential stressors, such as dominance rank, aggression received, food availability, or moving facilities for captive primates. It is vitally important that researchers engaging in non-human primate cortisol research properly select the sample type and quantification method best suited to answer their particular research questions.

It is also important that the quantification of cortisol and the subsequent reporting of methods and results obtained is done correctly and transparently so that other researchers are able to interpret and build upon previous results. In this dissertation, the past instances of non-human primate cortisol analyses are reviewed with a particular

focus on urinary analyses. A critical view is taken of past methods and means of reporting results, and suggestions for better practices are made. Researchers should be reporting ranges of raw values measured for cortisol in order to help establish expected values in specific species, as well as explicit justifications for protocol modifications if any are made.

A new method for assessment of urinary cortisol in bonobos (*Pan paniscus*) is validated and reported. A longitudinal study of captive bonobos at the Columbus Zoo and Aquarium contributed 154 urine samples for analyses over three field seasons (2012, 2013, and 2014). A commercially available cortisol EIA kit (Arbor Assays, Ann Arbor, MI) was determined to be appropriate for use in bonobos and subsequently used to test 154 urine samples. A diurnal cortisol rhythm was detected in bonobos for the first time. Individual differences were identified in AM and PM samples and will be the foundation for future behavioral association investigations.

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ACKNOWLEDGEMENTS

I would like to thank my committee for their time and assistance in aiding me in the completion of my dissertation, Klaree Boose for generously sharing her dataset for analysis in this project, and Ann Nacey Maggioncalda for funding the research presented here. A special thank you to my chair, Frances White, for giving me this opportunity to complete my PhD and for her enduring support throughout the process.

For Wes and Josie. Don’t let your dreams be dreams.

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CHAPTER I

INTRODUCTION

Primate Socioendocrinology: A focus on cortisol

Primate socioendocrinology is a term that has been used numerous times in non-human primate research since 1990 (Worthman, 1990). There seems to be no strict definition for primate socioendocrinology, as evidenced by the multitude of ways it has been defined and used. Such definitions range from simple explanations such as the measurement of social environment on the timing and degree of hormonally induced changes (Dixson and Nevison, 1997) to a way to integrate neuro-biological and socio-behavioral data (Gettler, 2014) to more specific definitions that seek to break away from biologically deterministic ideas of sociobiology. For example, one definition describes socioendocrinology as the study of hormones in the context of social behavior without a deterministic viewpoint that hormones dictate behavior, and recognition that hormones are under multiple selective pressures to regulate organisms' behaviors (Anestis, 2010).

For the purposes of this review, socioendocrinology will be defined as the study of the covariation between hormones and behavior and the mechanisms that affect the two, such as health, life history, and social status (Maggioncalda, 1995). Hormones and behavior have been shown to have the ability to influence one another in mammals. For example, a normally functioning stress response elicits physiological changes that allow an animal to quickly adapt to a transient threat with elevated heart rate and increased focus. However, the presence of a chronic social stressor, such as consistently receiving aggression from a higher ranking group member can influence the hormonal stress response and induce a change from a normal pattern of hormone release.

In primates, stress has been studied many ways in both wild and captive populations. Researchers have investigated how situations known to be stressful affect the hormones and health of primates. For example, the behavioral outcomes of stress are one focus of stress research, such as hair plucking in captive bonobos (Brand, in review). Researchers have investigated how situations known to be stressful affect the hormones and health of primates. Other approaches have investigated associations between an individual's rank within a group and the stress response, (Emery Thompson et al., 2010; Muller and Wrangham, 2004; Surbeck et al., 2012).

Inclusion of biomarker research in the field of primate socioendocrinology has vastly improved our understanding of how primates deal with stress and which situations are stressful as well as greatly improved our understanding of the development of the human stress response from an evolutionary perspective. However, the process of utilizing and refining biomarker testing is still relatively new and primatology as a whole could benefit greatly from several improvements in methods and means of reporting moving forward. Currently, primate studies that use cortisol testing are diffuse and unorganized in terms of methods used and how results are reported, and there is little standardization, discussion, or agreement on such methods. Standardizing methodology and reporting detailed results would enable the field to have data that is comparable between studies rather than simply within groups and also help to establish expected reference values for measured variables including cortisol in its various sample matrices.

In this dissertation, I will present a review of available literature on studies of cortisol and stress in primates, discuss how the field can move toward standardizing its approach to urinary hormone research, and present results from a longitudinal study of

captive bonobos at the Columbus Zoo and Aquarium (CZA) in Columbus, Ohio. Cortisol has been a focus of primate stress research for over 30 years. However the most commonly used method of testing- an enzyme immunoassay (EIA) using an antibody developed at a lab at University of California, Davis- will become obsolete in the near future, as the UC Davis lab has run out of the only batch of antibody ever produced. This presents primatologists with a unique opportunity to investigate new commercially available kits and to standardize or calibrate testing methods to enable comparison of results between studies in the future.

Commercially available kits yield several advantages to primatologists. They enable more researchers to undertake biomarker testing on their own since EIAs are relatively simple tests to conduct if one has access to the proper equipment. The sample cost is far cheaper than the currently employed methods, which include sending samples to labs that charge up to $1600 per sample for biomarker determination. In addition, do-it-yourself EIAs require far smaller sample volumes than sending samples for testing at outside labs. The established polyclonal anti-cortisol antibody method was developed at UC Davis 30 years ago and has been outmoded by new technology that uses newly developed, highly specific monoclonal cortisol antibodies. This enables commercially available kits on the market today to have high sensitivities to target analytes so that samples often require fewer steps in preparation for testing. And finally, since the kits are validated in-house by the manufacturer, little work is required when researchers want to use the kit in a new species. When performing an in-house, or home made, assay, considerable work is required by the researcher developing the assay to optimize an antibody concentration and sample dilution for use in a new species.

A commercially-available cortisol EIA kit from Arbor Assays (Ann Arbor, MI) was validated for use in bonobos and was used to measure cortisol in urine from bonobos (*Pan paniscus*) at the CZA over three field seasons. Urinary cortisol measures were used to reconstruct individual diurnal cortisol rhythms and test for associations with age, sex, and certain behaviors traditionally associated with stress in primates, such as aggression given or received. Individual differences in patterns of AM to PM diurnal cortisol rhythm were detected by this all-day sampling method. There were no sex differences in cortisol concentrations within the group and individuals did not significantly differ between field seasons.

Background

A hormone is defined as a biological substance that is produced in one tissue and acts globally in an organism. This mode of action is typically receptor-mediated – meaning the hormone induces its effects at the cellular level once bound with its target receptor. This can be accomplished by passively diffusing through the cell membrane and binding with nuclear receptors, receptor-mediated endycytosis from the cell-surface, or receptor-mediated cellular signaling once the hormone is bound to its receptor (Chen and Farese Jr, 1999). Chemicals can also act as autocrine (released and acts upon same tissue) or paracrine (released and acts upon nearby tissue).

There are four classes of hormones- steroid, amino acid, peptide/protein, and eicosanoid. Steroid hormones are those that are derived from the parent molecule cholesterol, and are the main focus of this review. Cholesterol is converted to pregnenolone in the inner mitochondrial membrane by cytochrome P450scc. Production

of pregnenolone is regulated by pituitary hormones (Leutenizing hormone/follicle stimulating hormone, adrenocorticotropic hormone). Cholesterol is the precursor for sex steroids (testosterone), glucocorticoids (cortisol), and mineralocorticoids (aldosterone) (Roberts, 1999). Steroid hormones diffuse through cell membranes and bind nuclear receptors to effect DNA transcription (Czaja, 1978). The molecular structure is highly conserved across many classes of animals (Evans, 2005).

Stress and Cortisol

Stress is a hot topic of biomedical and social science research and its study has exploded in growth in the past 60 years. One of the first researchers to talk about approaching stress from a scientific standpoint was Hans Selye, who began writing about it in the 1950s. An article written by Selye in 1973 in *American Scientist* included 9 references, all of his own works. Today, a PubMed search for the term 'stress' returns over 670,000 results. Stress has been studied in numerous contexts including psychosocial stress, oxidative stress, blood vessel shear stress, endoplasmic reticulum stress, stress disorders, and numerous other ways. It is studied at the biochemical level, cellular level, and at the organism level across many classes of animals in multiple environmental contexts including in lab animals, wild animals, zoo animals, and in human adults and children.

Early pioneering work in the study of physiological stress defined stress as “the nonspecific response of the body to any demand made upon it.” (Selye, 1973). The stress response is further explained as the body's reaction to challenges such as heat/cold or ingestion of sugar. While stress itself is a difficult word to define comprehensively,

scientific studies usually focus on examining the stress response, what triggers that response, and the outcomes of the response. The stress response can, therefore, be defined as the body's physiological response to challenge, either exogenous or endogenous. These challenges are stressors that elicit an adaptive response that causes the body to exist outside of homeostatic conditions for some amount of time.

The stress response can be measured objectively and quantitatively in vertebrates. Typically, the stress response consists of altered endocrine levels that elicit downstream physiological changes. These changes tend to occur in patterns and in many cases are highly conserved across many orders of animals, and therefore suitable for studies of both a predictive nature and from an evolutionary perspective.

The stress response itself is not maladaptive, *per se*. The word 'stress' carries with it a negative connotation, however the physiological processes that allow for adaptation to challenges to the system are both necessary and positive under non-pathological conditions. In humans, the stress response (as defined by Selye) allows most of us to live at sea level and take a trip to the Atacama Desert and become adapted to high altitude in a matter of days. We can train to run dozens of miles at once, lift objects multiple times heavier than our own bodies, and fight off disease. Humans can even adapt to a moderate amount of abuse including living without major health consequences for years while being overweight/obese, ingesting copious amounts of drugs or alcohol, getting little exercise, or living on an erratic sleep schedule.

The body's ability to adapt to challenges to the system, however, can become maladaptive if maintained over an extended time. If a stressor remains in place so that is chronically challenges the system and causes continued compensation/adaptation to

attempt to achieve normal function, negative health outcomes can result. While a few weeks of heaving drinking may leave an individual with a bad hangover, years of it can lead to cirrhosis of the liver. Years of distance running can cause arthritic damage to joints. Even non-physical stressors can cause lasting damage as when psychosocial stressors cause chronic deleterious alterations to the body's stress response.

Cortisol, a steroid hormone, is known colloquially as the “stress hormone.” However, cortisol can probably be more accurately thought of as an energy regulator. The cortisol response allows the body to direct its resources to fighting an impending threat. During this response, short term access to energy via gluconeogenesis is favored over long term processes like mounting an immune response or reproductive capabilities. In fact, it is from this gluconeogenic capability that cortisol derives one of its common descriptive names, ‘glucocorticoid.’ Up regulation of the pathways that produce cortisol concurrently result in inhibition of production of growth hormone, gonadotropin releasing hormone, and thyroid stimulating hormone, in effect diminishing growth and reproductive capabilities (Tsigos and Chrousos, 2002).

Cortisol is produced by the adrenal glands, which are located superior to the kidneys, and acts on tissues throughout the body. The glucocorticoid receptor (GCR) is present on the nuclei of virtually all cells in the body, and thus cortisol has a very wide range of biological activities (Lu et al., 2006). Cortisol is part of the chain of hormonal feedback of the hypothalamic pituitary adrenal (HPA) axis (Figure 1.1). The HPA axis is a series of endocrine glands that trigger successive releases of hormones culminating in production of glucocorticoids such as cortisol from the zona fasciculata of the adrenal cortex. The hypothalamus produces corticotropin releasing hormone (CRH), which

stimulates the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH), which in turn acts on the adrenal glands. The HPA axis operates by negative feedback, whereby increased concentrations of ACTH and glucocorticoids exert inhibitory effects on upstream endocrine glands.

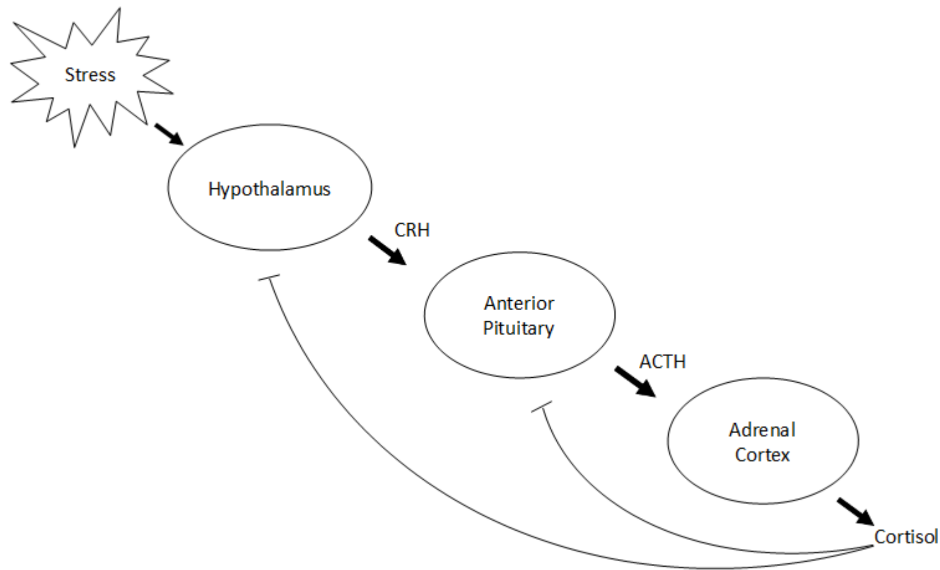

Figure 1.1. The HPA Axis, a classic negative feedback pathway.

The hypothalamus receives neurological input from the lateral aspect of the amygdala, which projects onto the central nucleus and the part of the brain involved with fear response. This exerts action on the hypothalamus which induces HPA axis response as well as an increase in sympathetic nervous system action (adrenaline) (Tsigos and Chrousos, 2002).

Cortisol can be enzymatically converted to cortisone, the biologically inactive form of cortisol, by conversion with the enzyme 11 β-hydroxysteroid dehydrogenase (11 β-HSD). There are two isoforms of 11 β-HSD, 11 β-HSD-1 and 2. Type 1 converts

cortisone to cortisol, and Type 2 inactivates cortisol via NAD-dependent dehydrogenase (Tomlinson and Stewart, 2001).

Altered cortisol production can produce pathological conditions. Both hypercortisolism (Cushing's) and hypocortisolism (Addison's) are serious, potentially life threatening diseases. Both conditions also have behavioral effects. Cushing's Syndrome is associated with depression and insomnia (Orth, 1995). Addison's adrenal insufficiency is also associated with depression and can present as anorexia and malaise as well, in addition to causing a range of health issues like sudden weight loss, weakness, hypotension, and dehydration (Ten et al., 2001).

Cortisol can also exert mineralocorticoid effects. Mineralocorticoid receptors (MR) are found in the kidneys, colon, heart and central nervous system. Aldosterone is the primary ligand of the MR, and is produced as a result of activation of the renin-angiotensin-aldosterone system in the kidneys, which is an enzymatic cascade involved in systemic blood pressure regulation. Angiotensin exerts vasoconstrictive effects in peripheral vasculature and mediates release of aldosterone, which in turn acts on MR to increase Na^+ reabsorption, decreases diuresis, and acts on the hypothalamus to produce vasopressin (anti-diuretic hormone) (Griendling et al., 1993). Vasopressin itself can also activate the HPA axis by inducing production of pro-opiomelanocortin in the anterior pituitary, thus stimulating release of ACTH (Lightman, 2008).

Though aldosterone is the primary ligand of the MR, cortisol has similar affinity for the MR. MR are present in non-epithelial tissue (Funder, 2005). Typically, cortisol does not have access to renal MR due to pre-receptor conversion to cortisone by 11 β-HSD (Tomlinson and Stewart, 2001), however non-epithelial cells lack this enzyme (Frey

et al., 2004). It is not yet fully understood how cortisol exerts action on MR, but it is suspected to be implicated in some disease states due to findings of decreased expression of 11 β-HSD2 in response to presence of shear stress, angiotensin II, and hypoxia (Frey et al., 2004).

Cortisol can be measured in blood, urine, feces, cerebrospinal fluid (CSF), saliva, hair, and tissue. Concentrations of cortisol fluctuate in a diurnal pattern, one that is highly conserved across species (Denver, 2009). Cortisol begins spiking upon awakening, reaches a plateau within 30-60 minutes of waking up, and then slowly drops to a nadir overnight. Minor fluctuations within this pattern exist when an individual experiences isolated stressors. However, when a chronic stressor exists, there can be changes in the entire diurnal curve. The diurnal curve can be dysregulated in several ways. Blunting happens when the awakening response, or morning spike, is diminished significantly. A total elevation of the curve is indicative of increased overall output of cortisol. The overall pattern can also be affected, such as an inversion of the pattern where peak cortisol is produced in the afternoon (Karlamangla et al., 2013).

In humans, dysregulation of the diurnal rhythm of cortisol production is associated with negative health outcomes such as elevated risk of cardiovascular disease, metabolic syndrome, increased incidence of overweight and obesity, and increased morbidity and mortality (Panter-Brick and Fuentes, 2009). In primates, the relationship between diurnal cortisol rhythm and health outcomes has been less studied.

In primates, stress has been studied in captive (laboratory or zoo) animals and in the wild. In captive primates, stress research has focused on topics such as the effects of captivity on stress hormones, rank and stress, pregnancy and cortisol levels (Fite and

French, 2000; Smith et al., 1999), and seasonality of stress hormone fluctuations (Cunha, 2007). In wild populations, the focus of stress research is often tied to dominance and rank (Abbott et al., 2003; Moscovice et al., 2015; Muller and Wrangham, 2004), aggression given or received (Surbeck et al., 2012), or the impact of changes in habitat such as deforestation (Jaimez et al., 2012) and exposure to humans (Vanlangendonck, N et al., 2015).

Studying stress in primates in both wild and captive populations has unique challenges and limitations. In captivity, simply being a captive primate is a stressor in and of itself (Segal, 1989). The animals are exposed to humans in ways they would not normally be in the wild, either on display for zoo patrons or in contact with researchers performing testing such as sedation or blood draws or simply feeding the captive animals. Despite such draw backs, there are advantages to conducting primate stress research in captive populations. Many aspects of their environment are relatively controlled, including group composition, diet, and overall daily routine. It is easier to tie biological samples to specific contributors, as opposed to attempting to collect something like fecal or urine samples from a canopy-dwelling wild primate. Additionally, sample types such as blood or saliva are much easier to obtain from captive primates and allow for a wide range of testing and for repeat-measures.

In wild primates, stress research does not have the issue of dealing with the stress of captivity inherent in captive-based studies. Wild primates are free-ranging and living under conditions that more closely resemble their natural, evolutionary context. However, it is more difficult to obtain biological specimens from wild-dwelling primates and the act of attempting to do so necessitates habituation of the group to the researcher's

presence in order to get and remain close enough for behavioral observations and sample collections. It is more difficult to determine the source of certain biological samples, such as feces or urine and the samples may become contaminated if they are deposited in an area where another individual had previously contributed his own sample. Some researchers have been successful with obtaining blood samples from wild primates, however this process involves darting, sedation, and caging which is all inherently stressful for the subject.

Review

Ways to Measure Cortisol

Cortisol has been an important biomarker of stress in humans for nearly forty years. It is also a well-studied hormone within primate socioendocrinology. Studies have been conducted with non-human primates from New World to Old World Monkeys and apes, laboratory animals to zoo animals to wild animals, and examined cortisol in numerous contexts from intra-group stress and dominance to post-parturition changes in maternal cortisol.

Performing the biochemical analysis to determine hormone concentrations in a biological sample is not particularly difficult, however it does require lab space with relatively expensive equipment. As a result, many primatologists have had their samples sent to labs like the Wisconsin National Primate Research Center's (WNPRC) Assay Services (Madison, WI) division for testing after they collect their samples. A significant proportion of primate socioendocrine research has gone through the WNPRC. There are other laboratories around the world that also perform the testing, such as the Max Planck

Institute in Leipzeig, Germany, the Human Biology Research Lab at Yale (New Haven, CT), and most recently within the University of Oregon's own Anthropology Department.

There are four main testing methods that have been employed for the determination of cortisol concentration in primate research studies- enzyme immunoassay (EIA), radioimmunoassay (RIA), high performance liquid chromatography (HPLC), and liquid chromatography tandem mass spectroscopy (LC-MS/MS). Each method carries its own advantages and limitations and will be discussed further below.

An immunoassay is a method for generating and measuring signal from very small quantities of target analytes by detection with antibodies. Antibodies can be developed against virtually any protein, hormone, cell, or virus. Antibodies are proteins produced by the immune system that target antigens to mark for destruction by immune cells. An antibody can be polyclonal or monoclonal. A polyclonal antibody is one that has multiple epitopes, or binding regions, against the same antigen, while monoclonal antibodies share the same epitope. Polyclonal antibodies are less specific than monoclonal and are prone to higher potential cross reactivity with molecules that share similar structure to target analytes.

Immunoassays can be structured in various ways, such as directly measuring the target analyte by labeled antibody or by indirectly calculating your target analyte's concentration by measuring a competitive binding target. Antibodies can be labeled with various signal molecules such as fluorophores, enzymes (as in EIA), radioactive tracers (RIA), chemiluminescent molecules, metals, microparticles, and numerous other labels that are less commonly used. In EIA, enzyme-labeled antibodies are bound to target analytes or competitors and immobilized to high protein-binding polystyrene microplate

wells, then an enzyme-labeled antibody binds immobilized target analyte and substrate is added to the wells to induce a color change that is proportional to the amount of enzyme-labeled target or competitor present. The color change is read by a microplate reader at a wavelength specific to the substrate used. Absorbance of light at that wavelength is calculated and reported as optical density (O.D.) units. This type of test is made quantitative by including a standard curve over a range of known concentrations and interpolating concentration based on absorbance of unknowns.

RIA operates on similar principles as a competitive EIA, except analyte is labeled with a radioisotope (typically 125-I or tritium), unlabeled antigen will displace radio-labeled antigen at antibody binding sites, and concentration is determined by a scintillation counter rather than absorbance of light on a plate reader. RIA is a highly sensitive and specific method for analyte determination with a very low per-sample cost. However, a major disadvantage of RIA as a method is that it requires handling and disposal of radioactive material, which often requires special permissions from the institution where the laboratory is located.

High-performance liquid chromatography (HPLC) is a method that employs liquid chromatography to separate component molecules in a solution by size. Liquid samples are injected into a stationary phase that can separate molecules by a number of chromatographic techniques depending on the type of column used (e.g., size exclusion, ion exchange, affinity, etc.). Samples elute at different times from the stationary phase depending on the type of column used and can be compared to retention times of known standards. HPLC can be used for quantitative and qualitative analysis of samples. The size of the peak produced as output is directly proportional to the amount of substance

comprising each peak (Sivasankar, 2012 Ch 13).

Mass spectroscopy (MS) is a technique used to determine the size, structure, and quantity of unknowns based on how they fragment when exposed to ionizing particles. MS is looked at as the gold standard in sample determination because of its extremely high sensitivity and specificity. Molecules break apart in specific and predictable ways, which are sorted by mass. The output of a MS test is a histogram of fragment sizes based on their relative abundance compared to the base peak, or the most abundant fragment ion, which is graphically depicted at a relative abundance of 100% for comparison to other daughter fragments. This method can be made quantitative by including a known amount of the target analyte and measuring the relative abundance of the unknown compared to the known quantity.

There are many configurations of MS that can be used for sample determination. The most commonly used method in primate research has been LC/MS-MS, which is HPLC coupled to tandem mass spec (Jurke et al., 2000; Surbeck et al., 2012). HPLC separates constituent molecules in a solution based on size, and tandem mass spec uses two mass analyzers to perform primary and secondary fragmentations, which allows for determination of the exact configuration of biomolecules such as proteins. (Sivasankar, 2012 Ch 10)

Cortisol in Primate Research, Sorted by Sample Matrix

There are numerous sample types in which cortisol can be measured in primate socioendocrinology research. Each sample type carries its own set of advantages and limitations with respect to ease of collection, temporal association of hormone to

behavior, reflection of central hormonal pathways, and available testing methods.

Blood

Cortisol is secreted from the adrenal glands into the blood where it is transported to target tissues. Cortisol exists in two forms in the blood- bound to carrier proteins such as albumin and corticosteroid-binding globulin (CBG), and as soluble "free" cortisol. Approximately 3-10% of total cortisol exists as free cortisol in humans (Coolens et al., 1987). The remaining 90-97% of cortisol is bound, primarily to CBG. Cortisol has a much lower affinity for albumin than CBG in many species. Total cortisol is defined as the combination of free and bound cortisol in blood.

It was long considered a truism that free cortisol was the more important measure because unbound cortisol would be biologically active according to the Free Hormone Hypothesis (Mendel, 1989), however this notion has recently been challenged. See (Levine et al., 2007) for a review. The Free Hormone Hypothesis has been challenged in part because CBG-bound cortisol has been shown to exhibit biological activity and the measure of bound cortisol itself has physiological relevance. CBG helps regulate the amount of free cortisol available to target tissues (Beishuizen et al., 2014) and may possibly be taken up directly by cells as a CBG-cortisol complex (Siiteri, et al. 1982). Interestingly, CBG enables cortisol to exert local anti-inflammatory effects by undergoing enzyme cleavage in the presence of neutrophil elastase, resulting in a decreased affinity of CBG for cortisol and delivery of the steroid to tissue where an inflammatory response is occurring (Pemberton et al., 1988).

Many studies assess either free or total plasma/serum cortisol levels, but this may

not be enough to get a full understanding of HPA axis function. Due to high inter-species variation in glucocorticoid production, plasma CBG concentration, and CBG affinity for cortisol, it may be necessary to include both free and total cortisol as well as quantifying CBG and how these values change under various conditions (Delehanty et al., 2015). Little is known about reference ranges of normal circulating cortisol levels in primates, but numerous studies have demonstrated elevated plasma values in plattyrhines (New World monkeys) compared to cattarhines (Old World monkeys and apes), and strepsirrhines (lemurs and lorises). It has also been shown that some plattyrhines have very little CBG or a CBG isoform with very low affinity for cortisol. In squirrel monkeys, free cortisol exists as up to 50% of the fraction of total cortisol due to low levels of CBG, and the bound fraction is predominantly bound to albumin (Gayrard et al., 1996). There is a wide range in plasma total and free cortisol in primate taxa (Chrousos et al., 1982).

In early studies of stress in wild olive baboons, researchers darted individuals to obtain serum samples and used the darting procedure as an induced stressor (Sapolsky, 1992, 1982). Because the animals need to be sedated to collect blood samples, some studies use this to examine the effects of sedation on primate stress response (Anestis et al., 2006). Most blood cortisol studies have used RIA to measure cortisol (Maestripieri et al., 2008; Saltzman et al., 1998; Saltzman and Abbott, 2009; Sapolsky, 1992, 1982). Although one study states they performed an enzyme immunoassay for testing, but cite the Saltzman et al., 1994 paper as their source for protocol, and that study used an RIA for cortisol determination. Many are treating the plasma or serum with an organic solvent to release cortisol from CBG and albumin in order to measure total cortisol (Sapolsky,

1992, 1982), while others make no mention of whether or not the plasma was pretreated prior to analysis (Cunha, 2007; Maestripieri et al., 2008; Saltzman et al., 1998; Saltzman and Abbott, 2009).

Serum or plasma cortisol assays are advantageous because of the likelihood of accurate reflection of central nervous system pathways since the blood is how cortisol is transported to target tissues. Other sample types are simply proxy measures for circulating hormone concentrations, whereas blood samples have the potential to give a more direct assessment. However, because cortisol is both diurnal and pulsatile, a single blood sample runs the risk of catching a peak of cortisol release into the blood stream. Blood samples also require significant processing for testing due to the complex nature of the sample matrix in order to extract cortisol and remove interfering substances. Researchers using blood as their sample matrix of choice in primate studies have the added issue of sample collection being a stressor in and of itself. Researchers should additionally determine whether free or total cortisol is being assayed and what conclusions can be drawn from studies in their subject species given the wide ranges of free and total cortisol and CBG between species.

Table 1.1. Primate studies reporting on plasma or serum cortisol in this review. Shown with ranges of cortisol concentrations and method used in determining cortisol concentrations.

Authors	Year	Species	Conc	Units	Method
Sapolsky	1982	*Papio anubis*	20-40	µg/dL	RIA
Sapolsky	1992	*Papio anubis*	13-30	µg/dL	RIA
Saltzman et al.	1998	*Callithrix jacchus*	100-500	µg/dL	RIA
Smith et al.	1999	*Pan troglodytes* and *Gorilla gorilla*	636-1250	nmol/L	chemilum
Sanchez et al.	2005	*Macaca mulatta*	10-35	µg/dL	EIA

Ziegler et al.	2005	*Callithrix jacchus*	100-250	µg/dL	RIA
Anestis et al.	2006	*Pan troglodytes*	20-120	µg/dL	RIA
Cunha et al.	2007	*Callithrix jacchus*	40-60		EIA
Maestripieri et al.	2008	*Macaca mulatta*	15-45	µg/dL	RIA
Saltzman et al.	2009	*Callithrix jacchus*	100-1000	µg/dL	RIA

Urine

Urine as a sample matrix for quantification of cortisol has several advantages in primate research. Urine concentrations represent a smoothed average of pulsatile secretions of cortisol transported in the plasma. Primates can be trained to void urine at specific times and into cups or a clean collection area (Anestis, 2005). There is little to no observable stress involved in the process of urine collection in this manner because collection is noninvasive. Hormone concentrations quantified from urine samples, unlike plasma/serum samples, are susceptible to changes in hydration status of the animal providing the sample. Therefore, urine samples should be normalized to some measure of the concentration of the urine. Creatinine concentration and specific gravity (SG) are the two most common methods of urine normalization in primate research. Creatinine is much more widely used, however recent evidence suggests there is a non-zero difference in the values determined from creatinine versus SG and that SG normalization may result in a more accurate reflection of true concentration (Anestis et al., 2009).

As a sample matrix, urine is relatively uncomplicated compared to plasma or serum due to the decrease in proteins and phospholipids, which can interfere with assay performance. In the newest types of commercially available assays, urine can be directly tested with minimal preparation (i.e., dilution) compared to the previously required

preparatory steps like elaborate extraction, hydrolysis, or solvolysis.

Cortisol is excreted by the kidneys and can be found covalently bound to glurcuronides and sulfates in urine. Hydrolysis with *Helix pomatia* gastric juices, which contains glucuronidase and sulfatase, breaks those bonds and makes cortisol more readily available for detection and is required in some assays. Cortisol is metabolized by the liver and metabolites are also excreted in urine. In a radiolabeled study of cortisol metabolism, peak radioactivity was measured in chimpanzee, macaque, and marmoset urine 5.5 hours after administration (Bahr et al., 2000).

Urine demonstrates a robust diurnal pattern shifted a few hours later than plasma cortisol, due to the time required for filtration and excretion in urine. Primate studies have controlled for diurnal variation using several different methods. Some researchers elect only to take samples at first morning void (Smith and French, 1997; Ziegler et al., 1996, 1995), at hourly intervals (Smith and French, 1997), generally collect morning samples (Bahr et al., 1998; Jurke et al., 2000; Maggioncalda et al., 2002), exactly marking time of sample and controlling for time-based variation statistically (Anestis, 2009; Anestis and Bribiescas, 2004; Kahlenberg et al., 2008; Muller and Lipson, 2003), or splitting samples by morning versus afternoon (Robbins and Czekala, 1997) to get a picture of overall diurnal variation. Time periods as short as one hour have been shown to produce significantly different cortisol concentrations within individuals, highlighting the importance of awareness of time of sample in study design (Anestis and Bribiescas, 2004). Similar to plasma cortisol, more variation is shown in morning cortisol concentrations versus afternoon concentrations (Muller and Lipson, 2003; Smith and French, 1997; Smith and French, 1997; Ziegler et al., 2004, 1996, 1995).

Urinary studies have investigated a wide range of associations between cortisol and stress, including both behavioral and energetic contributions. Some studies associated increased cortisol with administered anesthetic or restraint procedures performed on captive primates (Anestis, 2009; Ziegler et al., 1996). Contrary to many other studies, one study in wild female chimpanzees showed an increase in cortisol with age (Emery Thompson et al., 2010). Plattyrhines consistently had elevated cortisol levels compared to other groups of primates (McCallister et al., 2004; Tessa E. Smith and French, 1997; Tessa E Smith and French, 1997; Ziegler et al., 1996, 1995), which mirrors plasma and serum studies previously discussed in this review. One study tied cortisol levels to onset and maintenance of maternal behaviors in gorillas (Bahr et al., 1998). Results are mixed for associations of urinary cortisol with rank. In one study, rank in gorillas was not associated with urinary cortisol (Robbins and Czekala, 1997). However, other studies have found a positive correlation between rank and urinary cortisol (Muller and Wrangham, 2004) or an association between male rank only when there are females in estrus (Surbeck et al., 2012). In studies that report sex differences, females have higher cortisol than males (Tessa E Smith and French, 1997).

Creatinine is a metabolite of phospho-creatine and is excreted by the kidneys. Assuming normal renal function, creatinine is excreted in relatively constant amounts (Eaton and Pooler, 2013). However, this is dependent upon body size and muscle mass. Since excreted creatinine is influenced by body size, use of creatinine for normalization in studies of male and female primates in species with any degree of sexual dimorphism can be complicated. Researchers using creatinine in sexually dimorphic species should test for associations with body size, or age class as a proxy for body size. Creatinine is

measured by a simple colorimetric assay based on the Jaffe reaction.

SG is the ratio of the density of a urine sample to the density of water. SG may be less affected by health status and freeze/thaw cycles and therefore may be a more appropriate normalization measure. SG can be measured with an inexpensive point of care device that can be battery powered and therefore appropriate for field work (Anestis et al., 2009). Since SG is not dependent upon body size it should not be subject to sex differences in sexually dimorphic species in the same manner as creatinine.

In humans, 24 hour urine collection is the standard method for assessing adreno-cortical function. This involves collection of all excreted urine in a 24 hour period in one container to determine the total amount of cortisol produced over the course of one day. This type of measurement is extremely difficult, if not impossible in primate studies and there remains debate about the utility of spot urine collection to assess central HPA function. Two studies have done 24 hour collections in a limited number of species (common marmoset, long-tailed macaque, and chimpanzees) and determined total daily output of cortisol to range between 0.2-6.0 mg/day (Bahr et al., 2000; Layne et al., 1964).

Table 1.2. Primate studies reporting on urinary cortisol in this review. Shown with ranges of cortisol concentrations and method used in determining cortisol concentrations.

Authors	**Year**	**Species**	**CortConc**	**Units**	**Diurnal**	**Method**
Layne et al.	1963	*Pan troglodytes*	2.70-6.04	mg/day	n/a	Radio tracer
Crockett et al.	1993	*Macaca fascicularis*				
Ziegler et al.	1995	*Sanguinus oedipus*	10-60	µg/mg cr	FMV	Munro EIA
Smith & French	1996	*Callithrix kuhli*	5-40	µg/mg cr	hourly	
Ziegler et al.	1996	*Sanguinus oedipus*	8-20	µg/mg cr	FMV	Munro EIA

Smith & French	1997	*Callithrix kuhli*	15-40	µg/mg cr	FMV	Munro EIA
Smith and French	1997	*Callithrix kuhli*	7-50	µg/mg cr		Munro EIA
Robbins &Czekala	1997	*Gorilla gorilla*	400-900	ng/mg cr	am vs pm	RIA
Whitten et al.	1998	*Pan troglodytes*	3-14	µg/dL	none	RIA
Bahr et al.	1998	*Gorilla gorilla*	0.35-1.12	µg/mg cr	morning urine	RIA
Bahr et al.	2000	*Multiple*	0.2-2.75	µg/mg cr	n/a	HPLC
Jurke et al.	2000	*Pan paniscus*	400-1500	ng/mg cr	morning urine	RIA
Maggioncalda et al.	2002	*Pongo pygmaeus*	0.6-1.2	µg/mg cr	morning urine	RIA
Muller & Lipson	2003	*Pan troglodytes*	50-500	pmol/mg cr	time of sample	RIA
McCallister et al.	2004	*Sanguinus imperator*	10-55	µg/mg cr	morning urine	Munro EIA
Muller &Wrangham	2004	*Pan troglodytes*	200-550	pmol/mg cr	FMV & time	RIA
Ziegler et al.	2004	*Sanguinus oedipus*	?	% change	FMV	UV abs
Anestis and Bribiescas	2004	*Pan troglodytes*	10-100	pmol/mg cr	time of sample	RIA
Ramirez et al.	2004	*Papio hamadryas anubis*	2.5-4.75	µg/mg cr		Munro EIA
Anestis	2005	*Pan troglodytes*	?	ng/mg cr		RIA
Skurski	2006	*Gorilla gorilla*	4.82-351.52	ng/mg cr	morning urine	Munro EIA
Muller et al.	2007	*Pan troglodytes*	150-550	ng/mg cr	?	Munro EIA
Kahlenberg et al.	2008	*Pan troglodytes*	-100 to +200	hr vs ng/mg cr	time of sample	Munro EIA
Dittami et al.	2008	*Pan paniscus*				
Anestis	2009	*Pan troglodytes*	20-200	pmol/mg cr	time of sample	RIA
Emery Thompson et al.	2010	*Pan troglodytes*	-40 thru +60	hr vs ng/mg cr	time of sample	Munro EIA
Salvante et al.	2012	*Multiple*	0-4 (log)	ng/mL		multiplex
Surbeck et al.	2012	*Pan paniscus*	20-250	ng/mg cr	time of sample	lc/ms/ms

Jaimes et al.	2012	*Cercocebus albigena*				
Moscovice et al.	2015	*Pan paniscus*	100-200	ng/mg cr		Munro EIA
Squires et al.	unpub	*Pan paniscus*	7-800	ng/mg cr	am vs pm	Arbor EIA

Saliva

Cortisol can be measured in saliva from humans and primates. Salivary cortisol is highly correlated with free plasma cortisol, but not linearly correlated with total cortisol (Hellhammer et al., 2009). Changes in the ratio of salivary to total plasma cortisol are dictated by the amount of cortisol bound to CBG, which can be affected by things such as oral contraceptives and sex steroids. Salivary cortisol measures become significantly elevated once plasma CBG is saturated, which in turn quickly elevates plasma free cortisol (Hellhammer et al., 2009). Even in saliva, approximately 30% of cortisol is bound to CBG (Levine et al., 2007).

Salivary cortisol as a sample type for use in primate studies has several advantages. Its collection is non-invasive and relatively stress-free compared to other collection procedures (e.g., darting for plasma/serum collection). Typically, primates are trained to chew on a dental rope that is often treated with a sugar mixture like Kool-Aid to entice longer chewing on the rope. The rope is then collected and saliva is extracted by centrifugation. Presence of Kool-Aid does not affect measured cortisol in the saliva samples. An additional advantage to selecting salivary cortisol for primate studies is that there are several existing commercially available EIA kits specifically designed to measure salivary cortisol (Salimetrics, State College, PA). Though there are several reported successful uses of salivary collection in captive primates, some studies have

shown mixed results in training primates to provide saliva samples (Lutz et al., 2000).

Table 1.3. Primate studies reporting on salivary cortisol in this review. Shown with ranges of cortisol concentrations and method used in determining cortisol concentrations.

Authors	**Year**	**Species**	**Conc**	**Units**	**Method**
Fuchs et al	1997	Saimiri vanzolinii	15-30	pmol/mL	EIA (fluor)
Lutz et al	2000	Macaca mulatta	0.27-1.77	µg/dL	RIA
Tiefenbacher et al	2003	Saimiri vanzolinii	5-35	µg/dL	RIA
Hohmann et al	2008	Pan paniscus	0.11-3.66	ng/mL	EIA
Behringer et al	2009	Pan paniscus	3-15	ng/mL	
Wobber et al	2010	Pan troglodytes and Pan paniscus			RIA
Heintz et al	2011	Pan troglodytes	5.79-13.06	ng/mL	Munro EIA

Feces

As stated previously, the liver is responsible for metabolizing glucocorticoids. Because cortisol is largely metabolized before being excreted in feces, fecal glucocorticoid metabolites (fGCM) are often measured in addition to or in lieu of simply measuring fecal cortisol. Cortisol excretion in feces varies wildly between species, with some primates excreting no detectable cortisol and some in which cortisol is the primary excreted GC. Due to this type of variation, it is advisable for researchers to first test fecal samples by HPLC to determine the type and concentration ranges of fGCM present in fecal samples of their study population prior to employing an immunoassay to ensure the predominant fGCM are targeted for detection and analysis.

Fecal samples require a fair amount of pre-processing prior to HPLC or immunoassay determination of fGCM. Typically, a steroid extraction is performed with organic solvents (for review see Keay et al., 2006). Samples are often dried either by

lyophilization or speed vacuum to remove water. Dried samples can then be pulverized and a standard dry weight can be reconstituted in a set volume of buffer to be filtered/extracted and assayed. The process of drying is thought to normalize between sample differences in water excretion. Alternatively, samples can be wet extracted by filtering or centrifuging and running supernatant through C18 solid phase extraction columns. Special considerations are necessary for preserving and preparing field-collected samples due to the level of processing required prior to assay. Fecal samples are an appealing sample type for use in primate studies due to the relative ease of collection and the lack of stress involved in the process of collection. In studies of wild primates, habituation to the presence of researchers is often reported as a necessary precursor to sample collection.

Studies assessing fGCM levels have reported mixed results in whether or not diurnal variation is detectable. When diurnal variation in fecal cortisol or fGCM is noted, the peak values are detected in the afternoon (Murray et al., 2013; Sousa and Ziegler, 1998). Some studies were unable to detect diurnal variation in fGCM (Beehner and Whitten, 2004).

Table 1.4. Primate studies reporting on fecal cortisol in this review. Shown with ranges of cortisol concentrations and method used in determining cortisol concentrations.

Authors	**Year**	**Species**	**Conc**	**Units**	**Method**
Whitten et al.	1998	Pan troglodytes	2-8	ng/g	RIA/HPLC
Sousa and Ziegler	1998	Marmosets	50-400	ng/g	Munro EIA
Beehner and Whitten	2004	Baboons	20-45	ng/g	RIA
Heisterman et al.	2006	Multiple	0-12	µg/g	EIA/HPLC
Setchell et al.	2008	Mandrillus sphinx	1.77-2.0	ng/mg	
Arlet et al.	2009	Gray-cheeked mangabey	0-7 (log)	ng/g	Munro EIA

Setchell et al.	2010	Mandrillus sphinx	1.83-1.95 (log)	ng/g	Munro EIA
Weingrill et al.	2011	Pongo pygmaeus	0-1	µg/g	EIA
Wasserman et al.	2013	Colobus badius and Pan troglodytes	72-284	ng/g	Munro EIA
Murray et al.	2013	Pan troglodytes	19.8-22.7	ng/g	Munro EIA
Amrein et al.	2014	Pongo pygmaeus	0-3000	ng/g	
Vanlangendonck et al.	2015	Spider and Howler Monkeys	5-35	ng/g	Arbor EIA

Hair

The final sample type used in primate studies of HPA axis function is hair. Hair is a complex tissue type. It grows from a root embedded several millimeters within the epidermis and surrounded by sebaceous and eccrine glands as well as capillaries. Hair has an outer cuticle and inner medulla. Not all strands of hair are in the same phase of growth at the same time. The route of incorporation of cortisol into hair is not well defined at this time (Gow et al., 2010; Raul et al., 2004), however it is theorized that surrounding capillaries deposit plasma cortisol at the hair root during growth. It has been suggested that the free plasma cortisol, and not CBG-bound cortisol is the type most likely to diffuse into hair (Davenport et al., 2006). Free cortisol may diffuse into the growing hair shaft from capillaries surrounding the root of the hair (Cone, 1996).

Proponents of cortisol studies in hair suggest that hair as a sample type is free from the daily diurnal fluctuations as well as transient bursts in cortisol concentrations that might affect blood and saliva studies, and can therefore provide an even longer term picture of overall stress than even urine. Hair is assumed to grow at a fairly constant rate of approximately 1 cm per month in both humans and primates (Gow et al., 2010). Therefore, cortisol will theoretically be slowly deposited on the growing hair shaft dependent upon circulating cortisol concentrations and thus reflective of a longer time-

course of plasma cortisol.

In order to measure cortisol deposited into hair, hair must be cut, not plucked so as not to damage the skin. Hair is typically shaved from between the scapula of primate subjects. This shaving process can provide a pre-study cortisol level if repeat samples are to be taken from the same individuals at a future time and starts all individuals from the same time point in order to compare cortisol deposition in hair during the study period in the new hair growth. Animals are anesthetized with ketamine during the hair shaving procedure, but unlike other sample types, hair is not prone to impact from the distress of sample collection (Laudenslager et al., 2012). Making cortisol available for testing and quantification from hair shafts involves washing and/or extraction, however human studies of hair often employ harsh washes to ensure measurement is of substances deposited from blood and not inadvertently acquired environmentally. However, these washes may remove hormone molecules from hair shafts (Davenport et al., 2006). Since environmental deposition of cortisol (via hydrocortisone cream) is not likely to be an issue in primates, these harsh washes are unnecessary. Isopropanol is used to wash clipped hair. Extraction of cortisol from hair shafts is improved up to 3.5 times by pulverizing with a ball mill versus mincing with scissors (Davenport et al., 2006). Cortisol is then extracted into methanol, and extracts are dried and reconstituted for testing. Most studies are using a salivary cortisol EIA (Salimetrics, State College, PA) to measure hair cortisol.

Like plasma values, a cross-taxa comparison of hair cortisol values demonstrated that many small monkeys have cortisol values much higher than other larger bodied primates (Fourie and Bernstein, 2011). However, this particular study also reports mean

hair cortisol values in vervet monkeys of 1.26 + 0.39 ng/mg, which is more than an order of magnitude higher than the range of approximately 25-100 pg/mg reported in other studies in vervet monkeys (Fairbanks et al., 2011; Laudenslager et al., 2012, 2011), and more closely aligned with high values reported in small new world monkeys. The difference in reported concentrations in hair cortisol may be attributable to a difference in EIA kits used- Fourie and Bernstein (2011) used a kit from ALPCO Diagnostics, while the other groups all used a Salimetrics EIA. A recent study comparing testing methods for hair cortisol determination found similar elevated readings from ALPCO assay kits compared to Salimetrics. All immunoassays tested in that study measured higher than LC-MS/MS determination of hair cortisol, but all assays were correlated with LC-MS/MS in such a way that correction factors can be applied (Russell et al., 2015).

The application of hair cortisol studies usually involves long term or chronic stress research questions. In one study, novelty seeking behavior in vervet monkeys was associated with reduced cortisol in hair (Laudenslager et al., 2012), but this is used for a between individual comparison of long-term HPA axis function because no information was available about vervet monkey hair growth rate or rate of deposition of cortisol in hair. Several studies have found that hair cortisol increases after monkeys are moved from one facility to another (Davenport et al., 2006; Dettmer et al., 2012; Fairbanks et al., 2011). Several studies note an age related decline in hair cortisol, with highest levels in infancy (Dettmer et al., 2012; Laudenslager et al., 2012). Dettmer at al., also reported that infants that experienced early life stress and elevated cortisol levels were more likely to display anxiety-related behaviors later in life. A study of orangutan hair cortisol found that the hair can act as a time-sensitive measure of past stressors when the hair growth

rate is known (Carlitz et al., 2014), where different sections of the hair shaft display varying concentrations of cortisol that mapped onto a time-course evaluation of stressful events. Another study did not find differences in hair cortisol concentrations along the length of the hair shaft, which would seem to call into question the utility of hair as a long term, but time-specific measure of chronic stress (Davenport et al., 2006). Some researchers have hypothesized that cortisol may diffuse throughout the hair shaft causing averaged levels to be detected across the length of the shaft, but hair contains little water and there is no evidence to support this hypothesis at present.

Table 1.5. Primate studies reporting on hair cortisol in this review. Shown with ranges of cortisol concentrations and method used in determining cortisol concentrations.

Authors	Year	Species	Conc	Units	Method
Davenport et al	2006	Macaca mulatta	32.1-254.3	pg/mg	Salimetrics EIA
Fourie& Bernstein	2011	Cercopithecus aethiops and baboons	0.22-62	ng/mg	Alpco Salivary EIA
Fairbanks et al	2011	Cercopithecus aethiops	50-70 (mean)	pg/mg	Salimetrics EIA
Laudenslager et al	2011	Cercopithecus aethiops	25-100	pg/mg	Salimetrics EIA
Laudenslager et al	2012	Cercopithecus aethiops	40-70	pg/mg	Salimetrics EIA
Dettmer et al	2012	Macaca mulatta	150-200	pg/mg	Salimetrics EIA
Dettmer et al	2014	Macaca mulatta	25-200	pg/mg	Salimetrics EIA
Carlitz et al	2014	Pongo pygmaeus	9-108	pg/mg	IBL- Hamburg

Conclusions

Cortisol and its metabolites have been widely studied in primate socioendocrine research. Cortisol is an excellent way to measure stress in various sample matrices, but some matrices better reflect central stress responses than others.

Several different quantification methods have been employed to determine

cortisol concentrations. As primate socioendocrinology continues to grow as a field of research, more scientists will likely seek to quantify hormones in biological samples from primates. This can be accomplished by sending samples to the labs that already do this testing, or by using existing lab space at institutions where the researchers work. EIA is probably the easiest method for researchers new to hormone measurements to learn. It is relatively simple, cost effective (especially when compared to sending samples out for measurement), and there are an ever increasing number of commercially available kits for virtually any hormone of interest, usable in a wide range of species.

When conducting a stress study using cortisol as the objective measure of stress, researchers should be aware of the manner and mode in which they perform testing to ensure their measured cortisol values are reflecting the type of stress that relates to their research questions. For instance, if a research program wishes to investigate associations between intra-group bouts of aggression and daily cortisol fluctuations, urinary cortisol would likely be an appropriate measure. It can be temporally related to the stress-inducing behaviors. In contrast, if research questions are focused on links between a long-term stressor captive primates being moved between facilities or humans encroaching in the habitat of wild primates, a different approach might be more appropriate. Hair cortisol has the potential to provide long-term look-back periods for cortisol produced and that can be related to contemporaneous bouts of known stressors, however information is conflicting regarding whether or not the different spots on a hair produce enough variation in measured cortisol to reflect changes over time that correspond with growth rate of the hair. Alternatively, researchers could use a traditionally acute cortisol measure, but repeat sampling to obtain a long-term average of

am and pm values, such as urinary or fecal cortisol (or cortisol metabolites).

A limitation of cortisol-based stress research is the lack of understanding, particularly in primates, of how GC receptor polymorphisms and differential tissue expression of 11 β-HSD affect cortisol action in target tissues. Humans are known to have wide variation in GCR expression and action as well as tissue-specific effects of 11 β-HSD. A simple assessment of cortisol concentration obviously cannot take these considerations into account, and so appropriate caution is warranted in drawing conclusions from cortisol research.

CHAPTER II

TOWARD IMPROVING METHODS

Adding biomarker testing to a field like primatology has tremendous potential to add significant value to the types of behavioral and health research being done in the field. This is particularly true if results of socioendocrine research are comparable within and between species and labs conducting the studies. For this to be possible, improved accuracy and transparency in testing methods and reporting of results is necessary.

In the previous chapter, primate cortisol testing methods from the past several decades were presented in the style of a review. In this chapter, I will break down various aspects of these tests as reported, how reported results are or are not comparable between studies, potential procedural missteps, possible issues with documentation and publication of methods, and how the field as a whole can move toward a more systematic and methodological approach to these types of studies. While this discussion is narrowly focused on testing urinary cortisol, one could apply the suggestions to any type of biomarker testing conducted in primatological studies.

Currently, primate urinary cortisol research is uniquely situated to approach future studies with the goal of improving cross-comparability by standardization of methods. This is because the antibody most commonly used in primate cortisol EIAs, a polyclonal anti-cortisol antibody developed by Coralie Munro of UC Davis, is no longer available for sale (R. Cotterman, personal communication, March 7, 2016). While the possibility exists that some labs have a sizable enough stockpile of antibody to meet their needs for testing in the foreseeable future, labs new to testing cortisol will not be able to purchase

this antibody and, consequently, will be unable to replicate the most commonly published EIA protocol.

Therefore, the field of primate stress research could benefit greatly from adopting a more standardized method between labs around the world. This will allow for comparison of results between labs and species, while lowering cost of testing per sample compared to sending samples for testing in another lab, and give new labs the ability to incorporate EIA testing into their research programs. I propose that primatologists look to use commercially available EIA kits due to the pre-validated nature of the kits, the improved specificity of monoclonal antibodies, lower per-sample cost, and in many cases less sample preparation prior to analysis and smaller sample volume required for testing.

Attempts have already been made to suggest improvement of methods within this type of research (Anestis et al., 2009; White et al., 2010). This work seeks to build on previous efforts to improve methods and standardization in primatological research. In addition to simply adopting a field-standard EIA kit, I will propose several potential ways to improve upon the current methods for reporting of urinary cortisol data, such as inclusion of raw data concentration ranges and standardized concentration units, in order to ensure clarity and accuracy in results.

Why Change?

In a survey of 25 available primate urinary cortisol studies since 1993, one used HPLC, one used a multiplex assay, one used UV absorbance, two used LC-MS/MS, 10 used RIA, and 10 used the Munro antibody EIA for determination of cortisol concentration. There are relatively few labs performing their own assays for primate

hormonal analyses, however, of the labs that are using EIA to test for cortisol, the overwhelming majority use the Munro antibody and Ziegler protocol.

In a properly optimized and validated in-house EIA, there are many considerations to ensure the method has been developed and applied appropriately. Antibody dilution, sample dilution, incubation time, buffer selection, and enzyme/substrate selection should all be tested for assay optimization. Methods such as the "checkerboard test" allow researchers to vary two conditions simultaneously in a checkerboard pattern on a microplate to determine the most appropriate combination of factors such as antibody dilution and sample dilution (Cox et al., 2004). Ideally, the first instance of the use of an in-house assay in published research should include a description not only of the final method but also the ways the researchers arrived at selection of the assay's component factors. In a field like primatology where the same target analyte may be studied in dozens of species, in-house assays should go through sample and antibody dilution testing for each species tested due to varied ranges of expected concentrations in different species and sample types.

An advantage of using commercially available kits is that assay optimization has already been performed and researchers can demonstrate a kit's appropriateness of use in a particular species by identifying a proper sample dilution based on the expected concentration range of the target analyte and demonstrating parallelism in a pooled, serially diluted sample from several individuals in the species to the standard curve. Another consideration in order to determine if a particular kit/method is appropriate in a study species is whether or not measured results lie within the expected physiological range. This can be difficult to determine in some primate species because there is a lack

of available reference ranges. Without available reference ranges, researchers can compare to previously published results in the same species and sample matrix. However, this does not guarantee that measured values are correct, just that other experiments have produced similar values.

In light of these 'best practices' for assay optimization and method publication there are several issues with the most commonly used EIA method for urinary cortisol detection in primates. The first instance of publication of the Munro EIA protocol for use in primate urine was a 1995 paper on Cotton Top Tamarins (Ziegler et al., 1995). Normally, when a new EIA protocol is used for the first time, a full write up of the optimization and validation of the assay should be published. However, when this assay was developed and published, the only discussion in that publication about assay development was, “all urine samples were assayed for cortisol concentration by an enzyme immunoassay modified from a progesterone ELISA developed by Munro and Stabenfeldt (1984).” The paper then goes on to give a basic protocol for the assay without a single note of how the EIA was modified from the Munro and Stabenfeldt 1984 progesterone EIA. This is wholly inadequate for demonstration of optimization of an assay, particularly because the protocol was adapted from an assay for a totally different target analyte (progesterone) with a different antibody for a different sample type (plasma) (Munro and Stabenfeldt, 1984).

It should also be noted, in this publication and several others, the cross-reactivity of the Munro anti-cortisol antibody with cortisone is listed as 60%. In later studies, the cross-reactivity is reported as 5% with cortisone without any note about what caused the change. Though, some authors still cite a 60% cross-reactivity with cortisone as recently

as 2014. According to personal communication with the current lab director of the UC Davis lab that originally made the antibody, only one lot was ever produced and it will never be produced again. If the cross reactivity with cortisone has changed over time, it could possibly be due to a better purification process, however that is not stated in the literature. Also of note, the Munro antibody is at various times referred to as antiserum (Kahlenberg et al., 2008), an EIA provided by Coralie Munro (Moscovice et al., 2015), and purified antibody. It is not clear if researchers are performing their own purification or are simply unclear about what they have purchased, but the lab director specified that the original lot of antiserum was affinity purified and then lyophillized for long term storage. If it has already been purified, then the antibody being used is no longer called antiserum, but affinity purified antibody.

A potential issue with cross-reactivity being as high as the reported 60% with cortisone is that cortisone is present at levels up to ten-fold higher than cortisol in human urine. It is unclear the relative ratio of cortisol to cortisone in primates, but it possible it is present at concentrations high enough to skew results.

Further issues exist with the Ziegler 1995 protocol using the Munro antibody. The method uses horseradish peroxidase (HRP) as its enzyme. HRP is a great selection of enzyme for this type of assay and is capable of producing highly sensitive results with the appropriately paired substrate. However, the Ziegler protocol uses ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]) as substrate. ABTS yields a green reaction product when reacted with HRP that is measurable on a plate reader at 410 nm. According to numerous manufacturer instructions (Thermo, SigmaAldrich, KPI), ABTS has relatively low sensitivity compared to other available substrates and is appropriate for

detection of analytes in the 250 pg/well range (2.5 ng/mL assuming 100 uL per well). The Ziegler protocol lists an assay sensitivity of 4.3 pg/well (0.043 ng/mL assuming 100 uL per well), or 58 times lower than manufacturer specifications for ABTS. TMB (3,3′,5,5′-tetramethylbenzidine) would likely be a better selection of substrate for an assay requiring detection limits in the pg/mL concentration range.

The reason substrate selection matters in assay sensitivity is it dictates the amount of color development one is able to achieve with the reaction product after incubation with the enzyme. A particular wavelength of light specific to the substrate is aimed at the microplate wells in a plate reader and absorbance of light is measured. This is measured in optical density (O.D.) units. Deeper color development, such as that achieved by use of TMB, allows for more precision and more sensitivity across a lower range of concentrations. This is particularly true in a competitive or indirect EIA where enzyme is binding the capture antibodies that have not bound analyte in samples, thereby giving an inverse measure of concentration (i.e., more color development/higher O.D. values = lower concentration).

The 1995 Ziegler protocol has been adapted for use in many primate species, including gorillas (Skurski, 2006), chimpanzees (Emery Thompson et al., 2010 Kahlenberg et al., 2008; Muller et al., 2007), and bonobos (Moscovice et al., 2015) in urinary cortisol analyses. None of the chimpanzee or bonobo publications include information about antibody dilution. Several of the papers mention directly diluting urine without hydrolysis (Kahlenberg et al., 2008; Muller et al., 2007). None of the papers using the Ziegler 1995 protocol or any adaptation therein make mention of sample re-run criteria based on per cent coefficient of variance (%CV). While this does not mean

anything was necessarily done incorrectly in any of these implementations of the previously published protocol, there is very little information available for outside researchers to use if someone wanted to recreate the protocol and methods used in another lab.

Another potential source of error or variation in an adapted protocol based on the 1995 Ziegler paper can be found in the work with Disney gorillas in Florida (Skurski, 2006). In this particular adaptation, microplates are coated with 50 µL antibody solution (at 1:22,000 versus Ziegler's 1:85,000). Plates are then washed and 50 µL each of standards or samples, 50 µL of cortisol-HRP competitor, and 50 µL PBS buffer is then added to each well, for a total of 150 µL per well. There is no mention of plate blocking, though it is possible the PBS solution contains an appropriate blocking agent such as bovine serum albumin (BSA). If the plates are not blocked, it is possible for molecules to directly adsorb to the sides of the microplate wells, leading to non-specific binding and higher background noise in the assay (Figure 2.1). This method does not detail assay sensitivity or limit of detection, which are typically set as 2 standard deviations above the O.D. from multiple replicates of a zero standard or blank well, which would give a proxy measure of background noise in the assay. A lower sensitivity (higher limit of detection) would potentially indicate non-specific binding was happening. This could be solved by coating each plate with a volume of antibody at least equal to the volume of samples to later be used in the assay and then properly blocking the wells with slightly higher volume than will be added to the wells in other steps in the assay after the antibody has been adsorbed.

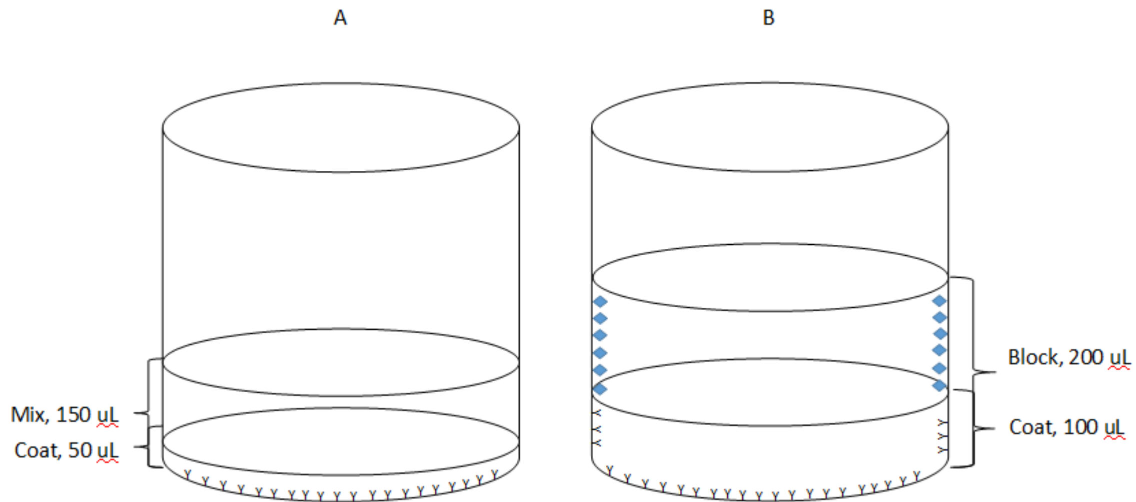

Figure 2.1. Cross section of microplate wells without (A) and with (B) blocking buffer (image not to scale). Well B represents available binding sites on polystyrene wells blocked by addition of blocking protein, while Well A represents a low volume of coating antibody (Y) used with higher volume of added sample and competitor mix, without blocking buffer. This can cause non-specific binding and higher noise.

As mentioned in the previous chapter, small new world monkeys (NWM) such as tamarins have very high levels of urinary cortisol relative to other primates. NWM cortisol is often reported in the µg/mg range compared to old world monkeys (OWM) and apes in the ng/mg range. In ideal conditions, an in-house assay provides the flexibility to optimize a standard curve concentration range to the expected values in a sample (Cox et al., 2004). However, the Ziegler 1995 protocol uses the same standard curve range for all species assayed, meaning that NWM urine is diluted up to 6400 times (Smith and French, 1997). Sample dilution is required to overcome matrix interference in urine, but if there is a justification for the extreme dilution used in NWM (such as particularly concentrated urine or high concentrations of interfering chemicals), it is not stated or cited in any publication. Diluting a sample so much magnifies minor errors in

measurement when the dilution factor correction is applied to the measured cortisol values. A difference of just 5 pg/mL measured in the assay is magnified to a difference of 32,000 pg/mL when corrected for dilution. A difference of 5 pg/mL is not likely to be of physiological significance, but 32,000 pg/mL could very well be representative of high enough concentrations to have a physiological effect.

In the NWM assays where samples are diluted 6400x, the standard curve is orders of magnitude lower in concentration than samples. This has the potential to compound sensitivity issues since ABTS is used as substrate in this assay lead to a strong recommendation that researchers reconsider sample dilution and standard range in the future. Alternatively, while commercial kits may not offer the flexibility to change the standard range, it may be appropriate to select kits from different manufacturers for different species depending on kit sensitivity and expected sample concentration range.

Other issues in the primate cortisol literature are of less direct consequence to measurement of cortisol, but nonetheless make it difficult for new researchers to follow the evolution of protocol and method development. For example, some papers published using the Munro antibody cite a Munro and Stabenfeldt 1985 publication, however the citation leads to an abstract from a conference that is about measuring plasma cortisol by EIA, but does not include detailed instructions about a protocol. The abstract is quoted in full below:

> A microtiter plate enzyme immunoassay (EIA) was developed for the determination of cortisol. The antibody was raised in a rabbit (R-Z) against cortisol-3-carboxysethyloxime (CMO):BSA and used at a titer of 1:15,000 (50 µL/well). The enzyme conjugate was horseradish peroxidase coupled to cortisol at C3 through a CMO bridge, and was used at 1:40,000 (50 µL/well). The average percent binding for standard displacement curves (n = 8) was 80.3% for 1 pg, 40.9% for 10 pg and 11.8% for 100 pg. The amount of cortisol that effected 50% displacement was 6.5 pg. The time of the assay is 2 h for the competitive reaction

> and 40 min for substrate reaction (substrate is ABTS).The results are read in a Dynatech Microelisa reader at 405 nm.
> Using a plasma extraction technique (ethanol), a comparison of the R-Z antibody with a commercial antibody (Miles) via RIA of cortisol resulted in a correlation coefficient of 0.998. A correlation coefficient of 0.998 was also obtained in comparing cortisol results obtained by EIA vs RIA, both using the R-Z antibody.
> The EIA was also tested for the assay of unextracted plasma. On a comparison of 32 samples, average cortisol values were 8.07 μg for extracted and 8.22 pg for unextracted samples (1 mL aliquot). The effect of volume of plasma (0.5 and 1.0 μL, n = 38) was also tested in an unextracted EIA. The average value for the direct assay of 0.5 μL aliquots was 7.56 μg/mL and for 1.0 μL, 7.71 μg/mL.

This abstract has been cited a total of 66 times. It appears in primate literature a total of 7 times. In some cases (Arlet et al., 2015, 2009; Arlet and Isbell, 2009), this article is cited as Munro and Stabenfeldt 1984, which is actually the reference to the progesterone assay that was altered in the Ziegler 1995 protocol. In one instance, the abstract is cited in a paper about testosterone (Arlet et al., 2011), not cortisol, as the origin of the anti-testosterone antibody development. In other cases (McCallister et al., 2004), it is cited as the reference for the antibody being raised in rabbits against a "steroid bovine albumin," which is then sometimes abbreviated (BSA). Typically, BSA specifically refers to bovine serum albumin, or albumin from cow blood. The term "steroid bovine albumin" can only be found in publications referencing this particular antibody. While it is correct that a steroid was coupled to BSA and injected into rabbits in the development of the anti-cortisol antibody, steroid bovine albumin is not the correct term for the abbreviation and could refer to any number of steroids coupled to BSA, not just cortisol.

What can be done?

Simply examining the literature on urinary cortisol testing in primates reveals numerous potential issues with methodology and reporting over the last twenty years. It is possible for the field to improve in these areas if a general consensus can be formed regarding adopting a commercially available kit and best practices for reporting methods and results of studies.

There are numerous kits available and pre-validated for use in urinary cortisol detection. Cortisol structure is identical across many species (Evans, 2005), therefore determining whether a kit is appropriate for use in a particular species and sample matrix will depend upon the detection range of the kit compared to the expected physiological range in the study species, as well as demonstration of parallelism between the standard curve and a pooled, serially diluted sample from multiple individuals. A serial dilution of a control-spiked sample, or a spike recovery experiment, can also demonstrate there is no interference from the sample matrix if expected and observed concentrations of measured cortisol are within tolerance limits (typically 90-110% recovery). This serial dilution can also serve to determine an appropriate dilution range for a sample matrix, which can be selected from any of the points in the range of dilutions that exhibit appropriate recovery and make it likely for samples to fall on the standard curve, thereby not requiring re-runs at more concentrated or dilute starting points.

Reporting Results

Another area that could be improved by consensus in the community of primatological researchers is the manner of reporting results of primate socioendocrine

research. As previously mentioned, there is a lack of available data about reference ranges for many biomarker concentrations in individual species for the various sample matrices used. All researchers certainly are not required to use the same kits or methods to analyze samples, however it would help to establish expected analyte concentrations if results can be repeated by different groups. Also, for the sake of transparency and ability to compare data between research groups, it would be helpful to have access to raw values for each individual analyte. In primate urinary cortisol studies, this would mean reporting the range of measured cortisol concentrations, dilution factor used, range of creatinine concentrations, and the range of normalized cortisol to creatinine values.

Since the most commonly used cortisol antibody is in limited supply and a new method will be required in the near future, it would be advantageous to have accurate data for comparison of raw values of the old method with potential new methods. For instance, in urinary cortisol studies there has not been a single study thus far that has reported the range of raw cortisol concentrations. Only the transformed µg or ng cortisol to mg creatinine values have been reported. In fact, most studies are not even reporting a range for the normalized cortisol values. The numbers included in this chapter were sometimes estimated by visually assessing concentration graphs included in the papers cited. Most studies do not report raw creatinine values, either.

It is vital that these raw values, and not the ratio, are reported so that researchers can know whether or not their measured values correspond to other measured ranges. When the ratio of cortisol to creatinine is the only value published, the possibility exists that researchers are obtaining different raw values and incorrectly assuming their values are in range if the ratios come out similar to previously published values. Furthermore,

some studies do not even report the cortisol to creatinine ratio, but a transformed time residual. Another minor complication in results comparison is the usage of different concentration units in the literature. Some studies report pg/mg while others use pmol/mg. The studies about urinary cortisol in apes that report concentration units in pmol/mg uniformly report values up to six orders of magnitude lower than researchers who report in the ng/mg range (Table 2.1). However, when the pmol/mg units are used, the numbers themselves appear to be within range of other urinary cortisol in apes. It is hard not to assume the concentration units were deliberately selected in order to distract from the abnormally low measured values. For ease of comparison, a single concentration unit should be adopted.

Table 2. 1. Ape urinary cortisol values converted to common units (ng/mg).

Authors	Year	Species	Cort Range	Units Reported	Convert ng/mg	Diurnal
Jurke et al.	2000	Pan paniscus	303.2-1558.4	ng/mg	400-1500	morning urine
Moscovice et al.	2015	Pan paniscus	102.7-226.3	ng/mg	100-200	morning urine
Squires et al.	unpub	Pan paniscus	7-800	ng/mg	7-800	am vs pm
Surbeck et al.	2012	Pan paniscus	44-309	ng/mg	20-250	time of sample
Whitten et al.	1998	Pan troglodytes	3-14	ug/dL	60-280**	none
Muller & Lipson	2003	Pan troglodytes	50-500*	pmol/mg	0.0018-0.0181	time of sample
Anestis and Bribiescas	2004	Pan troglodytes	10-100*	pmol/mg	0.0036-0.036	time of sample
Anestis	2009	Pan troglodytes	12-190*	pmol/mg	0.0043-0.069	time of sample
Anestis	2005	Pan troglodytes	?	?	?	time of sample
Muller	2004	Pan	200-	pmol/mg	0.072-0.2	FMV &

&Wrangham		troglodytes	550*			time
Muller et al.	2007	Pan troglodytes	150-550*	ng/mg cr	150-550	?
Kahlenberg et al.	2008	Pan troglodytes	-100 to +200*	hr vs ng/mg	?	time of sample
Emery Thompson et al.	2010	Pan troglodytes	- 40 thru +60*	hr vs ng/mg	?	time of sample
Skurski	2006	Gorilla gorilla	4.82-351.52	ng/mg cr	4.82-351.52	morning urine
Robbins & Czekala	1997	Gorilla gorilla	400-900*	ng/mg cr	400-900	am vs pm
Bahr et al.	1998	Gorilla gorilla	0.35-1.12	ug/mg cr	350-1120	morning urine

* Denotes values that were estimated visually from graphs because no ranges were reported in the publication. ** Denotes an estimated value normalized to a creatinine value of 0.5 mg/mL because the original reported numbers were direct cortisol concentrations. Diurnal column describes the manner in which researchers controlled for diurnal fluctuations. FMV = first morning void.

Expected Range of Bonobo and Chimpanzee Urinary Cortisol

While it is useful to know that a measured cortisol to creatinine ratio in a particular species corresponds with other published values using different methods, no calculations have been published to estimate the expected range of urinary cortisol values. For urinary cortisol in bonobos and chimpanzees, the expected range can be determined by calculating an estimated corresponding plasma cortisol value if a few variables are known or estimated. The glomerular filtration rate (GFR), or volume of blood filtered by the kidneys per minute has been empirically determined in chimpanzees to be 90 mL/min (Fanelli Jr and Weiner, 1973), which is very similar to a human with normally functioning kidneys, according to the National Kidney Foundation. The median reported daily output of urine in chimpanzees is 850 mL/day (Eder, 1996).

Using the numbers reported for average time for cortisol clearance of approximately 5.5 hours by Bahr et al., 2000 and the reported bonobo plasma cortisol value of approximately 100 µg/dL (1000 ng/mL) (Anestis et al., 2006) we can calculate a rough estimate of cortisol expected in urine over the course of 5.5 hours. Given a GFR of 90 mL per min, a chimpanzee will filter 29.7 L of blood in 5.5 hours. Assuming a steady concentration of cortisol for the ease of estimation, that blood will have 29,700 ng cortisol filtered by the kidneys. That is total cortisol, and free cortisol should exist as approximately 5% of total (range of 3-10%). Bahr also reported that 90% of H3 cortisol (functionally free) was excreted in urine of chimpanzees in 5.5 hours, which means approximately 1336.5 ng will be excreted in that time. In 5.5 hours, we can expect an average volume of urine equivalent to approximately 154 mL, providing an average expected urinary concentration of 8.7 ng/mL. Median raw urinary cortisol concentration measured in the CZA bonobos was 19.16 ng/mL, which is relatively close to the estimate provided. This is further evidence to support that the Arbor kit is appropriate for use in bonobo urinary cortisol studies. It has produced results that replicate published urinary cortisol values in chimpanzees and bonobos as well as producing values that are within the expected physiological range for urinary cortisol.

Methods

In this experiment, a commercially available cortisol EIA kit (Arbor Assays, Ann Arbor, MI, USA) was validated for use in bonobo urine. The Arbor Assay Detect-X EIA allows for direct measurement of urinary cortisol (with appropriate dilution) without hydrolysis, per manufacturer instructions. The Arbor Assays kit lists a sensitivity of 17.3

pg/mL and a limit of detection of 45.4 pg/mL. Manufacturer instructions dictate that urine should be diluted ≥ 1:8 in assay buffer. We performed a serial dilution of bonobo urine and determined an optimal dilution of 1:20, which puts our samples in a linear range of dilution and means most samples would fall within the measurable range of the standards. Samples were re-run when a CV was above 10% or there was less than 0.1 pg/mL absolute value difference between samples run in duplicate. The absolute value difference of 0.1 pg/mL was selected because it is10x the standard deviation of the mean of samples. Intra-assay CVs were 6.0% and 14.7% for high and low controls, respectively, according to the kit booklet, and inter-assay CVs were 7.2% and 10.9% for high and low controls, respectively. CVs will be determined experimentally in the future with the development of in-house high and low controls.

Creatinine concentrations were determined by commercially available kit (Arbor Assays, Ann Arbor, MI, USA) in a colorimetric enzyme reaction based on the Jaffe reaction.

Urine samples were collected from a population of captive bonobos housed at the CZA. Samples were collected during the summers of 2012, 2013, and 2014. Samples used in this preliminary analysis for curve parallelism were only from the 2012 field season. Urine was collected by CZA husbandry staff in accordance with CZA's existing urine collection protocol where individuals were trained to urinate on command through the mesh caging and into a sterile cup and/or pipetted up off of a clean floor. Samples were then placed into sterile Eppendorf tubes and labeled with subject, date, and time and immediately frozen in -4 degree Celsius freezer until shipment overnight on dry ice to the UO Snodgrass lab and stored in a -80 degree Celsius freezer until time of analyses.

Samples were taken from compliant individuals on a daily basis at varying times.

Data were analyzed using SAS/® software. Copyright © [2015] SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Results

Pooled urine samples showed parallelism to the standard curve (Table 2.2). Calculated concentration values for the standards and pooled serially diluted samples were $\log_{10}$ transformed to create a linear range and plotted against percent binding of the labeled cortisol competitor. The Arbor Assays Detect-X cortisol EIA is an indirect assay, so higher percent binding happens with lower concentrations. An ANCOVA was performed to test for parallelism of the log-transformed cortisol values and percent binding. The standards and pooled serially diluted samples were not significantly different (Figure 2.2).

Table 2.2. Pooled urine serial dilution results

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	3	3.83	1.28	991.38	<.0001
Error	8	0.01	0.001		
Corrected Total	11	3.84			

R-Square	**CoeffVar**	**Root MSE**	**conc Mean**
0.99	1.36	0.04	2.64

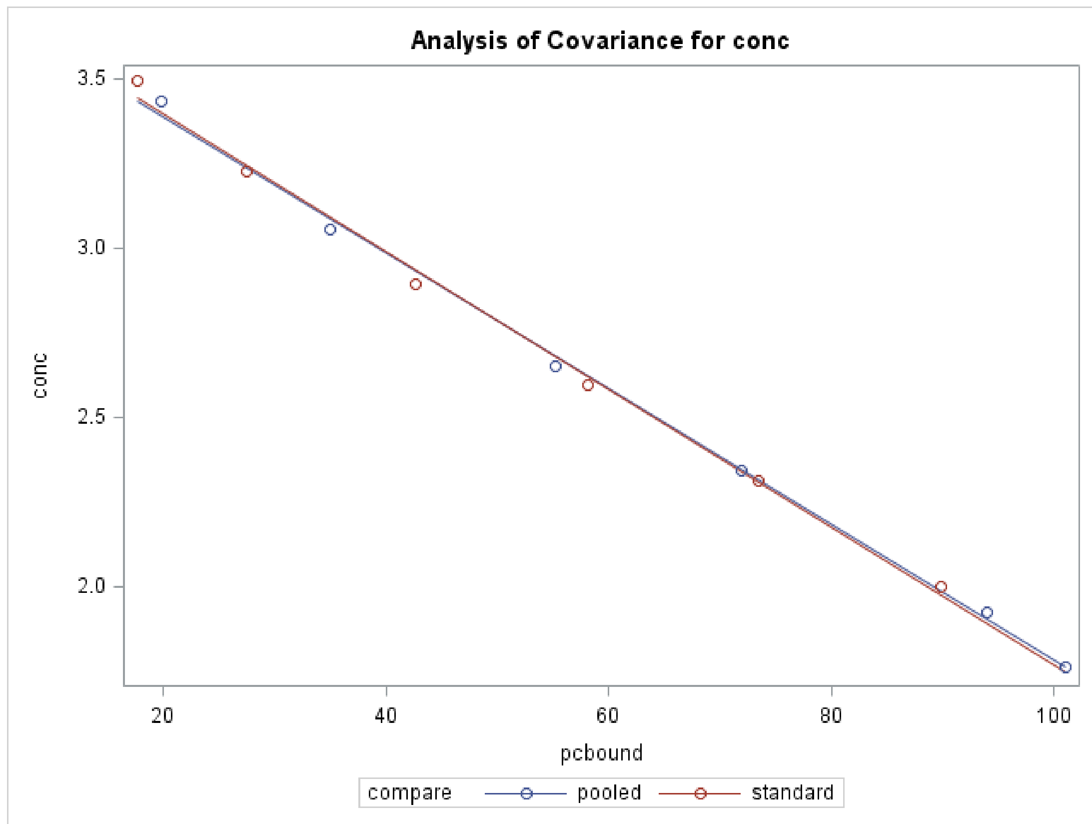

Figure 2.2. Plot of Standards and Pooled Serially Diluted Bonobo Urine

Creatinine concentrations were not normally distributed and were therefore $\log_{10}$ transformed. Prior to normalizing cortisol concentrations to the log-transformed creatinine, creatinine concentrations were analyzed to determine if values were associated with body size. Age class (infant, juvenile, sub-adult 1, sub-adult 2, and adult) was used as a proxy measure for body size due to the lack of available body mass data for each individual. There was a significant and positive association between creatinine and age class ($p < 0.001$, $r^2 = 0.05$). Individual creatinine concentrations were residual adjusted to control for body size effect. All cortisol values were normalized to log-transformed, residual adjusted creatinine concentration and are reported as ng cortisol per mg

creatinine.

For the samples used in this preliminary analysis, the range of raw cortisol values obtained was 1.1-225.3 ng/mL (mean = 60.1 ng/mL, median = 60.4 ng/mL). The range of corresponding creatinine values was 0.06-1.80 mg/mL.

Conclusions

It was determined that the Arbor Assays Detect-X cortisol EIA is an appropriate way to measure urinary cortisol in bonobos. This is the first use of the Arbor Assays EIA in bonobos. The cortisol to creatinine ratio measured in the CZA bonobos in this preliminary group of samples was within the range of reported cortisol to creatinine ratios measured by other groups with other methods. This, coupled with the demonstrated curve parallelism between a serially diluted pooled urine sample and the standard curve supports the use of this kit as an appropriate way to measure urinary cortisol in bonobos.

While there is no direct evidence that the Ziegler 1995 protocol is leading to incorrect results, the lack of available details about the published method and the manner in which results are typically published leave the strong probability for issues arising from running a cortisol EIA with this method. It might be easy to write these issues off as procedural inertia or simple misunderstanding, however the WNPRC has previously been alerted to improper procedure in measurement of urinary oxytocin and vasopressin in children (Fries et al., 2005). Their use of HPLC-UV to determine oxytocin and vasopressin concentration was not an appropriate method and led to results one million fold higher than ever before reported (Young and Anderson, 2010). The Fries paper was published *in Proceedings of the National Academy of the Sciences* and the million-fold

error has never been addressed. It has been cited 437 times at the time of writing this dissertation.

Researchers examining citation bias have suggested that a high number of citations in and of itself provides authority to the cited paper (Greenberg, 2009). Based on the citation trees in the urinary cortisol data examined in this chapter, it seems this phenomenon is alive and well within primatology research. Certain papers get repeatedly cited, sometimes improperly, to the point where it seems almost necessary to cite certain previous publication, even if the content is not exactly pertinent to the topic. This can provide a barrier to new researchers attempting to publish within small research communities because the people likely to be reviewers of new researcher's papers either are the authors of the repeatedly cited papers or the people who are engaging in the repeated citation trees that lead nowhere.

Citing an abstract as the source for the development of an antibody or protocol is not sufficient to provide the level of detail needed for an outside party to follow that citation and reproduce the results in their own lab. Similarly, citing an assay developed to detect one hormone and stating it was modified for use in detecting another hormone is not sufficient to demonstrate method validation. Primatologists can improve standards in these areas- both the testing methodology and publication of those methods- in order to make their work more reproducible, open, and transparent.

It would be nice to think that science operates in a purely objective truth-seeking fashion in that when errors are pointed out new methods are tested and adopted in order to improve accuracy. However, scientists are humans and it does not always happen that way. Considering that this group has already been told about an error in methodology and

failed to address or correct it, there is not much confidence any of the issues raised here will be addressed. However, since the antibody used in the assay is presumed to be in short supply, hopefully this will necessitate adoption of newer, more robust methods of cortisol analysis.

Beyond issues of protocol and reporting disparities between research groups, using a commercially available kit for cortisol testing also significantly lowers the price compared to sending samples for analysis at WNPRC. The Arbor Assays cortisol EIA cost approximately $8.13 per sample, accounting for running in duplicate and the creatinine assay cost for normalization. Sending samples to WNPRC costs either $40.38 per sample or possibly as high as $73.07 if samples are run in duplicate.

The Arbor method requires a total of 12 uL, after accounting for a 1:20 dilution factor, of urine per sample for cortisol and creatinine to each be run in duplicate. To have the cortisol and creatinine analyses done by WNPRC, 150 uL of urine is required for analysis in duplicate. Primate samples are hard to come by and therefore precious in terms of the types of data that can be gleaned from analysis. Conserving every last microliter is essential to the potential to expand the range of sample analyses conducted.

CHAPTER III

DIURNAL CORTISOL IN CAPTIVE BONOBOS

Introduction

Stress and Cortisol

Since Selye's seminal work in defining stress 50 years ago, researchers from widely varied backgrounds have studied stress in humans and animals. Stress can be defined as a challenge to the system that induces an adaptive response. Chronic stress is when the stressor is present for long periods of time and can lead to maladaptive responses, dysfunction, and negative health outcomes.

Cortisol is widely known as the “stress hormone” and is therefore often the focus of stress research. Molecularly, cortisol is identical across all vertebrates (Evans, 2005). The pattern of release of cortisol from the adrenal glands follows a diurnal pattern of spiking shortly after waking, reaching a peak within approximately 1 hour of awakening, and slowly returning to base levels overnight (Hellhammer, 2009). This is referred to the cortisol awakening response and the pattern is highly conserved in mammals and primates. Given the emerging knowledge about deleterious effects of dysregulated cortisol function in humans and the highly conserved nature of the awakening response, primates are an ideal choice of species to model stress in an evolutionary sense.

Using cortisol as a biomarker of stress in primates is not as straightforward as simply measuring cortisol values of different individuals, noting contemporaneous stressors, and comparing changes within and between individuals. The way in which cortisol is measured has a direct impact on the research questions that can be asked and the type of conclusions that can be drawn from stress research. Since cortisol fluctuates in

a diurnal pattern, time of sampling must be accounted for. This can be done by choosing a single time of day for sample collection, attempting to capture the entire awakening response by collected repeat samples, or taking samples across a range of times and including time of sample as a control statistically.

Primate researchers use multiple types of cortisol variables to relate to perceived stress. Most primate studies that use samples collected at specific times focus on inter-individual variability and any changes within each individual over time. Primate studies that collect samples from a range of times throughout the day typically focus on percent change in morning to evening values, area under the curve (analogous to a day's total output of cortisol), or slope of the curve between morning and evening values to assess the degree of stress in an individual.

From an evolutionary perspective, these studies enable primatologists to finally assess the physiological stress experienced by an individual as opposed to an inferred level of stress that the animal may be experiencing based on behavioral observations. Many social and environmental factors have been proposed to be stressful in primates including food shortage, captivity, and social rank, social stability and presence of allies. In order to apply these studies directly to the evolution of the hormonal basis of stress in humans, it is helpful to examine these factors in our closest relatives, the genus *Pan*.and to understand the social contexts that may be stressors in their social system. This, therefore, allows us to bridge the subjective and objective assessment of stress, and the social context of that stress, in our closest relatives.

Bonobos and the social context of stress

Along with the chimpanzee (*Pan troglodytes*), the bonobo (*Pan paniscus*) is our closest phylogenetic relative, existing today as a descendant of a common ancestor that the genera Homo and Pan shared approximately six million years ago. Consequently, information on the social organization and ecology of bonobos and chimpanzees are vital for reconstructing scenarios of early hominin sociality (Nunn and Van Schaik, 2001). But while the overwhelming majority of research projects studying the Pan species have focused on chimpanzees, bonobos have received considerably less attention. Until recently, scientists have focused on the violent nature of male chimpanzees, the lack of close association among female chimpanzees, and inferred parallels with early human societies. Humans, however, have an equally close relative in the bonobo or pygmy chimpanzee. Bonobo societies are based on peaceful cooperation and strong social bonds both between males and females and among females.

Despite being closely related, chimpanzees and bonobos have drastically different social systems. Chimpanzee males are highly bonded, more gregarious, and cooperate with male relatives to defend a communal range that includes the feeding ranges of several unrelated females (Goodall et al., 1979; Nishida, 1979). Female chimpanzees are rarely affiliative towards each other and live in semi-solitude with their young in overlapping core areas (Wrangham and Smuts, 1980; Wakefield, 2008; Newton-Fisher, 2006). Unlike chimpanzees, bonobo communities are based on strong social ties among unrelated females and long-term bonding between individual males and females. Bonobos associate in parties based around unrelated, allied females, their offspring, and related but independent males (Kano, 1992; White, 1996). Male bonobos do not form the

tight bands that are associated with the male cooperative killing behavior of chimpanzees. Instead, bonobo aggression is mild. Disputes and social tensions are often diffused through sexual behavior. Female sociality in bonobos is unique among apes and indeed all other non-human primates (Furuichi 1987; White 1996). Unlike chimpanzees in which the communities are centered around highly bonded males that are territorial and aggressive within and between communities (Wrangham 1999), bonobo central social units consist of allied, unrelated females (Kano 1992; White 1996). These female associations appear to be relatively stable with greater membership turnover occurring among males and females without infants. Evidence suggests that some males may rely on their mothers to help them gain rank and facilitate entrance into the social network of bonded females (Furuichi 1997).

Bonobo females, unlike chimpanzee females, have considerable social influence over males which has been described as female dominance (Parish 1996) or female power (White and Wood 2007). Males rarely aggress against females (White and Wood 2007) and male aggression is rarely followed by mating (sexual coercion). The mating behavior of bonobos is unusual among primates and differs greatly from chimpanzees because of the socio-behavioral function associated with increased sexual activity (White 1996). Recent findings (Boose and White 2012) highlight two important elements in the mating pattern of bonobos. First, male dominance rank, determined through agonistic male-male interactions is important in male mating frequency, where females prefer to mate with the dominant male. Second, context dependent rank, based on the presence of high rank mothers, is also important in male mating success, where females are most receptive to solicitations of copulations from males with high-ranking mothers in the

group. This structure of dominance rank, with high ranking females and male rank depending on context, is very different from the strictly male-dominance system in bonobos.

Methods

Study population

Behavioral observations and urine samples were collected from a group of captive bonobos housed at the CZA. The study is part of a larger, ongoing longitudinal sociobehavioral research collaboration between the University of Oregon and CZA. The urinary and behavioral data for this project were collected in the summers of 2012-2014. The zoo's population of bonobos was in flux during this time. Group composition changes when infants are born and when members immigrate/emigrate. Season-specific group descriptions are provided in Table 3.1. Overall, 156 urinary samples were analyzed for cortisol and creatinine.

Table 3.1. Group composition and urine sample number by year.

	Males	**Females**	**AM Samples**	**PM Samples**
2012	6	7	14	14
2013	7	5	32	35
2014	7	6	36	25

Urine Collection

Urine samples were collected during the summers of 2012, 2013, and 2014. Urine was collected by CZA husbandry staff in accordance with CZA’s existing urine

collection protocol where individuals were trained to urinate on command through the mesh caging and into a sterile cup and/or pipetted up off of a clean floor. Samples were then placed into sterile Eppendorf tubes and labeled with subject, date, and time and immediately frozen in -4 degree Celsius freezer until shipment overnight on dry ice to the UO Snodgrass Lab and stored in a -80 degree Celsius freezer until time of analyses. Samples were taken from compliant individuals on a daily basis at varying times.

Cortisol EIA

Free urinary cortisol concentrations were measured with a commercially available cortisol EIA kit (Arbor Assays, Ann Arbor, MI). All manufacturer kit instructions were followed, including sample preparation instructions. Urine samples were spun at 8g for 5 minutes to pellet any detritus present in samples. Kit instructions suggest to dilute urine at least 1:8. A serial dilution of pooled bonobo urine was used to determine the linear range of recovery that was most likely to fall within the limits of the standard curve, and a 1:20 dilution was selected for testing purposes. Samples, standards, and controls were assayed in duplicate. Any samples that fell above or below the curve were re-run with higher (1:40) or lower (1:10) dilution factors. Previous analysis of this kit has determined it is appropriate for use in bonobos based on a pooled sample serial dilution exhibiting parallelism with the standard curve as presented in the previous chapter of this dissertation.

Urinary creatinine concentrations were calculated in order to correct for variation in hydration status within and between individuals. A commercially available colorimetric kit (Arbor Assays, Ann Arbor, MI) based on the Jaffe reaction was used.

Samples with >10% CV or an absolute value difference of less than 0.10 ng/mL were re-run. Inter assay and intra assay CVs were xx and xx, respectively (determined by control CVs from 3 different plates). Plates were read on a Biotek plate reader at 410 nm and raw data was analyzed in MyAssays software, as recommended by the kit manufacturer. Standards were fit to a 4-PL curve and samples within 10-90% binding were considered in range for the assay. The kit sensitivity is reported to be 17.3 pg/mL and the limit of detection is 45.4 pg/mL.

All protocols were approved by the University of Oregon Animal Care and Use Committee as well as the CZA (IACUC # 11-10RA).

Statistical Analyses

Cortisol values were split by morning (AM) and afternoon (PM). A nested analysis of variance was performed to test whether individual differences were consistent between sexes (i.e., whether males and females differed in cortisol concentrations) with individuals nested within sex. There were no sex differences in AM or PM cortisol concentrations so further analyses were performed without separating by sex.

A two-way ANOVA was performed with AM/PM and individuals as main effects, with a test for whether there was a significant interaction between time of day and individual. A significant interaction term shows inconsistency of response in that the AM/PM cortisol concentration change is different in magnitude and/or direction between individuals (Sokal and Rohlf, 2011).

Creatinine concentrations were not normally distributed and were therefore $\log_{10}$ transformed to normality. Creatinine concentrations were analyzed using regression to

determine if values were associated with body size. Age class (infant, juvenile, sub-adult 1, sub-adult 2, and adult) was used as a proxy measure for body size due to the lack of available body mass data for each individual. There was a significant and positive regression of creatinine on age class ($p = 0.02$, $R^2 = 0.05$ for females; $p = 0.001$, $R^2 = 0.16$ for males). This relationship was, therefore, statistically removed and the individual creatinine concentration residuals were calculated. These residuals were non-normally distributed and were transformed using $\log_{10}$ to normality. All cortisol values were normalized to this log-transformed, residual adjusted creatinine concentration and are reported as ng cortisol per mg creatinine. Infants were excluded from analyses of cortisol concentrations.

Data were analyzed using SAS/® software. Copyright © [2015] SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Results

The range, mean, median, and standard deviation of raw cortisol, raw creatinine, and cortisol normalized to creatinine are reported in Table 3.2.

Table 3.2. Group raw cortisol and creatinine values across three field seasons.

	Creatinine (mg/mL)	Cortisol (ng/mL)	Cort/Cre (ng/mg)
Low	0.067	1.55	4.33
High	1.80	67.91	412.36
Mean	0.34	21.76	88.87
Median	0.23	19.16	59.09
St. Deviation	0.28	16.08	82.78

There were no significant differences between sexes, so all analyses were split only by AM/PM. There were no significant differences between field seasons; therefore all analyses included data from all three field seasons. Individuals had significantly different values in both AM and PM cortisol concentrations (Tables 3.3 and 3.4), morning values were significantly higher than afternoon values, and there was a significant interaction term (p = 0.019), indicating that individuals differ in the pattern of AM to PM change in cortisol concentrations (Figure 3.1).

Table 3.3. Individual AM differences.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	15	3.82	0.25	3.46	0.0002
Error	66	4.86	0.07		
Corrected Total	81	8.69			

R-Square	**CoeffVar**	**Root MSE**	**logCortCreatRP Mean**
0.44	6.21	0.27	4.37

Table 3.4. Individual PM differences.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	16	3.28	0.20	2.58	0.0045
Error	57	4.53	0.08		
Corrected Total	73	7.81			

R-Square	**CoeffVar**	**Root MSE**	**logCortCreatRP Mean**
0.42	6.98	0.28	4.04

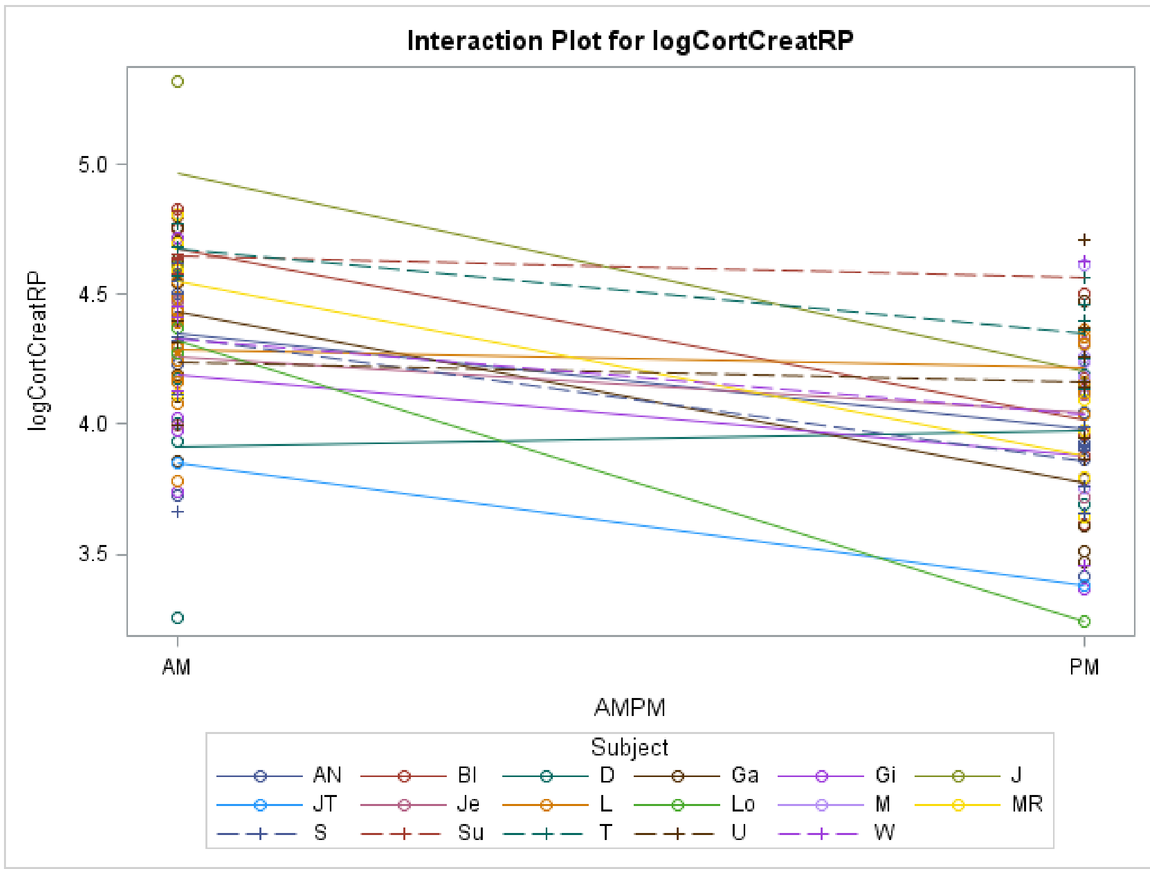

Figure 3.1. Individual changes from mean AM to PM values across all field seasons.

AM and PM cortisol values were not significantly different between age classes (juvenile, sub adult 1, sub adult 2, and adult). However, the difference between AM and PM values did differ between age classes. Sub adult 1 and sub adult 2 had much larger separation between AM and PM cortisol values (Figure 3.2 and Table 3.5).

Mean AM cortisol values were higher than mean PM values for the group (Figure 3.3) and mean AM and PM values across all field seasons were plotted by individual (Figure 3.5) and individual per cent change in AM to PM values were calculated to identify dysregulation (Figure 3.6). All samples used in this analysis were plotted against time of sample and show more variation in AM than PM concentrations (Figure 3.4).

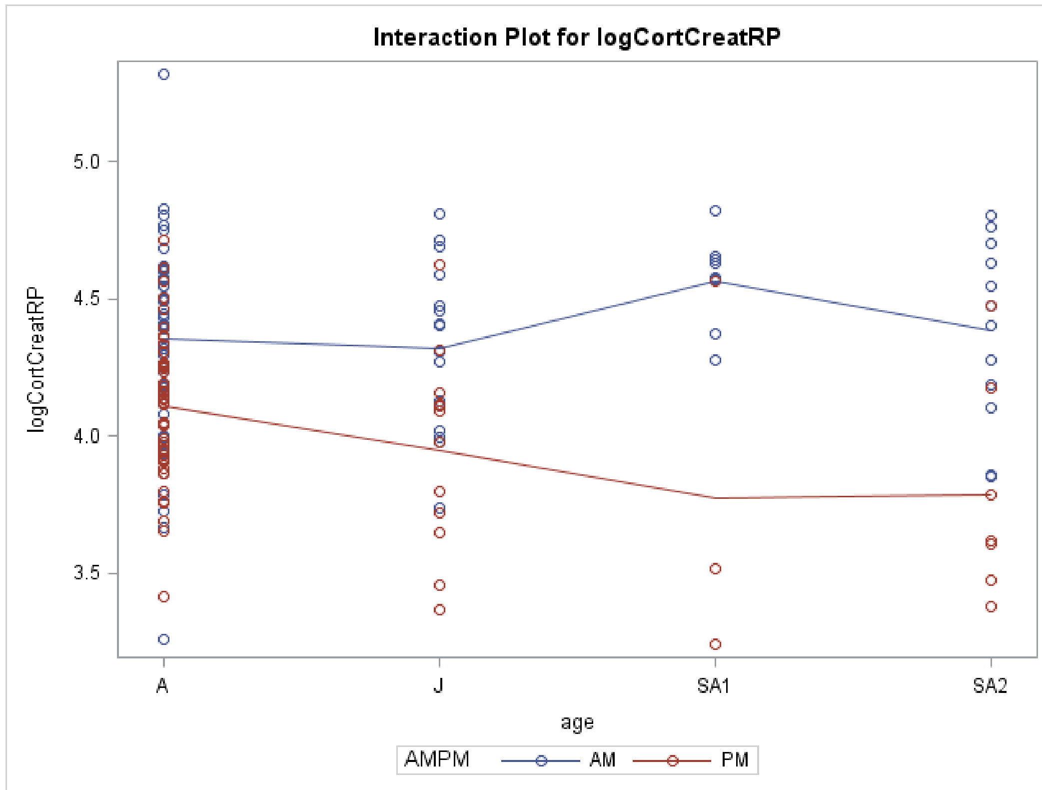

Figure 3.2. Age class differences in AM to PM cortisol changes. SA1 and SA2 have largest AM to PM change.

Table 3.5. Summary of two-way ANOVA of AM vs PM differences between age classes.

Source	DF	Sum of Squares	Mean Square	F Value	Pr> F
Model	7	5.74	0.82	8.02	<.0001
Error	148	15.12	0.10		
Corrected Total	155	20.85			

R-Square	CoeffVar	Root MSE	logCortCreatRP Mean
0.28	7.58	0.32	4.22

Figure 3.3. Group mean AM versus PM across all field seasons. Error bars are SEM.

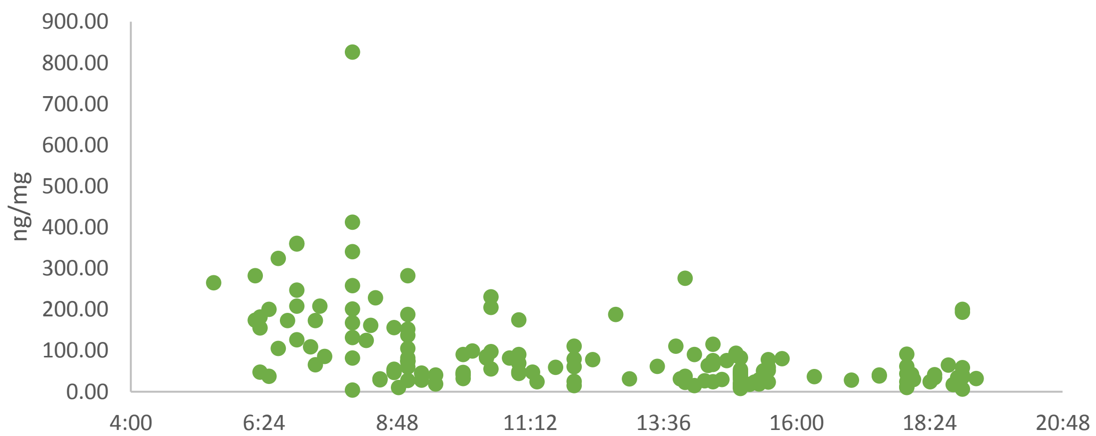

Figure 3.4. Group cortisol concentrations versus time of sample.

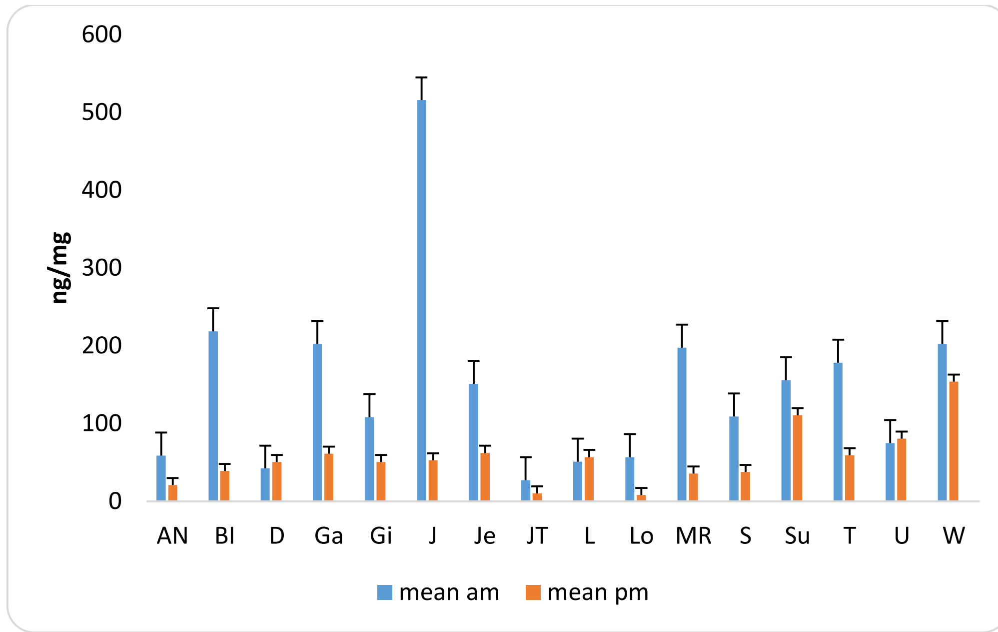

Figure 3.5. Individual mean AM and PM values across all field seasons.

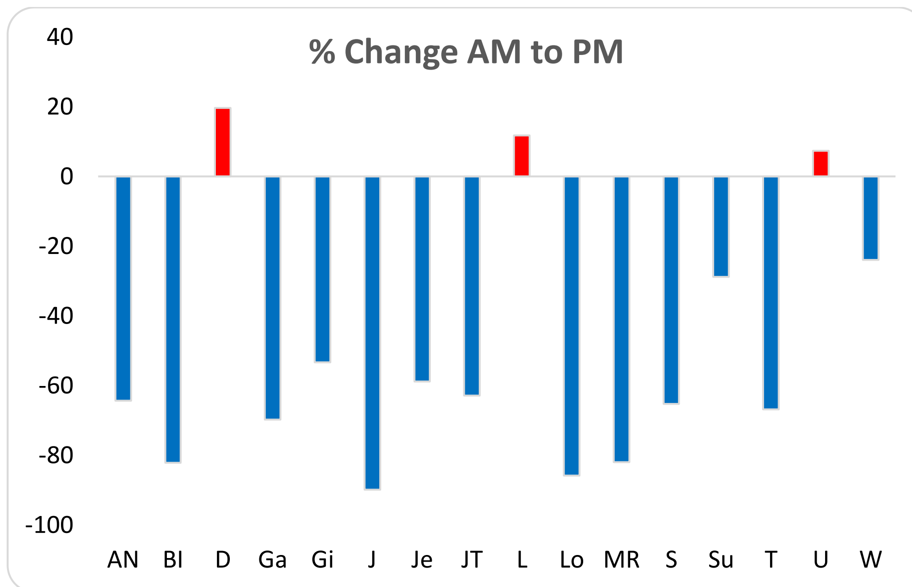

Figure 3.6. Individual percent change in mean AM to PM cortisol concentrations across the study time period.

Data were tested for outliers by fitting each individual's cortisol values to a

normal curve and detecting which samples fell outside the 95% confidence interval (Figures 3.7 and 3.8). One individual who had the highest measured cortisol value of all samples in all field seasons (>800 ng/mg) (Figure 3.9) would normally have that sample removed or adjusted to 2-3 standard deviations above the mean. However, outliers were identified not to remove or alter within the data set, but rather to identify potentially stressful instances within individuals.

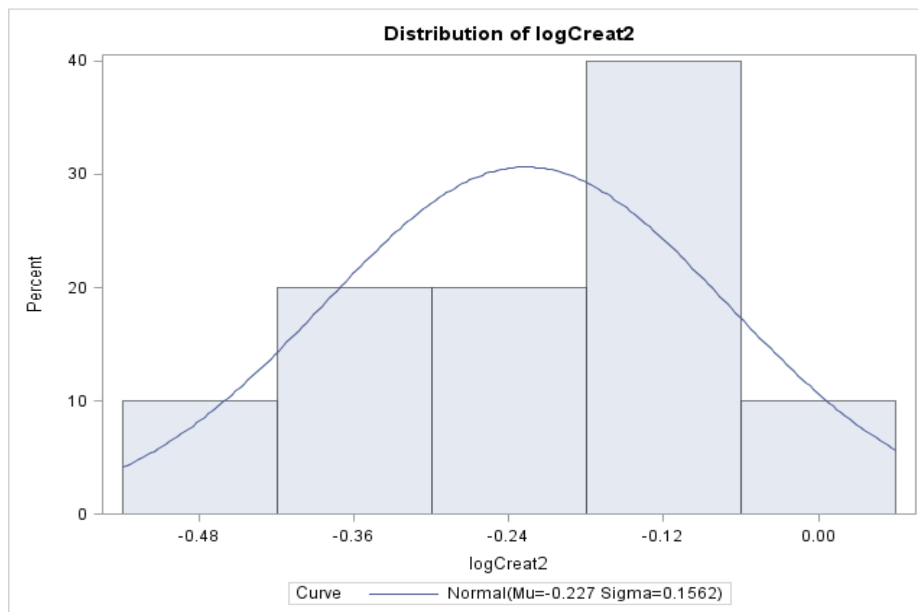

Figure 3.7. Histogram of cortisol values fit to normal curve for one individual (Gander).

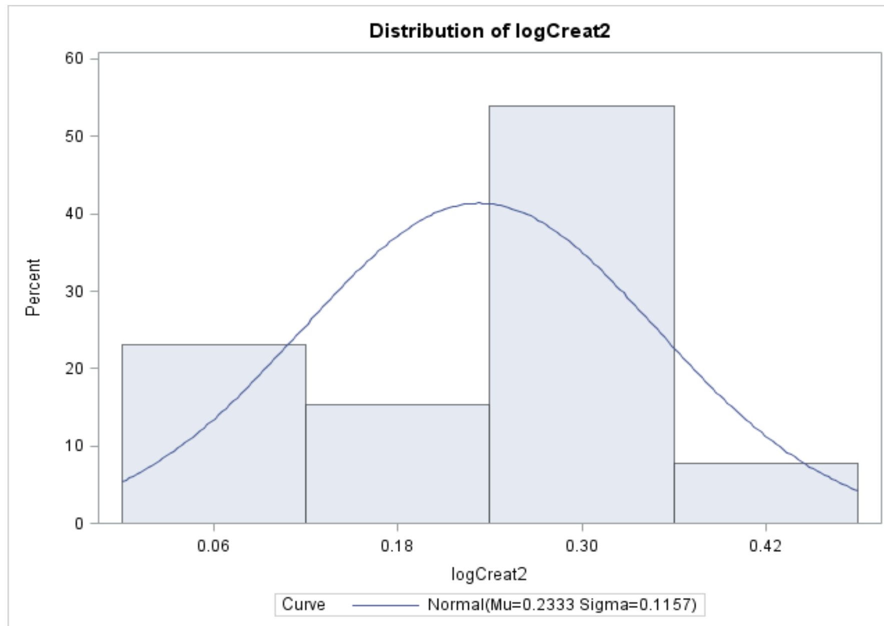

Figure 3.8. Histogram of cortisol values fit to normal curve for one individual (Lady).

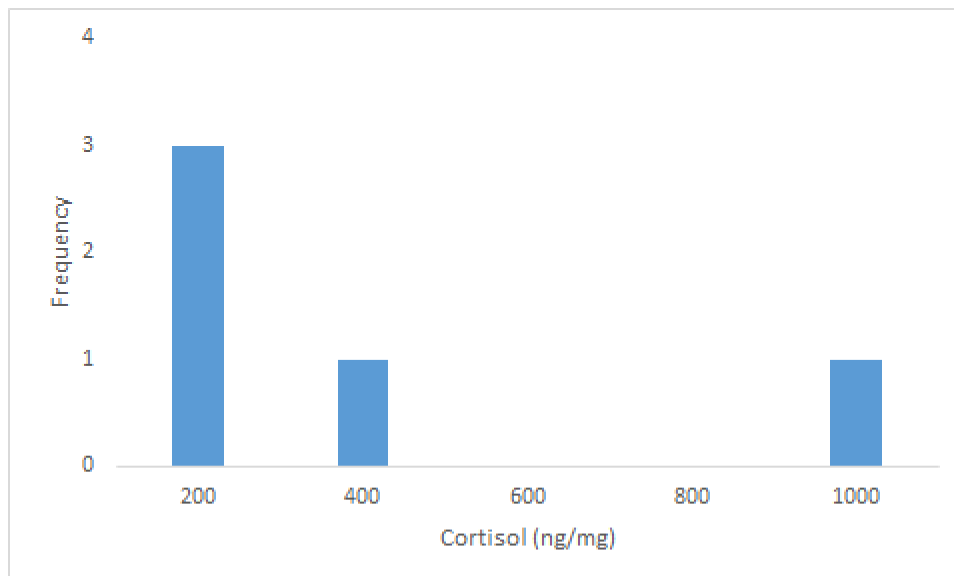

Figure 3.9. Histogram of cortisol concentrations for one individual (Jimmy) that had the highest single cortisol value measured in all seasons (826.57 ng/mg).

Conclusions

This study is the first to demonstrate the existence of a diurnal pattern of cortisol production in bonobos. The existence of the diurnal pattern is not unexpected given that humans and lower order primates have been known for quite some time to exhibit the same type of cortisol production pattern (i.e., peak in the early morning and a slow drop throughout the day). This is also one of the first studies to present longitudinal hormonal data from captive bonobos. This is a valuable data set that will aid in our understanding of hormonal patterns and the stress response in our closest living relatives.

This preliminary analysis of data from the bonobos at the CZA shows there are individual differences in cortisol levels- in the morning, in the evening, and differences in the morning to evening changes.

Previous studies have reported wildly variable levels of cortisol, sometimes attempting to mask differences of up to six orders of magnitude by hiding the differences behind an alternative concentration units. This study reports raw values obtained for cortisol, creatinine, and the normalized cortisol/creatinine ranges obtained across three field seasons of urine collection. This study is also the first of its kind to use a commercially available kit from Arbor Assays to quantify urinary cortisol in bonobos. We previously demonstrated that this kit was appropriate for use in bonobos, and in this preliminary data analysis we obtained a range of cortisol concentrations consistent with several other studies, as well as falling within range of an estimate of expected urinary cortisol in bonobos based on the calculation presented in the previous chapter.

It is interesting there were no sex differences. This may be because of the unique nature of bonobo social organization or may reflect that, although males and females may

respond to different stressors, both sexes experience stress. Most importantly, this analysis shows the importance of controlling for covariant factors. In the original data, there was an apparent sex difference but this was removed by controlling for body size. Many studies have reported that there was no body size effect on creatinine values, but our data indicates that a slight effect may still be present. In this analysis, the amount of variance in creatinine concentrations explained by variance in body size was only 5% in females and 16% in males, but statistical removal of this effect of body size changed the interpretation of the final results and so size can confuse the results if it is not accounted for. Alternatively, the existence of body size influence on creatinine may be reason enough to consider using alternative measures of urine concentration that are not influenced by body size, such as specific gravity, to correct urinary hormone values for hydration status.

These results also show constant and consistent individual differences in AM and PM values and in the change from AM to PM. This demonstrates that cortisol levels may be very useful measures to assess an individual's social stress levels because, despite common housing, food and other environmental conditions, there were still detectable inter-individual stress differences. Additionally, we suggest that rather than removing or altering outliers that may exist in a cortisol data set, researchers might leave these values as measured in order to detect stressful events.

Diurnal rhythm considerations were previously mainly handled by controlling time of sample collection by study design. However, study designs that only take AM samples are missing a piece of the puzzle that might help identify patterns of stress in primates. Many human studies employ some form of test for dysregulated cortisol

rhythms, and these patterns are strong predictors of negative health outcomes in humans. If primatologists are correct in their assessments of which behaviors are causing a stress response for species like bonobos, tests for dysregulated cortisol rhythms may hold similar predictive power.

Taking urinary samples from any point throughout the day has potential added value for both captive and wild primate studies. Opportunistic collection may make sample collection easier since it can happen at any time throughout the day. For study designs that seek to temporally associate behavioral data with cortisol measures, samples from multiple times throughout the day allow for a wider range of behavioral data incorporation. In the previous chapter, 16 urinary cortisol studies focusing on apes were reviewed. Only about half of those studies employed sampling throughout the day. In monkeys, it is even less common to take samples from various times.

This information is important for captive management to help zoo keepers make decisions about best practices for keeping bonobos. Since captivity is a stressor in and of itself, it is important for zoos to understand how they can minimize stress by controlling the environment and habitat of captive primates. The CZA keeps their bonobos in a unique fashion meant to mimic their natural fission-fusion style of social structure the bonobos would engage in if they were wild-living primates. Some zoos may find benefit in limiting human viewing hours or altering feeding practices in order to minimize stress within the group. In some cases, long term cortisol and behavioral tracking has been used to identify habitual aggressors, or bullies, within a group. In the CZA bonobos, one female bonobo was selected to be transferred to a zoo in Jacksonville, due in part to her constant harassing behavior toward the low-ranking male. Retrospective analysis of the

low-ranking male's cortisol values indicate he went from a pattern suggestive of dysregulated cortisol function to a more normal pattern after the aggressive female emigrated.

This study has numerous limitations, some of which can be addressed in future follow up studies. Our sample sizes for the analyses in this study were on the small side (n = 156) for three field seasons. For some individuals in the group it was more difficult to obtain urine samples than others. We used three field seasons worth of data, split by morning and afternoon sample times, in order to determine overall mean AM/PM patterns. While our data supported the hypothesis that bonobos would demonstrate patterns of diurnal cortisol production that mirror those found in humans and other primates, it is possible our method of demonstrating this pattern is inadequate. It is possible that some urine samples were collected at times that were particularly high-stress times, especially in the individuals we have deemed to have dysregulated cortisol rhythms, and that these isolated stressful incidents might be influencing our conclusion that longer term patterns of cortisol are dysregulated overall.

Future studies will analyze additional samples, as well as incorporate social and behavioral data and hormonal analyses of testosterone and oxytocin. Since there is so little known about bonobo urinary cortisol levels and how those relate to behavior, the cortisol concentrations presented in this study should help establish evidence in support of a reference range for bonobo urinary cortisol.

APPENDIX A

ZIEGLER 1995 CORTISOL EIA PROTOCOL

100 uL R4866 (Munro Antibody) were added to each well at 1:22,000 dilution and incubated for 6 hrs at room temp.

Replace antibody with 150 uL PBS/BSA solution and store at -20C.

Dilute urine samples 1:1000 with assay buffer.

50 uL sample and standard mixed with 250 uL cortisol:HRP (at 1:62,500 in PBS), add 100 uL to each well

Standards were prepared in a range of 1000 to 10 pg, n = 6

Standards, samples, and conjugate were incubated for 2 hrs at room temp in humid

chamber and then the plate is washed 5x.

100 uL ABTS: 0.5 M H_2O_2 in citrate buffer, pH = 4.0, was added to each well an incubated 1 hour at room temp in a humid chamber.

The reaction was stopped with 0.15 M HF, 6.0 mM NaOH, and 1.0 M EDTA

Plates were read at 410 nm with a plate reader.

Protocol taken from Ziegler et al., 1995

APPENDIX B

MUNRO ANTI-CORTISOL ANTIBODY CROSS REACTIVITIES

Raised against cortisol-3-CMO-BSA per American Biochemical. Cortisol standard Sigma H001 Hydroxycortisone

Steroid	**% Cross Reaction**
Cortisol	100
Prednisolone	9.9
Prednisone	6.3
Compound S	6.2
Cortisone	5.0
Corticosterone	0.7
Desoxycorticosterone	0.3
21-deoxycortisone	0.5
11-desoxycortisol	0.2
Progesterone	0.2
17a-hydroxypregnenolone	0.2
Pregneneolone	0.1
Androstenedione	0.1
Testosterone	0.1
Androsterone	0.1
Dehydroepiandrosterone	0.1
Dehydroisoandrosterone-3-sulfate	0.1
Aldosterone	0.1
Estradiol-17B	0.1
Estrone	0.1
Estriol	0.1
Spironolactone	0.1
Cholesterol	0.1

Data provided by the Clinical Endocrinology Lab, UC Davis. (R. Cotterman, personal communication, March 4, 2016).

APPENDIX C

ARBOR ASSAYS URINARY CORTISOL EIA PROTOCOL

WEB INSERT 12Feb15

ASSAY PROTOCOL

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.

2. If you are using the 1 by 8 well strip plate version of the kit, K003-H1 or -H5, determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.

   Pipet standards or samples down the plate strip columns (A to H) to ensure maximum use of the strip wells.

   The use of any wells in the whole plate versions of the kit, K003-H1W and K003-H5W will not allow use of unused parts of that plate in a later assay.

3. Pipet 50 µL of samples or standards into wells in the plate.

4. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.

5. Pipet 50 µL of Assay Buffer into wells to act as maximum binding wells (Bo or 0 pg/mL).

6. Add 25 µL of the DetectX® Cortisol Conjugate to each well using a repeater pipet.

7. Add 25 µL of the DetectX® Cortisol Antibody to each well, **except the NSB wells**, using a repeater pipet.

8. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour.

9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

10. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.

11. Incubate the plate at room temperature for 30 minutes without shaking.

12. Add 50 µL of the Stop Solution to each well, using a repeater pipet.

13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

14. Use the plate reader's built-in 4PLC software capabilities to calculate cortisol concentration for each sample.

NOTE:

If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

www.ArborAssays.com

For sample prep in this project, we diluted bonoboo urine samples 1:20 and prepared assay buffer solutions according to manufacturer instructions.

APPENDIX D

MUNRO CORTISOL PROTOCOL

CORTISOL EIA

Day 1:

1. Plate coating

- Use NUNC Maxisorb plates
- Add 50 µl antibody stock (
- 1:85, -20ºC) to 5 ml coating buffer (working dilution 1:8500)
- Add 50 µl per well of antibody solution to plate.
- **do not coat column 1** - start at A2 and go down each column (see plate map).
- Pipet all solutions in this order.
- Tap plates gently to ensure that coating solution covers bottom of well
- Label, cover with acetate plate sealer and leave overnight (no less than 12 hrs) at 4ºC.

Day 2:

2. Standards

- Standard values used are: 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg/well.
- Dilute standard working stock (20 ng/ml or 1000 pg/well) serially (2-fold) by using 200 µl stock plus 200 µl EIA buffer.

3.Samples/controls

- Dilute urine or fecal samples in dilution buffer to appropriate dilution.
- Prepare High and Low control

4. HRP

- Cortisol HRP working dilution is 1:20,000.
- Add 25 µl of HRP working stock to 5 ml EIA buffer to make the working dilution (keep this solution cool)

5. Plate washing

- Wash the plate five times with wash solution.
- Blot the plate on paper towel to remove excess wash solution.

6. Plate loading

- Pipet 50 µl of standard, control and standard per well as quickly and accurately as possible, according to plate map.
- Add 50 µl of diluted cortisol HRP (step 4) to all wells that contain standard, control, or sample. Avoid splashing.
- No more than 10 minutes should pass during this process
- Cover plates with acetate plate sealer and incubate at room temperature for exactly 1 hour.

7. Plate washing

- Wash the plate five times with wash solution.
- Blot the plate on paper towel to remove excess wash solution.
- Plates are fairly stable at this point and can be left upside down on bench top until all plates are washed (no more than 20 minutes)

- Prepare ABTS substrate immediately before use (within 20 min).
- Combine 40 µl 0.5 M H2O2, 125 µl 40 mM ABTS and 12.5 ml substrate buffer, and mix well.
- Add 100 µl ABTS substrate to all wells that contain standard, control, or sample.
- Cover with plate sealer and incubate at room temperature with shaking.
- Plate color development will vary based on age of HRP and/or Ab, but should be no greater than one hour.

9. Plate reading

- Optical density (OD) of 0 wells should read 1 or less.
- Optimal readings for 0 wells: > 0.7 to < 1 OD.
- Read at 405 nm (reference 540 nm).

<u>CORTISOL STOCK PREPARATIONS</u> – per plate

1. Antibody

- Dilute cortisol R4866 at a dilution of 1:85 by adding 24 µl of stock to 2 ml of coating buffer.
- Aliquot 300-400 µl into O-ring vials and store at -20°C.
- Store antibody stock at -80°C.

2. HRP Conjugate

- Dilute cortisol-horseradish peroxidase (HRP) 1:100 by adding 25 µl of stock to 2.475 ml EIA buffer for a working stock and store at 4°C.
- Store HRP stock at -80°C.

3. Standards

- Weigh out 1 mg cortisol (Sigma Diagnostics) and add to 1 ml ETOH for a 1 mg/ml primary stock.
- Dilute 1 mg/ml primary stock 1:100 by adding 100 µl to 10 ml ETOH for a 10 µg/ml secondary stock.
- Dilute 10 µg/ml secondary stock 1:500 by adding 100 µl to 49.9 ml of EIA Buffer for a 20 ng/ml (1000 pg/well*) working stock.
- Aliquot working stock and store all stocks at -20°C.

* a well is equal to 50 µl, the amount used in the assay.

4. Controls

- Use urine or extracted fecal samples with a high corticoid levels to make controls.
- Make a pool of high corticoid level urine or extracted feces (~20 ml).
- Serially dilute pool and run on assay.
- Find the dilutions that bind at ~70% and ~30%.
- Use the pool to make up two separate stocks for low and high controls using the dilutions that bound at 70% and 30% respectively.
- Make up enough controls to run on at least 500 assays (for this you may need more than the 20 ml used for the pool. Any species will do so long at the urine has high corticoid levels.)

Protocol provided by personal communication with UC Davis lab director (R. Cotterman, March 7, 2016). This cortisol EIA protocol was developed by Coralie Munro.

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