Hair Cortisol in Wild and Captive Primates: Environmental Effects and Behavioral Phenotypes

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Dedication

To my family, my friends, my mentors “I can no other answer make but thanks, and thanks, and ever thanks...” (Shakespeare, Twelfth Night).

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General

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Chapter 5, 6 and 7, and Appendix 2

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Appendix 1

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Abstract of Dissertation

Hair Cortisol in Wild and Captive Primates: Environmental Effects and Behavioral Phenotypes

Cortisol archived in hair represents circulating concentrations averaged over the growth period of the hair. Measurement of cortisol in hair is finding wider application in animal studies of stable behavioral trends or the effects of chronic stressors. In primatology the method has only been applied to a small number of species, and only in captivity. The goal of this dissertation was first, to assess whether patterns of hair cortisol reflect biologically meaningful variation within and between wild and captive non-human primate species, and second, to apply the method to the study of behavioral and environmental effects on stress in wild non-human primate populations. These studies also make methodological contributions to the use of hair as a tool in behavioral endocrinology.

I used enzyme-linked immunosorbernt assay techniques (ELISA) to measure cortisol extracted from hair samples from 653 individuals, representing 17 primate species. First, I demonstrated that hair cortisol concentrations reflect known phylogenetic, species, and age related patterns in circulating cortisol. Second, using a cross-sectional population level approach, I showed that populations of wild baboons (anubis – *Papio anubis*, hamadryas – *P. hamadryas* and their hybrids) in the Awash National Park, Ethiopia, differed in chronic cortisol profiles, specifically, I found that male hybrids have elevated and relatively variable hair cortisol. This may reflect the destabilizing effect of hybridization on bio-behavioral stress complexes. Hamadryas populations sampled during a drought in 1973, exhibited elevated hair cortisol consistent with evidence of severe nutritional stress in these

populations, while both mixed hamadryas and hybrid populations exhibited variation in basal cortisol profiles consistent with hybrid dysregulation. Females of these populations did not exhibit similar patterns in hair cortisol. I demonstrated that variation in hair cortisol in wild juvenile anubis baboons is marked by infant hypercortisolism and a sharp decline in cortisol with age. These results further suggest maternal effects on offspring development, in that light-for-age juvenile males exhibited elevated hair cortisol compared to heavy-for-age animals. Finally, an analysis of vervet monkey (*Chlorocebus aethiops*) hair cortisol, in samples from populations occupying habitats with varying levels of human disturbance, found that male animals in habitats with high levels of human disturbance have elevated cortisol concentrations.

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Introduction

Hormones are the inter- and intra-cellular messenger molecules that regulate bodily function in multicellular organisms (Nelson, 2005; Norman and Litwack, 1997). In vertebrates, they influence all aspects of homeostasis, growth, development, life history and behavior. Although hormonal mechanisms regulate adaptive functions, if endocrine axes are chronically activated they can result in physiological health costs with consequences for fitness (e.g. Chrousos, 2000; Cohen, 1992; de Kloet et al., 1999; Das et al., 2010; Epel, 2009; Ondrak et al., 2011; Sapolsky, 1992a, 2004, 2005). This feature of hormone systems, and their function in relation to behavior and the external environment, can be used to objectively assess the costs associated with certain behaviors, life history stages, reproductive strategies or environmental challenges (e.g. McEwen, 2000; Stearns, 1989; Wingfield et al., 1990). Such perspectives provided by this approach are used to understand fitness trade-offs and model the evolution of life-history and behavioral traits (e.g. Lochmiller and Deerenberg, 2000; Taylor et al., 2000; Zera and Harshman, 2001).

This dissertation focuses on the principal hormonal axis involved in the physiological stress response, the hypothalamic-pituitary-adrenal (HPA) axis (Boron and Boulpaep, 2003; Nelson, 2005). The activation of this axis is typically measured by quantification of corticosteroids, specifically glucocorticoids (GCs; Sapolsky, 1992a, 2004; 2005; Terio et al., 2004; Whitten et al., 1998a; Yamada et al., 2007). These hormones are produced by the adrenal gland (Hiller-Sturmhöfel and Bartke, 1998; Lehoux et al., 1987) as part of the body's response to any challenge to homeostasis (i.e. stress response – Selye, 1936). They are of particular interest in behavioral endocrinology because, although adaptive in

the short term, if chronically maintained at high concentrations they can result in severe health consequences and impact fitness (e.g. Chrousos, 2000; de Kloet et al., 1999; Sapolsky, 1992a, 2004, 2005). Biological and behavioral interactions with this endocrine axis are likely key targets of selection.

Steroid hormones (including cortisol) are usually quantified in physiological substrates such as blood, saliva, feces or urine (e.g. Anestis, 2005; Rehbinder and Hau, 2006). However, each of these substrates carries their own methodological and logistical limitations depending on the research goals (e.g. whether studying physiological reactivity or stable behavioral trends) or research conditions (e.g. captive studies versus field studies). Although relatively stable, cortisol may deteriorate rapidly in these biological media (e.g. Gard and Hanson, 2005; Stroud et al., 2007; Miki and Sudo, 1998) as blood and excreta themselves are prone to decomposition and microbial activity. These substrates therefore need to be treated, frozen, and desiccated or cortisol must be extracted soon after collection in order to preserve biologically meaningful concentrations and variation (Dabbs, 1991; Galama et al., 2004; Groschl et al., 2001; Hankinson et al., 1989). Additionally, blood, saliva, urine and feces record variation in cortisol concentrations over the short term (minutes, hours to days, e.g. urine and feces – Bahr et al., 2000), which are acutely affected by circadian variation (Turtin and Deegan 1974) and recent experiences of the individual. When researchers are interested in measuring basal or chronic cortisol concentrations then multiple samples of blood, saliva, urine or feces need to be collected in order to determine time-averaged cortisol concentrations. Hair has recently been recognized as an archive of cortisol concentrations averaged over several weeks or months during which hair grows (e.g. macaques -

Davenport et al., 2006; 2008; various primates - Fourie and Bernstein, 2011a; humans - Kirschbaum et al., 2009; Webb et al., 2010). A single hair sample can therefore be used to determine the basal or chronic cortisol concentrations characteristic of an individual, representative of a longer period of time than more traditionally collected substrates can offer (Davenport et al., 2006).

Hair cortisol analysis is increasingly being used to characterize cortisol phenotypes in animals and humans which have proven to be useful in the study of the endocrine correlates of stable behavioral tendencies (e.g. personality/temperament – Clara et al., 2008; Laudenslager et al., 2011), cognitive performance (e.g. Dettmer et al., 2009), chronic disease (e.g. Thomson et al., 2011), pain (e.g. van Uum et al., 2008), psychological trauma (e.g. Steudte et al., 2011a) or as a clinical indicator of disease risk (e.g. Pereg et al., 2011). Variation in hair cortisol specifically reflects biological variation in chronic circulating cortisol in relation to stable or chronic behavioral, physical or environmental factors rather than short term stressors which can be more accurately studied using traditionally collected substrates.

Cortisol is relatively stable within the hair matrix, facilitating easy storage and transport of samples in the field (e.g. Bechshoft et al. 2011; Fourie and Bernstein, 2011a; Chapter 5 and 6). However, the quantification of cortisol concentrations from hair using enzyme-lined immunoassay (ELISA) techniques and its application to field based animal studies is relatively new and several methodological and technical questions remain to be resolved with respect to the use of this methodology for the quantification of cortisol: 1) in species that have not previously been examined, and 2) those samples collected under field conditions. More specifically, assay and biological validation of this technique is

lacking for most non-human primate species. The universality of the application of this technique and its ability to recover biologically relevant variation in cortisol concentrations in non-human primates, therefore remain unclear. Due to a paucity in hair growth data for non-human primates, little is known about the potential sources of between sample variation, the length of time over which cortisol accumulates in a hair sample and the temporal stability of cortisol archived in hair. This dissertation examines some of these issues in species for which there exists no information.

This dissertation examines variation in cortisol concentrations among several non-human primate species (specifically *Papio* sp. baboons and their hybrids, and *Chlorocebus aethiops*, vervets) using a cross-sectional population level approach to assess relationships between species-specific behavioral tendencies, hybridity, sex, development, environmental variables and physiological stress. I examined variation in basal cortisol concentrations among populations of baboons (*Papio anubis* and *P. hamadryas*) and their natural hybrids in the Awash National Park, Ethiopia. These *Papio* species are behaviorally strongly contrasted (Jolly and Phillips-Conroy, 2003; Jolly et al., 2008; Kaplan et al., 2001). Anubis baboons follow the more generalized savannah baboon social and behavioral program (Bercovitch, 1987; Bercovitch and Strum, 1993; Charpentier et al., 2008; Easley et al., 1989; Johnson, 1987; Packer, 1979a; Packer, 1995; Smuts and Watanabe, 1990; Strum, 1982) whereas hamadryas baboons exhibit distinct behavioral patterns and a social organization characterized by hierarchically organized social units (Abegglen 1984; Colmenares et al., 2006; Kummer, 1968a, 1984, 1995; Schreier and Swedell, 2009). These differences have been interpreted in terms of differences in male reproductive evolution related to differences in the evolutionary

ecology and environments of these populations (Dunbar, 1988; Henzi and Barrett, 2003, 2005; Kummer, 1990, 1995). I explored whether these wild populations exhibited differences in physiological stress levels, evidence of which might suggest differential costs associated with divergent socioreproductive strategies. Furthermore, I considered sources of individual variation in basal cortisol concentrations, such as relative body mass, and habitat stress. This investigation of hair cortisol-behavior relationships was then extended to an exploration of anthropogenic variables and stress in vervet monkeys from differentially disturbed habitats in South Africa.

Following this brief introduction, the first chapter reviews HPA axis functioning, and the second reviews baboon social behavior, and the third reviews hair hormone analysis. These review chapters provide the necessary background within which subsequent chapters are contextualized, and are followed by five chapters organized as manuscripts in which 1) the potential of hair cortisol analysis to recover biologically relevant variation in cortisol concentrations is examined (Chapter 4), and 2) hypotheses regarding species, social group, hybrid and individual variation in basal cortisol concentrations are examined in baboons and vervets (Chapters 5-8).

Chapter Goals and Hypotheses

Review Chapters

Chapter 1: Stress and the Hypothalamic-Pituitary-Adrenal Axis.

This chapter briefly describes the history of physiological stress as a concept and reviews the functioning of the various components of the endocrine stress axes. The role of glucocorticoids in the stress response is reviewed and the pathological consequences of sustained elevated levels of these hormones are discussed. Finally, the basis of psychosocial stress and stress coping mechanisms are reviewed.

Chapter 2: Baboon Social Behavior.

This chapter gives a general review of the *Papio* baboons, briefly discussing specific and sub-specific classification of the genus, and intra- and inter-specific variation in behavior and the evolutionary scenario thought to be the basis of species-specific socio-behavioral profiles. The chapter then reviews the social and behavioral dynamics of anubis, hamadryas and hybrid baboons with emphasis on the populations in the Awash National Park, Ethiopia.

Chapter 3: Hair Hormone Analysis.

This chapter reviews the history of hair hormone analysis from its earliest days to its present applications. Mechanisms of steroid sequestration into the hair shaft, factors affecting sequestration, patterns of hair growth, the stability of steroids in hair and the importance of these issues for hair steroid analysis are reviewed. Methods of extraction and quantification of cortisol in hair are discussed. Finally, the advantages of hair cortisol analysis and its novel application and potential is reviewed and discussed.

Biological Validation Chapter

Chapter 4: Hair cortisol levels track phylogenetic and age related differences in hypothalamic-pituitary-adrenal (HPA) axis activity in non-human primates.

In this chapter I tested the hypothesis that if cortisol archived in hair reflects biologically relevant variation in circulating cortisol concentrations then hair cortisol concentrations should show the same patterns of biological variation with age and species/phylogeny as has been demonstrated with cortisol concentrations measured previously in serum, saliva, urine or feces. Three predictions were examined.

1) Infant and young juvenile Guinea baboon (*Papio papio*) and vervet (*Chlorocebus aethiops*) monkey hair cortisol concentrations reflect hypercortisolism relative older age classes.
2) Hair cortisol concentrations decline rapidly and asymptotically with age.
3) Hair cortisol concentrations reflect the well known phenomenon of platyrrhine hypercortisolism compared to catarrhine and strepsirrhine primates.

Baboon and Vervet Chapters

Chapter 5: Hair cortisol concentrations in populations of two socially divergent baboon species (*Papio anubis*, *P. hamadryas*) and a population of their natural hybrids: evidence of hybrid hypercortisolism.

In this chapter I assessed variation in hair cortisol concentrations among three baboon populations (anubis, hamadryas and hybrid baboons) in the Awash National Park, Ethiopia by testing predictions regarding possible sources of variation. Two sub-hypothesis, regarding the source of male inter-specific variation in hair cortisol concentrations were examined.

1) The *Social Stress Hypothesis* suggests that inter-specific differences in basal cortisol concentrations result from species differences in male sociosexual behavioral styles which result in differential social stress loads. Alternatively, as an extension of H_1, I suggest that while the two baboon species may differ in the levels of social conflict and uncertainty, each has evolved bio-behavioral mechanisms for coping with social stressors, lowering their effect on chronic cortisol concentrations, but that these mechanisms are disrupted by the biological and social effects of hybridization.
2) The *Ecological Stress Hypothesis* proposes that inter-specific differences in basal cortisol concentrations are mainly a product of environmental factors, especially shortage of food during periods of low habitat productivity. This hypothesis

predicts that cortisol concentrations within and between populations will be negatively associated with rainfall.

Chapter 6: Hybridity and sex effects on inter-specific variation in hair cortisol concentrations in populations of wild baboons in the Awash National Park, Ethiopia

In this chapter I extended the paradigms put forward in Chapter 5 to an examination of additional populations with different histories of admixture and hybridization, and to females of these populations. The samples used in this examination of inter-specific differences in basal cortisol concentrations were collected during a severe drought, were older than the samples that were analyzed in Chapter 5, and had been sterilized with an ethanol rinse during importation. Therefore, before evaluating inter-specific differences and association of hair cortisol, the use of these samples was validated by examining known age related changes in hair cortisol in a juvenile subsample and comparing the observed patterns to those obtained from more recent, untreated, samples of the same populations. Following this, three hypotheses concerning inter-specific hair cortisol variation were tested.

1) I hypothesized that because hamadryas baboons were observed to exhibit the most severe symptoms of nutritional stress that anubis and hybrid baboons were relatively less affected by the drought. This suggested that hamadryas baboons would show elevated basal cortisol concentrations compared to anubis and hybrid populations.

2) Alternatively, I hypothesized that although the drought would have an effect on physiological stress that inter-specific differences in basal cortisol profiles would instead reflect the species-specific patterns found previously. Additionally, because the hamadryas population analyzed here has experienced significant asymmetric introgression from anubis baboons, I predicted that this population would exhibit hybrid-like variation in hair cortisol.
3) I further hypothesized that females are more sensitive to ecological stress compared to males, and predicted that females would show elevated basal cortisol concentrations in response to the drought compared to male conspecifics.

Chapter 7: Variation in basal cortisol during the juvenile period in wild anubis baboons.

In this chapter I examined sources of variation in basal cortisol concentrations among juvenile male and female anubis baboons. Ontogenetic variation in hair cortisol concentrations were described for both males and females of this species. The hypothesis that variation in body mass relative to age predicts basal cortisol concentrations was tested, while controlling for the effects of rainfall, since low relative body mass in juvenile baboons is associated with the stress effects of low maternal rank on offspring. Therefore it was predicted that if low relative body mass reflected socio-ecological stress than juveniles with low relative body mass would also exhibit elevated basal cortisol.

Chapter 8: Variation in vervet (*Chlorocebus aethiops*) hair cortisol concentrations reflect ecological disturbance by humans.

In this chapter I extended the application of hair cortisol analysis to wild populations of vervet monkeys in order to assess the impact of human activity on chronic cortisol concentrations and to assess the potential of hair cortisol analysis as a conservation biology tool. Two sub-hypothesis were examined.

1) The *Behavioral Stress Hypothesis* suggests that human impacts on vervet ecology result in high stress loads for vervets. Populations experiencing high levels of human disturbance and interaction were predicted to exhibit elevated basal cortisol concentrations.
2) Alternatively it was hypothesized that the negative impacts of human activity are mitigated by the positive impacts of living in proximity to humans (i.e. *Energetic Stress Hypothesis*). I suggest that vervets benefit from the proximity of humans through increased dietary quality resulting in lower energetic stress loads. This hypothesis predicted that vervet populations characterized by lower levels of human disturbance and reduced access to human foods would exhibit higher stress loads, reflected in basal cortisol levels, compared to animals with regular access to human food.

Chapter 1: Stress and the Hypothalamic-Pituitary-Adrenal Axis

General Adaptation Syndrome

The word "stress" was first used in biology in the 1930s to describe the concept of challenges to homeostasis, which was still poorly understood at the time (e.g. Selye 1936). Hans Selye developed the idea of stress as a biological condition based on ideas put forward by Claude Bernard (1878) and Walter Cannon (1915, 1926, 1929a). Bernard (1878) and later Cannon (1926) proposed that organisms strive to maintain constant internal states. Cannon coined the term "homeostasis" which is now widely used to describe the maintenance of constant internal conditions in organisms. Cannon also introduced the idea of challenges to the physical body and mechanisms of responding to such challenges (Cannon, 1915; 1929b). The "Fight or Flight Response" was a term coined by Cannon (1915, 1929b) to describe the integrated physiological and emotional response to a threat. Cannon observed that interactions between the sympathetic nervous system and the adrenal system were key in affecting changes in blood sugar availability, blood flow and blood clotting which prepared and facilitated the "violent display of energy" necessary to deal with an eminent threat. By the 1950s, thanks to the efforts of Selye (1950, 1956) the concept of biological stress had found wider usage and had generated considerable interest.

A stressor can be defined as any challenge, real or imagined, to homeostasis; stress is the condition brought about by a stressor. As a medical student, Selye noticed that people suffering from different diseases often exhibited similar symptoms such as intestinal

disturbances, loss of appetite, fever and inflammation (Selye, 1956). He performed a series of experiments on rats, which were subjected to various stressors, such as excessive exercise, cold, surgical shock, and noxious substances (e.g. Selye, 1936). Regardless of the nature of the stressor, rats exhibited the same basic biological changes as a result of the stressor. Based on these observations Selye (1936) proposed that organisms possessed a stereotypic, non-specific response to stress which he termed the "General Adaptation Syndrome" (GAS – Selye 1936). Stress is adaptive when it effectively helps an organism cope with a stressor by enhancing certain physical and mental functions ("eustress" – Selye, 1975) but when the condition persists, the stress response exhausts the body's resources and becomes maladaptive ("distress" – Seyle, 1975). "Distress" as Selye termed it, or chronic stress, can cause a host of negative physical and mental pathologies.

It is now known that the non-specific stereotypical stress response proposed by Selye (1936) and the simple polarized behavioral stress response model of Cannon (1915, 1929b - Fight or Flight") are over-simplifications and that both physiological and behavioral responses to stress are more complex and subtle than were previously thought. However these early models of stress were fundamental as founding principals in stress research and in generating great interest in the field. Subsequent decades of research have resulted in rapid progress in understanding the physiological basis of stress and its consequences. The next section describes the physiological basis of the stereotypical stress response.

Hypothalamic-Pituitary-Adrenal Axis

The principal endocrine axis implicated in the biological response to real or perceived challenges to homeostasis is the Hypothalamic-Pituitary-Adrenal (HPA) axis (Boron and Boulpaep, 2003, see Figure 1.1). The hypothalamus connects the nervous system to the endocrine system through the pituitary gland (Dampney, 2011; Hiller-Sturmhöfel and Bartke, 1998; Hodson et al., 2010) and the hypothalamus receives input from various regions of the brain (Chrousos, 2009; Dampney, 2011; Marano et al., 2011; Papadimitriou and Priftis, 2009), this includes areas of the brainstem, which controls, and processes information relating the autonomic (e.g. Dampney, 2011; Kalsbeek et al., 2010, Saper et al., 2005) and central nervous system (Chrousos, 2009; Dampney, 2011; Marano et al., 2011). The hypothalamus is also richly connected to parts of the limbic system, which controls and processes information relating to functions such as emotion (Etkin et al., 2011; Papez, 1937), behavior, memory, and smell (e.g. Hiller-Sturmhöfel and Bartke, 1998; Saper, 2008). Important brain regions, which communicate with the hypothalamus, include the amygdala and hippocampus. Impulses are sent from these brain regions to the hypothalamus which activates the sympathetic and parasympathetic nervous system and sets in motion hormonal cascades which target specific organs and organ systems (Boron and Boulpaep, 2003; Dampney, 2011, Saper, 2008). The HPA axis is also sometimes referred to the Limbic-Hypothalamic-Pituitary-Adrenal (LHPA) axis, emphasizing the importance of the limbic forebrain in the functioning of this endocrine axis.

The initial and immediate neuroendocrine response to a stressor is mediated by the hypothalamus through the activation of the sympathetic nervous system (Boron and Boulpaep, 2003; Dampney, 2011; Saper, 2008). Impulses generated by the hypothalamus activate the sympathetic nervous system, which acts via the splanchnic nerves (Bornstein

et al., 1990; Llewellyn-Smith and Anthony, 2011) to stimulate the adrenal medulla to secrete epinephrine and norepinephrine (e.g. Engeland, 1998; Tsuchimochi et al., 2010). Epinephrine and norepinephrine are catecholamines which act as both hormones (transported to target tissues via blood) and neurotransmitters (transmitting signals from neurons to target cells; Boron and Boulpaep, 2003). Norepinephrine is also secreted by the noradrenergic neurons of the locus coeruleus in the brainstem, where it acts exclusively as a neurotransmitter (e.g. Berridge and Waterhouse, 2003; Sara, 2009). The action of epinephrine and norepinephrine is therefore very rapid and constitutes the body's first phase, immediate response to a challenge (Sapolsky et al., 2000). Epinephrine and norepinephrine affect almost all tissues in the body by acting on adrenergic and noradrenergic receptors. The nature of the effect of epinephrine and norepinephrine on cells depend on the type and tissue specific expression of these receptors.

Epinephrine causes vasoconstriction (e.g. Floraz et al., 1988; Thomas, 2011; Vedung et al., 2010; Widegren et al., 2010), increases heart rate (e.g. Brown et al., 1979; Fuchs, 1938; Harris et al., 1967; Jensen et al., 2011; Jouven et al., 2009), and can have opposite effects on muscle tone, either stimulation contraction or relaxation of muscle fibers depending on adrenergic receptor type expression (e.g. Kohjitani and Obara, 2002; Litvinov et al., 2009; Nakada et al., 2007; Marsden and Meadows, 1970; Wuest et al., 2009). In addition epinephrine also increases the availability of energy by rapidly limiting the secretion of insulin by the pancreas (e.g. Black et al., 1982; Peterhoff et al., 2003; Zhang et al., 2009), stimulating glycolysis in muscle tissue (e.g. James et al., 1999; Levy et al., 2008; Raz et al, 1991; Sarpeshkar and Bentley, 2010) and glycogenolysis in muscle and liver tissue along with several other metabolic effects to increase immediate energy

availability (e.g. Bentham et al., 1990; Cherrington et al., 1984; Dufour et al., 2009; Gjedsted et al., 2011; Matthews et al., 1990). Norepinephrine also affects blood flow to muscles (e.g. Hoy et al., 2009; Pagan et al., 2009), heart rate (e.g. De Backer et al., 2010) and energy availability (e.g. Hutchinson et al., 2011) as well as influencing amygdala function by affecting mood, attentiveness, emotion and memory formation and consolidation (Berridge and Waterhouse, 2003; e.g. Bouret and Sara, 2005; Gibbs, 2008; Groch et al., 2011).

One of the main targets of hormones secreted by the hypothalamus is the pituitary gland (see Figure 1.1). Neurons in the supraoptic nucleus and paraventricular nucleus of the hypothalamus synthesize vasopressin and corticotropin-releasing-hormone (CRH) which are released in response to a stressor and act directly on the pituitary gland (Boron and Boulpaep, 2003; Dampney, 2011; Hiller-Sturmhöfel and Bartke, 1998). CRH and vasopressin act together on the anterior and posterior pituitary, respectively, to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Boron and Boulpaep, 2003: Hiller-Sturmhöfel and Bartke, 1998).

ACTH is secreted into the blood stream and carried to the adrenal glands where it acts on adrenocortical cells by increasing the bioavailability of cholesterol (the basic molecule from which all steroids are synthesized: Lehoux et al., 1987; Stocco and Clark, 1996), transport of cholesterol to mitochondria (e.g. Brownie et al, 1972; Jefcoate et al., 1987) and activating some of the enzymes involved in the synthesis of glucocorticoids (GCs) by the cells of the middle cortical layer (*zona fasciculata*) of the adrenal cortex (e.g. Lehoux et al., 1987). ACTH therefore stimulates the rapid production of GCs. The principal GC secreted as part of stress response by the adrenal cortex in humans, non-human primates,

and many other mammals is cortisol (Hiller-Sturmhöfel and Bartke, 1998; Sapolsky, 2000; 2004). In most non-mammalian vertebrates and rodents, corticosterone is the principal stress hormone secreted by the adrenal cortex (e.g. amphibians – Moore and Jessop, 2003; birds – Bokony et al., 2009; chondrichthyean fishes - Wendelaar-Bonga, 1993; rats - Katyare et al., 2003; reptiles – French et al., 2008). This, second phase "hormonal" component of the HPA axis stress response, although only manifesting in biological effects after the first phase "neurotransmitter" component of the HPA axis stress response, maintains many of the effects of the initial immediate stress response mediated by the neurotransmitters, epinephrine, and norepinephrine (Sapolsky et al., 2000).

GCs play a central role in energy metabolism. This effect is mainly achieved through acting on the liver (Thompson and Lippman, 1974) to regulate gluconeogenesis (synthesis of glucose from non-carbohydrate carbon substrates: e.g. Exton et al., 1972; Khani and Tayek, 2001) by acting on the expression and sensitivity of enzymes involved in this process (e.g. Exton, 1979; Suebert et al., 1968), and extra-hepatically by regulating the availability of substrates for gluconeogenesis. In particular GCs increase the plasma availability of amino acids (e.g. Asterita, 1985; van der Boon et al., 1991) by inhibiting protein synthesis in peripheral tissues, facilitating protein breakdown in muscle tissue (e.g. Di Pasquale, 2008; Rannels et al., 1978; Shoji and Pennington, 1977; Steele, 1975; Tomas et al., 1979; Yoshioka et al., 2005) and down-regulating the transport of amino acids to skeletal muscle (e.g. Krause-Friedman, 1984). Fatty acids are also released as a result of lipolysis of adipose tissue under the influence of GCs (e.g. Djurhuus et al., 2002; Xu et al., 2009), and these are also used as substrates for gluconeogenesis. GCs also

inhibit the uptake of glucose into muscle and adipose tissue (e.g. Ngo et al., 2009), which enhances and maintains blood glucose levels, counteracting the hypoglycemic effects of insulin and thereby sustaining the initial effects of catecholamines on energy availability necessary to sustain physical reactions to stress (Munck and Faray-Fejes-Toth, 1995).

Glucocorticoids generally affect the immune system by inhibiting the synthesis of cytokines (viz. immunomodulating agents which act as inter-cellular signaling molecules: e.g. Clerici et al., 1997; Kirwan et al., 2010; Kunz et al., 1994; Lekander et al., 2009; Munck and Faray-Fejes-Toth, 1995; Radomski et al., 1990; Wiegers and Reul, 1998; Wu et al., 1995) as well as inhibiting their secretion and functional efficacy, thereby down-regulating immune function and reducing inflammatory responses (e.g. Kirwan et al., 2010). This reaction, although stereotypical, is not necessarily universal and indiscriminate, but can act locally (e.g. Gilmour et al., 2006; Hardy et al., 2008; Sandeep and Walker, 2001). The immunosuppressive and anti-inflammatory effects of cortisol can affect specific target sites where inflammation is detected (and communicated to the hypothalamus via vagal sensory nerve fibers) and can be terminated when appropriate (e.g. Rook et al, 2000). Additionally, it is thought that suppression of certain immune components during a stress response by glucocorticoids protects the body from potential autoimmune reactions which may be precipitated by a universal indiscriminate (unregulated) immune response, and can enhance the efficacy of the body to respond immunologically to potential infectious insults (Sapolsky et al., 2000).

Blood pressure, heart rate and cardiac output are rapidly modified by the catecholamines in response to a stressor; CRH and ACTH are also known to have direct effects on cardiac function (e.g. Brown and Fisher, 1983, 1986; Fisher et al., 1982; Kang

et al., 2011; Lorenz et al., 2008; Vale et al., 1993) and are implicated in the initial modulation of cardiac function in response to stress. GCs have similar effects on cardiovascular function (e.g. Pirpiris et al., 1993; Sambhi et al., 1965). However, rather than being the main effectors of changes in cardiovascular function, GCs enhance and prolong the effects of catecholamines on cardiovascular (Cooper, 2009; Fowler and Cleghorn, 1942; Fritz and Levine, 1952; Grunfeld and Eloy, 1987; Ramey et al., 1951; Sapolsky and Share, 1994) and respiratory tissues (Barnes, 2012; Chong et al., 1997; Scherrer et al., 1997) by inhibiting catecholamine reuptake (Dailely and Westfall, 1978; Gibson, 1981; Hill et al., 2011), enhancing tissue sensitivity to catecholamines (Barnes, 2012; Collins, 1988; Cooper, 2009; Sakaue and Hoffman, 1991), and inducing certain enzymes involved in catecholamine synthesis (Barnes, 2012; Kennedy and Ziegler, 1991; Wurtman and Axelrod, 1966). Up-regulated cardiovascular function is adaptive in facilitating and sustaining physical muscoskeletal reactions to stress.

Biological reproductive processes are disrupted as part of the stress response during the initial rapid phase of the neurophysiological stress cascade due to the antagonistic effect of CRH on gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Breen et al., 2008; Dudas and Merchenthaler, 2002; Gambacciani et al., 1986; Kalantaridou et al., 2010; Michopoulos et al., 2009; Ono et al., 1984; Rivier and Vale, 1984; Rivier et al., 1986; Sirinathsinghji et al., 1983; see Figure 1.2). CRH is also implicated in the activation of the autonomic nervous system (Arlet et al., 2003; Nijsen et al., 2000), through which it may directly affect reproductive function (e.g. Saenz et al., 1988). LH is essential to normal biological sexual functioning in males and females. GCs have broadly similar effects on reproductive function. GCs

down regulate GnRH release by the hypothalamus (e.g. Belhadj et al., 1989; Ciechanowska et al., 2010; Dubey and Plant, 1985; Oakley et al., 2009), and consequently inhibit the GnRH mediated release of LH and FSH from the pituitary (Baldwin et al., 1991; Breen and Karsch, 2004; Man et al., 1982; Pierce et al., 2009; Ringstrom et al., 1991; Sakura et al., 1975; Sapolsky, 1985) as well as reduce gonadal sensitivity to LH (e.g. Cumming et al., 1983; Hayasi and Moberg, 1990; Orr and Mann, 1992; Sapolsky, 1985; Whirledge and Cidlowski, 2010). Biological reproductive functions are energetically expensive, especially in females; temporary disruption of these processes focuses energetic resources for use in functions more relevant for immediate survival (Wasser and Barash, 1983).

During the stress response, catecholamines in concert with glucocorticoids appear to enhance memory formation and consolidation, and learning primarily through action on the basolateral amygdala and hippocampus, brain centers associated with memory and learning (Cahill et al., 1994; McGuach, 1989; Roozendaal et al., 2006; 2009; Sapolsky, 2003; Sapolsky et al., 2000; Schwabe et al., 2011). A slight elevation in GC concentrations from basal levels (e.g. in response to a mild stressor) appears to enhance cognitive functions whereas more pronounced elevations in GC concentrations appear to decrease hippocampal excitability, impairing cognitive capacity relating to the hippocampus, especially memory retrieval (Diamond et al., 1992; Joels, 2001; Joels and de Kloet, 1992; McEwen, 2007; Oei et al., 2007; Pavlides et al., 1994; Sapolsky, 2003; Vaher et al., 1994; Wolf, 2009). In the case of severe or chronic stressors where hippocampal activity is impaired under the action of GCs it appears that GCs may cause hypertrophy of neurons in the amygdala which may positively affect memory formation

(e.g. Vyas et al., 2003) but may also result in increased expression of fear and anxiety related behaviors (McEwen, 2007).

The effects and concentrations of glucocorticoids, specifically cortisol, are in part mediated by cortisol binding globulin (CBG, e.g. Dhillo et al., 2002). Low binding capacity of CBG and tissue GC receptor insensitivity may result in elevated free, bio-available cortisol concentrations. In such cases high cortisol concentrations may not be indicative of stress, but reflect normal bio-available concentrations necessary in order to affect tissues. Variation in expression of receptor sensitivity and CBG binding capacity is thought to be the basis of hypercortisolism among New World primates (e.g. Pugeat et al. 1984). The biological effects of cortisol may also be suppressed by increased CBG secretion. CBG binds to cortisol making it biologically inactive (e.g. Dhillo et al., 2002).

Stress, Glucocorticoids and Health

Allostatic Load:

As described above, GCs are critical to sustaining a number of adaptive metabolic, immune and cardiovascular responses to challenges to homeostasis. The role of GCs in mediating changes to these systems is not uniform and can be permissive (i.e. facilitating the action of certain stress mechanisms: e.g. cardiovascular stress response), suppressive (down-regulating certain stress responses to avoid them being damaging to the organism; e.g. immune function), stimulatory (i.e. directly elicit the stress response mechanism: e.g. energy metabolism) or preparative (i.e. prepares or primes certain mechanisms for a stress

response; e.g. metabolic function – Sapolsky et al., 2000). This shows that the role of GCs in the physiological stress response is varied and dynamic, and affects multiple systems in the body. The stress response, rather than simply maintaining a steady physiological and metabolic state, modulates internal processes, up-regulating some, and down-regulating others within an acceptable operating range which can sustain the organism. This state of maintaining stability through change is termed "allostasis" (Sterling and Eyer, 1988).

Frequent changes to metabolic and physiological processes in order to maintain homeostasis, although not great in magnitude (i.e. within the acceptable operating range), imply heightened activation of certain endocrine axes, and constant physiological action on organs and organ systems will eventually lead to "wear and tear" of those endocrine mechanisms and organs. The frequency of change needed in order to maintain stability is termed the "allostatic load" (McEwen and Stellar, 1993) and is used to describe the amount of "wear and tear" on internal systems resulting from the pursuit of homeostasis. Allostatic load is a useful concept used for understanding and modeling the pathological consequences of frequent or sustained challenges to homeostasis (e.g. McEwen, 2000). This model of the effects of stress on health does not suppose failure of the stress response to maintain homeostasis and necessarily the dysregulation of adaptive physiological processes (e.g. chronic elevation of stress hormones). On the contrary, this model assumes the maintenance of homeostasis, but that in doing so the body's stress response mechanisms are frequently activated. For instance, using an example from McEwen and Steller (1993), an individual may be able to cope successfully with the stresses associated with the working week (i.e. successfully maintain homeostasis during the week in the face of constant challenge). However, the working week constitutes a

period of high stress and the weekend constitutes a period of relatively low stress. The body takes time to return to a basal state and insults to the immune system may only manifest during the low stress period. The repeated cyclical nature of the work week and weekend are themselves elements of overall allostatic load (i.e. changing states) and are associated with risks to cardiovascular function in the long term (e.g. Bodis et al., 2010; Friedman et al., 1958; McEniery et al., 2010).

GC Effects on Physiological Health and Memory:

As discussed, GCs are among the principal hormones secreted as part of the body's stress response. Chronic stress and high allostatic load are associated with elevated GC concentrations. Chronically or frequently elevated GC concentrations can result in damage to internal systems resulting in negative physical and cognitive health consequences. GCs promote insulin secretion (Clore et al., 2009; Polonsky and O'Meara, 1995; Rafacho et al., 2010), and although designed to promote energy availability by counteracting the effect of insulin (i.e. a permissive function of GCs), increased insulin secretion is designed to prepare the body for a future insult (i.e. a preparative function of GCs – Sapolsky et al., 2000). However if elevated GC concentrations are chronically maintained, then the combined effect of these hormones promotes body fat deposition (e.g. Epel, 2009; Ondrak et al., 2011; Purnell et al., 2008; Vicinnati et al., 2009; Warne, 2009), muscle wastage (e.g. Hu et al., 2009; Kayali et al., 1987; Sapolsky et al., 2000) and the formation of atherosclerotic plaques (e.g. Brindley and Rolland 1989; De Santis et al., 2011; Fantidis, 2010; Juster et al., 2009; Margeirsdottir et al., 2010; Sapolsky et al.,

2000). Chronic stress and the effects of elevated GC concentrations on energy metabolism are therefore implicated in the exacerbation and onset of diabetes (Byung-Wan et al., 2010; Cox and Gonder-Frederick, 1991; Haggiof et al., 1991; Heraclides et al., 2009; Juster et al., 2009; Surwit et al., 1991) and heart disease (e.g. Chandola et al., 2008; Dimsdale, 2008; Juster et al., 2009; Kivimaki et al., 2006; Stoney et al., 2007).

Chronic elevation of GCs promotes sustained high blood pressure, vasoconstriction, and elevated heart rate, among other cardiovascular effects (e.g. Dodt et al., 2009; Ong and Whitworth, 2011; Panoulas et al., 2008; Sapolsky et al., 2000; Sapolsky, 2005). Chronic or frequent activation of these cardiovascular stress responses will result in hypertension and damage to coronary arteries resulting in myocardial infarction (e.g. Brindley and Rolland 1989, Chi and Kloner, 2003; Nijm and Jonasson, 2009; Pereg et al., 2011).

Similarly, chronic or frequent elevation of GCs can lead to immunosuppression increasing susceptibility to infection (e.g. Cohen, 1992; Das et al., 2010; Dhabhar, 2006; Elftman et al., 2010; Han et al., 2011; Jamieson et al., 2010; Sheridan et al., 1994). Chronically elevated concentrations of GCs can also have significant negative effects on cognitive mechanisms and behavior (e.g. Brown, 2009; Damsted et al., 2011; Lupien et al., 2009; Marin et al., 2011; Sapolsky, 1999). GCs decrease neural plasticity (e.g. Diamond and Park, 2000; Foy et al., 1987; Krugers et al., 2006; Sapolsky, 2010; Yaniv et al., 2008), and neural excitability in the hippocampus (e.g. Abraham et al., 1996; Damsted et al., 2011; Joels and de Kloet, 1992; Beck et al., 1994). Prolonged exposure to high GC concentrations results in atrophy of neural processes (e.g. retraction of dentritic processes – e.g. Damsted et al., 2011; Woolley et al., 1990; Sapolsky, 2010; Sunanda et al., 1997),

which, if sustained, will eventually result in apoptotic cell death (i.e. programmed cell death) in the hippocampus (e.g. Brown et al., 2004; Damsted et al., 2011; Sapolsky, 1992a; 1999; 2003; 2010; Sapolsky et al., 1985) leading to compromised memory function.

Stress, and by extension GCs, can result in significant detrimental health effects if high allostatic loads or chronic stress is maintained. Therefore, stress and the control of the stress response is an important target of selection, shaping both biological and behavioral adaptations.

Stressors

Lack of Control and Uncertainty:

Stressors can be generally divided into two main groups, physical stressors and psychological stressors. Physical stressors include any stressor which directly affects or threatens to affect the physical body, such as temperature, hunger, thirst, pain, infection or being chased by a predator. Psychological stressors result from the interaction between the physical environment and internal mental processes. Psychological stressors need not be real and can be imagined but still elicit the same physiological response as a physical stressor. For instance, a fear of sharks while swimming in the ocean will initiate the same physiological stress response as would be initiated if one was really attacked by a shark. Furthermore the nature and magnitude of responses to stress can be greatly influenced by both genetic (e.g. DeRijk, 2009; Fairbanks et al., 2011; Gentsch et al., 1988; Hage et al.,

2009; Norman et al., 2012) and developmental (e.g. Glover et al., 2010; Hage et al., 2009; Meaney et al., 1994; Tyrka et al., 2009) idiosyncrasies of the individual.

The two most important components of psychological stress are lack of control and lack of predictability (Basset and Buchanan-Smith, 2007; Bouchard et al., 2010; Foa et al., 1992; Koolhaas et al., 2011; Meier et al., 2008; Sapolsky, 1994; 2005; 2011). Various studies in a number of animals have demonstrated the effect of control (or the lack thereof) on stress and GC secretion (e.g. dogs – Des-Beech et al., 1983, fish – Galhardo and Oliveira, 2009; humans – Brier et al., 1987; Diener et al., 2009; Meier et al., 2008, non-human primates – Hanson et al., 1976; Sapolsky, 2005; rats – Amat et al., 2005; Coover et al., 1973; Weis 1971a, b, c, 1984) . Typically one animal is passively exposed to a stressor while a second is given some measure of control over the stressor. Experiments consistently show that individuals with more control over the stressor exhibit lower GC concentrations and fewer pathological symptoms resulting from stress. However, the relationship between stress and control is context dependant and may also be affected by personality characteristics and sex (e.g. Meier et al., 2008; Shors et al., 2007).

Similarly, uncertainty or lack of predictability is a potent source of psychological stress. Animals which receive information about the commencement or cessation of a stressor exhibit lower GC concentrations than animals that experience the commencement or cessation of the stressor randomly (Basset and Buchanan-Smith, 2007; Des-Beech et al., 1983; Grillon et al., 2008; Hennesy et al., 1977; Oka et al., 2010; Tyler et al., 2007; Weis, 1984). Predictability and control are inter-linked concepts. A greater degree of predictability may result in feelings of greater control over the stressor, such that one

might prepare for the stressor. Conversely, greater control over the physical and social environment may result in greater predictability over the nature and timing of stressors.

Stress Coping Mechanisms:

Outlets for frustration resulting from a stressor can greatly influence the magnitude and duration of the stress response. Outlets for frustration may involve displacing aggression onto a subordinate, self directed behaviors, or physical activity. Such secondary responses to a stressor, such as attacking a weaker individual after one has been attacked by a stronger individual (downward directed aggression), does not in any way deal directly with the original stressor (being attacked by a stronger/more dominant individual) but feelings of control are restored which attenuate the physiological stress response (e.g. baboons - Ray and Sapolsky, 1992; Sapolsky, 2011; Sapolsky and Ray, 1989; Virgin and Sapolsky, 1997; fish - Overli et al., 2004; Galhardo et al., 2008; Munro and Pitcher, 1985). Depriving animals of such outlets, such as displacing aggression, eating, drinking, or physical exercise considerably elevates stress markers (Azrin et al., 1966; Eastwood et al., 2008; Gray et al., 1978).

An important behavioral mechanism for attenuating the stress response is mediated through prosocial interactions. A large body of work shows that close physical contact, inter-individual bonds, and social support are powerful attenuators of the physiological stress response (e.g. Abbott et al., 2003; Carter, 1998; Chen et al., 2011; DeVries, 2002; Gunnar et al., 1981; Kirschbaum et al., 1995; Luckow et al., 1998; Taylor, 2006; Taylor et al., 2000; Thorsteinsson and James, 1999). Similar to physical displacement behaviors,

prosocial strategies attenuate the magnitude and duration of the stress response post-stressor rather than directly addressing the source of stress. Prosocial interactions may restore feelings of control and predictability thereby ameliorating feelings of anxiety and fear and associated physiological stress. In macaques, infants that were removed from their mothers showed ameliorated stress levels when exposed to social contact from peers (Boccia et al., 1997; Laudenslager et al., 1990). Similarly, physical contact or proximity (by humans or pets) has been shown to result in decreased stress levels and enhanced recovery in hospitalized infants and patients (Coakley and Mahoney, 2009; Feldman et al., 2009; Field, 2010; Kramer, 1990; Kulkarni et al., 2010; Olson et al., 1992; Turner et al., 1998). Research in wild and captive animals has shown that subordinate animals only show relatively elevated stress levels compared to other group members when lacking social support structures (e.g. Abbot et al., 2003; Sapolsky et al., 1997). Such patterns are often species-specific. For instance, subordinate animals of non-human primate species characterized by few opportunities for social support (e.g. few kin available in social group) have higher levels of relative stress than subordinates in non-human primate species which exhibit kin or non-kin based affiliative social networks or coalitions (Abbot et al., 2003; Clarke et al., 1988). Lack of conspecifics with which to engage with in socially affiliative ways may result in significant stress (social isolation; Abbot et al., 2003; Sapolsky et al., 1997).

Both males and females in a number of primate species form strong same-sex and cross-sex alliances/relationships, sometimes consisting of non-kin. Challenge to any one individual may elicit a response from one or more members of the affiliative network in defense of the victim (e.g. anti-harassment hypothesis - Altmann 1980; Lemasson et al.,

2008; Smuts, 1985) or provide the victim with a range of potential partners as social outlets for the frustration caused by the stressor (e.g. grooming, proximity). Individuals that belong to such affiliatively bonded networks may have greater control and predictability over the outcome of interactions with individuals outside the affiliatively bonded network and therefore incur relatively low stress costs compared to socially isolated individuals. For instance, both male and female baboons form "friendships" (Altmann, 1980; Lemasson et al., 2008; Silk, 2006; Smuts, 1985; Weingrill, 2000). Friendship relationships are characterized by high levels of non-aggressive interactions between two individuals (i.e. grooming, physical proximity, tolerance, reciprocity – Silk, 2006; Smuts, 1985), "Friendships" are associated with low levels of stress when both partners are together and is a term typically reserved for affiliative relationships between non-kin (Silk, 2006). Among baboons, females with male friends may gain protection for themselves and their offspring (Altmann 1980; Busse and Hamilton 1981; Lemasson et al., 2008; Palombit, 2008; Palombit et al., 1997; Smuts, 1985; van Schaik and Dunbar 1990) and males may secure future reproductive opportunities without having to aggressively compete with other males (i.e. an alternative non-rank based reproductive strategy – Virgin and Sapolsky, 1997) and provide protection to offspring they may have sired (Moscovice et al., 2010; Smuts, 1985). Male primates also form alliances with other males (coalitions) when competing for females and dominance rank with another, usually higher ranking, party (e.g. Bissonnette et al., 2009; Noe and Sluitjer, 1995). Having coalition partners may increase the predictability and control over social interactions. Furthermore, coalitionary behavior ties in to general ideas about social adeptness and connectedness which are both associated with ameliorated stress in non-human primates

and humans (e.g. Brent et al., 2011; Sapolsky et al., 1997; Yeh and Inose, 2003). Affiliative and prosocial relationships can have profound health consequences: socially bonded individuals are healthier and live longer than socially isolated ones (Bruhn, 2009; House et al., 1988).

The importance of friends and affiliative bonding in mediating the stress response has been integrated into a coherent model of female affiliative behavior; the "tend and befriend model" (Taylor et al., 2000). This research emphasizes significant sex differences in the social strategies males and females of various mammalian species employ in the face of a stressor. Taylor et al. (2000) suggest that female stress coping responses rely on maternal affiliative neurophysiological mechanisms. These maternal mechanisms (e.g. nurturing behaviors) are elaborated as adaptive responses to stress. For instance, female stress coping mechanisms more often involve seeking physical contact, often with offspring, and seeking out social contact with other females (e.g. Jordan and Revenson, 1999; Matud, 2004; Tamres et al., 2002; Taylor, et al., 2000; Wittig et al., 2008). Males tend to less often seek prosocial outlets for stress and may employ more "fight or flight" based behavioral responses (Baum and Grunberg, 1991; Bull et al., 1972; David and Lyons-Ruth, 2005; Luckow et al., 1998; Repetti, 1989; Turton and Campbell, 2005).

The purpose of stress coping mechanisms is to return feelings of control and predictability, either by physically exerting control (e.g. displacement aggression), or by having it socially affirmed by friends and allies. Among humans men and women may exhibit some differences in the way in which they achieve this. Males tend to employ more problem-focused strategies and women employ a wider range of strategies (e.g.

Tamres et al., 2002) and tend to use more emotion focused strategies (e.g. Jordan and Revenson, 1999; Matud, 2004; Ptacek et al., 1994).

Summary

Vertebrates exhibit a universal generalized physiological response to stress. This physiological response is mediated by the HPA axis. GCs, are the principal adrenal hormones involved in preparing, affecting, facilitating, and sustaining the bodies' response to stress. Frequent activation of the HPA axis results in high allostatic load and chronically elevated GC concentrations, which have a negative impact on physiological, neural and cognitive health, with implications for fitness. Stress and the control thereof is therefore a target of selection. Psychosocial stress is an important source of physiological stress in vertebrates and results primarily from uncertainty and a lack of control over the social and physical environment. Animals exhibit various stress coping mechanisms which can significantly increase predictability and control, thereby avoiding stress and/or restore feelings of predictability and control thereby ameliorating the physiological expression of stress.

Tables and Figures

Figure 1.1: Flow diagram summarizing the neuroendocrine stress response. ACTH = Adrenocorticotropic Hormone, CRH = Corticotropin Releasing Hormone, E = Epinephrine, GCs = Glucocorticoids, NE = Nor-Epinephrine, VP = Vasopressin.

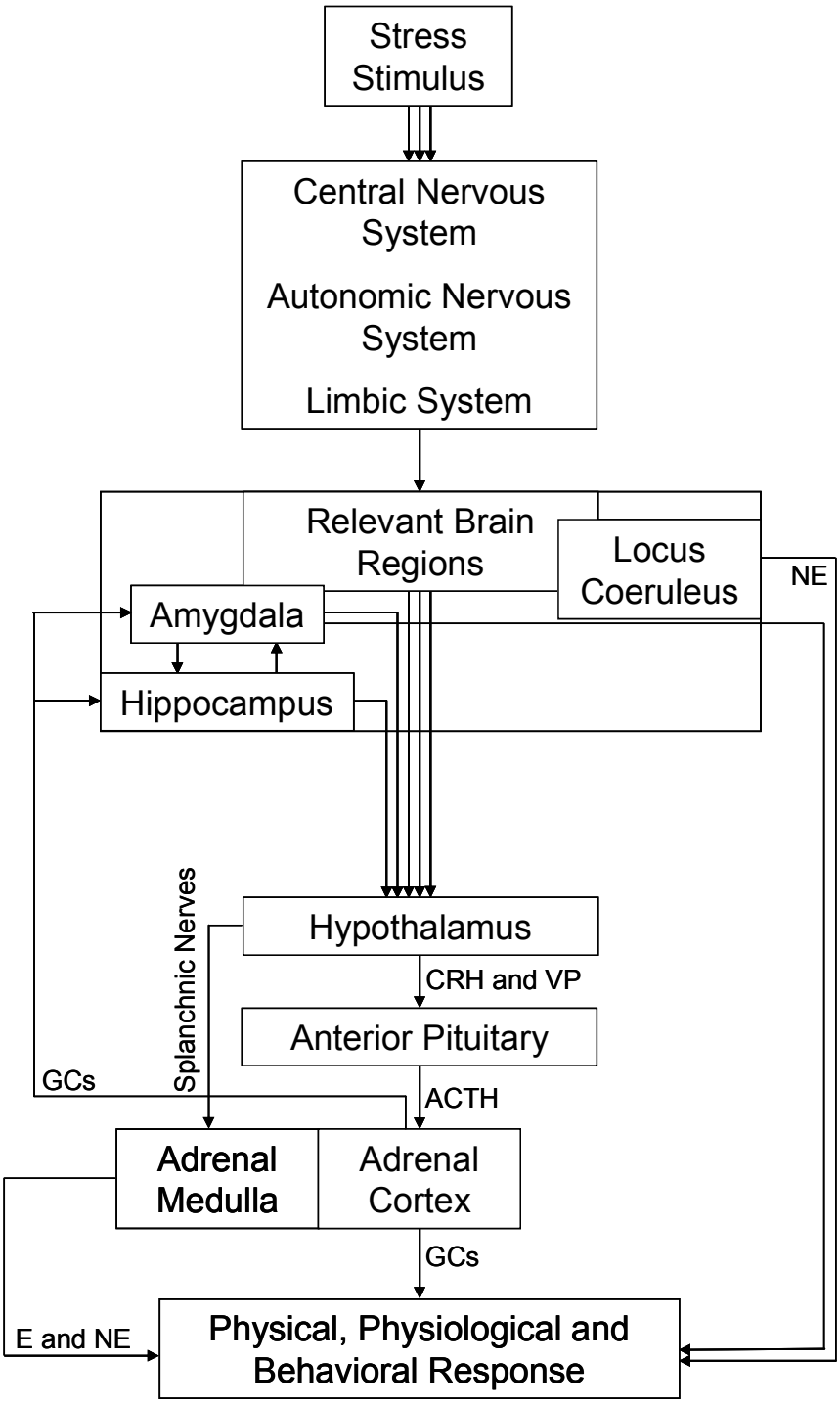
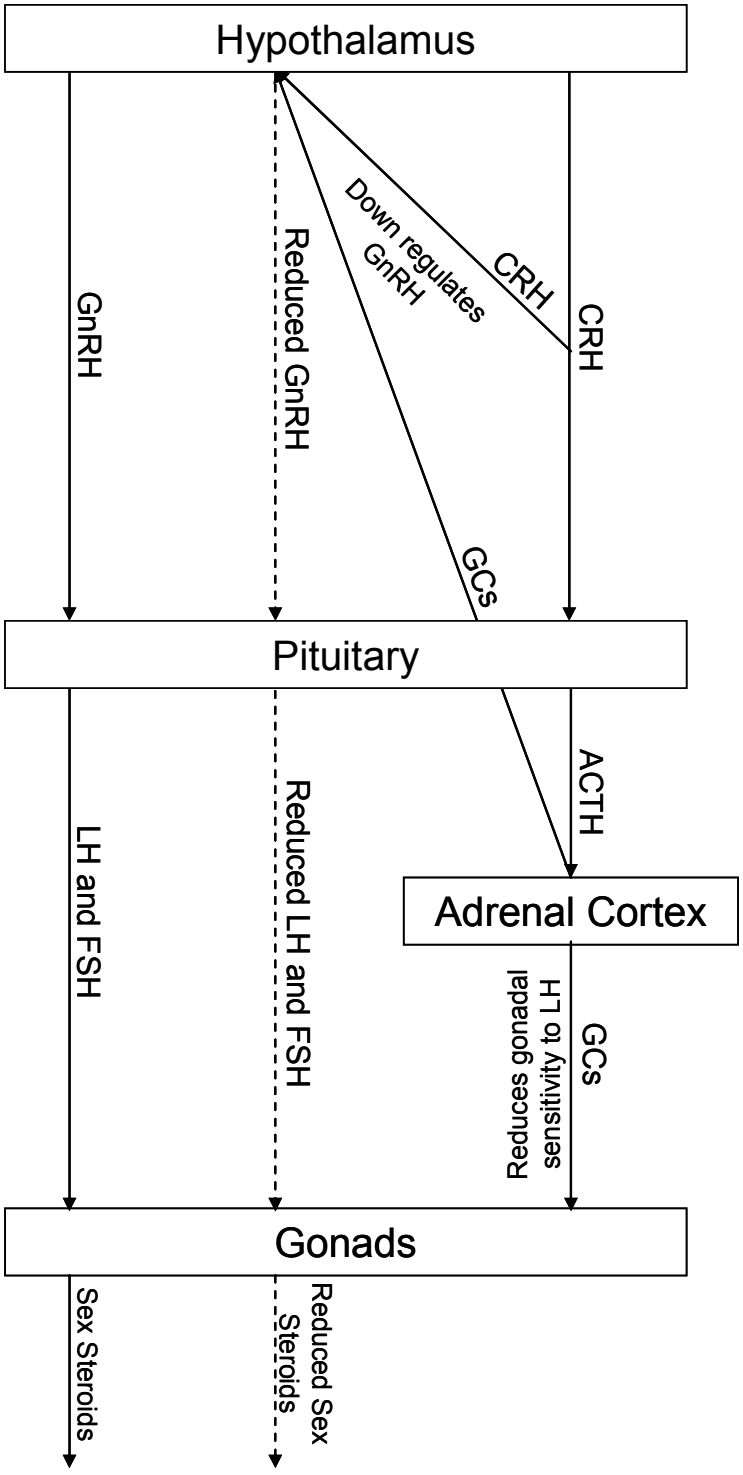


Figure 1.2: Flow diagram of the effects of the endocrine stress response on the hypothalamic-pituitary-gonadal axis. ACTH = Adrenocorticotropic Hormone, CRH = Corticotropin Releasing Hormone, FSH = Follicle Stimulating Hormone, GCs = Glucocorticoids, GnRH = Gonadotropin Releasing Hormone, LH = Luteinizing Hormone.



Chapter 2: Baboon Social Behavior

Baboon Behavior

The *Papio* baboons represent a diverse but closely related genus of medium-sized terrestrial primates (Jolly, 1993) which diverged from their sister genus, *Theropithecus*, around 3.5-4 million years ago (Delson, 1993; Jablonski, 1993; Zinner et al. 2009). The earliest *Papio* fossils (Benefit, 1999; Broadfield et al., 1994; McKee, 1992) and greatest diversity of fossil *Papionin* species (as many as 12 species – e.g. Brain, 1981; Broom, 1940; Delson, 1992, 1993; Freedman, 1957, 1970; 1976;) are found in Southern Africa, suggesting it as the likely place of evolution of the genus during the Pliocene (Benefit, 1999; Broadfield et al., 1994; McKee, 1992). This view is further supported by mitochondrial evidence which consistently show Southern African *Papio* types to possess the most ancient mitochondrial haplotypes (Newman et al., 2004; Zinner et al., 2009). The taxonomic status of the *Papio* baboons remains a topic of debate and has not been fully resolved (Hill, 1970; Jolly, 1993; Zinner et al., 2009). There are five generally accepted allotaxa, representing geographically and morphologically (and behaviorally – Henzi and Barrett, 2003, 2005) distinct types, which are variably considered subspecies (Frost, 2003; Jolly, 1993) or species (Groves, 2001; Grubb et al., 2003; Jolly, 2007). Here I consider the five types as full species (chacma – *Papio ursinus*, anubis – *P. anubis*, yellow – *P. cynocephalus*, Guinea – *P. papio* and hamadryas – *P. hamadryas*; after Groves, 2001; Grubb et al., 2003; Jolly, 2007). A number of morphological subspecies are also recognized (e.g. anubis subspecies, chacma subspecies, yellow subspecies and

the Kinda baboon, see Figure 2.1 for geographic distribution of types and sub-types), some of which are supported by recent molecular research (e.g. Phillips-Conroy et al., 2009; Sithaldeen et al., 2009; Zinner et al., 2009, 2011). Anubis subspecies have been described (Elliot, 1913) and are likely given their wide distribution, and specific cases of population isolation (i.e. Saharan Massif populations - Bigourdan and Prunier, 1937; Dekeyser and Derivot, 1960; Magin, 1990; Monod, 1963), but they remain poorly described and studied (Jolly, 1993). It has been suggested that the Kinda baboon may be recognized as a full species (*P. kindae*: Zinner et al., 2009; 2011) based on geographic, morphological (Frost et al., 2003), behavioral (Phillips-Conroy et al., 2009) and genetic (Burrell, 2008; Phillips-Conroy et al., 2009; Zinner et al., 2009, 2011) differences with other *Papio* species. Notwithstanding geographic, morphological and behavioral differences between the *Papio* species, all species can hybridize and produce fertile offspring (e.g. anubis-hamadryas – Kawai and Sugawara,1976; Kummer, 1971; Nagel, 1973; Phillips-Conroy, 1978; anubis-yellow- Alberts and Altmann, 2001; Samuels and Altmann, 1986, chacma-kinda – Jolly et al., 2011); the *Papio* baboons even hybridize with their sister taxon, *Theropithecus*, in the wild producing fertile offspring (Dunbar and Dunbar, 1974a; Jolly et al., 1997).

It is generally acknowledged that most baboon taxa are capable of considerable behavioral flexibility resulting in variation in behavior among populations of the same species, and individuals within populations (e.g. Byrne et al., 1989; Hall and DeVore, 1965; Mori et al., 2007; Ray and Sapolsky, 1992; Sapolsy and Share, 2004; also see Chapman and Rothman, 2009). Nevertheless, like the macaques (*Macaca* sp.: Gachot-Neveu and Menard, 2004; Thierry et al., 2000; 2004), baboon species differ markedly in

social organization and behavioral styles (Barton et al., 1996; Cheney and Seyfarth, 2007; Henzi and Barrett, 2003; 2005; Swedell and Leigh, 2006). For instance, hamadryas social structure, reproductive strategies, and behavior are strikingly different from the social structure, reproductive strategies, and behavior of "savannah" baboons (i.e. anubis, chacma and yellow baboons).

Hamadryas baboons exhibit a hierarchically structured social system (Abegglen 1984; Colmenares et al., 2006; Kummer, 1968, 1984, 1995; Schreier and Swedell, 2009) and a form of male philopatry (Abegglen 1984; Hammond et al., 2006; Hapke et al., 2001; Kummer 1968; Swedel et al., 2011), unlike the general pattern of male dispersal among savannah baboons (Smuts, 1985; Strum, 1987; Packer ,1979a; Phillips-Conroy and Jolly, 2004). The smallest basic hamadryas social unit is the one-male-unit (OMU), consisting of a single male and several females, to which the leader claims exclusive access, and their offspring. These OMU's form the basis of larger hierarchically organized social units (e.g. clans, bands and troops). Male-male social interactions among clan members are relatively non-aggressive, often affiliative and ritualized (Colmenares, 1990; Jolly et al., 2008; Kummer, 1968), however males are constantly vigilant against abduction of females by other males (Pines and Swedell, 2011). Hamadryas tenure as OMU leader is stable for an extended period of time (3-6 years – Abegglen, 1984; Sigg et al., 1982) compared to other taxa with similar OMU mating structures (e.g. gelada baboons, 6 -9 months – Dunbar, 1979). There is some intra-specific variability in the nature and occurrence of the various levels of social organization. For instance, the hierarchical nature and strictness of OMU and clan units are less pronounced in Saudi-Arabian hamadryas than in Ethiopian hamadryas (Biquand et al., 1992; Mori et al., 2007).

Guinea baboons exhibit a "soft" version of the hamadryas-typical, hierarchically organized social units (Anderson and McGrew, 1984; Galat-Luong et al., 2006; Maestripieri et al., 2007; Partzelt et al., 2011). Among Guinea baboons, female membership to one-male units appears to be fluid; females maintain social bonds with relatives belonging to different OMUs, while males do not exhibit aggressive herding behavior towards females and generally interact non-aggressively with other males (Galat-Luong et al., 2006; Maestripieri et al., 2007).

Anubis (*P. anubis*), yellow (*P. cynocephalus*) and chacma baboons (*P. ursinus*) live in multi-male-multi-female societies, characterized by female philopatry and male dispersal (Alberts and Altmann, 1995a; Alberts et al., 2003; Bergman et al., 2005; Bulger and Hamilton, 1988; Hall, 1962; Smuts, 1985; Silk et al., 1999). In these societies males aggressively compete for dominance status, which determines access to females (Alberts et al., 2003; 2006; Bulger, 1993; Hausfater, 1975; Weingrill et al., 2000). Females also exercise a degree of mate choice (Bercovitch, 1991; 1995; Higham et al., 2009; O'Connell and Cowlishaw, 1994). Even among these species, subtle differences in reproductive strategies and sociosexual behavior have been noted (Henzi and Barrett, 2003, 2005).

For instance, chacma males tend to have short alpha tenures, compared to anubis and yellow baboons (Collins et al., 1984; Palombit et al., 2000), during which reproductive skew is high (Bulger, 1993; Weingrill et al., 2000), followed by periods of relatively low rank, little reproduction, and a shift to offspring protection (Beehner et al., 2009; Henzi and Barrett., 2003; 2005; Henzi et al., 1999). Infanticide is also relatively more common among chacma baboons than among anubis and yellow baboons (Broom et al., 2004;

Palombit, 2003). Male anubis and yellow baboons are also known to form coalitions in order to compete with rival males for dominance or access to females (Bercovitch, 1988; Henzi and Barrett, 2003; Noe, 1986; Noe and Sluijter, 1995; Packer, 1977). Coalition behaviors have not been described in chacma baboons, even under demographic and social conditions that should favor the expression of the behavior (Henzi and Barrett, 2003; Henzi et al., 1999), and even though this species has been the focus of several intensive long term studies (e.g. Cape Peninsula, Drakensberg, De Hoop, Mkuze, Moremi, Suikerbosrand). This has lead researchers to speculate whether the extent of chacma behavioral plasticity precludes the expression of the behavior under natural conditions (Henzi and Barrett, 2003). Anubis, chacma and yellow baboons are also known to form cross-sex bonds, described as "friendships" (Lemasson et al., 2008; Palombit et al., 1997; Silk, 2002; Smuts, 1985), however chacma friendships are thought to be qualitatively different to those described in anubis and yellow baboons (Henzi and Barrett, 2003).

The extent to which these species-specific behavioral tendencies are learned or innate (i.e. suggesting a genetic basis for differences) remains relatively poorly understood. Observations based on wild baboons in the Awash National Park (ANP) of Ethiopia suggest that hamadryas baboon behavior is relatively fixed (Bergman and Beehner, 2004; Bergman et al., 2008; Kummer et al., 1970). Hamadryas and anubis baboons form a natural species border in the ANP, along which cross-species immigration occurs resulting in natural hybridization (Aldrich-Blake et al., 1971; Brett et al., 1977,1982; Jolly and Brett, 1973; Jolly, 1993; Jolly and Phillip-Conroy, 2003; Kummer, 1968a,b,1995; Nagel, 1973; Phillips- Conroy and Jolly, 1981, 1986; Phillips-Conroy et

al., 1986; Shotake, 1982; Shotake et al., 1977; Sugawara et al., 1982). This natural hybridization has resulted in the creation of social groups dominated by phenotypically diverse multi-generational hybrids (hybrid troops) sandwiched between the phenotypically more homogenous parent populations. Cross-species immigrant males continue to express the specific behavioral profiles consistent with their external phenotype. For example, male hamadryas immigrants to anubis troops attempt to herd anubis females, with only temporary success (e.g. Phillips-Conroy et al., 1991, 1992). Most significantly, hybrid offspring exhibit behavioral tendencies consistent with their external phenotype. In other words, a hybrid animal that looks more hamadryas-like exhibits hamadryas-like behavior and animals that look more anubis-like exhibit more anubis-like behavior; animals of intermediate phenotypes exhibit behavioral aspects of both parent species (Bergman and Beehner, 2004; Bergman et al., 2008). This is true of both males and females (Bergman and Beehner, 2004; Bergman et al., 2008). This suggests that species-specific behavioral profiles are relatively fixed and may have a strong genetic basis (Bergman and Beehner, 2004; Bergman et al., 2008; Kummer et al., 1970.).

Male socioreproductive strategies are thought to be principally shaped by female dispersal on the landscape and demographic factors, whereas female behavior is principally shaped by ecological factors such as resource availability, predator pressure, and infanticide avoidance (e.g. Barton et al., 1996; Dunbar, 1988, 1992; Henzi and Barrett, 2003, 2005; Hill and Dunbar, 1998). For instance hamadryas baboons tend to be restricted to more marginal arid habitats than anubis baboons (Biquand et al., 1992; Phillips-Conroy and Jolly, 1986). Under such conditions it is more effective for females

to form smaller foraging parties and disperse over the landscape thereby increasing foraging efficiency while sacrificing protection from predation afforded by large social groups (e.g. Altmann, 1974; Barton et al., 1996; Dunbar, 1988; Janson and Goldsmith, 1995; Kummer, 1990, 1995). Males can not monopolize access to females that are so spatially dispersed and instead focus on subgroups of females, hence the origin of the hamadryas OMU (Dunbar, 1988; Kummer, 1990, 1995). A similar socioreproductive pattern is reproduced by chacma baboons under similar ecological constraints (Anderson, 1981; Barton et al., 1996; Byrne et al., 1989). Anubis baboons generally inhabit more productive habitats, where it is advantageous for females to forage in larger, more cohesive groups, since competition for resources is less intense than when resources occur at low densities and are scattered. When females are more densely associated on the landscape, males that are effectively able to assert dominance over the whole group will secure reproductive access to a large pool of females at any one time, outweighing the reproductive benefits of controlling access to only a few females (Aldrich-Blake et al., 1971; Altmann, 1974; Barton et al., 1996; Dunbar, 1988; Henzi and Barrett, 2003, 2005; Janson and Goldsmith, 1995; Kummer, 1990, 1995; Wiley and Poston, 1996). Such evolutionary scenarios likely underlie differences in baboon behavioral programs.

Anubis, Hamadryas and Hybrid Baboon Social Behavior

Anubis Baboons:

Anubis baboons in the ANP exhibit social and behavioral tendencies similar to those described for other populations of this species. The anubis social environment is structured around male and female dominance hierarchies (Bercovitch, 1987; Bercovitch and Strum, 1993; Charpentier et al., 2008; Easley et al., 1989; Johnson, 1987; Packer, 1979a; Packer, 1995; Smuts and Watanabe, 1990; Strum, 1982) within groups that can range in size between 15 and 150 individuals (Dunbar and Dunbar, 1974; Henzi and Barrett, 2003; Ray and Sapolsky, 1992; Rowel, 1966). Males disperse from their natal groups around eight years of age (Alberts and Altmann 1995a; Alberts et al. 1992; Bergman et al. 2005; Packer, 1979b; Phillips-Conroy and Jolly, 2004), although dispersal of juveniles in this species is known to occur with some frequency (Alberts and Altmann, 1995a, b, 2001). Female rank is inherited within matrilines (Engh et al., 2009; Johnson, 1987). Matrilineal dominance rank is relatively stable within a group (Barton and Whiten, 1993; Cheney and Seyfarth, 2007; Hausfater et al., 1982; Silk, 2002; Smuts, 1985) but both adult and juvenile females can experience changes their dominance rank (Engh et al., 2009; Johnson, 1987). Females tend to associate with other females within their matriline; female rank and matrilineal group rank confer advantages in agonistic social interactions (support from male and female siblings – Engh et al., 2009; Silk et al., 2009) as well foraging (Barton and Whiten, 1993; Smuts, 1985) with possible consequences for reproductive success (Garcia et al., 2006; 2009). Females invest in kin and non-kin social interactions forming female alliances which provide social support in agonistic interactions. Females that are socially connected may enjoy better reproductive success (Silk et al., 2009). Female dominance rank in savanna baboons is known to have stress consequences for females as well as their offspring. Specifically, lower ranking females

may experience greater social and energetic stress which they may pass on to their offspring (Altmann and Alberts, 2005; Onyango et al., 2008).

Male rank is associated with mating success (Alberts et al., 2003; 2006; Bulger, 1993; Hausfater, 1975; Weingrill et al., 2000). Dominance is therefore actively and aggressively contested (Cowlishaw and Dunbar 1991; Packer 1979a). Males form short intense consortships with ovulating females during which they maintain exclusive access in order to increase the probability of paternity. The ability of a male to ensure paternity also rests on experience (Daspre et al., 2009; Smuts, 1985; Weingrill et al., 2000), as external female ovulatory cues are not exact and premature consorting or premature cessation of consortship may not result in conception. Consortships are often interrupted by other males or male coalitions (Bercovitch, 1988; Smuts and Watanabe, 1990), and a female may have multiple consorting partners during a single receptive cycle (Ransom, 1981; Smuts, 1985). Females also exercise mate choice and the degree to which a consorting male will successfully enter into a consortship and defend a female in estrus may depend on the relationship the male has cultivated with the female (i.e. familiarity and "friendship" – Smuts, 1985), and what information regarding ovulation females allow males to gain access to (Higham et al., 2009). However, subordinate males do secure mating opportunities by employing a number of non-dominance related strategies (Bulger, 1993; Cowlishaw and Dunbar, 1991; Smuts, 1985). These include male-female friendships and male coalitions (Alberts et al., 2003; 2006; Lemasson et al., 2008; Noe and Sluitjer, 1990 Palombit et al., 1997; Silk, 2002). Male anubis social environments are dynamic and males aggressively compete for dominance, must adjust to changes in the dominance hierarchy, female choice, new unknown adult males (immigrants) or new

social groups when emigrating. This suggests that the male anubis social milieu may be characterized by frequent social instability and unpredictability resulting in high social stress loads for males, but participation in social competition and the resulting stress laods are offset by potential access to a large pool of reproductive females.

Hamadryas Baboons:

Hamadryas baboons exhibit a strikingly different social and reproductive system and some of the concepts used to describe anubis, chacma, and yellow baboon social structure may have no relevant analogues in hamadryas baboons. Hamadryas baboons live in a hierarchically structured society where the OMU is the lowest basic social group (Abegglen 1984; Colmenares et al., 2006; Kummer, 1968a, 1984, 1995; Schreier and Swedell, 2009). An OMU consists of one male and several females with whom he is exclusively bonded (Kummer, 1968, 1995). A number of OMUs which are habitually associated may form a clan (Abbeglen, 1984; Schreier and Swedell, 2009). Male clan members are thought to be related (Abegglen 1984; Kummer 1968; Sigg et al., 1982). A number of associated clans form larger social units known as bands and a number of bands may congregate on the same sleeping cliffs to form troops (Abegglen 1984; Kummer, 1968).

Males do not typically actively compete for females, but instead respect each other's claims to a harem of females (Bachmann and Kummer, 1980; Kummer, 1968; Kummer et al., 1974) although abductions and takeovers do occur. As a consequence, the typical linear dominance hierarchy as described in other baboon species does not exist among

hamadryas baboons. Male dominance relationships are more subtle and variable and remain poorly defined (Romero and Castellanos, 2006). Juvenile males leave their natal OMU and attach themselves as "followers" to other OMUs within their patrilineal clan (Abegglen, 1984; Sigg et al., 1982). The juvenile and sub-adult followers sneak copulations with females of their adopted OMU (Kummer, 1968; Sigg et al., 1982). Studies of wild and captive populations suggest that extra-OMU mating and paternity accounts for around 10 to 30% of offspring (Smith et al., 1999; Yamane et al, 2003, Zinner et al., 2006). Juveniles as young as four years old have been shown to mate and reproduce successfully (Zinner et al., 2006).

These data suggest that precocial sexual maturity may be a significant part of hamadryas reproductive life history (Jolly and Phillips-Conroy, 2003; Zinner et al., 2006). Hamadryas males experience early testicular maturation (~4 years – Jolly and Phillips-Conroy, 2003) and reach near full adult testicular size by the time they are sub-adults (Jolly and Phillips-Conroy, 2003). Adult hamadryas males have smaller testes than the males of other species (Jolly and Phillips-Conroy, 2003). Males disperse from their mothers at around the same time as the onset of testicular maturation (~2 years – Sigg et al., 1982).

Females disperse from their natal OMU at between 1.5-3.5 years old and tend to transfer within the clan (around half of all females transfer within the clan – Sigg et al., 1982; around 80% - Swedell et al., 2011) and band (around 70% females transfer within the band - Swedell et al., 2011). Females mainly disperse through male takeovers of OMUs (Swedell et al., 2011) or through abductions (Pines and Swedell, 2011). Female dispersal therefore appears to be forceful and social rather than spatial (Swedell et al.,

2011). Male hamadryas are known to disperse (Phillips-Conroy et al., 1991, 1992), although the norm appears to be for males to remain within their natal clans and bands (Swedell et al., 2011).

Hamadryas males attain their own OMUs through four main strategies: the initial unit strategy (i.e. separating a juvenile female with whom a relationship had been cultivated as an OMU follower), inheritance strategy (i.e. gradual replacement of aging leader male), challenge strategy (i.e. deposing aging or injured leader male) or opportunistic strategy (i.e. assuming leader role in case of leader disappearance or absence – Pines et al., 2011, Swedell et al., 2011). After loss of the OMU, hamadryas males have limited reproductive opportunities and usually continue to follow their former OMU. Male hamadryas social dynamics are marked by less overt aggressive competition, and male-male interactions are usually affiliative or ritualized. These characteristics suggest that unlike male anubis the male hamadryas social environment may be associated with lower social stress loads than anubis, but hamadryas males only have reproductive access to a small number of females throughout their reproductive careers.

Anubis x Hamadryas Hybrid Baboons:

Most hybrids in the Awash are not first generation hybrids but have at least one hybrid parent. Hybrid phenotypes are therefore very diverse. This is the result of crossing and back-crossing over multiple generations (Bergman and Beehner 2004; Bergman et al., 2008; Phillips-Conroy and Jolly, 1986). Hybrid troop H on the left bank, below the Awash Falls on the Awash River (see Figure 2.2) has been the subject of the most

intensive investigations of behavior in a hybrid troop and almost all of the behavioral information available for hybrid troops are based on investigations of this troop. As previously noted, hybrid behavior in both males and females reflects the phenotype of the animal (Bergman and Beehner 2004). A comparison of phenotypic hybridity in males with behavioral tendencies support these observations and show (with some exceptions) that phenotypically more hamadryas-like males tend to behave in a more hamadryas-typical fashion (Bergman et al., 2008). Hybrid phenotype is characterized using an index constructed using eight morphological characteristics relating to hair color, length, pattern, as well as skin patches and colors. Behavior appears to correlate with the overall index rather than with any one characteristic making up the index.

Hybrids exhibit a variety of social agendas (Bergman and Beehner 2004; Bergman et al., 2008; Sugawara, 1979) and appear to lack cohesion at the troop level (Bergman and Beehner 2004). Hybrid troop H exhibits an anubis-typical multi-male-multi-female social organization, whilst containing several hamadryas-like OMUs (Bergman and Beehner 2004; Sugawara, 1979). The OMUs in troop H can be divided into "strict" and "loose" OMUs. "Strict" OMUs more closely follow the pure hamadryas condition in that female seem to be exclusively bonded to the leader male. "Loose" OMUs exhibit more fluid female membership, where females always transitioned between OMUs and never out of an OMU into the anubis-like social segment (Bergman and Beehner 2004; Bergman et al., 2008). Additionally, males rarely exhibited hamadryas-typical herding behavior, with one exception. Unlike what is seen in anubis groups, male dispersal appears to be rare and the norm is for males to remain in their natal group. One consequence of this trend is that pairwise relatedness in group H has increased over time as neither males nor females

disperse (Bergman and Beehner 2004; Woolley-Barker, 1999). Anubis-like linear dominance hierarchies among males also appear to be absent as males rarely interact and male-male aggression is uncommon. Females appear to mate exclusively within their consortship or OMU during a given cycle (i.e. hamadryas-like) but may consort with different males over successive estrus cycle (i.e. promiscuous anubis-like mating - Bergman and Beehner 2004). Although male-male aggression appears to be in frequent, the diversity of male social agendas in hybrid groups may be a source of social uncertainty for male and could be a source of social stress.

Analyses of reproductive success suggest that the most phenotypically intermediate males did not exhibit an obvious reproductive disadvantage compared to phenotypically more anubis- or hamadryas-like males within hybrid group H (Bergman et al., 2008). It is speculated that hybrid females may actually prefer the intermediate males, since these males form close social bonds with females without restricting their ability to interact with female relatives in the larger group. Although successful in a context where almost all group members are hybrids, evidence suggests that hybrids would be at a considerable disadvantage in anubis and hamadryas troops where persistent harassment by anubis males rapidly turn over consortships (Beyene, 1998; Nystrom, 1992) and access to females in hamadryas groups are severely limited as all females belong to an OMU (Sugawara, 1982, 1988) and can only be attained through a protracted strategy or brute force (Pines et al., 2011, Swedell et al., 2011). This may account for the low rate of male emigration observed in this group.

Summary

The *Papio* baboons consist of several closely related species with a wide distribution across Africa and the Arabian Peninsula. The species are morphologically distinct and, notwithstanding intra-specific variation, exhibit species typical behavioral trends and socioreproductive strategies. *Papio* baboon species are also known to hybridize at contact zones between species. In the ANP, Ethiopia, a socially and spatially distinct hybrid population has developed at a contact zone between anubis and hamadryas baboons. Anubis baboon society is characterized by multi-male-multi-female social groups, structured around male and female dominance hierarchies, male dispersal, female philopatry and polygynandrous mating. Male anubis aggressively contest access to females resulting in a relatively unstable social hierarchy, which may imply high social stress loads for males of this species. Hamadryas baboons exhibit hierarchically structured social units of which the smallest basic unit is the OMU. Males are philopatric, traditional dominance hierarchies as seen in anubis baboons are not present and males follow a number of protracted social strategies in order to gain access to females. Male-male interactions are generally non-aggressive and ritualized, suggesting low social stress loads for males of this species. Hybrid social groups are characterized by the lack of social cohesion and social norms, and the presence of multiple behavioural programs in both males and females. Hybrid animals typically exhibit behavioural propensities reflecting their external phenotype resulting in graded variation in the expression of anubis- or hamadryas-like behavior. The lack of stable social norms in hybrid groups may be a source of social stress.

Tables and Figures

Figure 2.1: Map showing the distribution of six Papio baboon species; anubis/olive (*Papio anubis*), chacma (*P. ursinus*), hamadryas (*P. hamadryas*), Guinea /red (*P. papio*), kinda (*P. kindae*) and yellow (*P. cynocephalus*), as well as yellow (typical; yellow – *P. c. cynocephalus*, and Ibean yellow baboon – *P. c. ibeanus*) and chacma (Cape chacma – *P. u. ursinus*, gray footed chacma – *P. u. griseipes*, and Ruacana chacma – *P. u. ruacana*) subtypes which may be supported by molecular evidence (e.g. Phillips-Conroy et al., 2009; Sithaldeen et al., 2009; Zinner et al., 2009, 2011). Anubis subspecies are not included as they remain relatively poorly described and have not been reconciled with geographic haplotype distributions for the species. Species and subspecies distributions are based on Kingdon, 1997, and mitochondrial haplotype distributions reported by Sithaldeen et al., 2009 and Zinner et al., 2011.

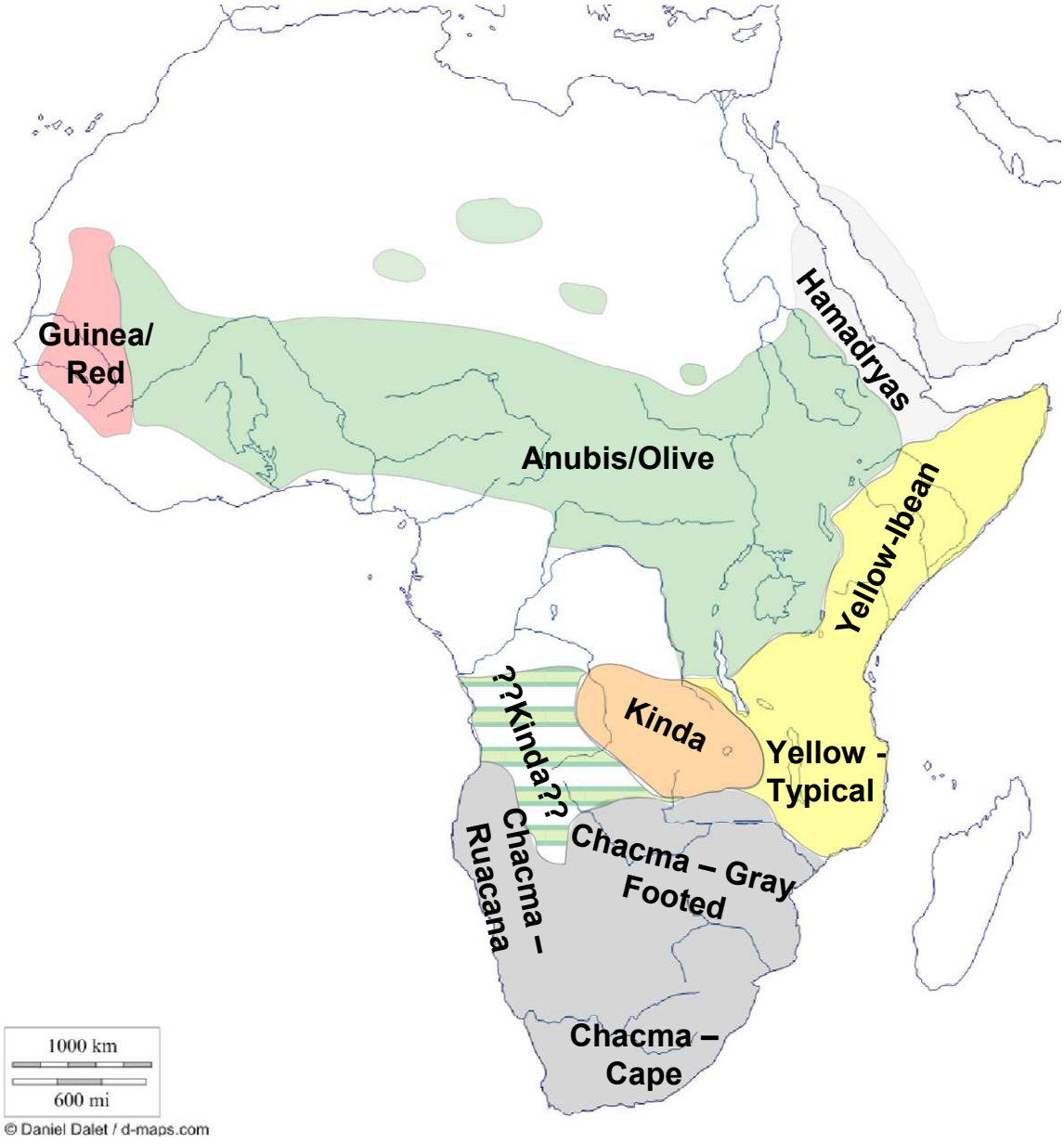
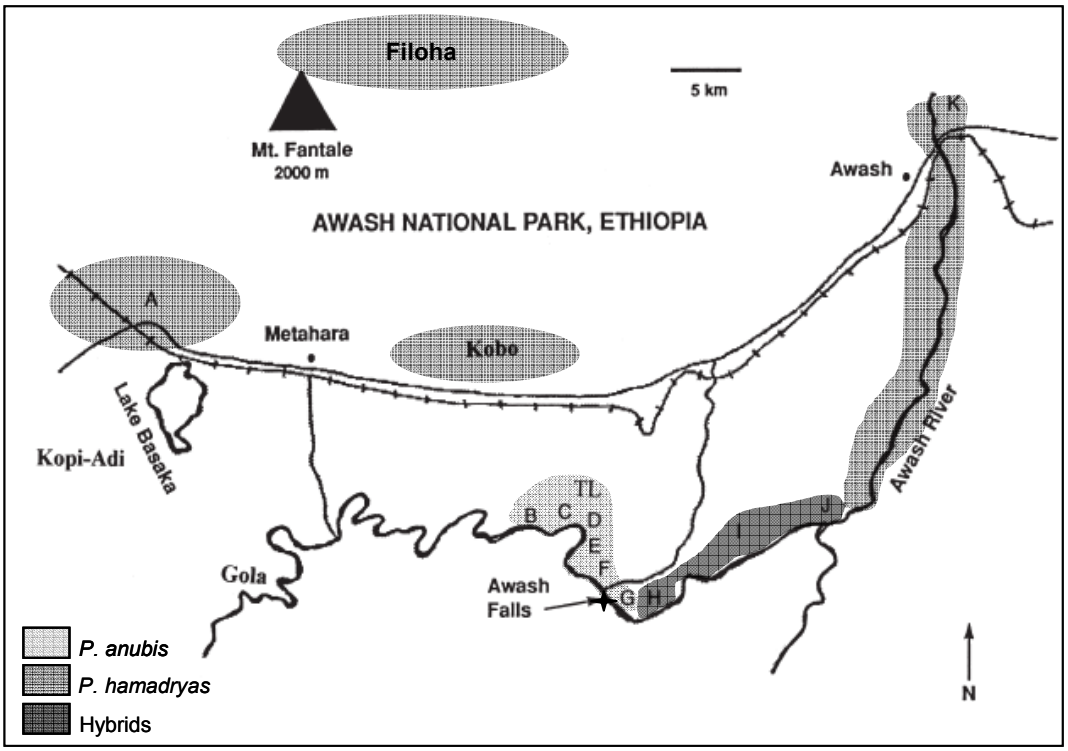


Figure 2.2: Map showing the distribution of anubis, hamadryas and hybrid populations within the Awash National Park, Ethiopia.



Chapter 3: Hair Hormone Analysis

From Toxicology to Endocrinology

During the early 1900s it was observed that hair recorded exposure to arsenic (e.g. Knecht and Dearden, 1901; Roberts, 1902; Ross, 1902; Underhill, 1914). Hair arsenic analysis was thereafter often used as a diagnostic tool to determine exposure to arsenic (e.g. Bourret et al. 1953; Gault et al., 2008; Griffon and Barbaud 1951; Harrington et al., 1978; Young and Ralphs, 1942). These initial observations were followed by research which focused on protein and amino acid composition of hair (Beveridge and Lucas 1944a, 1944b, 1944c; Block, 1939; Lucas and Beveridge 1940; Rimmington 1929; Rossouw and Wilken-Jorden 1935; Wilson and Lewis, 1927; Ritter, 1917; Smuts et al., 1932). Research identifying and quantifying a great variety of xenobiotics (e.g. vanadium – Mountain et al. 1953; iodine – Leblond 1954; copper - Goss and Green 1955; iron – Green and Duffield 1956; lead – Jaworowski et al. 1966; mercury – Skerfving 1974) in hair proliferated and hair soon became a focal matrix to monitor exposure to environmental pollutants (e.g. Jervis et al. 1971; Kijewski and Lange 1977; Liu et al., 2009; Ohmori et al. 1981; Schramm, 2008; Seifert et al., 2000). Not until the early 1980s did researchers begin to routinely identify and quantify organic compounds and their metabolites in hair (although see Goldblum et al. 1954). This research was mainly driven by the development of drug-use detection and monitoring techniques (e.g. Arnold and Puschel, 1981; Baumgartner, 1989; Baumgartner et al., 1979; 1981; Valente et al., 1981). Three main research groups pursuing hair drug analysis (i.e. USA- Baumgartner,

Germany – Arnold, Italy – Valente) were pivotal in designing the first radioimmunoassay (RIA) systems for the quantification of drug metabolites archived in hair, which formed the basis for future enzyme-linked immunosorbent assays (ELISA).

The first reports identifying and quantifying hormones in hair were made by a Hungarian group (Faredin et al., 1966; Julesz et al., 1966, 1967). They reported the detection and quantification by RIA of seven androgens and androgen metabolites in human axillary hair. There appears to have been little subsequent interest in hair hormone analysis, as evidenced by a paucity of publications related to the technique until 1988 when Lui et al. (1988) reported the detection of progesterone in the hair of cows and Nixon et al. (1988) reported the quantification of odorous androgens in the axillary hair of men by gas chromatography. There then appears another short hiatus in the publication of hair hormone research until 1996 when Hold et al. (1996) reported on the detection of an anabolic steroid in hair by mass spectrometry (also see Kintz 1998; Kintz et al. 1999a, b). This was followed by reports by Gliexner and Meyer (1997), Schrerer et al. (1998) and Yang et al. (1998) who reported the measurement of endogenous steroids in hair by high performance liquid chromatography (HPLC)/mass spectrometry and ELISA methods. Until recently the majority of hair steroid research focused on the detection of banned performance enhancing substances and their metabolites in hair (e.g. Anielski, 2008; Anielski et al. 2005; Boyer et al. 2007; Deshmuk et al., 2010; Gaillard et al., 1999, 2000; Hold et al. 1999; Kintz 1998; Kintz et al. 1999b; McKinney, 2009; Segura et al. 2000). For reasons of accuracy and legal requirements in sports drugs testing, these studies mainly employed chromatographic and mass spectrometric methods to quantify hormones and their metabolites. More recently, emphasis has been placed on detecting

normal endogenous circulating concentrations of various steroids. With this, the focus shifted away from androgens and to corticosteroids (although see Choi et al., 2000 – estrone and estradiol). In 2000 Cirimele et al., (2000) reported the identification of ten corticosteroids in human hair by Liquid Chromatography– Ion-Mobility Spectrometry (LC-IMS) (also see Bevalot et al., 2000). A number of subsequent studies undertook the quantification of corticosteroids in hair. Again the initial interest seems to have been as a method to identify the use of banned substances in professional sport and the emphasis has been on using HPLC and mass-spectrometric methods (Boumba et al., 2006; Gaillard et al. 1999, 2000; Raul et al. 2004).

The first report of using ELISA to detect endogenous steroid hormones in hair was by Gliexner and Meyer (1997) (estradiol and testosterone, also see Appendix 2). Later Koren et al. (2002) used commercially produced kits, designed for the quantification of cortisol in human saliva and testosterone in human serum or plasma, to determine cortisol and testosterone levels in hyrax *(Procavia capensis*) hair. Before Gliexner and Meyer (1997) and Koren et al. (2002) there appears to be little precedent for using ELISA methods other than that RIA had been used previously (Faredin et al. 1966; Julesz et al. 1966, 1967) and that ELISA methods (and the development of ELISA kits) had been widely used in drug detection in hair (Cirimele et al., 2004; Cooper et al., 2003; Gleixner et al., 1996; Nakahara et al., 1989; Pujol et al., 2007; Speihler, 2000; Sweeney et al., 1998). In 2006, Davenport and colleagues published a rigorous methodological paper in which they explored the optimal wash and extraction procedures for cortisol quantification by ELISA, and provided biological validation of the technique and the assay system using a primate model (*Macaca mulatta* - Davenport et al. 2006). In doing so they provided the

first explicit validation data for the use of commercially produced ELISA kits designed for the quantification of cortisol in human saliva. This paper seems to have become the source of the growing interest in using hair as a substrate to determine cortisol concentrations and doing so using commercially produced ELISA kits. Since then a growing number of papers reporting the quantification of cortisol in the hair of a number of species has been published. The emphasis now appeared to shift to the use of hair steroid analysis as a diagnostic tool of stress related to disease, mental disorders, or ecological factors (Detternborn et al., 2010; Karlen et al., 2011; Macbeth et al., 2010; Martin and Reale, 2008; Stuedte et al., 2011a, b; Thomson et al., 2009, 2010; Yamada et al., 2007).

Currently two prominent groups have emerged as major proponents of the technique as a diagnostic tool in clinical medical research. The first of these to emerge is a Canadian collaboration based at the University of Western Ontario under the direction of Stan van Uum and Gideon Koren from the Hospital for Sick Children in Toronto (Gow et al. 2010; Pereg et al. 2010; Sauve et al. 2007; Thomson et al. 2010; van Uum et al. 2008; Webb et al. 2010; Yamada et al. 2007; also see Thomson et al. 2009; Appendix 2 - testosterone). The second is a German group based at the Technical University of Dresden under the direction of Clemens Kirschbaum (Dettenborn et al. 2010, 2011; Kirschbuam et al. 2009; Stalder et al. 2010, 2011; Stuedte et al. 2010, 2011a, b). Additionally, researchers in various other fields have successfully applied the technique in the study of animal behavior (e.g. bears - Becshoft et al. 2010; Macbeth et al. 2010; caribou - Ashley et al. 2011; ground squirrels - Martin and Reale 2008; hyraxes - Koren et al. 2002; macaques - Davenport et al. 2006; Dettmer et al., 2009; Feng et al., 2011;

marmosets - Clara et al. 2008; various primates - Fourie and Bernstein, 2011a; vervet monkeys - Fairbanks et al. 2011; Lauderslager et al., 2011). Yet others continue to investigate the technical and methodological complexities of the technique (e.g. validation using feline and canine models – Accorsi et al. 2008; time course of steroids to expression in hair using a guinea pig model – Shen et al. 2009a; effects of hair pigmentation using a canine model – Bennet and Hayssen 2010).

Mechanisms for the Incorporation of Hormones into the Hair Shaft

There are three primary mechanisms through which xeno- and endobiotics are thought to enter the hair shaft; (i) blood, (ii) glandular secretions and sweat and (iii) exogenous contamination. Only the first two mechanisms are generally seen as principal mechanisms through which significant amounts of endogenous steroid hormones enter the hair shaft.

Steroid hormones in circulation diffuse into the hair follicle from neighboring capillaries and are incorporated in the hair shaft during growth (Cone 1996; Henderson, 1993; Kikura and Nakahara, 1998; Villain et al. 2004, Figure 3.1). If this mechanism is the only or main mechanism of steroid incorporation into the hair shaft then it may be expected that serial differences in steroid concentrations along the hair shaft should be detectable, assuming that the steroids are relatively immobile once fixed in the hair matrix. Indeed such evidence does exist which suggests variation in sequestered steroids concentrations along the hair shaft (e.g. Kirschbuam et al. 2009; Gao et al., 2010; Thomson et al., 2010; Webb et al. 2010).

Recent evidence suggests that glandular secretions, specifically sweat, may be a previously unappreciated contributor of steroid hormones to the hair shaft (Anielski 2008; Henderson, 1993; Thieme et al. 2003; Figure 3.1). Not all mammals sweat but most produce oily secretions (sebum) from sebaceous glands in the skin usually associated with the hair follicle. The ducts of the sebaceous glands (and apocrine glands) empty into the hair follicle and onto the emerging hair, bringing the glandular secretions to the skin surface (Eurell et al. 2006). Hormones contained in the sweat or glandular secretions diffuse into the hair shaft. Since the hormone is in a mobile phase while in sweat or glandular secretions, steroid hormones may diffuse into the hair shaft at any point along the shaft and may be mobile within the shaft for some time. Additionally, sweat or glandular secretions once having penetrated the hair shaft may change steroid hormones already trapped in the hair matrix into a mobile phase and facilitate diffusion along the length of the hair. In taxa or body areas where hair is likely to be bathed in such secretions, the steroid signal may become homogenized along the hair shaft. Recent evidence in a number of taxa report no significant difference in cortisol concentrations between proximal and distal parts of the hair (Accorsi et al. 2008; Anielski 2008; Bennett and Hayssen 2010; Davenport et al. 2006; Macbeth et al., 2010). However, evidence in humans suggests that cortisol concentrations decrease towards the distal parts of the hair (Kirschbuam et al. 2009). Contrary to the results of Kirschbaum et al. (2009) Bennett and Hayssen (2010) find a pattern of higher cortisol levels in the distal segments of dog hair.

Different parts of the body contain different distributions, combinations and densities of cutaneous glands (apocrine and eccrine sweat glands and sebaceous glands – Boucsein, 2012; Folk and Semken, 1991; Goldsmith, 2003; Sarkany and Gaylarde, 1968;

Schaller and Plewig, 2003; Thiele et al., 1999), which secrete directly onto the skin or into the hair follicle and may contribute differentially to the hormones sequestered in hair. For instance, eccrine sweat glands perform thermoregulatory functions in humans and non-human primates, whereas apocrine sweat glands perform thermoregulatory functions in other animal that sweat to stay cool (e.g. horses). Eccrine sweat glands tend to be widely dispersed over the human body, and open up directly onto the skin, whereas apocrine sweat glands in humans are found at their greatest densities in the axilla and anogenital regions, and secrete a more viscous form of sweat directly into the hair follicle (Folk and Semken, 1991; Goldsmith, 2003; Schaller and Plewig, 2003). Non-human animals may also have modified apocrine (usually sebaceous) glands which excrete pheromone containing substances used as olfactory cues (Bakshi, 2010; Johnson, 1973; Thiessen and Maureen, 1976; Zouboulis et al., 2008).

Additionally, different parts of the body may be exposed to the environment in different ways. For example, head hair is exposed to sunlight and a variety of temperatures and ambient humidity, whereas axial and axillary hair may rarely be exposed to the effects of sunlight, and experience constant temperatures and humidity. Sauve et al. (2007) show that although mean cortisol concentrations for hair from different parts of the scalp do not differ significantly; hair from the posterior vertex yielded the lowest inter-aliquot coefficient of variation (~15%) compared to other regions of the scalp (nape, anterior vertex, temporal etc., mean CV = ~30%), suggesting that hairs from the posterior vertex show greater homogeneity in growth cycle compared to other parts of the scalp. It is thought that this is because the proportions of hair in different life-cycle phases differ between regions of the scalp. Hairs from the posterior vertex show

greater uniformity in growth phase and therefore reflect similar periods of growth and endocrine activity, hence the lower inter-aliquot variability (Sauve et al., 2007). Similarly it has been shown that human hair cortisol concentrations determined from arm, leg and scalp hair differ significantly (Sharpley et al., 2010a, b). Hair from different body regions in the grizzly bear (*Ursus arctos)* has been shown to archive different concentrations of cortisol (Macbeth et al., 2010; also see baboons - Appendix 1). It is therefore important to control for anatomical region when collecting hair samples.

Whatever the exact mechanism of steroid deposition and incorporation into the hair shaft, a growing body of evidence shows that hair cortisol concentrations reflect averaged circulating levels of the hormone. A number of researchers have shown a clear correlation between cortisol (Accorsi et al. 2008; Bennett and Hayssen 2010; Davenport et al. 2006; Fourie and Bernstein, 2011b; Sauve et al. 2007), estradiol, progesterone and testosterone concentrations (Gleixner and Meyer 1997; Yang et al. 1998) determined from hair and averaged concentrations of these same hormones determined from serum, saliva, urine, and feces.

Research suggests that the extent of steroid deposition and incorporation into the hair shaft is related to its melanin affinity and lipophilicity (i.e. the ability of steroids to bond with- or attach to melanin and fats, oils and lipids). These extrapolations are made mainly based on research on the incorporation of drugs into hair (Gygi et al., 1996; Hubbard et al., 2000; Kikura and Nakahara, 1998; Kulaga et al., 2009; Slawson et al., 1996). Shen et al. (2009a) find that lipophilicity was not a major predictor of androgen and androgen metabolite incorporation into the hair shaft. They suggest the presence of hydroxyl groups and the physical properties of the molecule (particular structure) rather than

lipophilicity were the principal factors affecting hormone metabolite sequestration into the hair shaft. The presence of hydroxyl groups are known to inhibit the incorporation of drugs into hair (Nakahara et al. 1995; Nakahara and Hanajiri 2000). The physical properties of the molecule appear to affect its ability to bind to melanin. For instance Shen et al. (2009a) suggest that the physical characteristics (e.g. double bond groups) of certain drugs or steroids enhance their ability to be involved in electron-transfer reactions which may facilitate binding between electron rich molecules (drugs/steroids) and electron accepting structures on the surface of the melanin polymer.

Melanin levels may affect the incorporation of certain steroids into the hair shaft (e.g. Hold et al. 1996; Shen et al. 2009a), though evidence for a melanin effect on the sequestration of glucocorticoids has been somewhat inconsistent (e.g. Bennett and Hayssen 2010; Macbeth et al., 2010; Raul et al. 2004). Low melanin hair (i.e. white hair) should contain less hormone than melanin rich hair (i.e. black hair). However, this will depend on whether the hormone of interest has high melanin affinity (e.g. estradiol, testosterone – Sauer and Anderson, 1994; estradiol – Jacobsohn and Jacobsohn, 1992) or not. Hormones with low melanin affinity may more easily be incorporated and fixed in low melanin hair. Data are still lacking in this regard. Most studies comparing steroid levels in hair of different colors have done so using glucocorticoids (especially cortisol) (e.g. Bennett and Hayssen 2010; Raul et al. 2004) with little work on other steroids (estradiol, testosterone - Gleixner and Meyer 1997). With regard to cortisol, some researchers find no effect of hair color on cortisol concentrations archived in hair (e.g. Raul et al. 2004; Sauve et al. 2007); whereas Bennett and Hayssen (2010) are the only researchers to date that have reported higher cortisol concentrations in pheomelanin

(yellow) hair compared to eumelanin (black) hair in dogs. Only Gliexner and Meyer (1997) report hair color effects on testosterone and estradiol in the hair of cattle. They find that darker hair appears to sequester more testosterone than lighter hair, whereas the relationship is inconsistent with regard to estradiol. Raule et al. (2004) used human hair samples and the lack of clear differences may be related to the effects of hair treatment or washing, factors which they do not appear to address explicitly but are known to affect cortisol (Hamel et al., 2011; Li et al., 2012; Sauve et al. 2007) and drug metabolite concentrations in hair (Yegles et al. 2000). However, even when controlling for hair treatments (e.g. dying) Sauve et al. (2007) could not demonstrate differences in hair cortisol levels in hair of different colors. The results of these studies, although not always consistent, suggest that cortisol may show low affinity for melanin (or its incorporation into the hair shaft may be unaffected by melanin) and that testosterone's sequestration into the hair shaft is positively affected by melanin (consistent with Sauer and Anderson 1994 – melanin affinity of growth promoters).

Hair Growth Patterns and Hair Hormones

It is necessary to consider patterns of hair growth when quantifying hormone concentrations from hair. The nature of hair and its growth patterns may differ across regions of the body and among sexes and species. In humans, head hair grows continuously but asynchronously. This means that different hairs are in different phases of growth (anagen phase – active growth, catagen phase – maturation, telogen phase – resting, see Figure 3.2). At any time 15% of human scalp hair is in the telogen phase and

85% either in the anagen or catagen phases (Ebling, 1987; Harkey 1993; Saitoh et al., 1970). Hair on other parts of the human body also follows this kind of asynchronous growth although the percentages of hair in different growth phases may differ; additionally, human body hair growth is not continuous as is scalp hair (Ebling, 1987; Harkey 1993; Saitoh et al., 1970). It follows that hairs of different ages will record different periods of endocrine activity, and it is therefore recommended to sample hair from the same region of the body when samples are being collected from several individuals in order to control for variations in the composition of hair in different growth phases on different parts of the body and scalp (Bost, 1993; Sachs, 1997).

Small aliquots (i.e. aliquots consisting of a few hairs and therefore of low weight) of asynchronously growing hair will result in higher inter-aliquot variability than using large aliquots (i.e. aliquots consisting of a many hairs and therefore of higher weight – Fourie and Bernstein 2011b; Appendix 1). Evidence derived from the hair of an adult zoo kept *Pithecia pithecia* female suggests that hair growth in this species is also asynchronous (Fourie and Bernstein 2011b; Appendix 1). Low weight aliquots exhibit high inter-aliquot variability (CV = ~40% in 3mg samples) which declines as sample weight increases (CV=9% in 20mg samples) and the number of individual hairs included in the aliquot increases (10mg = 71 hairs, 20mg = 142 hairs). Based on the length and weight of the hairs, aliquot weights of more than 10mg are required in order to maintain coefficients of variation to around 10-15% for this species (Fourie and Bernstein 2011b, Appendix 1).

Conversely, small aliquots can be used if hair growth is synchronous, as intra-aliquot variability will be low because all individual hairs reflect the same period of endocrine

activity. Further, knowing whether animals experience synchronous versus asynchronous hair growth affects the types of questions that can be answered. For instance, it may be possible to trace seasonal changes in endocrine function in animals that exhibit synchronous hair growth and seasonal molts (e.g., eastern chipmunks – Martin and Reale 2008) but not in animals with asynchronous hair growth. Aliquot size is therefore essentially constrained by two factors: 1) the number of hairs needed to adequately estimate the mean hair hormone concentration for a population of hairs, and 2) the minimum raw weight of hair needed to extract enough hormone to be accurately quantified given the sensitivity of the specific assay system.

Very few data are available on hair growth patterns, rates of hair growth, or patterns of dermal gland distribution in animals other than humans (e.g. Bost, 1993; Boucsein, 2012; Sachs, 1997; Sarkany and Gaylarde, 1968; Thiele et al., 1999; Williams et al., 2011). Most detailed data on other animals come from those which are routinely used as model organisms in research (i.e. mice - Andreasen and Engelbreth-Holm, 1953; Mardaryev et al., 2010; Paus et al., 1989; Stenn, 2001 and rats - Ansell et al., 2010; Couchman et al. 1990; Durward and Rudall, 1949; Johnson, 1958; Plikus and Chuong, 2008, see also guinea-pigs – Dawson, 1930; Jackson and Ebling, 1971). Humans and guinea pigs show asynchronous ("mosaic") hair growth (e.g., Harkey 1993; Jackson and Ebling, 1971, see Figure 3.2), whereas rats exhibit synchronous hair growth (e.g., Plikus and Chuong, 2008, see Figure 3.2). No information could be found for hair growth patterns in non-human primates, and that which is reported here (Appendix 1) suggests asynchronous hair growth and it is expected that other primate species most will likely exhibit asynchronous hair growth as well.

Besides hair growth patterns there is a similar paucity of information on hair growth rates in non-human primates. In order to determine at least rough estimates of hair growth rates in a range of primates (*Allenopithecus nigroviridis*, *Chlorocebus chrysogaster*, *Theropithecus gelada*, *Gorilla gorilla gorilla*, *Hylobates gabriellae*, *Symphalanges syndactylus*), time to full regrowth of hair was visually monitored in a number of captive primates (11 individuals) at the San Diego Zoo from which hair samples had been collected (Fourie and Bernstein 2011b; Appendix 1). Hair growth rates were found to vary between 3.5 cm per month (e.g. *Theropithecus gelada*) to 0.66 ± 0.08 cm per month (e.g. *Cercocebus chrysogaster*). Hair regrowth to pre-sampling pelage conditions took between 3 and 7 months, with an average of ~5 months for cercopithecines (Appendix 1). The preliminary data suggest considerable variability within and between taxa in hair growth rates. Variation among taxa may suggest adaptive differences in hair growth biology related to the climates that the species evolved in. For instance *Theropithecus gelada* live in relatively cool climates at high altitudes, and long fast growing coats may be more advantages in such climates compared to the shorter slow growing coats of *Cercocebus chrysogatser*, a species confined to tropical areas. These data were collected from zoo-kept individuals and hair growth rates may be faster compared to wild conspecifics due to differences in dietary ecology. Hair growth rates in some individuals may also be affected by contraceptive implants placed immediately below the sample area (Phil Giralte, pers. comm.). However, these data *are* suggestive of the minimum temporal ranges of endocrine activity that may be archived in fully mature non-human primate hair. A sample consisting of multiple hairs can be conservatively considered to include information on cortisol production for multiple five month periods. A sample of

two hairs consisting of a hair which has been mature (and in the resting phase for some time, perhaps a few months) and a newly mature hair (i.e. just completed the growth and maturation phase) represent two different overlapping five month periods of endocrine activity, and cortisol extracted from such a sample therefore reflects basal cortisol production averaged over these two overlapping five month periods. In other words, any given hair sample (assuming an asynchronous hair growth pattern) probably reflects cortisol concentrations averaged over a time period in excess of five months. Using a simple model of hair growth, based on unpublished data of the proportions of hairs in different growth phases from the head of wild baboons, it is estimated that a sample of hair consisting of many individual hairs could represent an average of a time interval of as much as or in excess of 12 months (Chapter 5). Stable isotope research (Fourie et al. 2008, in preparation) using hair sampled from wild baboons show that multi-hair homogenized samples yield stable isotope signals of diet which correlate well with rainfall data collected over a 12-month period preceding sampling, matching well with the estimation of the simple hair growth model. Multi-hair homogenized samples may plausibly reflect endocrine activity over a similar period.

Temporal Stability of Steroid Hormones in the Hair Matrix

No research has explicitly examined the temporal stability of steroids sequestered in hair. However, at least three studies report some results regarding the concentration of steroids in the hair shaft in relation to time. Kirschbaum et al (2009) report that cortisol concentrations in human scalp hair declined along the shaft of the hair. Hair segments

that were older than six months showed asymptotically declining cortisol concentrations until the lower limit of detection by the assay was reached. They report that similarly low levels of cortisol were measured in hair that was approximately 20 years old. However, Macbeth et al. (2010) find no evidence of deterioration of cortisol in hair samples that were one year old or which were exposed to the external environment for 18 days. Webb et al. (2010) report the successful extraction and quantification of cortisol from the hair of Peruvian mummies (circa 1000 AD). They detect variation in cortisol concentrations along the hair shaft of individuals and among hair from different individuals. They argue that this variation reflects biological variation in cortisol concentration within and among individuals. They find no asymptotic decline in cortisol concentrations along the hair shaft and hair cortisol concentrations were not universally low. Similarly, Davenport et al. (2006), Bennet and Hayssen (2010) and Macbeth et al. (2010) do not find differences in hair cortisol concentrations between the proximal and distal portions of the hair shaft in macaques, dogs, and grizzly bears. Further, evidence shows that hair cortisol concentrations measured in hair collected from wild baboons over an eight year period, several decades ago, showed no time effect (see Chapter 4, 5 and 6).

These conflicting findings regarding the stability of steroids in the hair shaft may be due to differences in how the hair is treated during life. Kirschbuam et al. (2009) used human samples which in all probability have been washed or treated regularly. Water temperature, duration of soak, detergent and ultra violet light applied under experimental conditions appears to bleach the cortisol signal in hair (Hamel et al., 2011; Li et al., 2012). Regular hair treatment may systematically leach steroids sequestered in hair (Hamel et al., 2011; Li et al. 2012; Sauve et al. 2007; although see Yang et al. 1998).

These effects may be principally of interest with regard to human hair. Regardless of the effects of artificial treatment of hair on sequestered hormone concentrations, it stands to reason that hair must provide a particularly stable environment for steroids once they have become fixed in the hair matrix. Hair, especially in wild animals, is exposed to widely fluctuating temperatures, levels of moisture, precipitation and solar radiation, all of which can degrade or leach steroid molecules. Nonetheless a number of steroids and their metabolites have been successfully measured in the hair of wild animals, and these have been shown to reflect biological variation in circulating steroid concentrations. (Becshoft et al. 2010; Fourie and Bernstein, 2011a; Koren et al. 2002; Macbeth et al. 2010; Martin and Reale 2008). More research is required to explicitly establish how external factors may impact the preservation of steroid molecules in the hair shaft.

Cortisol: Extraction for quantification by ELISA/RIA

Most recent protocols for the extraction of cortisol from hair for quantification by ELISA appear to be variations on a protocol published by Davenport et al. (2006), though Koren et al. (2002) were already using a similar extraction protocol. The most novel aspect of the Davenport et al. (2006) protocol was the introduction of a washing step to remove exogenous contaminants and hormone-containing sebum using a solvent that minimized leaching of sequestered cortisol. Davenport et al. (2006) advocate the use of a large multi-carbon molecule alcohol. Larger molecules are less capable of penetrating the hair shaft, whilst being adequate to dissolve and remove exogenous steroids and oils. Davenport et al. (2006) found that rinsing hair samples using isopropanol (C_3H_8O)

removed exogenous contaminants without significant leaching of endogenous cortisol. Optimal extraction of cortisol was then achieved by incubating hair samples in methanol (CH_4O), a single carbon alcohol. If large samples are available, the samples can be homogenized using a ball mill and aliquoted out (e.g. D'Anna-Hernandez et al., 2011; Davenport et al. 2006; Dettenborn et al., 2010; Fairbaink et al., 2011; Hamel et al., 2011; Kirschbaum et al. 2009; Raul et al., 2004). Decreasing the particle size increased the amount of cortisol extracted (Davenport et al. 2006). Alternatively, hair samples can be reduced by mincing using a pair of scissors (Fourie and Bernstein, 2011a; Gow et al., 2011; Koren et al., 2002; Sharpley et al., 2010a, b; Thomson et al., 2010; van Uum et al., 2008). Some authors also include a short sonication step (30min) (e.g. Anielski, 2008; Kalra et al., 2007; Koren et al. 2002; also see Fourie and Bernstein, 2011a and Appendix 1. Incubation times are typically between 16 and 24 hrs at ~50^{o}C (see also Gow et al. 2010 for summary). Independent assessment of cortisol extraction showed that incubation in a sonic bath for four hours can also significantly increase cortisol yield (based on techniques employed in food science – e.g. Albu et al., 2004; Shinor et al. 2004; Wu et al., 2001), while sonication for longer periods may decrease measurable cortisol, possibly due to prolonged exposure to high temperatures (Appendix 1).

Advantages of Hair over Traditional Hormone Bearing Substrates

Hair has a number of advantages over traditional hormone bearing substrates which make it attractive to researchers interested in time averaged measures of circulating steroid concentrations. First, a single hair sample can be used to determine averaged basal

circulating cortisol concentrations (Accorsi et al. 2008; Bennett and Hayssen 2010; Davenport et al. 2006; Fourie and Bernstein, 2011b; Sauve et al. 2007). Research has shown that within-individual variation in hair cortisol concentration over time remains relatively stable suggesting that hair cortisol concentrations reflect stable endocrine phenotypes (Stalder et al., 2011). To derive averaged/basal measures of endocrine activity from traditional substrates, multiple samples collected over long periods of time are needed. Hormone concentrations in some substrates, like blood and saliva, are especially sensitive to circadian variation in hormone concentrations (e.g. cortisol - Daly et al., 2011; Santiago et al., 1996; Strahler et al., 2010; Turtin and Deegan 1974; van Cauter et al., 1996). In order to determine basal hormone concentrations or to characterize hormone phenotypes from these substrates, multiple samples need to be collected at the same time of day; this can be especially challenging under field conditions. Additionally, substrates which record short term variation in hormone concentrations are sensitive to acute fluctuations in hormone concentrations resulting from the stress of trapping, sedation and sampling (Anestis et al., 2006; Crokett et al., 1993; Delehanty and Boonstra, 2009; Delgiudice et al., 1990; Lynn and Porter, 2008). Hair hormone concentrations are not affected by circadian variations or short term acute variation resulting from external stressors. Basal cortisol concentrations and phenotypic endocrine characterizations (e.g. Dettenborn et al. 2010; Fairbanks et al. 2011; Kirschbuam et al. 2009; Stalder et al. 2010; Stuedte et al. 2010) can be made using a single hair sample. This also means that hair is not an appropriate substrate if research questions focus on physiological reactivity and short term effects.

A second advantage to using hair rather than traditional substrates is the lack of special needs for storage and shipment. Other types of samples need to be preserved in alcohol, immediately dried or frozen upon collection, and need to be shipped and stored under specific conditions in order to prevent deterioration of hormones and their metabolites. Salivary, serum, fecal and urine glucocorticoid concentrations are also variably sensitive to changes in temperature and pH (Chen et al., 1992; Garde and Hansen, 2005; Gouarne et al., 2004; Kubasik et al., 1982; MacKichan and Jusko, 1979; Olson et al., 1981). The stability of hormones (of at least cortisol – Chapter 4 and 5; Macbeth et al., 2010; Webb et al., 2010) in the hair matrix means that previously overlooked sources of stress information can now be accessed from archived collections, or from trappings planned for other purposes (e.g. population monitoring, radiocollaring, veterinary intervention). It is now possible to examine cortisol in historical populations (possibly even archeological populations – Webb et al., 2010) by obtaining museum and biomaterials collections. Hair samples can also be simply collected and transported from remote areas at little cost by non-specialists suggesting applications for conservation and stress monitoring of animal populations (see Chapter 8). For instance, in a recent study of polar bear (*Ursus maritimus*) populations in East Greenland, researchers collected hair samples provided by subsistence hunters in order to assess the potential of the technique in the physiological monitoring of wildlife populations (Bechshoft et al. 2011).

Hair can also be collected non-invasively using or adjusting existing techniques for the non-invasive collection of hair for molecular research (Améndola-Pimenta et al., 2009; Bremner-Harrison et al., 2006; Frantz et al. 2004; Kendall and McKelvey, 2008; Scheppers et al. 2007; Valderrama et al. 1999). Techniques using adhesive coated

substrates which animals (specifically primates) are enticed to rub against or handle resulting in the capture of hairs typically only collect few individual hairs (Valderama et al., 1999; see also Améndola-Pimenta et al., 2009 for glue-dart method). Especially in larger animals with long, thick coarse hairs, only a few hairs may be needed for the quantification of cortisol concentrations (e.g. five hairs – grizzly bears – Macbeth et al., 2010). Smaller animals, especially ones that live in burrows, holes, nests or can be enticed to maneuver through a restricted space forcing animals to rub against hair snares, which can be used to collect enough hair for cortisol quantification (Bremner-Harrison et al., 2006; Frantz et al. 2004; Scheppers et al. 2007, though the technique has also been adapted to large and medium sized non-burrowing animals – Clevenger and Sawaya, 2010; Kendall and McKelvey, 2008). Such approaches may be of specific interest to researchers interested in monitoring stress in wildlife populations (e.g. Bechshoft et al. 2011; Macbeth et al., 2010; Martin and Reale, 2008; Sheriff et al., 2011).

Summary

The quantification of endogenous steroids in hair using ELISA methods is a relatively new technique finding rapid application in both human and animal studies. Current research has focused principally on cortisol sequestered in hair. Hormones enter the hair shaft through diffusion from blood supplying the follicle during hair growth and glandular secretions which may cover the hair. Hormones sequestered in hair therefore reflect circulating levels averaged over the growth period of the hair. Hair growth patterns and hair growth rates significantly affect the length of the time period averaged

in a sample of hair. Cortisol is stable in the hair shaft over long periods of time and can be extracted using simple alcohol extraction methods and can be quantified using ELISA methods. Hair as a hormone bearing substrate has several advantages over blood, saliva, urine and feces as an averaged measure of endogenous circulating steroid (specifically cortisol) concentrations, which can be measured using a single sample, hair does not require specific storage and shipment conditions, and hair can be collected non-invasively.

Tables and Figures

Figure 3.1: Diagram of the hair follicle, the blood supply to the hair follicle, the sebaceous gland, apocrine sweat gland and eccrine sweat gland. The diagram demonstrates the relationship between the hair shaft and vascular and glandular sources of hormones which are sequestered into the hair shaft. Drawing by Cecilia Reifschneider – Pigment Art Studio (pigmentartstudio.com).

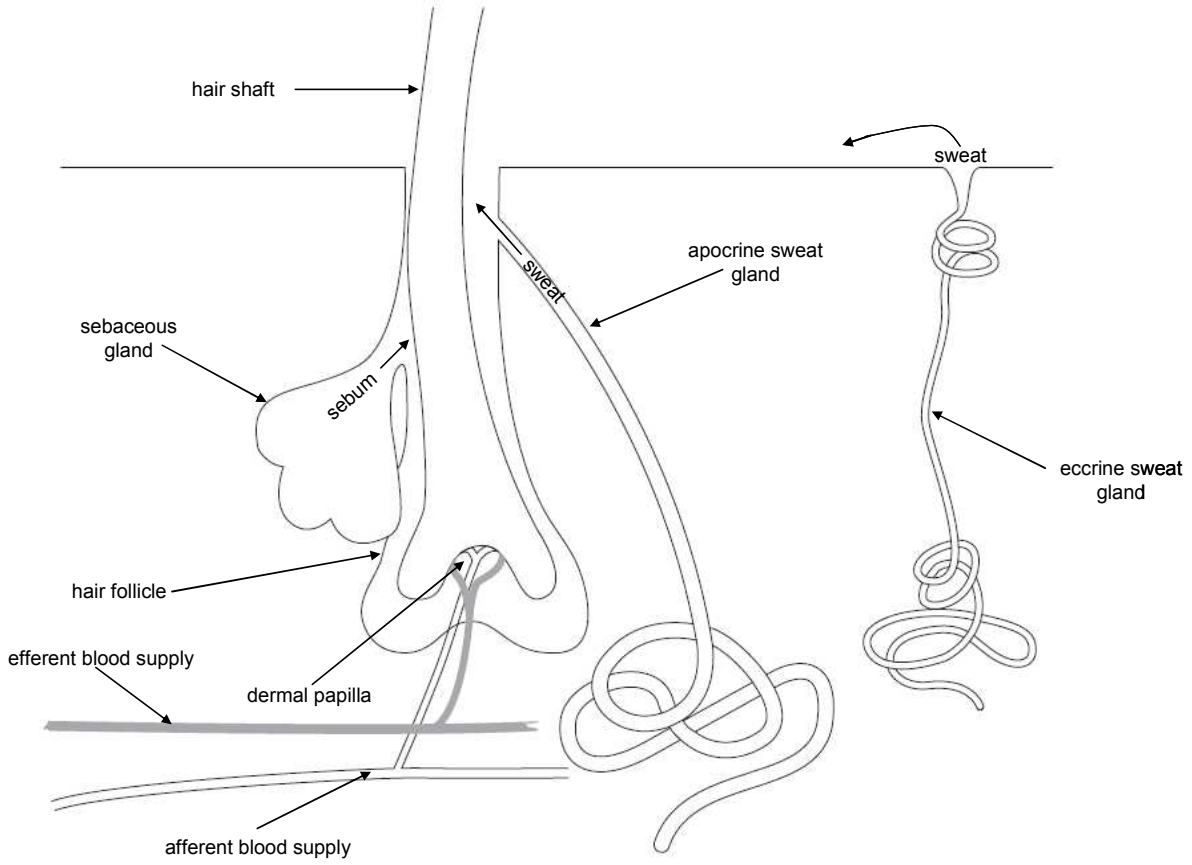
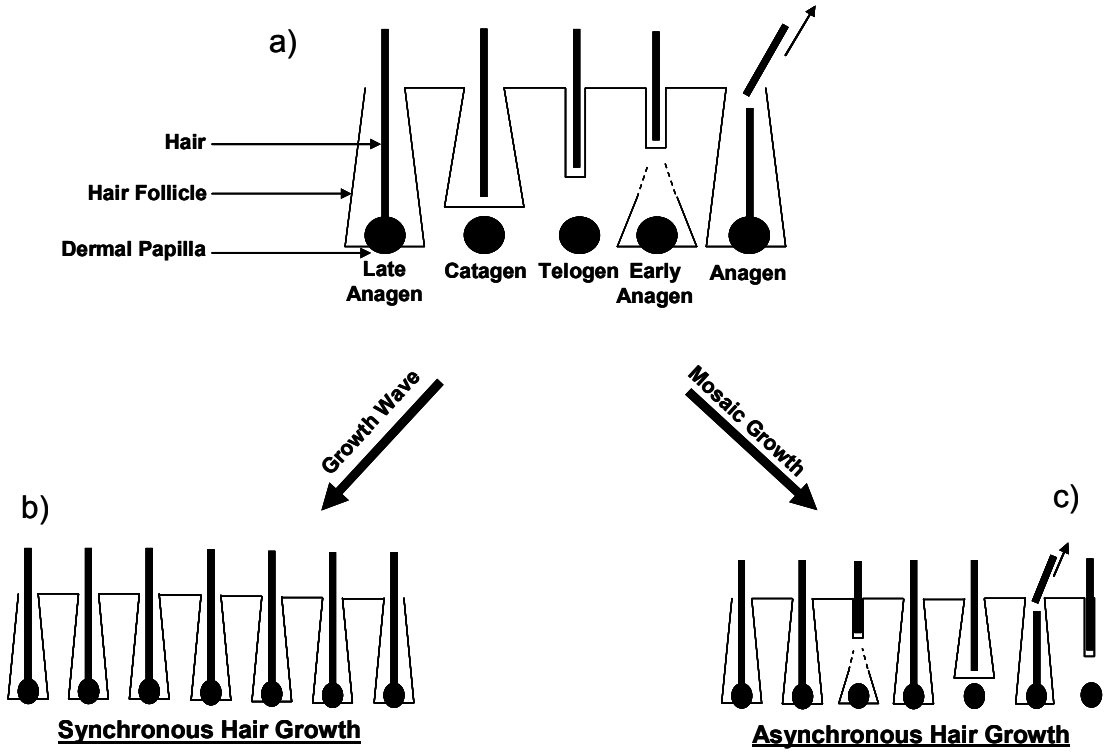


Figure 3.2: Schematic diagram of the hair growth cycle (a) and the two basic hair growth patterns (b and c). Diagram (a) shows the different stages in the hair growth cycle, while

diagram (b) shows that in a synchronous hair growth pattern the growth phases of all hairs are harmonized, while diagram (c) shows that a mosaic hair growth pattern results in individual hairs being in different hair growth stages.



Chapter 4: Hair cortisol levels track phylogenetic and age related differences in hypothalamic-pituitary-adrenal (HPA) axis activity in non-human primates

ABSTRACT

Hair has been shown to archive a uniquely time averaged signal of endocrine activity, and holds attractive advantages for both laboratory and field research. Prior research has explored the potential of hair hormone analysis to examine hormone-behavior relationships. To date, no research has focused on the potential of the technique to investigate age-related changes or taxon differences in endocrine function. It is known that non-human primate infants of many taxa exhibit high cortisol levels after parturition, which rapidly decline with age. It has also been shown that hypercortisolism generally characterizes platyrrhine (New World monkey) endocrine function. These endocrine trends have been characterized using cortisol levels determined from serum, plasma, and feces. Here I tested whether cortisol levels determined from hair recover similar phylogenetic and age related patterns in endocrine function in non-human primates. In order to test whether hair cortisol reflect infant hypercortisolism with significant age-related decline, hair cortisol levels were measured in samples from wild vervet monkeys (*Chlorocebus aethiops*) and captive Guinea baboons (*Papio papio*), ranging in age from infants through juveniles. Further, in order to test whether platyrrhines exhibit significantly higher hair cortisol levels compared to strepsirrhines and catarrhines, and therefore faithfully recover similar signals as more traditionally used substrates (e.g., serum) do, hair cortisol levels were quantified in adult female hair samples collected from

a broad range of nonhuman primate taxa. Results confirm that hair cortisol levels accurately reflected known phylogenetic and age related patterns of circulating cortisol levels. Therefore, these results suggest that hair may be an ideal hormone-bearing substrate for research focused on the examination of population endocrine profiles, cross-sectional studies of endocrine function, and taxon variation in hormone levels, as well as stable behavioral trends.

INTRODUCTION

Over the past few decades, a number of studies have reported the extraction and measurement of endogenous glucocorticoids and androgens from hair (e.g. Gleixner and Meyer, 1997; Koren et al., 2002; Lui et al., 1988). Refined protocols for use in enzyme linked immunosorbent assay (ELISA) procedures have also recently been published (e.g. Davenport et al., 2006). Lipid solubility and low protein binding favor the incorporation of the unbound (free) fraction of steroids into the hair shaft over the protein-bound fraction (Raul et al., 2004). It is thought that the free fraction of the hormones in circulation diffuses into the hair follicle from neighboring capillaries and is incorporated into the hair shaft during growth (Anielski, 2008; Cone, 1996; Villain et al., 2004). Therefore, hormone levels quantified from the hair shaft reflect averaged circulating levels accumulated over the growth period of the hair. Various studies have confirmed that the concentration of hormone (progesterone – Yang et al., 1998; testosterone and estradiol – Lui et al., 1988; Yang et al., 1998; cortisol – Accorsi et al., 2008; Davenport et al., 2008) measured in hair is significantly correlated with averaged concentrations of the same hormone calculated from multiple samples from more traditionally-used substrates, collected over the corresponding period of hair growth (plasma – Gleixner and Meyer, 1997; serum – Yang et al., 1998; saliva – Davenport et al., 2006; feces – Accorsi et al., 2008).

One of the principal advantages of using hair is that only a single sample is needed to provide an averaged measure of endocrine activity. Traditional substrates (e.g. serum, saliva, feces) are acutely sensitive to changes in endocrine function, which may be

induced through trapping, handling, or environmental disturbance associated with sample collection. Additionally, circadian patterns of endocrine function may also affect the measured hormone levels. Therefore, multiple samples may be required to establish basal hormone profiles. Hair is therefore not suited to studies of short-term hormonal fluctuations or responses but is particularly suited for tracking chronic hormone levels. Another major advantage of hair as a substrate, especially to field researchers, is that hair can be stored and transported under ambient non-specific conditions. It appears that steroid hormones (at least cortisol) may be relatively stable in the hair matrix under a range of conditions for a long period of time (~1000 years – Webb et al., 2010).

These properties of hair are of special interest to researchers interested in understanding the hormonal correlates of stable behavioral patterns (Clara et al., 2008; Martin and Reale, 2008) and of life history, developmental and biological differences among individuals and populations.

Here I report novel hair hormone data, derived from a diverse non-human primate sample. These data are used to examine hair as a potential tool to be employed in investigations of developmental and biological differences in endocrine function. Specifically, I tested whether hair cortisol levels faithfully reflect known age-related and phylogenetic variation in endogenous cortisol levels in non-human primates in order to demonstrate the utility of hair as a stable, durable (e.g. Webb et al., 2010), and potentially non-invasively collected (e.g. Frantz et al., 2004; Scheppers et al., 2007; Valderama et al., 1999) substrate to examine several aspects of development and biology in both wild and captive animals. It is well known that hormone levels change during ontogeny. In particular, among non-human primates, the infant and juvenile period is characterized by

higher circulating levels of glucocorticoids (marmosets –Pryce et al., 2002; baboons – Castracane et al., 1981; Gesquiere at al., 2005; western lowland gorillas – Stoinski et al., 2002; mountain gorillas – Robbins and Czekala, 1997; chimpanzees – Anestis and Hasselschwert, 2006). Glucocorticoid profiles that span infancy through juvenile phases are particularly well described in baboons and marmosets (marmosets – Pryce et al., 2002, baboons – Castracane et al., 1981; Gesquiere at al., 2005). These studies showed that infant baboons and marmosets have higher glucocorticoid levels than all older age classes, and that this rapidly declines with age. If hair archives circulating levels of cortisol, then similar age-related variation in hair cortisol levels is expected. Therefore, the first aim of this paper was to measure cortisol levels in hair samples derived from a captive population of Guinea baboons (*Papio papio*) and wild populations of vervet monkeys (*Chlorocebus aethiops*) spanning young infants to juveniles. These data are used to examine whether hair cortisol levels vary predictably with age. Specifically, it was predicted that infants have significantly higher hair cortisol levels than older age classes and that hair cortisol levels show a decline with age.

Other biological differences in hypothalamic-pituitary-adrenal (HPA) axis function also exist among primates. Circulating cortisol levels are known to differ among several higher taxonomic groups of primates: the strepsirrhines (i.e. lemurs), the catarrhines (i.e. Old World monkeys, apes and humans), and platyrrhines (i.e. New World monkeys) (Chrousos et al., 1982; Pugeat et al., 1984). Specifically, platyrrhine taxa are generally characterized by hypercortisolism relative to strepsirrhine and catarrhine taxa. Data also suggest that strepsirrhines have somewhat lower cortisol levels than catarrhines, although this difference has not been reported as statistically significant (Chrousos et al., 1982). It

appears that platyrrhine cortisol receptors are relatively insensitive to cortisol, and therefore they maintain exceptionally high circulating levels of the hormone (Chrousos et al., 1982). Variation among taxa has also been noted, although the basis for these differences has not been extensively investigated (e.g. Chrousos et al., 1982). Chrousus et al. (1982) showed that humans have low plasma cortisol levels relative to chimpanzees and Old World monkeys, and that northern owl monkeys (*Aotus trivirgatus*) have plasma cortisol levels that are nearly six times lower than those of other platyrrhines (Chrousos et al., 1982). Therefore, the second aim of this paper was to measure cortisol levels in the hair of adult female animals from several strepsirrhine, catarrhine, and platyrrhine taxa. These data are used to test the prediction that platyrrhines have higher hair cortisol levels than strepsirrhines and catarrhines. If hair cortisol recovers similar signals as circulating cortisol, then these data should mirror taxonomic variation in HPA axis activity and elevated cortisol levels in platyrrhines relative to strepsirrhines and catarrhines.

MATERIALS AND METHODS

Animals and Collection of Hair Samples

Approximately 50 to 100mg of hair was shaved (using hair clippers) or cut (using scissors) from the back or shoulder of each individual. Hair was cut as close to the skin as possible without causing injury. All hair samples were stored and shipped in labeled paper envelopes under ambient non-specific conditions.

Hair samples used to test for age-related changes in cortisol levels were obtained from male and female wild caught vervet monkeys (*Chlorocebus aethiops*) and captive Guinea baboons (*Papio papio*) (Table 4.1). The vervet samples derive from an unrelated research initiative during which individuals from several free-ranging populations across South Africa were trapped and anesthetized, and various biometric data and biological samples were collected (see Brett et al., 1982; Turner, 1981; Turner et al., 1997) for details pertaining to trapping, anesthetization, and data collection procedures). Animals were aged based on dental eruption and dental wear data and assigned to one of nine age categories (see Turner et al., 1997). Hair from zoo kept Guinea baboons was supplied by the Museum de Besancon, France. Animals were sampled opportunistically during annual veterinary exams. Exact ages were available for all individuals. Only infant and juvenile phases (i.e. vervets: age class 1 to 5, and Guinea baboons: 0-72 months) are used for the present analysis. Guinea baboons are divided into three age categories for statistical comparison (infants – 0-24 months, young juveniles – 24 -48 months and older juveniles – 48-72 months, see Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000).

Hair samples used to compare hair cortisol levels among platyrrhine, strepsirrhine, and catarrhine taxa were derived from a number of sources (Table 4.2). Although few data exist showing systematic differences in male and female primate cortisol levels, preliminary analyses of a larger data set suggest that significant sex differences in HPA axis activity may exist in some taxa (Chapter 5). In order to control for the effect of possible sex differences only adult females are used in the comparison of hair cortisol levels among higher taxonomic groups. Hair samples of Nancy Ma's night monkeys (*Aotus nancymaae*), ring-tailed lemurs (*Lemur catta*), white-faced sakis (*Pithecia*

pithecia), Coquerel's sifakas (*Propithecus verreauxi coquereli*), and cotton-top tamarins (*Saguinus oedipus*) were clipped from cadavers originating from the Duke Lemur Center and Santa Ana Zoo. Hair samples of golden-bellied mangabeys (*Cercocebus chrysogaster*) and western lowland gorillas (*Gorilla gorilla gorilla*) were opportunistically collected during annual veterinary exams at the San Diego Zoo. Collaborating researchers facilitated the opportunistic collection of hair samples during the course of their research involving common marmosets (*Callithrix jacchus*), tufted capuchins (*Cebus apella*), and patas monkeys (*Erythrocebus patas*) housed at the NIH Primate Center in Poolesville, Maryland. Specifically, marmosets and capuchin monkeys are used in behavioral studies and have identifying patches shaved onto their backs; whenever these identifying marks were made the hair was collected. Patas monkey hair samples were collected during bi-annual tuberculosis tests and veterinary examinations. Wild chacma baboon (*Papio ursinus*) samples were provided by the National Zoological Gardens of South Africa. These hair samples were collected opportunistically during the course of an unrelated field study. Animals were trapped and anesthetized using procedures similar to those outlined in Rogers and Kidd (1993).

Extraction of Cortisol from Hair

10-mg aliquots were weighed out into labeled 5-ml borosilicate culture tubes. Samples were twice washed in 3ml isopropanol (C_3H_8O) for 3 minutes (after (12)) and let dry in a fume hood for 24hrs. The samples were then minced into 1-2mm pieces using a pair of surgical scissors. The minced samples were then extracted in 4ml of HPLC grade

methanol (CH_4O). Samples were incubated in a sonic bath at 50Hz at 55^oC for 240min, removed, and incubated on a reciprocating shaker at 100rpm at room temperature for a further 20 hours.

At the end of extraction, the tubes were centrifuged at 4000rpm at 4^oC for 5 minutes. 3.5ml of the extract was then aliquoted into labeled polypropelene tubes and allowed to dry under a stream of air in a fume hood. The dry extract was then reconstituted in successively smaller volumes of methanol (2ml, 1ml, and 500µl) and dried down under air in order to concentrate the dry extract at the bottom of the tube. Finally, the dry extract was reconstituted in 500µl of ELISA phosphate buffer solution (pH 7) and stored at -20^oC. The mean extraction efficiency for all taxa was 95±5%.

Cortisol Assay

Hair cortisol concentrations were quantified using commercially produced ELISA kits designed to measure the free cortisol in human saliva (ALPCO Diagnostics, Salem, NH). 50µl of reconstituted sample was run in duplicate as directed by the assay protocol. When needed, samples were diluted (either 1:8 or 1:16 dilutions were used for common marmoset, tufted capuchin and cotton-top tamarin samples) so they could be read in the optimal range on the standard curve. Sample results were converted from ng/ml to ng/mg of hair. Mean intra-aliquot variance in cortisol levels for all taxa was 11±7% and had a range of 2% to 21%.

The manufacturer reported cross-reactivity as follows: cortisol = 100%, prednisolone = 13.6%, corticosterone = 7.6%, deoxycorticosterone = 7.2%, progesterone = 7.2%,

cortisone = 6.2%, deoxy cortisol = 5.6%, pednisone = 5.6%, dexamethasone = 1.6%. The range of detection for this kit is 1-100 ng/ml. Mean intra- and inter-assay coefficients of variation were less than 10% (n = 11) and 15% (n = 23) respectively. The assay produced a mean recovery value for all taxa of 88±6%. The immunospecificity of the assay for hair cortisol for each of the sample taxa was demonstrated by showing significant parallelism between sample dilution curves (neat, 1:2, 1:4, 1:8, 1:16) and a standard dilution curve (modified t-test for comparing linear regressions, $p<0.05$, Zar, 1999).

Statistical Analysis

Cortisol values were not normally distributed. Therefore, non-parametric techniques were employed in the analysis of the data. When necessary, cortisol data were log transformed in order to facilitate graphical representation. Statistical comparisons between age classes and taxon groups were made using the untransformed cortisol data. Although Castracane et al. (1981) describe some differences in male and female yellow baboon glucocorticoid levels with age, they do not find that these differences are significant. Their findings also suggest that both sexes show broadly similar age-related variation in glucocorticoid levels. In order to allow greater statistical power for testing the prediction that elevated hair cortisol levels in infants and an age-related decline in cortisol irrespective of sex, male and female values were pooled, however descriptive statistics for each sex were calculated separately.

To graphically assess the relationship between age and hair cortisol levels, locally weighted scatter-plot smoothing (LOWESS) was applied. LOWESS smoothing is a non-parametric model that fits a smoothed non-linear regression model to localized subsets of the data. In order to test the prediction that infants have higher hair cortisol levels than older age classes, the relationship between age and hair cortisol concentrations were analyzed using a linear regression analysis and age categories are statistically compared using a Kruskal-Wallis analysis of variance (KW-ANOVA). Due to the nature of the sample set, the Conover-Inman test (CI) was used to make post hoc pairwise comparisons (Day and Quin, 1989; Spurrier, 2006). In order to test the prediction that platyrrhines have higher hair cortisol levels than strepsirhines and catarrhines, the three groups were compared using a KW-ANOVA; pairwise comparisons were made using a CI test. An alpha level of 0.05 was set for all statistical tests, which were run on the SYSTAT 13 statistical software package (Systat, Inc., San Francisco, CA).

RESULTS

Hair Cortisol in Non-Human Primates

The LOWESS fit shows that both male and female Guinea baboons experience a dramatic decline in hair cortisol levels from infancy through the early juvenile stages (0 to ~30 months), after which hair cortisol levels plateau (Figure 4.1). Similarly a LOWESS fit to the vervet data shows that both males and females show a steep decline in hair cortisol levels from age class 1 to 3, after which males, but not females, appear to

show an increase in hair cortisol levels during age classes 4 and 5 (Figure 4.2). Mean and standard deviation values for hair cortisol levels for all sex, age, and taxon categories are presented in Tables 4.1 and 4.2. Linear regression analyses find a significant negative relationship between age and hair cortisol concentrations for vervets ($F_{1,47} = 14.593$, adjusted $r^2 = 0.221$, $p<0.001$) and Guinea baboons ($F_{1,17} = 24.759$, adjusted $r^2 = 0.569$, $p<0.001$; see Figures 4.1 and 4.2). Both vervet and Guinea baboon age classes differed significantly from one another (vervet – KW-ANOVA: $H_{4, 49} =15.9$, $p<0.005$; Guinea baboon – KW-ANOVA: $H_{2, 19} =9.1$, $p<0.05$). The post hoc CI test showed that age class 1 vervets (i.e. <6 months old; n=7, 3.09±1.54ng/mg) had significantly higher hair cortisol levels than age classes 2 (n=13, 1.33±0.38ng/mg, $p<0.05$), 3 (n=5, 0.91±0.13ng/mg, $p<0.001$), 4 (n=15, 1.18±0.40ng/mg, $p<0.005$) and 5 (n= 9, 1.19±0.45ng/mg, $p<0.005$). The CI test also showed that age class 2 had significantly higher hair cortisol levels than age class 3 ($p<0.05$). No significant differences in hair cortisol levels were found for any other pairwise comparisons of vervet age classes. A CI test found that age class 1 (n=8, 3.19±1.35ng/mg) Guinea baboons had significantly elevated hair cortisol levels compared to both age classes 2 (n=6, 1.75±0.37ng/mg, $p<0.01$) and 3 (n=5, 0.61±0.35, $p<0.01$). The CI test found no significant difference between age class 2 and age class 3 ($p=0.4$).

A KW-ANOVA showed that significant differences existed between strepsirrhines, catarrhines, and platyrrhines (KW-ANOVA: $H_{2, 83} =40.5$ $p<0.005$). A CI test showed that platyrrhines (n=20, 44.39±45.39 ng/mg) had significantly higher hair cortisol levels than strepsirrhines (n=5, 0.76±0.44 ng/mg, $p<0.001$) and catarrhines (n=68, 1.31±0.56 ng/mg, $p<0.001$). The CI test also showed that strepsirrhines had

significantly lower hair cortisol levels than catarrhines ($p<0.05$). Hair cortisol levels reflect a great range of variation among the non-human primate taxa analyzed (see Figure 4.3).

DISCUSSION

The results of these analyses show that age-related cortisol profiles recovered from the hair of Guinea baboons and vervets are similar to those reported for feces and plasma in yellow baboons and marmosets (Castracane et al., 1981; Gesquiere et al., 2005; Pryce et al., 2002). Infants in both the vervet and Guinea baboon samples had significantly higher hair cortisol levels than older age classes and hair cortisol levels declined with increasing age. Although hair cortisol results are broadly similar to those reported for yellow baboons and marmosets, some differences can be noted. An increase in male vervet hair cortisol levels is apparent in age classes 4 and 5, roughly corresponding to 1.5 to 2.5 years of age. Although hair cortisol levels for age class 4 and 5 individuals are not significantly higher than age classes 2 and 3, it may signal the start of a trend associated with male sexual maturation. Whitten and Turner (2009) report that male vervets as young as two years old can have fully descended testes and can start reproducing by 3.5 years of age. Male reproductive maturation is associated with increasing testosterone levels (Nelson, 2005), which in turn may be associated with the onset of reproductively relevant behaviors, such as dominance competition and male-dispersal (e.g. Beehner et al., 2009, 2006; Woodroffe et al., 1995). Male-male competition and dispersal could be stressful for young animals, and therefore are likely to

be associated with an elevation in cortisol levels (e.g. reproductive competition and male-dispersal – Bergman et al., 2005). The onset of such behaviors may therefore explain a gradual increase in male cortisol levels. Further investigation overlaying the timing of life history milestones onto patterns of endocrine activity may be useful in assessing the reproductive and behavioral ontogenetic correlates of the observed elevation in male cortisol levels in this species and may also be useful in comparing patterns among different populations (e.g. Beehner et al., 2009; Turner et al., 1997; Whitten and Turner, 2009).

Known differences in endocrine function between platyrrhines, strepsirrhines, and catarrhines are reflected in hair cortisol levels and are consistent with previous reports based on serum and fecal cortisol levels (Chrousos et al., 1982; Pugeat et al., 1984). Although previous comparisons show that strepsirrhines tend to have lower cortisol levels than catarrhines (Chrousos et al., 1982), the results presented here suggest that strepsirrhines have significantly lower hair cortisol levels. Further research is needed to confirm this result. Additionally, Chrousos et al. (1982) report relatively low cortisol levels for northern night monkeys when compared to other platyrrhines. Northern night monkeys had plasma cortisol levels more similar to those typically seen in catarrhines. Cortisol levels measured in Nancy Ma's night monkey hair samples suggest comparable results.

Platyrrhine hypercortisolism is thought to be due to a general tissue resistance to cortisol, specific to this clade. Additionally, Pugeat et al. (1984) have shown that cortisol-binding-globulin (CBG) in new world primates had a much lower binding capacity compared to that of old world primates resulting in higher circulating levels of free-, bio-

available cortisol. It should also be noted that differences in CBG excretion, metabolism and clearance among taxa, sexes and age cohorts may also account for differences in the free cortisol measured in hair. CBG levels may respond dynamically to stress and may differ in the temporal span of their effect to that of cortisol. Davenport et al. (2008) have shown that decreased levels of free cortisol may not necessarily reflect decreased adrenocortical function, but may be as a result of increased CBG levels. These caveats are important to note for all studies focusing on variation in free cortisol levels and need to be further explored to examine the sources of higher level variation (e.g. taxon) in cortisol levels that cannot be directly related to stress.

CONCLUSIONS

A recent resurgence in interest in hair as a hormone-bearing substrate has resulted in protocols being validated in a number of mammalian species, including macaques (Davenport et al., 2006), humans (e.g. Dettenborn et al., 2010), hyraxes (Koren et al., 2002), cats, dogs (e.g. Accorsi et al., 2008), marmosets (Clara et al., 2008), ground squirrels (Martin and Reale, 2008), and bears (e.g. MacBeth et al., 2010). These studies have also shown that hair can be used to study the endocrine correlates of a range of behavioral characteristics (e.g. Clara et al., 2008; Dettenborn et al., 2010; Martin and Reale, 2008) and pathological conditions (Stalder et al., 2010; Stuedte et al., 2010). The results presented here further validate the technique in a taxonomically broad range of non-human primates from both wild and captive populations. Importantly, this study demonstrates the potential of biomaterials collections (e.g. cadavers, hair collections –

museums/universities) as potential sources of endocrine information. The results show that besides behavior and pathology, the technique is also suited for examinations of physiological differences among taxa and changes in endocrine function with age. Traditionally, such studies require repeated sedation of animals to collect biological samples or repeat collection of fecal or urine samples in order to determine time averaged estimates of hormone levels. Such basal hormone levels can be determined from a single hair sample, circumventing the often logistically and financially demanding nature of repeat sampling. When research is focused on stable behavioral trends, long term effects or cross-sectional examination of population endocrine profiles, hair may be an ideal candidate substrate from which to determine cortisol or other steroid hormones.

Tables and Figures

Table 4.1: Table showing the number of individuals for Guinea baboons and vervet monkeys by sex and age class and the mean ± one standard deviation (STD) hair cortisol levels for each age sex group. All hair cortisol values are reported ng/mg.

	Age Class						
Guinea baboons	1 (12-24 months)	Hair Cortisol (ng/mg)	2 (24-48 months)	Hair Cortisol (ng/mg)	3 (48-72 months)	Hair Cortisol (ng/mg)
Male	5	3.1±1.23	4	1.94±0.27	2	1.56±0.45
Female	3	3.34±1.82	2	1.38±0.16	3	1.64±0.37
Total	8	3.19±1.35	6	1.75±0.37	5	1.61±0.35

	Age Class									
Vervets	1 (<0.5 years)	Hair Cortisol (ng/mg)	2 (0.5-1 year)	Hair Cortisol (ng/mg)	3 (1-1.5 years)	Hair Cortisol (ng/mg)	4 (1.5-2 years)	Hair Cortisol (ng/mg)	5 (2-2.5 years)	Hair Cortisol (ng/mg)
Male	3	3.60±2.04	7	1.50±0.43	4	0.90±0.14	8	1.34±0.48	7	1.28±0.48
Female	4	2.71±1.22	6	1.12±0.18	1	0.9	7	1.00±0.17	2	0.88±0.18
Total	7	3.09±1.54	13	1.33±0.38	5	0.91±0.13	15	1.18±0.40	9	1.19±0.45

Table 4.2: Table showing the number of individuals and origins of all strepsirrhine, catarrhine, and platyrrhine taxa used in analyses and mean ± one standard deviation (STD) hair cortisol levels for each taxon. All hair cortisol values are reported ng/mg.

Taxa	n	Hair Cortisol (ng/mg)	Wild/Captive	Origin
Strepsirrhines				
Lemur catta	3	0.84±0.57	captive	Duke Lemur Center
Propithecus verreauxi coquereli	2	0.63±0.30	captive	Duke Lemur Center
Total	5	7.6±4.40		
Cattarrhines				
Chlorocebus aethiops	23	1.26±0.39	captive	multiple locations, South Africa
Cercocebus chrysogaster	3	0.72±0.11	wild	San Diego Zoo
Erythrocebus patas	8	1.58±0.32	captive	NIH Primate Center
Papio papio	12	1.93±0.32	captive	Museum de Besancon, France
Papio ursinus	7	1.10±0.12	wild	Suikerbos Rand, South Africa
Gorilla gorilla gorilla	5	0.22±0.07	captive	San Diego Zoo
Total	68	1.31±0.56		
Plattyrrhines				
Aotus nancymaae	2	1.82±1.44	captive	Santa Ana Zoo
Callithrix jacchus	12	62.30±50.48	captive	NIH Primate Center
Cebus apella	4	26.04±11.19	captive	NIH Primate Center
Pithecia pithecia	1	3.26	captive	Santa Ana Zoo
Saguinus oedipus	1	29.14	captive	Santa Ana Zoo
Total	18	44.39±45.39		

Figure 4.1: Guinea baboon hair cortisol plotted against age, showing the age related decline in male and female hair cortisol from infancy through the early juvenile phases. LOWESS Curves have been fitted to the data.

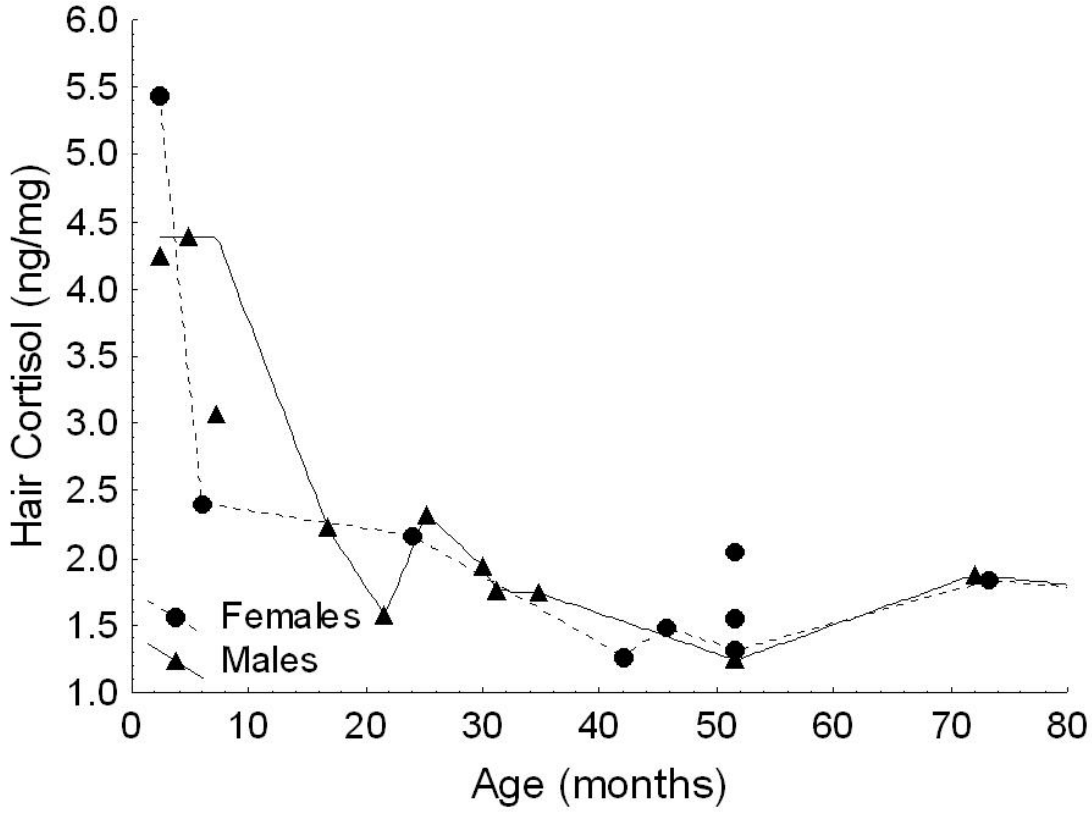


Figure 4.2: Vervet hair cortisol plotted against age class, showing the age related decline in male and female hair cortisol from infancy through the early juvenile phases (approximately 1 to 18 months old). LOWESS Curves have been fitted to the data.

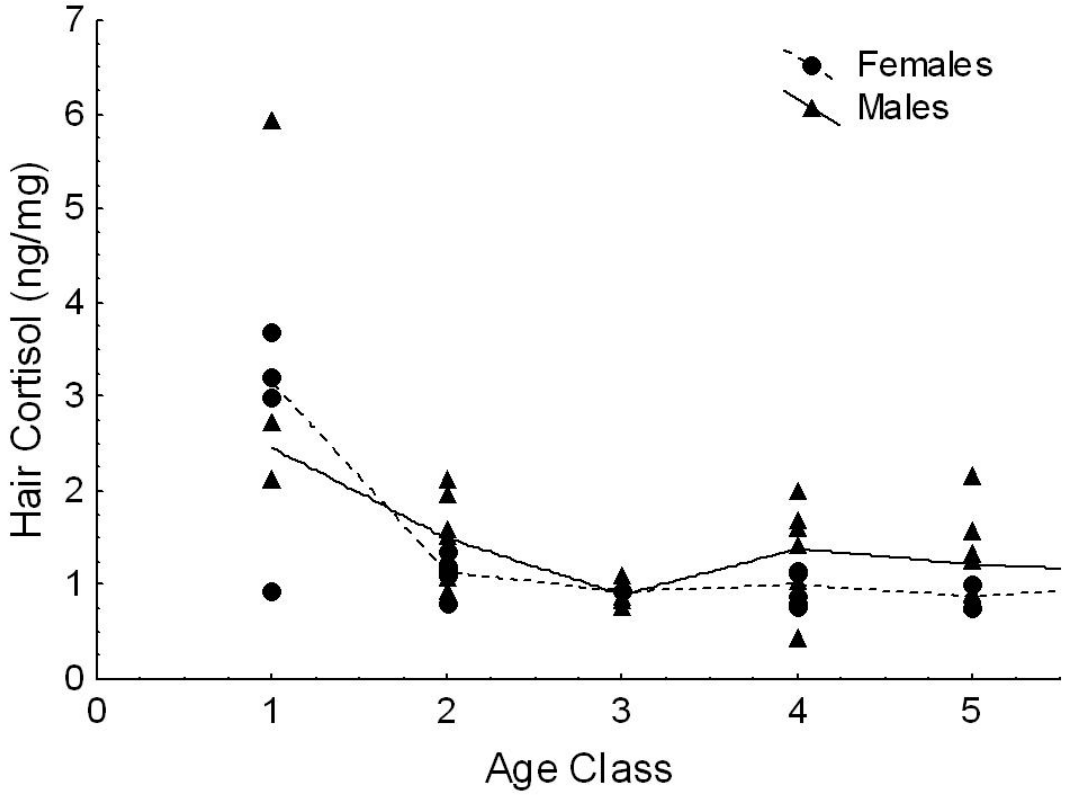
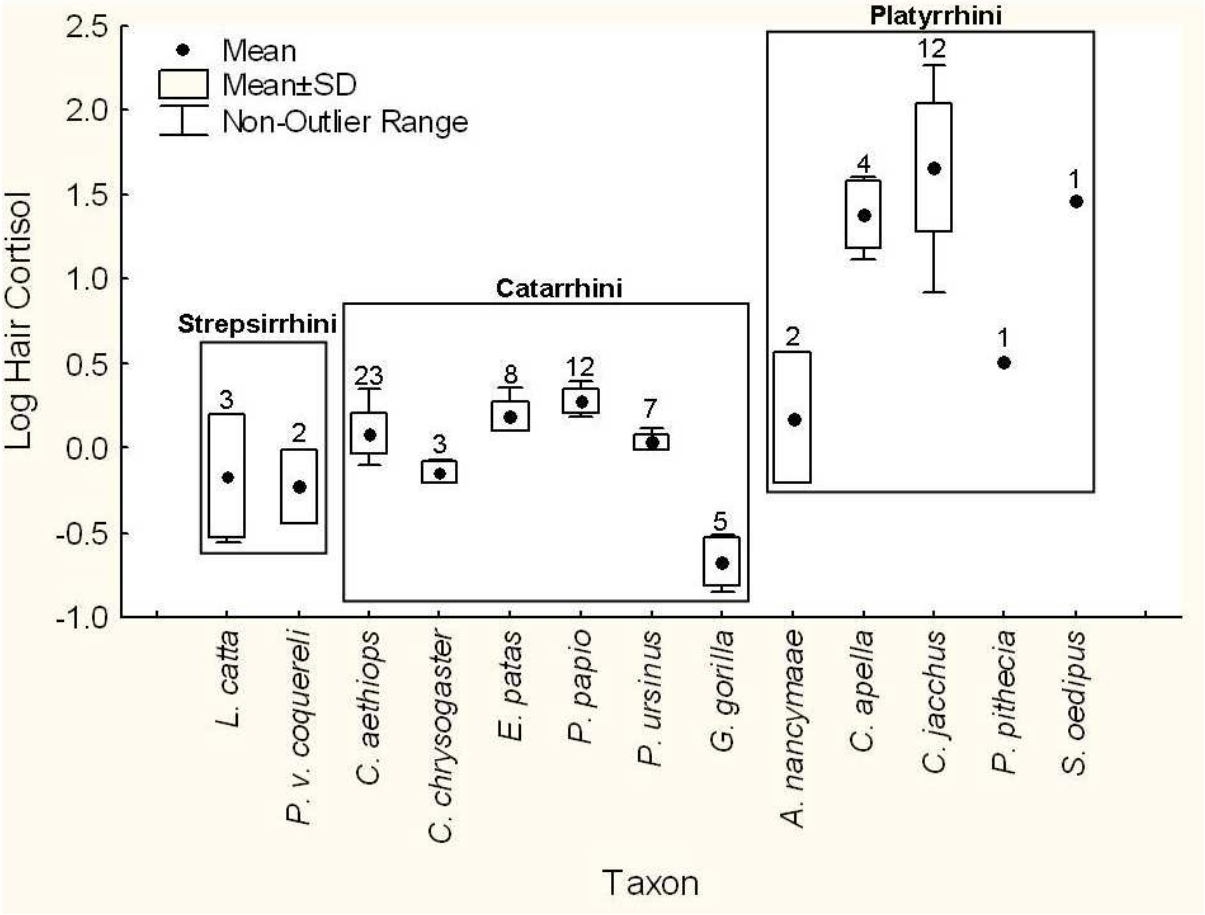


Figure 4.3: Box and whisker plot comparing the hair cortisol levels of adult female strepsirrhine, catarrhine and platyrrhine taxa. The solid circles represent mean hair cortisol values, the boxes represent one standard deviation and the whiskers represent the non-outlier range. The number of samples for each taxon is indicated by the numbers above the whiskers.



Chapter 5: Hair cortisol concentrations in populations of two socially divergent baboon species (*Papio anubis*, *P. hamadryas*) and a population of their natural hybrids: evidence of hybrid hypercortisolism.

ABSTRACT

I examined variation in basal cortisol concentrations among wild male *Papio* baboon populations by testing hypotheses relating to social and environmental factors associated with stress. Hair cortisol concentrations were quantified in a cross sectional sample of adult males from populations of anubis baboons (*Papio anubis*), hamadryas baboons (*P. hamadryas*) and their natural hybrids, sampled in the Awash National Park, Ethiopia. Using a multivariate generalized linear mixed model (GLMM) approach I assessed the significance of species affinity, variation in age, and variation in individually calculated regional rainfall, while controlling for the effects of individual identity and collection season, in order to assess whether population difference in male hair cortisol concentrations correlate to suggested effects of 1) the stability and predictability of species-specific male social environments, 2) the destabilizing effects of hybridity on bio-behavioral stress complexes, and 3) rainfall. Variables found to significantly contribute to the explanatory power of the model were further analyzed using *post hoc* tests of pairwise comparisons, which controlled for the effects of all other variables in the model, tests of homogeneity of variance, and correlation analysis. Hybrids males exhibited the highest and most variable hair cortisol concentrations, while the parental populations did not differ from one another. This suggestins that anubis and hamadryas did not

physiologically express differences in species-specific social stress loads, but that co-adapted bio-behavioral stress coping mechanisms may have been dismantled and recombined in hybrids resulting in variable and destabilized hybrid stress phenotypes. The model was unable to find an effect of regional rainfall on hair cortisol variation.

INTRODUCTION

Animals respond to stressors through a stereotypical physiological stress response (Selye 1950). In mammals this physiological stress response is mediated through the hypothalamic-pituitary-adrenal (HPA) axis (Nelson, 2005). One of the principal steroid stress hormones secreted as part of the physiological stress response by the adrenal cortex in most mammals is cortisol (Hiller-Sturmhöfel and Bartke, 1998; Sapolsky, 2000, 2004). Although a rise in cortisol may be adaptive in the short term, if chronically maintained, high concentrations can have negative physical and mental health effects (Byung-Wan et al., 2010; Chrousos, 2000; Damsted et al., 2011; Das et al., 2010; de Kloet et al., 1999; Epel, 2009; Nijm and Jonasson, 2009; Sapolsky, 1992a, 2004, 2005). Both the physical and the social environment of humans and other primates can be a source of significant stress (Sapolsky, 2000; 2005; Sapolsky et al., 1997; Shively, 2012; Tamashiro et al., 2005).

Cortisol is traditionally measured in blood (e.g. McConway and Chapman, 1986) and saliva (e.g. Heintz et al., 2011), collected from restrained or tranquilized animals (e.g. Sapolsky 1992b), or feces (e.g. Behie et al., 2010) and urine (e.g. Jaimez et al., 2011) which can be non-invasively collected. Unlike cortisol derived from more traditional substrates, hair cortisol is unaffected by acute fluctuations in circulating cortisol resulting from the capture, sedation and the sampling process, or other events preceding sampling (Anestis et al., 2006; Crokett et al., 1993; Delehanty and Boonstra, 2009; Delgiudice et al., 1990; Lynn and Porter, 2008). Hair records circulating cortisol concentrations averaged over the period of hair growth (Accorsi et al. 2008; Bennett and Hayssen 2010;

Davenport et al. 2006; Fourie and Bernstein, 2010b; Sauve et al. 2007). It has been examined in a variety of mammals (e.g. bears - Becshoft et al., 2010; Macbeth et al., 2010; caribou - Ashley et al., 2011; ground squirrels - Martin and Reale, 2008; humans – Dettenborn et al., 2010; hyraxes - Koren et al, 2002; macaques - Davenport et al., 2006; marmosets - Clara et al., 2008; various primates - Fourie and Bernstein., 2011a; vervet monkeys - Fairbanks et al., 2011). In primates, individual hairs may record endocrine activity over an approximately five month period (captive cercopithecines - Fourie and Bernstein, 2011b; Appendix 1). Hair growth in non-human primates is probably asynchronous (Fourie and Bernstein, 2011b; Appendix 1), and therefore a sample consisting of multiple hairs represents a record of cortisol production for multiple disparate five month periods. In other words, a single hair sample consists of multiple individual hairs, each representing disparate and overlapping five month periods of endocrine activity, because some hairs are in the resting phase (telogen), others in the active growth phase (anagen), some in the transitional phase (catagen) and some are ready to molt (exogen - Ebling, 1987; Harkey 1993; Saitoh et al., 1970). The average period of time represented in such a sample therefore almost certainly exceeds the estimated five months it takes for an individual hair to grow to its mature length, and probably reflects circulating cortisol concentrations averaged over an extended period of time.

Social stress in animals is principally born from unpredictability and a lack of control in their social environment (Basset and Buchanan-Smith, 2007; Bouchard et al., 2010; Foa et al., 1992; Koolhaas et al., 2011; Meier et al., 2008; Sapolsky, 2005, 2011). One way through which animals are able to increase social control and predictability is

through cultivating and relying on inter-personal social relationships (Abbott et al., 2003; Carter, 1998; Chen et al., 2011; DeVries, 2002; Gunnar et al., 1981; Kirschbaum et al., 1995; Luckow et al., 1998; Taylor, 2006; Taylor et al., 2000; Thorsteinsson and James, 1999). The degree to which some individuals in the social hierarchy experience and are able to mediate social stress through prosocial relationships follow species-specific patterns in non-human primates.

For instance, subordinate animals of different non-human primate species differ in relative basal cortisol concentrations (i.e. time-averaged cortisol concentrations) according to the amount of species typical social support such low-ranking animals receive (Abbott et al., 2003). Differences in levels of stress-related hormones have also been demonstrated between closely related species that differ consistently in behavioral style. The bonnet macaque (*Macaca radiata*), a socially stable, gregarious and affiliative species, has been shown to have lower concentrations of corticotrophin-releasing-hormone (CRH), a neuropeptide directly involved in the physiological stress response, than the behaviorally volatile pigtail macaque (*M. nemestrina*) (Rosenblum et al., 2002).

However, most research investigating species differences in stress physiology has been carried out on captive animals, often in a laboratory setting with artificially constituted groups of unnatural age-sex composition (e.g. Abbott et al., 2003; Clarke et al., 1986, 1988; Rosenblum et al., 2002). In such contexts, animals may be unable to mitigate stress by some of the means that are available in the wild. These include physical adjustment (e.g. moving away from an attacker) or taking advantage of support from a relative or ally. Different patterns of stress and its consequences may emerge under

natural conditions where animals theoretically have a greater degree of control over their social environment.

The *Papio* baboons represent a diverse but closely related genus of medium-sized terrestrial primates (Groves, 2001; Grubb et al., 2003; Jolly, 1993, 2007). Like the macaques, baboon species differ markedly in social organization and behavioral styles (Barton et al., 1996; Cheney and Seyfarth, 2007; Henzi and Barrett, 2003, 2005; Swedell and Leigh, 2006). In the Awash National Park (ANP), Ethiopia, anubis (*Papio anubis*) and hamadryas (*P. hamadryas*), two morphologically and behaviorally divergent taxa form a species border. Anubis baboons mainly inhabit the productive floodplain along the course of the Awash River, above the Awash Falls, while hamadryas groups inhabit dryer *Acacia* and *Grewia* scrubland habitats down stream from the falls and next to Lake Basaka and the Filoha thermal springs near Mount Fantale (Abule et al., 2007; Nagel, 1973; Swedell, 2002; Swedell et al., 2008; see Figure 2.2).

Anubis baboons live in multi-male, multi-female social groups ("troops" - Bercovitch, 1987; Bercovitch and Strum, 1993; Charpentier et al., 2008; Easley et al., 1989; Johnson, 1987; Packer, 1979a; Packer, 1995; Smuts and Watanabe, 1990; Strum, 1982). Male dispersal at maturity is the norm (Alberts and Altmann, 1995a; Alberts et al., 1992; Bergman et al., 2005; Packer, 1979b; Phillips-Conroy and Jolly, 2004), and at least in other baboon species in which it occurs, is known to be highly stressful for the dispersing animals (Alberts and Altmann, 1995a; Alberts et al., 1992; Bergman et al., 2005). Establishing and maintaining social status in a new troop involves often agonistic interaction with unrelated and perhaps unfamiliar animals. Short-term male reproductive success in anubis baboons is heavily influenced by dominance rank (Cowlishaw and

Dunbar, 1991; Packer, 1979a), which is aggressively contested, resulting in relatively frequent instability in the male hierarchy, with rank-specific stress consequences (e.g. Ray and Sapolsky,1992; Sapolsky, 1992b; Virgin and Sapolsky, 1997). Emigration, integration into a new social group, and aggressive dominance competition are all likely sources of unpredictability, and hence social stress, for male anubis baboons.

By contrast, male hamadryas baboons are normatively philopatric and females disperse (typically within clan and band structures - Hammond et al., 2006; Pusey and Packer, 1987; Swedell et al., 2011), and thus spend their adult lives among related animals of both sexes. Hamadryas society is multi-layered, with bands (equivalent to troops of other species) divided into "clans", whose male members are habitual associates and may be close relatives (Abegglen, 1984; Kummer, 1968a; Sigg et al., 1982). Most male-male interactions are affiliative, structured and ritualized (Colmenares, 1990, 1991; Fraser and Plowman, 2007; Kummer, 1968a). At the lowest level of hamadryas baboon social organization are one male units (OMUs), each consisting of a single adult male with several females and their young offspring. An OMU leader male exercises continuous vigilance to defend exclusive mating access to females of the group, and thus fathers most of its infants (but also see extra OMU paternity – Kummer, 1968a; Sigg et al., 1982; Smith et al., 1999; Yamane et al, 2003, Zinner et al., 2006). While the OMU leader's status is "respected" by other leaders (Bachmann and Kummer, 1980; Jolly, 1963; Kummer, 1968a; Kummer et al.,1974), it is under constant threat of takeover from "bachelor" males (Pines et al., 2011, Swedell et al., 2011), and infanticide often occurs after a take-over (Swedell and Saunders, 2006).

In the ANP, Ethiopia, at the contact zone between hamadryas and anubis baboon populations, cross-migration, interbreeding and backcrossing over many generations have produced a diverse hybrid population (Phillips-Conroy and Jolly, 1986) not unlike that observed at other zones of contact between baboon species (Alberts and Altmann, 2001; Jolly et al., 2010; Zinner et al., 2009). This population occupies the dry canyon habitat below the Awash Falls, down stream from the anubis population (Nagel, 1973). One group of hybrids ("Group H", see Figure 2.2) was observed periodically over several decades (Bergman and Beehner, 2004; Bergman et al., 2008; Sugawara, 1979), and found to have a social structure that included some hamadryas-type "strict" OMUs, some males and females without any OMU affiliation, and some "loose OMUs", with features of both hamadryas OMUs and anubis "friendships" (Bergman and Beehner, 2004; Bergman et al., 2008). While some anubis, hybrid, and hamadryas individuals migrated in and out of Group H, most of the adult males were natal. Group H males exhibit relatively affiliative, tolerant relationships within and between sexes, and male-male agonistic interactions appear to be rare, to the extent that thus far it has not been possible to calculate male dominance within this group (Bergman and Beehner, 2004; Bergman et al., 2008).

I explored social and environmental sources of variation in hair cortisol concentrations among anubis, hamadryas (specifically the Filoha population) and the hybrid group H (see Figure 2.2) by testing hypotheses using a cross-sectional population level approach.

I tested the *Social Stress Hypothesis* (H_1) that inter-specific population differences in hair cortisol concentrations reflect suggested differences in the levels of social stresses males are likely to experience in different social milieus (H_{1a}). This hypothesis predicts

that anubis males will exhibit elevated hair cortisol concentrations compared to hamadryas and that hybrids will exhibit intermediate concentrations. Interacting in stressful social contexts incurs a high physiological cost in terms of chronic stress, but this is countered by the reproductive benefits gained from participation. Previous studies have assumed that the principal source of stress for breeding-age males in savannah baboon societies is dispersal (Alberts and Altmann, 1995a; Alberts et al., 1992; Bergman et al., 2005), followed by life within an unstable, competitive dominance hierarchy, unmitigated by ties of kinship and familiarity (Sapolsky, 1992b, 1993, 1995; Sapolsky and Mott, 1987; Sapolsky and Ray, 1989; Sapolsky et al., 1997). This describes the reproductive life-history of anubis baboons and predicts higher chronic cortisol concentrations in the male anubis population than in male hamadryas population, while the behaviorally mixed and intermediate hybrid population is expected to exhibit similarly intermediate chronic cortisol concentrations. On the other hand, even in the absence of dispersal and an expressed male-male dominance hierarchy, hamadryas males experience the social stresses generated by attempting to form and defend an OMU, which may incur relatively higher stress costs than in anubis, resulting in higher hair cortisol concentrations. Alternatively, as an extension of H_1, the two baboon species may differ in the levels of social conflict and uncertainty that typifies the male social milieu, each species has evolved biological and/or behavioral mechanisms for coping with potential stressors, lowering their effect on chronic cortisol concentrations, these bio-behavioral mechanisms are disrupted in hybrid populations (H_{1b}). This hypothesis therefore predicts that male anubis and hamadryas populations will exhibit similar basal cortisol concentrations whereas hybrids will exhibit elevated and more variable hair

cortisol concentrations. In the hybrid population, species-specific, co-adapted stress complexes would presumably be dismantled by genetic recombination resulting in disrupted and more variable bio-behavioral complexes than either parent population. Given the behavioral heterogeneity of hybrid society, any one bio-behavioral stress complex may not be able to adequately respond to social stresses resulting in elevated physiological stress in this population compared to parent populations.

I tested the *Ecological Stress Hypothesis* (H_2), that population differences in basal cortisol principally reflect differences in ecological stress related to the rainfall experienced by each population prior to trapping and sampling. Previous research has shown that variation in rainfall within the normal range can result in changes in habitat productivity that can be a source of significant stress for baboons (e.g. Gesquiere et al., 2011). It is predicted that animals and populations (i.e. within and between population variation) which experienced lower rainfall over the period preceding sampling will show higher basal cortisol concentrations compared to animals and populations that experienced higher rainfall.

Although populations differ in aspects of habitat quality (e.g. hybrid and hamadryas populations inhabit poorer habitats than anubis in the ANP), habitat quality is a stable feature to which a population responds in such a way that population density does not exceed the carrying capacity of the habitat. These populations are well established and presumably demographically adjusted to their respective habitats. Indeed, this principal is thought to underlie the evolution of hamadryas society (Dunbar, 1988; Henzi and Barrett, 2003, 2005; Kummer, 1990, 1995), possibly the smaller hamadryas body size, and is further reflected by the dynamic response in population density and foraging strategy in

baboons (Anderson, 1981; Barton et al., 1996; Byrne et al., 1989; Henzi and Barrett, 2003, 2005). Therefore differences in habitat quality are not in themselves expected to affect chronic cortisol levels, but may be expressed as a response to rainfall. Therefore rainfall is expected to be the most salient predictor of ecological stress in these populations.

MATERIALS AND METHODS

Hair Samples

Hair samples of dental adults (i.e. males with third molars and canines in full occlusion - Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000) were collected and analyzed. Month age estimates were also available for each individual; these were based on patterns of occlusal wear (see Phillips-Conroy and Jolly, 1988 and Phillips-Conroy et al., 2000 for a description of the methods; see Tables 5.1 and 5.2). Samples were taken from anubis (n = 37, troops C, D, E, F) and hybrid (n = 20, troop H) troops which inhabit the left bank of the Awash River (Phillips-Conroy and Jolly, 1986); hamadryas samples (n = 17, troop Filoha, Figure 2.2) come from a group which inhabits an area around the Filoha hot-springs (Swedell, 2002). Cross-specific immigrants in the hybrid, anubis and hamadryas groups were not included in the present analyses as these would represent a non-native stress profile in the host population. Therefore all males from anubis populations were clearly anubis-like and all males from the hamadryas population were clearly hamadryas-like. All males from the hybrid population were

hybrids and all pure anubis and hamadryas (i.e. possible cross-specific immigrants) were excluded. Seventy four samples were collected from 72 animals captured during five field seasons spanning seven years between 1993 and 2000 (see Table 5.1). Two hybrid males (11006 and 11009) were sampled in two different field seasons. Animals were captured using manually operated cage traps and anesthetized (as described by Brett et al., 1982). Approximately 50 to 100mg of hair was clipped from between the shoulder blades as close to the skin as possible without causing injury. Samples were stored and shipped in paper envelopes under non-specific ambient conditions.

Rainfall experienced over the 12 months prior to capture were calculated from rainfall records kept by the Merti Metahara Sugar Estate next to Lake Basaka north of the Awash River (Tables 5.1 and 5.2, and see Figure 2.2). Although not specific to each site, these rainfall estimates are considered representative of the rainfall experienced by the region encompassing the study sites, which lie within 10 (Awash Falls – see Figure 2.2 to 30km (Filoha) of the Merti Metahara Sugar Estate.

Data from hair sampled from the head of anubis, hamadryas and hybrid baboons from the ANP in Ethiopia show that about 30% of the hair is in the active growth phase (anagen) and that 70% is in the inactive phase (catagen and telogen) (Jolly and Phillips-Conroy, unpublished data.). Assuming a five month growth period to reach mature length, this would suggest that approximately six percent of hairs are renewed every month. Using this information a simple model of hair growth was created. Every month a new wave of hair (six percent) emerges. Therefore each new wave of hair differs from the previous month's cohort by a month (see Figure 5.1). Therefor, multiple mature temporally overlapping cohorts of hair are present in a population of hair at any point in

time, and a mature cohort may take up to 20 months to be completely replaced (assuming constancy in hair growth throughout the year). Any given population of hair should contain elements that record biological information for the recent past as well a continuum that reaches back in excess of 12 months in the past, and therefore reflects biological information which has been averaged over this entire period. A caveat of this model is that it assumes equal proportions of different cohorts of resting hairs, this may not be the case and older hair may make a smaller contribution to the average. Nonetheless, the period of biological information recorded in a sample of primate hair appears to show a strong relationship with rainfall for the 12 month period preceding sampling. This is based on stable isotope research (Fourie et al. 2008, in preparation) on hair samples from baboons from the ANP, which finds that multi-hair homogenized samples produce a record of diet that correlates well with rainfall data collected over a 12-month period, by showing that animals that experienced low rainfall over that period exhibit a shift to isotopically distinct fallback foods (Fourie et al. 2008, in preparation). I therefore used this rainfall parameter as a covariate for variation in hair cortisol concentrations.

Hair Sample Preparation

Hair samples were stored in paper envelopes at ambient laboratory temperature (~24^{o}C) in closed cardboard containers. Cortisol is especially stable while sequestered in the hair matrix (Bennet and Hayssen, 2010; Davenport et al. 2006; MacBeth et al, 2010; Webb et al., 2010). The sample preparation protocol was modified from the basic version

generally used for cortisol extraction for quantification by enzyme immunoassay (e.g. Davenport et al., 2006; Sauve et al., 2007; Yamada et al., 2007). Hair samples (10mg) were washed twice in 3mL isopropanol (C_3H_8O) and dried in a fume hood under a stream of air for approximately 16 hours until incipient dryness (after Davenport et al., 2006). The wash procedure removed exogenous contaminants and sebum. Samples were weighed out into ~10mg aliquots, placed in labeled borosilicate tubes and cut into approximately one to three millimeter pieces using a pair of fine surgical scissors (after Sauve et al., 2007, Yamada et al., 2007). Four milliliters of methanol (CH_4O) were then added to each tube, which was then incubated at room temperature on a reciprocating shaker at 100rpm for 16 hours. The samples were centrifuged for 10 minutes at 4000rpm and 3.5mL of the resulting supernatant was pipetted into labeled polypropylene tubes. The supernatant was dried under a stream of air in a hot water bath at 60^{o}C in a fume hood. The dried mass was then reconstituted in 500µL of assay dilution buffer (0.1M $NaPO_4$, 0.149M NaCl, pH 7.0) and stored at -20^{o}C. All samples were assayed within one week of being extracted minimizing the effect of possible degradation of cortisol in the stored extracts.

Cortisol Enzyme Immunoassay (EIA)

A cortisol ELISA was used to measure cortisol extracted from hair samples and relied on a polyclonal cortisol antibody generated against cortisol-3-carboxymethyloxime (R4866, 1:20,000; C. Munro, University of California, Davis; Munro and Lasley 1988). The antibody cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%,

cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone and 11-desoxycortisol (C.J. Munro, pers. comm.). Assay sensitivity was 3.90 pg/well. Intra-assay and inter-assay coefficients of variation were less than 10% (n=15) and 15% (n=21) respectively. The assay was independently validated for anubis, hamadryas and hybrid baboons by showing (1) parallelism between a serial dilution of pooled sample extract and a serial dilution of a known amount of cortisol and (2) recovery of more than 90% of a known amount of cortisol added to sample extracts before analysis (Appendix 3 and 4).

Statistical Analysis

Variation in hair cortisol concentrations were examined using a general linear mixed model (GLMM). Hair cortisol concentrations were log transformed before being entered into the model in order to satisfy assumptions of normality. Significance for all tests was set to alpha = 0.05. Preliminary analysis showed that hair cortisol concentrations showed a positive relationship with age. I subsequently evaluated the age structure of the three samples using a Kruskal-Wallis one way ANOVA, followed by a Kolmogorov-Smirnov test in order to make pairwise comparisons. The continuous variable age was included in the GLMM as a fixed effect. The categorical variable "species" and the continuous variables rainfall (in mm) and age (months) were included in the model as fixed effects. Individual identification and collection season were included as random effects in the model. Categorical variables which were found to significantly explain variation in hair cortisol concentrations were then evaluated using the multiple pairwise comparisons

function in SYSTAT 13 (Systat, Inc., San Francisco, CA). This function allows for pairwise comparisons of groups in categorical variables in the GLMM (using Tukey's Honestly Significant Difference – HSD) using standard errors from the whole model, which control for the effects of all other variables in the model. The relationship of continuous variables with cortisol were further examined using Pearson's product moment correlations.

I tested the second part of the prediction of the *Social Stress Hypothesis* that hybrids show greater variability in hair cortisol concentrations than parent species, by first testing for homogeneity of variance using Hartley's Fmax test (Hartley, 1950) using the untransformed cortisol concentrations. Log transforming the data would reduce variance, thereby undermining the efficacy of the test. I then made multiple pairwise comparisons among the variances of the three populations using a Tukey-type multiple comparisons test for differences among variances using the natural logarithms of the variances calculated from the untransformed data (as per Zar, 1999, but also see Levy, 1975a, b).

RESULTS

A Kruskal-Wallis one way ANOVA showed that the age distributions within the three populations differed significantly ($H_{2,74}$ =10.045, p =.007). A Kolmogorov-Smirnov test showed that both anubis (mean±1SD =119 ± 26 months old) and hamadryas (mean±1SD =128 ± 40 months old) populations had significantly more younger males than the hybrid (mean±1SD =157 ± 39 months old) population (anubis-hybrid, $p<.05$; hamadryas-hybrid,

$p<0.05$) and but did not differ in age structure from one another ($p>0.1$; see Tables 5.1 and 5.2).

The GLMM indicated that the fixed effects species and age explain a significant portion of the variation in hair cortisol concentrations (Table 5.3). Regional 12 month rainfall did not significantly explain variation in hair cortisol concentrations in the model. Neither individual identity nor the field season during which animals were sampled appeared to significantly explain variation in hair cortisol concentrations in the model.

Post hoc pairwise comparisons show that the anubis males tended to have significantly lower (Tukey's HSD: anubis – hybrid; $t=-3.179$, $p=0.006$) hair cortisol concentrations than male hybrids, whereas male hybrids and male hamadryas hair cortisol did not differ significantly (Tukey's HSD: hamadryas – hybrid; $t=-1.371$, $p=0.088$, Figure 5.2). Male anubis did not differ significantly in hair cortisol concentrations compared to male hamadryas (Tukey's HSD: anubis – hamadryas; $t=-1.208$, $p=0.453$). Hartley's Fmax test (Hartley, 1950) found that variances differed significantly ($Fmax_{(0.05, 3, 15)} = 3.54$, $Fmax_{(3, 16)} = 3.775$, $p<0.05$) among the three male populations. The Tukey-type multiple comparisons test for differences among variances (Levy, 1975a, b; Zar, 1999) found that hybrid hair cortisol concentrations ($n=20$, mean±1SD =44.851 ± 26.946 ng/mg) were significantly more variable than anubis ($n= 37$, mean±1SD =25.155 ± 15.064 ng/mg; hybrid-anubis: $q_{(0.05,\infty,3)}= 3.314$, $q = 4.495$, $p<0.05$) and hamadryas ($n= 17$, mean±1SD =30.438 ± 13.869 ng/mg; hybrid-hamadryas: $q_{(0.05,\infty,3)}= 3.314$, $q = 3.920$, $p<0.05$) hair cortisol concentrations. Anubis and hamadryas did not differ from one another in this respect (hybrid-anubis: $q_{(0.05,\infty,3)}= 3.314$, $q = 0.555$, $p>0.05$, Table 5.2 and Figure 5.2).

Pearson's correlation tests showed that the linear relationship between age and hair cortisol concentrations in the hybrid population neared significance ($r = 0.431$, $p = 0.058$, $y = 1.210 + 0.003*x$), the anubis ($r = 0.267$, $p = 0.118$; $y = 1.120 + 0.002*x$) and hamadryas ($r = 0.191$, $p = 0.463$, $y = 1.223 + 0.002*x$) populations both showed non-significant trends for cortisol concentrations to increase with age (see Figures 5.3 , 5.4 and 5.6).

DISCUSSION

The GLMM and *post hoc* tests of variance found that the male hybrid population exhibited significantly higher hair cortisol concentrations than the anubis population and significantly more variance in hair cortisol compared to both parent species. The H_{1a} suggested that species differences in social stress loads would be associated with chronic cortisol levels, and predicted higher hair cortisol in anubis and intermediate hair cortisol in hybrids. These results do not meet the predictions of this hypothesis. The extension of the *Social Stress Hypothesis* (H_1), that co-adapted bio-behavioral stress complexes are disrupted and destabilized by hybridization and the social dynamics of hybrid groups (H_{1b}), which predicted higher and more variable hair cortisol in the male hybrid population, is partially supported by these results. The results suggest that not withstanding the possible effects of individual differences in dominance rank and socioreproductive status (which are note known for these individuals), or differences in age (which is controlled for in the model), on individual cortisol concentrations, that male anubis and hamadryas may be physiologically and behaviorally adapted to the demands

of their specific social milieus so that any differences in social stress loads are not physiologically expressed at the population level.

The GLMM did not find that rainfall significantly explained variation in hair cortisol concentrations within or among populations. This result does not support the *Ecological Stress Hypothesis* (H_2) that population differences in basal cortisol principally reflect differences in ecological stress related to rainfall experienced by each population prior to trapping and sampling. This hypothesis predicted that animals and populations which experienced relatively low rainfall would exhibit relatively elevated hair cortisol. Such trends were not observed in the current data set. Environmental factors are likely to be important sources of variation in cortisol concentrations in these populations, as has been demonstrated in other wild baboon populations (Crockford et al., 2008; Gesquiere et al., 2011). It may be that the rainfall data available for use in this model do not capture site specific environmental stresses related to local rainfall. Though, none of the sites are in excess of 30km of the station where rainfall was measured. Both the anubis and hybrid sites are within 10km or less from Merti Metahara. It is unlikely that significant variation in rainfall occurs over such a short distance in the absence of any geographical barriers. Additionally, none of the 12 month regional rainfall estimates were below the normal yearly average for the Awash region of Ethiopia (400 to 500 mm). This would suggest that local environments were not unusually stressed throughout the relevant 12 month period. In the absence of a sustained environmental stressor, like a drought, the effect of short lived fluctuations in environmental productivity on circulating cortisol concentrations may not reflect in cortisol concentrations averaged over an extended period of time, as is found in hair.

The covariate age also significantly contributed to explaining hair cortisol variation in the model. All three male populations show a trend of increasing hair cortisol concentrations with age; although the linear dependence of the association was only nearly significant for hybrid males (see Figures 5.3, 5.4 and 5.5). Older individuals are better represented in the hybrid sample (See Figures 5.3, 5.4 and 5.5, and Table 5.1 and 5.2) and the hybrid sample contains some of the oldest animals. These results are consistent with data from humans (Dodt et al., 1994; Goudochnikov, 2011; Kern et al., 1996; Swaab et al. 1994) and other animals (Erwin et al., 2004; Palazzolo and Quadri, 1987; Sapolsky, 1992a; Sapolsky and Altmann, 1991), and similar findings have been described in wild yellow baboons (*P. cynocephalus* - Sapolsky and Altmann, 1991), which show that circulating cortisol concentrations generally increases with senescence. It is thought that cortisol secretion increases with senescence due to blunting of glucocorticoid receptors in the hypothalamus inhibiting tonic regulation of adrenocortical function with age. These effects may be further exacerbated in baboons as males tend drop in rank as they age (Beehner et al., 2005; Noe and Sluitjer, 1995). Unless aging males are able to maintain social networks, low rank may result in significant stress (Abbott et al., 2003; Bergman et al., 2005; Sapolsky et al., 1997; Sapolsky and Mott, 1987).

The results show that when controlling for the effects of age, rainfall and field season that the male hybrid population exhibited an elevated basal cortisol profile compared to their parent species. This implies that male hybrids may experience relatively greater stress loads. This stress may emanate from inherent uncertainty in the hybrid social milieu itself due to variability in the extent to which troop members attempt to follow

hamadryas-like or anubis-like social agendas (Bergman and Beehner, 2004; Bergman et al., 2008; Sugawara, 1979). The social structure of the group is also liable to modification as hamadryas and anubis individuals migrate in or out adding an additional layer of social uncertainty. Physiological mechanisms for coping with potentially stressful situations, and preventing them from adding to the social stress load, may be less effective in individuals whose genetic makeup has been disrupted by hybridization. Hybridization results in novel gene combinations, gene duplication and gene silencing, all of which may have significant effects on the individual hybrid phenotype (see Burke and Arnold, 2001 for review). A better understanding of the parent genotype and variability in the hybrid genotype, especially in gene regions associated with the relevant endocrine markers and/or behavioral agendas are needed to assess the basis of such biological differences in endocrine function (e.g. Rogers et al., 2004). While the results imply that there may be a physiological cost associated with hybridity, it remains unclear whether this implies associated fitness costs. Current evidence suggests that the hybrid males of group H experience reproduce success (Bergman et al., 2008), and the hybrid sample used here contains a greater number older males compared to the anubis and hamadryas samples. All animals of groups H was trapped, so differences in age profiles are not a result of sampling biase in group H, but could reflect a sampling bias in anubis and hamadryas popultaions in that older males may just be easier to trap. Not with standing possible sources of sampling bias, it still shows that hybrids can live to relative old ages. Further research quantifying male reproductive rates, parasite load, and immune function among these three populations may speak to the adaptive significance of the observed patterns in basal cortisol concentrations.

All the anubis baboon populations so far surveyed in north-central Ethiopia and Eritrea carry hamadryas-related mitochondria (Burrell et al., 2008; Szmulewicz et al., 1999; Zinner et al., 2009), attesting to a long history of hybridization and asymmetrical male anubis introgression into hamadryas populations (Hapke et al., 2001; Wildman et al., 2004). At the active hybridization front in the Awash National Park, both hamadryas and anubis populations experience cross-species immigration (Nystrom, 1992; Phillips-Conroy and Jolly, 1986; Phillips-Conroy et al., 1991, 1992). While it is likely that in most cases the deleterious influences of hybridity and social instability introduced by behaviorally distinct immigrants may be swamped and selected out of the general population by competition with dominant species-specific norms (e.g. Beyene, 1998; Nystrom, 1992; Phillips-Conroy et al., 1992; Sugawara, 1982, 1988), some hamadryas populations in the Awash National Park show phenotypic evidence of significant introgression from the anubis population (Phillips-Conroy and Jolly, 1986, especially group A and K which is substantially mixed). If substantial introgression has occurred in those populations then similar destabilization of the stress phenotype may be expected. Further research extending the current paradigm to other populations forming part of the Awash hybrid zone complex may contribute to our understanding of hybrid fitness, and the selective advantage of the anubis phenotype in admixed populations.

CONCLUSION

To my knowledge this is the first research to utilize hair hormone analyses to examine stress biology in wild primates. I report the recovery of biologically meaningful hair

cortisol concentrations from archived hair collections. The results suggest that cortisol concentrations are relatively stable within the hair matrix over extended periods of time (e.g. Macbeth et al., 2010; Webb et al., 2010), as the model did not find that hair cortisol concentrations were significantly affected by the relative age of the sample. This research demonstrates the utility of hair hormone analysis to address theoretical questions in primate research and suggests the exploitation of hitherto un-utilized potential archives of endocrine information. The technique is especially suited to track relatively stable environmental and social phenomena in relation to organism stress at the population level and is already finding application in conservation biology (Beschoft et al., 2010; Macbeth et al., 2010; Martin and Reale, 2010), while advancing our understanding of the endocrine basis of stable behavioral traits (Fairbanks et al., 2011; Clara et al., 2008; Martin and Reale, 2008). The technique also demonstrates the potential to be applied non-invasively in wildlife.

Tables and Figures

Table 5.1: Table of animals used in this study along with details of individual population, troop and field season associations, estimated age (in months), the regional rainfall calculated for each individual for the twelve months prior to capture and sampling and the hair cortisol concentrations determined for each animal. Animals that were sampled multiple times are marked with an asterisk.

ID	Population	Troop	Field Season	Age (months)	12 Month Rainfall (mm)	Cort (ng/mg)
10900	Anubis	C	1993	111	616.0	11.1
10805	Anubis	C	1993	154	619.7	18.9
10783	Anubis	C	1993	173	621.5	11.3
10914	Anubis	C	1993	170	623.3	38.8
10988	Anubis	C	1993	102	625.2	15.2
10874	Anubis	D	1993	83	606.8	6.4
10815	Anubis	D	1993	84	612.3	23.8
10760	Anubis	F	1993	82	740.4	59.4
10878	Anubis	D	1995	106	438.7	44.6
10934	Anubis	F	1995	95	600.3	28.4
10991	Anubis	F	1995	167	600.3	8.4
10992	Anubis	F	1995	182	600.3	19.4
11230	Anubis	D	1998	95	504.6	9.4
11232	Anubis	D	1998	97	504.6	24.0
11233	Anubis	D	1998	96	504.6	14.6
11234	Anubis	D	1998	96	504.6	12.2
11235	Anubis	D	1998	108	504.6	20.5
10868	Anubis	D	1998	162	504.6	19.4
11238	Anubis	D	1998	95	504.6	17.9
10688	Anubis	D	1998	225	503.1	69.6
10968	Anubis	D	1998	134	498.5	15.3
10815	Anubis	D	1998	144	478.8	26.7
10620	Anubis	D	1998	225	494.2	36.5
11053	Anubis	D	1998	112	494.2	25.3
11254	Anubis	D	1998	94	494.2	14.6
10947	Anubis	D	1998	135	494.2	51.5
11036	Anubis	D	1998	98	494.2	14.4
10955	Anubis	D	1998	135	501.8	12.8
11057	Anubis	D	1998	146	501.8	14.9
11260	Anubis	D	1998	95	501.8	45.5
11084	Anubis	D	1998	96	501.8	14.3
11263	Anubis	D	1998	130	505.7	16.0
11224	Anubis	E	1998	104	507.7	26.5
11232	Anubis	D	2000	121	495.2	41.6
11260	Anubis	D	2000	107	492.7	30.6
10912	Anubis	D	2000	173	490.1	41.9
11242	Anubis	D	2000	197	488.9	28.9

Table 5.1: continued…

ID	Population	Troop	Field Season	Age (months)	12 Month Rainfall (mm)	Cort (ng/mg)
11111	Hamadryas	Filoha	1997	96	471.9	20.5
11113	Hamadryas	Filoha	1997	97	471.9	7.7
11116	Hamadryas	Filoha	1997	106	471.9	29.3
11121	Hamadryas	Filoha	1997	163	472.9	29.7
11124	Hamadryas	Filoha	1997	124	472.9	38.6
11128	Hamadryas	Filoha	1997	148	472.9	14.9
11132	Hamadryas	Filoha	1997	101	472.9	25.5
11135	Hamadryas	Filoha	1997	131	476.1	43.5
11137	Hamadryas	Filoha	1997	98	476.1	50.5
11142	Hamadryas	Filoha	1997	155	476.1	44.2
11144	Hamadryas	Filoha	1997	108	477.2	56.2
11152	Hamadryas	Filoha	1997	97	477.2	12.6
11156	Hamadryas	Filoha	1997	103	479.3	15.4
11157	Hamadryas	Filoha	1997	112	479.3	27.0
11160	Hamadryas	Filoha	1997	107	479.3	41.9
11341	Hamadryas	Filoha	2000	175	467.3	26.9
11350	Hamadryas	Filoha	2000	105	467.5	33.0
10889	Hybrid	H	1993	102	530.5	40.1
*11006	Hybrid	H	1995	168	530.5	24.1
*11009	Hybrid	H	1995	96	530.5	39.8
11010	Hybrid	H	1995	103	530.5	17.0
11018	Hybrid	H	1995	147	520.8	17.0
11030	Hybrid	H	1995	161	511.2	58.1
10891	Hybrid	H	1995	146	511.2	28.5
11038	Hybrid	H	1995	160	511.2	65.6
11167	Hybrid	H	1998	176	539.9	37.3
11019	Hybrid	H	1998	118	539.9	30.0
11029	Hybrid	H	1998	218	539.9	39.5
11018	Hybrid	H	1998	183	539.9	44.1
11017	Hybrid	H	1998	209	539.9	46.7
11020	Hybrid	H	1998	111	539.9	29.6
11173	Hybrid	H	1998	149	536.8	55.9
11038	Hybrid	H	1998	196	536.8	125.0
11030	Hybrid	H	1998	197	536.8	102.2
*11009	Hybrid	H	1998	116	532.2	31.6
*11006	Hybrid	H	1998	204	532.2	28.4
11196	Hybrid	H	1998	175	530.7	36.6

Table 5.2: Descriptive statistics for hair cortisol concentrations, age and regional rainfall for the 12 month period preceding trapping for each animal for the adult male anubis, hamadryas and hybrids.

Statistic	Anubis	Hamadryas	Hybrids
n	37	17	20
mean cortisol (ng/mg)	25.155	30.438	44.850
±1 standard deviation (ng/mg)	15.064	13.869	26.946
mean age (months)	127.811	119.176	156.750
±1 standard deviation (months)	39.466	25.708	38.747
mean 12 month rainfall (ml)	534.482	474.291	531.052
±1 standard deviation (ml)	63.767	3.732	9.894

Table 5.3: Results of the GLMM assessing the effects of predictor variables on hair cortisol concentrations. Significant results are highlighted in bold.

GLMM	d.f.	Standardized β	F	t	p
Intercept	4	0.344		4.419	0.012
Type II Test for Fixed Effects					
Species (anubis, hamadryas, hybrid)	**2, 64**		**5.132**		**0.009**
Age (months)	**1, 64**		**4.781**		**0.032**
12 Month Rainfall (mm)	1, 64		0.344		0.56
Random Effect Predictions					
Individual Identification	1, 64			0	1
Field Season	5, 64			0	1

Figure 5.1: Diagram illustrating the simple model of hair growth used to estimate the possible period of endocrine activity averaged in a multi-hair sample of baboon hair. The first column estimates the number of hairs of a cohort of hairs surviving in the population of hair assuming a six percent replacement rate. The second column gives the growth phase that the hair is in. Each subsequent column represents a cohort of hair, the different colors designate non-overlapping blocks of endocrine activity recorded in the population of hair, each block represents a five month period.

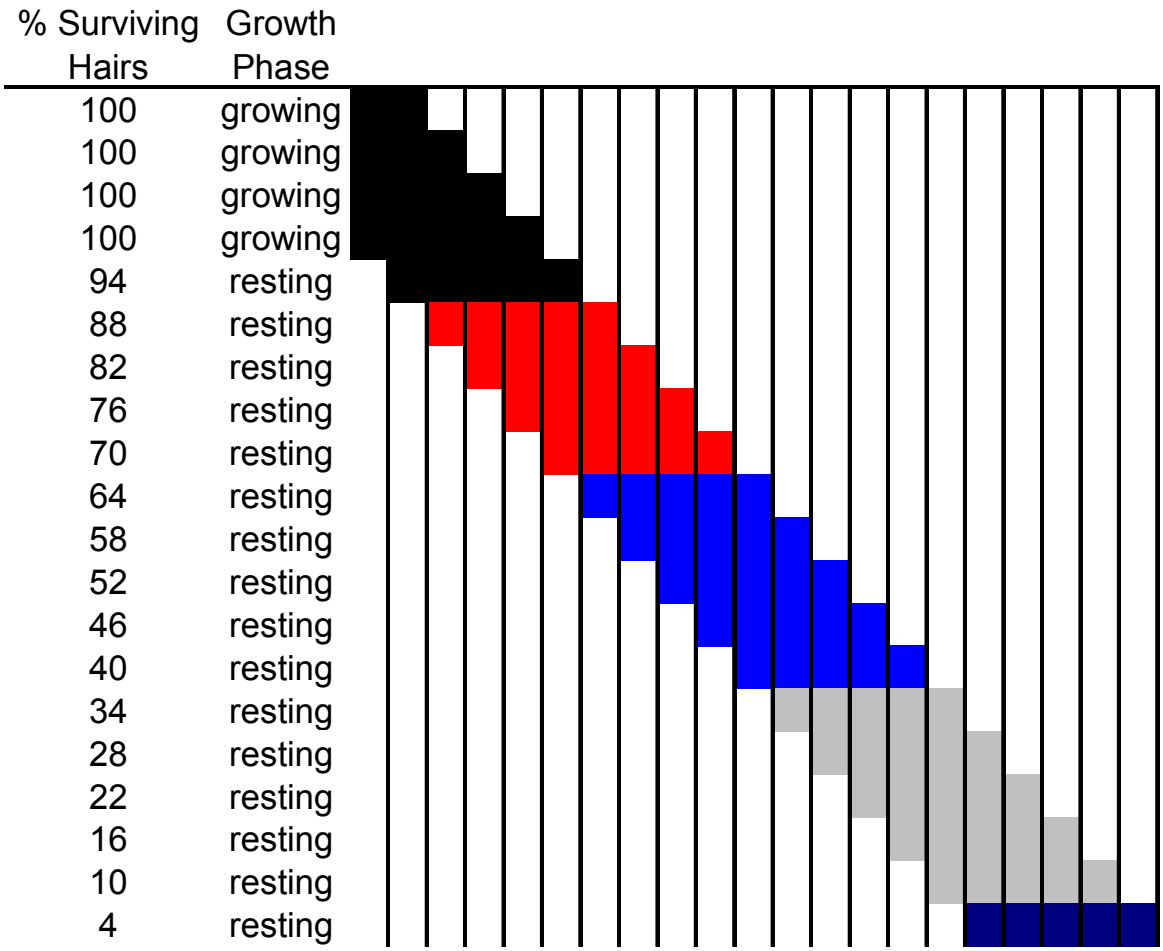


Figure 5.2: Plot of mean (black triangles) ± one standard deviation untransformed hair cortisol concentrations. Significance values above the bars are calculated for pair wise differences (using a Tukey's HSD test) using the standard errors from the GLMM model. The significance values below the bars are calculated for pairwise comparisons of variance of each population using a Tukey-type test for differences in variance.

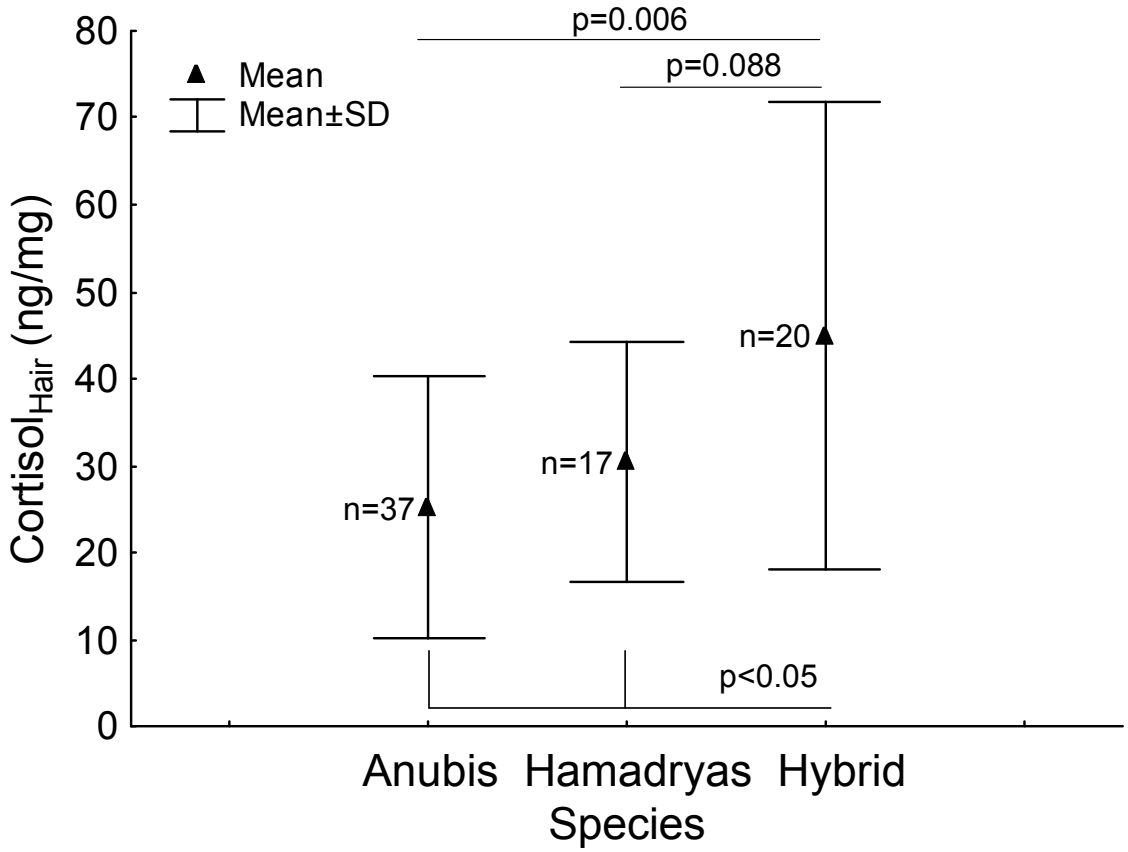


Figure 5.3: Figure showing a near significantly positive relationship between age and cortisol in male hybrids.

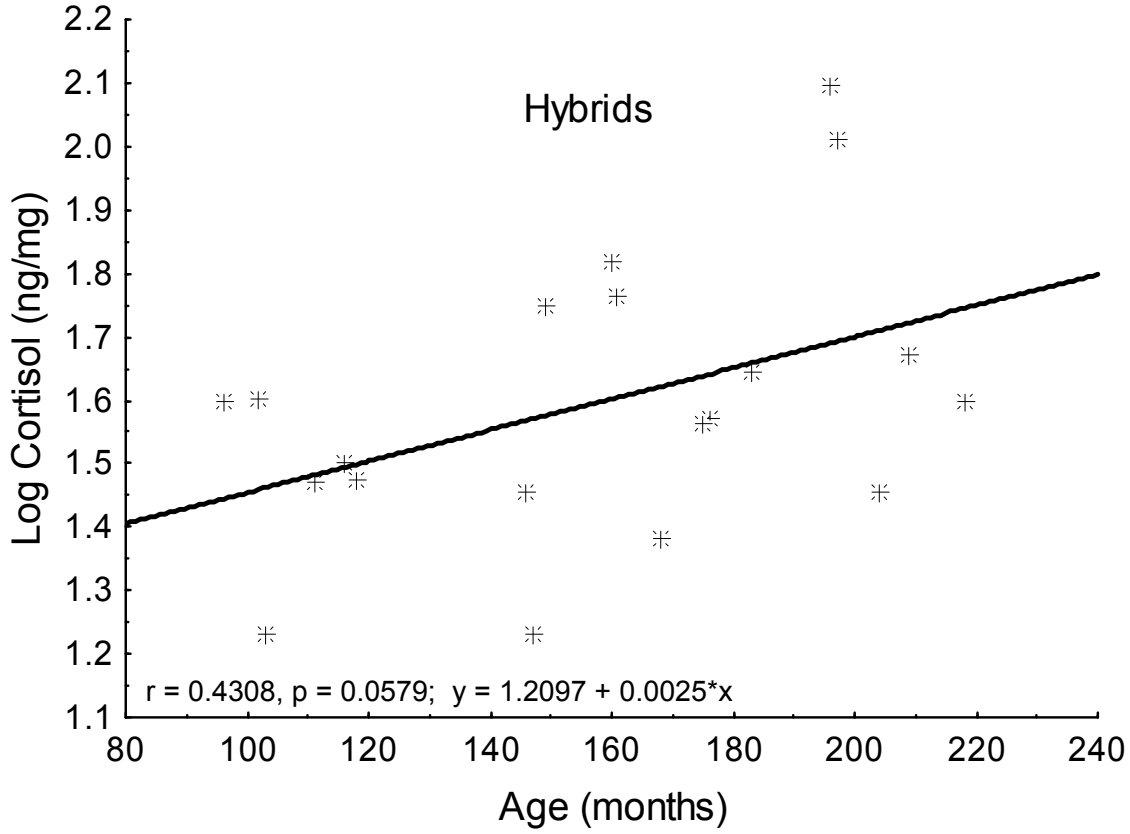


Figure 5.4: Figure showing the relationship between age and cortisol in male anubis.

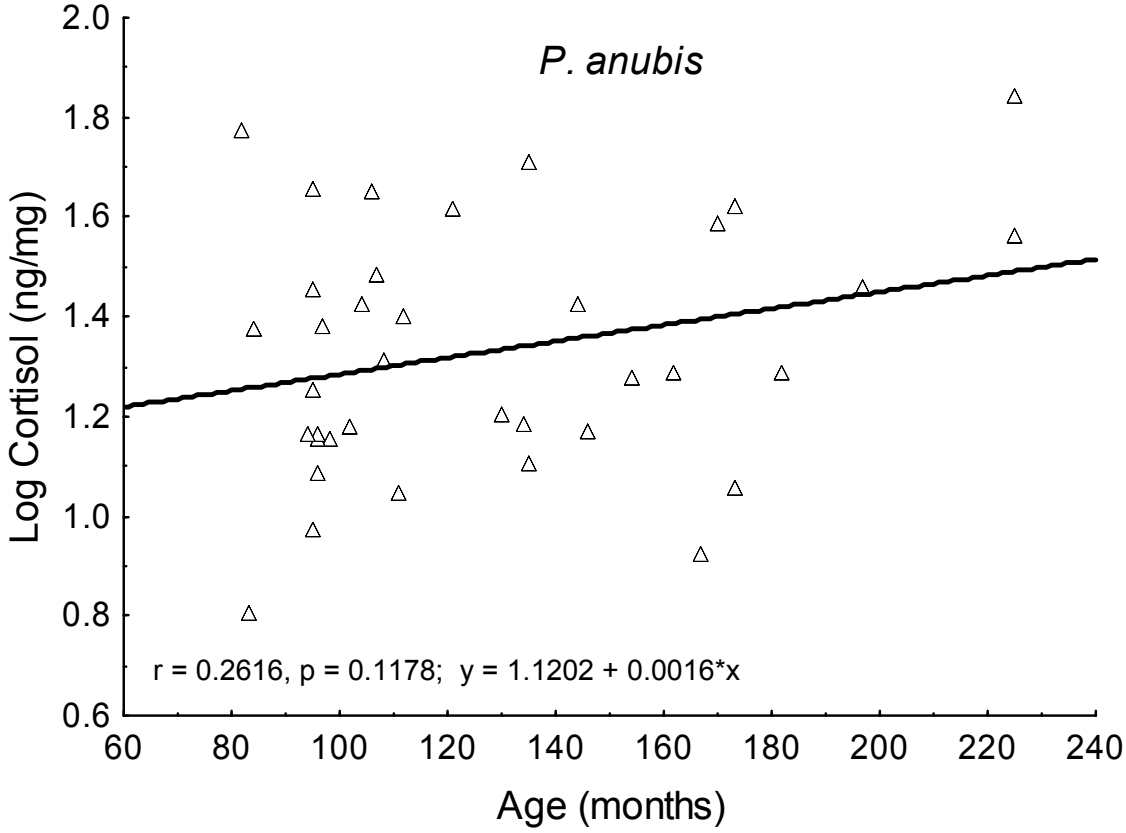
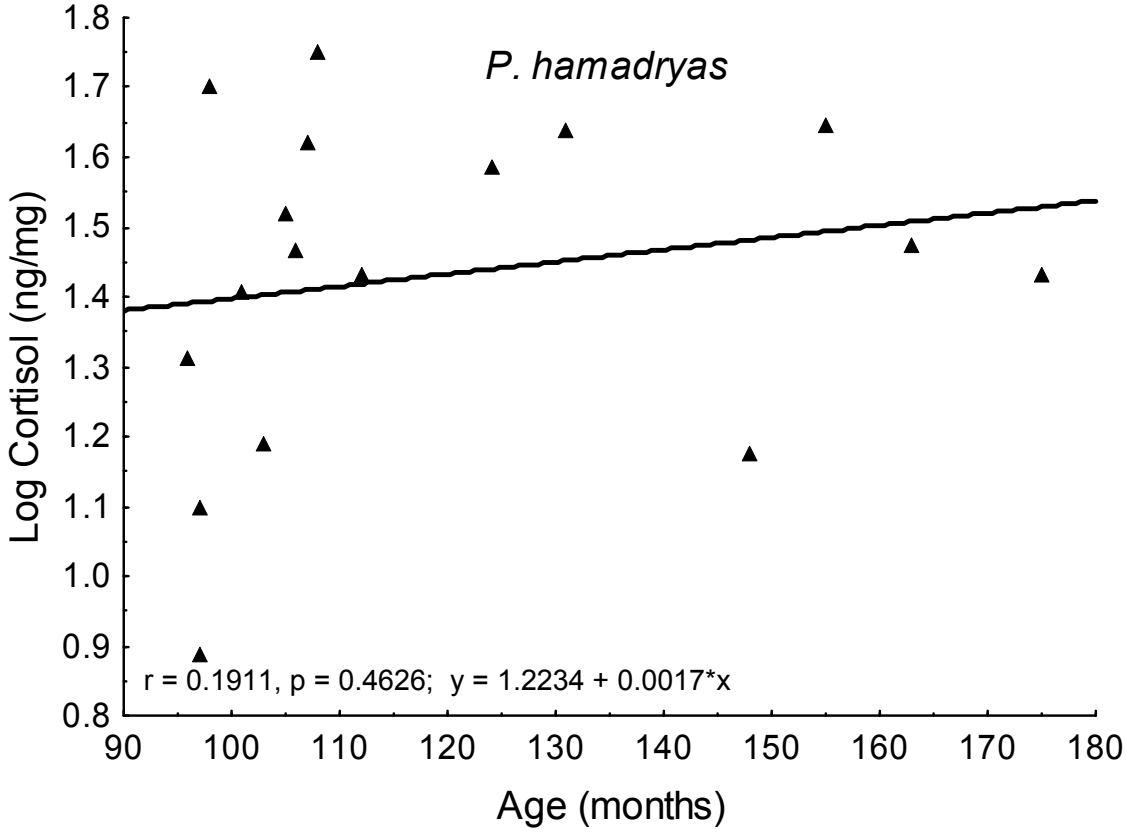


Figure 5.5: Figure showing the relationship between age and cortisol in male hamadryas.



Chapter 6: Hybridity and sex effects on inter-specific variation in hair cortisol concentrations in populations of wild baboons in the Awash National Park, Ethiopia

ABSTRACT

Research suggests that adult male hybrid baboons in the Awash National Park, Ethiopia, exhibit elevated basal cortisol concentrations compared to parent species (*Papio anubis* and *P. hamadryas*) due to hybrid disruption of bio-behavioral complexes. This paradigm was extended to an investigation of male and female baboons and additional populations within the hybrid zone by examining variation in basal cortisol. Animals were sampled during a drought in 1973. It was observed that hamadryas baboons exhibited particularly poor body condition during this time, and I predicted that hamadryas baboons would exhibit elevated basal cortisol concentrations. Alternatively, inter-specific differences in basal cortisol concentrations were previously demonstrated to be associated with hybridity in these populations. Therefore, it was predicted that hybrid populations would exhibit elevated basal cortisol. Basal cortisol concentrations were determined by quantifying time averaged cortisol concentrations archived in hair. Variation in hair cortisol was analyzed using a generalized linear mixed model. Variables found to be significant contributors to this model were subjected to *post hoc* analyses by making pairwise comparisons, tests of difference in variance and correlation analysis. The model found that both sex and species significantly explained variation in hair cortisol. Both male hamadryas and hybrid populations exhibited significantly elevated hair cortisol compared to anubis. Male hamadryas-like hybrids had higher hair cortisol than

intermediate phenotypes, and anubis-like phenotypes had the lowest hair cortisol concentrations. Female hair cortisol did not differ among taxa. However, all female cohorts had significantly elevated hair cortisol compared to anubis males.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis (Nelson, 2005) is the principal endocrine axis responsible for maintaining homeostasis in the face of an external stressor. Cortisol is one of the principal hormones produced by this axis (Hiller-Sturmhöfel and Bartke, 1998; Sapolsky, 2000, 2004). Both psychosocial (Eberhart et al., 1983; Manogue et al., 1975; Sapolsky, 2005, 2011; Shively et al., 1997) and physical (Foley et al., 2001; Pride, 2005; Saltz and White, 1991; Strier et al., 1999) stressors can result in elevated cortisol concentrations. The short term effects of cortisol are adaptive but if chronically maintained at high levels they can have negative health effects (Byung-Wan et al., 2010; Chrousos, 2000; Damsted et al., 2011; Das et al., 2010; de Kloet et al., 1999; Epel, 2009; Nijm and Jonasson, 2009; Sapolsky, 1992a, 2004, 2005). For this reason cortisol is often used as a measure of the physiological costs associated with particular social, reproductive and/or environmental conditions (e.g. Gesquiere et al., 2011; Kirschbuam et al., 2009; Saltzman et al., 1994; Sapolsky, 1992b). Here I examined variation in the basal cortisol concentrations among populations of two closely related but behaviorally divergent primate species and their natural hybrids. Specifically, in the previous chapter it was found that the basal cortisol profile of a wild male hybrid baboon population (*Papio anubis* x *P. hamadryas*) appears to be more variable than that of either parent population (Chapter 5), and higher than that of that of male anubis baboons, suggesting a physiological cost associated with hybridity (see Burke and Arnold, 2001 for review). Hybridization will dismantle and recombine parental bio-behavioral stress complexes, possibly disrupting their adaptive effectiveness and resulting in a greater variety of stress

phenotypes. Here I examine whether similar patterns of variation among anubis, hamadryas and hybrid baboons, as has been previously described (Chapter 5), were present in both male and female populations of anubis, hamadryas and hybrid baboons sampled at an earlier point in time during a severe drought.

I use a relatively novel methodology in primate research to measure basal cortisol concentrations by quantifying cortisol archived in hair. Cortisol is accumulated in the hair shaft during growth and hair cortisol therefore reflects a time averaged measure of circulating cortisol (Accorsi et al., 2008; Bennett and Hayssen, 2010; Davenport et al., 2006; Fourie and Bernstein, 2010a, b; Sauve et al., 2007). I used a simple model of hair growth, which is explained in detail in the previous chapter (Chapter 5), to estimate the period of time likely to be archived in the baboon hair samples used here, which is estimated to reflect a period of as much as twelve months. Cortisol archived in hair appears to be remarkably stable over time (Chapter 5; Macbeth et al., 2010; Webb et al., 2010). Samples collected between 20 and 12 years ago (Chapter 5) and samples from archeological contexts have shown to preserve biologically relevant variation in cortisol concentrations, and with proper validation historical collections may be safely used. I used these time averaged measures of cortisol to examine variation in basal cortisol concentrations among wild baboon populations.

In the Awash National Park (ANP), Ethiopia, two socially divergent baboon species coexist. During 1973 to 1974 the region experienced a drought. Samples collected during this period are treated separately as the effects of the drought on circulating cortisol need to be taken into account. Even though populations differ in behavioral patterns, and evidence suggests that these differences may be associated with differences in chronic

cortisol levels among adult males (Chapter 5), differences in population-specific habitat productivity during this drought period may affect how species-specific cortisol profiles are expressed. The drought and its possible effects are therefore discussed.

During the drought, green vegetation was nearly completely absent from the park. The usually evergreen riparian forest strip was also severely affected (Phillips-Conroy and Jolly, 1986). Browsing ungulates experienced the highest mortality, whereas species adapted to arid conditions, associated with the more open thorn scrublands appeared to be less affected. Hamadryas baboons (*Papio hamadryas*) of the ANP appeared to exhibit more severe signs of nutritional stress than anubis and hybrid animals (Phillips-Conroy, 1978; Phillips-Conroy and Jolly, 1986). During the drought period hamadryas baboons showed the most dramatic loss of body mass compared to animals from anubis and hybrid populations. Hamadryas males showed an approximately 16% decrease in body mass from non-drought conditions to drought conditions, whereas anubis only showed an approximately 4.5% decrease in body mass (Phillips-Conroy et al., 1991), while hybrid males showed an approximately 7.7% change in body weight (Jolly and Phillips-Conroy, unpublished data). Hamadryas baboons (*P. hamadryas*) are generally associated with more arid environments than anubis, and are found in the mainly arid thorn scrub habitats in the ANP (Biquand et al., 1992; Phillips-Conroy and Jolly, 1986; Swedell et al., 2008). Anubis are almost exclusively associated with the more productive flood plain habitat, which stretches above the Awash Falls to the west (see Figure 2.2). The hybrid population inhabits the canyon zone below the Awash Falls. This habitat is considerably less productive than the flood plain above the falls, but is of a better quality than that inhabited by hamadryas populations further down stream (Nagel, 1973), around the

alkaline Lake Basaka and the extinct volcano, Mount Fantale (Phillips-Conroy and Jolly, 1986; Swedell et al., 2008, Sugawara, 1979, 1982; Shotake, 1982, see Figure 2.2). The differences in relative quality among the anubis, hamadryas and hybrid habitats are likely to have been accentuated by the drought and hamadryas baboons appeared to be most affected by the accentuated relative poverty of their habitats.

Anubis, hamadryas and hybrid populations also differ markedly in social structure and behavior. Anubis baboons form multi-male, multi-female social groups referred to as "troops" organized around dominance hierarchies (Bercovitch, 1987; Bercovitch and Strum, 1993; Charpentier et al., 2008; Easley et al., 1989; Johnson, 1987; Packer, 1979a; Packer, 1995; Smuts and Watanabe, 1990; Strum, 1982). Anubis males disperse from their natal groups whereas females are philopatric (Phillips-Conroy and Jolly, 2004). Reproduction among savannah baboons (i.e. anubis and yellow baboons) is promiscuous and influenced by male dominance (Alberts et al., 2003; 2006; Bulger, 1993; Hausfater, 1975; Weingrill et al., 2000) as well as affiliative relationships that males cultivate with females (Alberts et al., 2003; 2006; Lemasson et al., 2008; Noe and Sluitjer, 1990 Palombit et al., 1997; Silk, 2002). Males compete for access to estrus females and defend access to an estrus female over a short consortship period.

Hamadryas baboons (*P. hamadryas*) live in multi-layered societies. In hamadryas society the band, which is equivalent to troops in other baboons, is divided into "clans", which consist of a number of associated one male units (OMUs) made up of a single leader male and several females with whom he is exclusively bonded (Abegglen, 1984; Colmenares et al., 2006; Kummer, 1968, 1984, 1995; Schreier and Swedell, 2009). Male members of a clan are thought to be related (Abegglen, 1984; Kummer, 1968; Sigg et al.,

1982). Males do not typically leave their natal clans whereas females more often are forcibly transferred between OMUs and clans (Pines and Swedell, 2011; Swedell et al., 2011). Males actively, and often aggressively, herd their females. Females primarily bond with the OMU leader (Abegglen, 1984; Kummer, 1968; Sigg, 1980), although they do also exhibit some female bonding (Swedell, 2002).

Along the Awash River, at the contact zone between anubis and hamadryas populations a multi-generation inter- and back-crossed hybrid population exists (Aldrich-Blake et al., 1971; Brett et al., 1977, 1982; Jolly and Brett, 1973; Jolly, 1993; Jolly and Phillip-Conroy, 2003; Kummer, 1968a, b, 1995; Nagel, 1973; Phillips- Conroy and Jolly, 1981, 1986; Phillips-Conroy et al., 1986; Shotake, 1982; Shotake et al., 1977; Sugawara et al., 1982). The behavioral profile of this population is diverse and exhibits characteristics of both parent species (Bergman and Beehner, 2004; Bergman et al., 2008). Male hybrid behavioral agendas typically reflect their external phenotype. In other words, hamadryas-like males try to form OMU's, anubis-like males form consortships and mate promiscuously, while males of intermediate phenotypes exhibit behavioral aspects of both parent species often forming "loose" OMUs with fluid membership (Bergman and Beehner, 2004; Bergman et al., 2008). Cross immigrant males also continue to express species typical behavior within the host group (Nystrom, 1992; Phillips-Conroy et al., 1991, 1992). This suggests that male behavioral strategies may be genetically programmed (Bergman and Beehner, 2004; Bergman et al., 2008).

Similarly females also exhibit behavioral strategies reflective of their external phenotype (Beehner and Bergman, 2006). However, unlike male behavioral strategies, female behavior, whether anubis, hamadryas or hybrid appears to be more flexible and in

part modulated by the male behavioral program. For example, female hamadryas which have been artificially transferred to an anubis group do not form strong inter-sex bonds or attempt associate with a single male, and female anubis in hamadryas groups conform to the OMU structure (Kummer et al., 1970). Females appear to be able to adapt their behavior to conform to the dominant male behavioral agenda suggesting that unlike males female behavior may be more generalized, and less rigidly set than that of male conspecifics. However, even given this apparent plasticity in female behavior, hamadryas anubis females still appear to prefer their species typical behavioral agendas and vary in preferences in accordance with external phenotype, as seen in males. In hybrid group H, hamadryas-like hybrid females tended to associate with a single male, whereas anubis-like hybrid females tended to mate promiscuously (Bergman and Beehner, 2004; Bergman et al., 2008). Presumably, female hamadryas and anubis would show similar species typical preferences for forming intra-sex bonds, with female anubis engaging participating in extended female networks within the group and female hamadryas preferentially forming bonds with the OMU leader.

In the ANP two separate hamadryas baboon groups occur which show signs of significant anubis introgression. Hamadryas group A occupies an area north of Lake Basaka which lies to the north east of the hybrid zone, while group K occupies an area along the Awash River downstream from the hybrid population (see Figure 2.2). The majority of animals in both these groups are hamadryas-like hybrids; a significant minority exhibits a more "pure" hamadryas phenotype (Phillips-Conroy and Jolly, 1986).

The hybrid population in the Awash is back-crossed over many generations and continues to experience inter-crossing from hamadryas and anubis populations.

Behavioral phenotypes are therefore diverse and social and behavioral norms are disrupted in hybrid society. Social interactions in such an environment may be accompanied with a high degree of unpredictability and possibly psychosocial stress. The social environment may be further destabilized with the immigration and emigration of anubis and hamadryas males. Male anubis and hamadryas populations do not appear to differ in their basal cortisol profiles suggesting that they are adapted to the social stresses imposed by their respective social environments (Chapter 5). The basal cortisol profile of the male hybrid population suggests the effects of a destabilized social environment and a distinct cortisol phenotype. The heterogeneous social milieu typical of hybrid groups may be less disruptive to females as they are less constrained in their social interaction by a fixed behavioral program than males. Females in this context appear to able to follow their preferred behavioral agenda and exercise a significant amount of choice as they move between the variably loosely or strictly defined OMUs and the anubis-like polygynandrous segment of the hybrid group (Bergman and Beehner, 2004; Bergman et al., 2008).

Females experience greater energetic constraints related to reproduction than males (Trivers, 1972; Williams, 1975). Various stages of female reproduction are known to be associated with increased cortisol (e.g. third trimester of pregnancy – Kirschbuam et al., 2008; lactation – Hoffman et al., 2010). Due to the energetic costs associated with female cycling, gestation and lactation, females may be physiologically more sensitive to ecological stressors than males. Nutritional stress in female primates may lead to disruption of reproductive function (Bronson, 1995; Ellison, 2001; Knott, 2005; Koenig et al., 1997) whereas it rarely affects male biological reproductive function (Ellison and

Panter-Brick, 1996). Females also experience greater mortality (e.g. lemurs and baboons) during periods of severe ecological stress (e.g. Gould et al., 1999; Hamilton, 1985).

Given these differences in social environments and habitat associations among the three populations, hybrid effects, and sex differences in possible vulnerability to environmental stresses, I examined variation in basal cortisol concentrations in the sample using a cross-sectional population level approach by testing hypothesis about potential sources of variation.

I tested the hypothesis that hamadryas baboons were the physiologically most stressed population during the drought (H_1). This hypothesis predicted that hamadryas baboons would show elevated basal cortisol concentrations compared to anubis and hybrid populations. Body mass data suggests that hamadryas baboons were more acutely affected by the drought, experiencing more acute energetic stresses, which should be expressed by elevated basal cortisol concentrations.

The alternative hypothesis, that population differences in basal cortisol concentrations reflect the dysregulating effect of hybridity on bio-behavioral stress complexes (H_2), predicted that male and female cohorts of the hybrid and hamadryas populations would exhibit higher and more variable basal cortisol concentrations than conspecifics in the anubis population. Previously it was shown that elevated basal cortisol profiles, characterized by significant inter-individual variability in hair cortisol, are associated with the hybrid population in the ANP, possibly reflecting the destabilizing effect of hybridization on social and biological stress complexes (Chapter 5). These hybrids, some of which derive from the same troop as was sampled in Chapter 5, are expected to exhibit

similarly elevated hair cortisol. The hamadryas populations used in this study also show signs of hybridization with anubis, it is expected that the basal cortisol profiles of these animals will be elevated due to the nutritional stress associated with the drought but also that basal cortisol levels will show the variability typical of hybrids. These predicted patterns are expected in both males and females.

I tested the hypothesis that females are more sensitive to ecological stress compared to males (H_3). It was predicted that females would show elevated basal cortisol concentrations compared to male conspecifics, reflecting the greater ecological stresses felt by females in the face of the drought due to the increased energetic demands associated with female reproduction.

Lastly, I examined the relationship between external phenotype in hybrids and basal cortisol concentrations in a subsample of hybrid animals for which data on external phenotype was available in order to further explore the variability in hybrid hair cortisol concentrations.

MATERIALS AND METHODS

Samples

This study focused on variation in adult animals (i.e. animals with third molars in full occlusion - Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000), however for the purposes of biologically validating the use of the current sample set I additionally measured hair cortisol concentrations in animals ranging from infants to older juveniles

(see Tables 6.1, 6.2, and 6.3 for sample list and Table 6.4 for basic statistics of the adult sample). Month age estimates, based on patterns of occlusal dental wear and eruption were available for all animals used in this study (Phillips-Conroy and Jolly, 1988 and Phillips-Conroy et al., 2000). I focused specifically on adults because 1) all biologically meaningful age classes are not equally well represented in the whole sample and 2) hair cortisol varies substantially with age which complicates analysis and interpretation of inter-specific differences. However I take advantage of the known age related variation in cortisol concentrations in baboons (Castracane et al., 1981, Fourie and Bernstein, 2011a; Gesquiere et al., 2005) to validate the use of this historical hair collection. All samples in the present study derive from anubis (troops: B, C, D, E and F), hamadryas (groups A and K), and hybrid (troops: G, H, I and J) groups that were trapped and sedated (as described by Brett et al., 1982) during the 1973 field season. Hair (50 to 100mg) was clipped from between the shoulder blades, taking care not to injure the skin.

All animals were not necessarily trapped in short succession but over the extent of the field season. This means that individual animals experienced different amounts of rainfall in the period preceding capture and sampling. In order to assess and control for the effects of rainfall on hair cortisol concentrations the amount of rainfall each individual had experienced in the 12 months preceding capture was calculated. This temporal span was picked as it matches well with a previous estimate of the period of endocrine activity recorded in a multi-hair sample of asynchronous growing hair in non-human primates (Chapter 5) and because previous analysis showed that dietary patterns archived in stable isotope ratios in hair correlate well with variation in rainfall measured over a 12 month period (Fourie et al., 2008, in preparation). Rainfall data representative of rainfall for the

ANP was provided by the Merti Metahara Sugar Estate (see Figure 2.2). All sites are located within 30km of Merti Metahara and these rainfall estimates are considered representative of the rainfall experienced by the ANP, encompassing the study sites.

Hybrid index (HI) data were available for a subset of the hybrid animals (n = 33). These were determined using a range of physical characteristics to calculate a hybrid index score, which scored the phenotypic character of animals on a continuous scale from 1 to 0, where 1 denotes a pure anubis phenotype and 0 denotes a pure hamadryas phenotype (see Phillips-Conroy and Jolly, 1986).

Sterilization and Biological Validation

The samples utilized in this study were sterilized with an ethanol (C_2H_6O) rinse prior to shipping for use in unrelated research. Samples were then shipped and stored under ambient conditions in paper envelopes. Ethanol sterilization of the hair samples may result in leaching of endogenous glucocorticoids from the hair. I biologically validated the use of these samples before further evaluation of hair cortisol concentrations by investigating whether age related variation in circulating cortisol concentrations known to occur in baboons are reflected by the samples (i.e. infant hypercortisolism and age related decline in cortisol – Castracane et al., 1981, Fourie and Bernstein, 2011a; Gesquiere et al., 2005). For this purpose I quantified the cortisol concentrations in a subset of male animals ranging from infants (dental age class 1, Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000) to older juveniles (dental age class 3), from samples that were ethanol sterilized (i.e. the 1973 sample set) and samples that were not treated (1993-

2000 sample set – Chapter 5; see Table 6.1 for sample breakdown). I examined whether the treated and untreated hair samples returned similar age-related patterns of cortisol concentrations and set the criterion of acceptance that both samples should show a significant decline in hair cortisol with age.

Hair Sample Preparation

Hair samples were prepared and cortisol was extracted using the method described in Chapter 5.

Cortisol Enzyme Immunoassay (EIA)

The same assay system and procedures were used as described in Chapter 5.

Statistical Analysis

For biological validation I conducted a simple linear correlation between hair cortisol concentrations and age (estimated in months – Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000) which was tested using the Pearson's product-moment correlation coefficient. All analyses were performed using SYSTAT 13 (Systat, Inc., San Francisco, CA) and significance was set to $p < 0.05$.

Variation in hair cortisol concentrations was examined using a multivariate general linear mixed model (GLMM) to evaluate the significance of different variables in

explaining hair cortisol variation. Species, sex and the interaction between these two categorical variables were included as fixed effects in the model. I also included individual age estimates (in months) and the regional rainfall experienced during the 12 months prior to capture and sampling as fixed effects in the model. Troop identity was included in the model as a random effect. I did not include individual identity as a random effect in the model as no individual was sampled more than once. Random effects are effects are not likely to be replicated in subsequent analyses or studies. In other words, an effect of age or rainfall on hair cortisol concentrations would be considered an effect that could be replicated in a subsequent study and generalized to the population, whereas troop affinity or individual identity are unlikely to be repeated in subsequent studies and represent potential sources of random variation unique to the sample. First, a model was created which included all the fixed effects and troop as random effect. The model was then optimized by a backward stepwise process, where the fixed effect that explained the least variance was removed from the model. This process was repeated until elimination of additional variables no longer increased the explanatory power of the model; random effects were not excluded. I used the Akaike information criterion (AIC) as an indicator of the relative explanatory power of the model. The model resulting from the backward stepwise elimination of variable resulting in the lowest AIC value was selected as the preferred model.

The GLMM analysis was followed by *post hoc* tests which further examined fixed or random effects that were found to significantly explain hair cortisol variation in the model. The GLMM module in SYSTAT 13 (Systat, Inc., San Francisco, CA), allows for post hoc pair wise comparisons to be made of categorical fixed effects using standard

errors generated from the model to control for all other fixed and random effects in the model. The Tukey's Honestly Significant Difference (HSD) test was used for all *post hoc* testing using fixed effects. All continuous variables that significantly explained variation in the model were further examined using Pearson's correlations.

The prediction that hybrids and/or mixed populations exhibit more variation in chronic cortisol concentrations than phenotypically more homogenous populations was tested by using Hartley's Fmax test (Hartley, 1950), a test for homogeneity of variance using the untransformed cortisol concentrations. These data were left untransformed as Hartley's Fmax test tests for differences in variance. The aim of the test is to examine whether the variance among multiple populations is homogeneous, and by log transforming the data the inherent variance is reduced and normalized. Multiple pairwise comparisons between population variances were then made using a Tukey-type multiple comparisons test for differences among variances using the natural logarithms of the variances calculated from the untransformed data (as per Zar, 1999; but also see Levy, 1975a, b).

Variation in hybrid hair cortisol concentrations relative to external phenotype, as expressed using hybrid index scores, was further explored using Pearson's correlation. A subset of hybrid animals (19 males and 14 females) was used for this analysis as hybrid index scores were not available for all animals from the hybrid population.

RESULTS

Biological Validation

Results showed that both treated and untreated male anubis (treated: n= 39, r^2 = 0.126, p = 0.025; untreated: n = 59, r^2 = 0.139, p = 0.004) and hamadryas (treated: n = 23, r^2 = 0.173, p = 0.048; untreated: n = 16, r^2 = 0.327, p = 0.021) samples showed a significant decline in hair cortisol concentrations from infancy through the juvenile period as was predicted if the treated samples retained a biologically relevant signal of hair cortisol (Figures 6.1 and 6.2). Hybrids did not show any age related variation in hair cortisol concentrations derived either in treated (n = 37, r^2 = 0.016, p = 0.463) or untreated samples (n = 17, r^2 = 0.076, p = 0.286; see Figure 6.3).

GLMM

The fixed effects species, sex and the interaction between the two effects were found to significantly explain variation in hair cortisol concentrations in the model. After backward stepwise elimination of non-significant variables and assessment of AIC values for each model, the final model included species, sex and the interaction between species and sex, as well as the random effect troop affiliation. Neither age nor 12 month regional rainfall was found to significantly explain variation in the model. The random effect, troop, did not significantly contribute to variation in the model (see Table 6.5).

Post Hoc Tests

I further analyzed the interaction between species and sex in the model using the Tukey's HSD test. Pairwise comparisons showed that the male hamadryas (n = 22, mean±1SD = 22.970 ± 15.221 ng/mg) and hybrid (n = 22, mean±1SD = 22.970 ± 15.221 ng/mg) populations had significantly elevated hair cortisol profiles compared the anubis population (n = 22, mean±1SD = 22.970 ± 15.221 ng/mg), but did not differ from one another (see Table 6.6 and Figure 6.4). Female populations did not differ significantly from one another in hair cortisol concentrations (see Table 6.4 and 6.6), showing association between hybridity and elevated hair cortisol, suggesting that hybridization may have different consequences for males and females. All female populations had significantly elevated hair cortisol profiles compared to the male anubis population, and did not differ significantly from the hamadryas and hybrid populations (see Table 6.6).

Variances in hair cortisol concentrations differed significantly among male anubis, hamadryas and hybrid populations (Hartley's Fmax test: $Fmax_{(0.05, 3, 20)} = 2.95$, $Fmax_{(3, 24)} = 5.651$, $p<0.05$). A Tukey-type multiple comparisons of variances test (Levy, 1975a, b) found that male hamadryas and hybrid populations had significantly greater variation in hair cortisol concentrations compared to male anubis (anubis – hamadryas: $q_{(0.05,\infty,3)}= 3.314$, $q = 6.275$, $p<0.05$; anubis-hybrid: $q_{(0.05,\infty,3)}= 3.314$, $q = 4.046$, $p<0.05$, Figure 6.2). Male hamadryas and hybrids did not differ in inter-individual variation in hair cortisol concentrations ($q_{(0.05,\infty,3)}= 3.314$, $q = 1.661$, $p<0.05$). Variability in female hair cortisol concentrations did not differ between the anubis, hamadryas and hybrid populations (Hartley's Fmax test: $Fmax_{(0.05, 3, 15)} = 3.54$, $Fmax_{(3, 13)} = 2.449$, $p>0.05$).

Hybrid Index

Neither male nor female hybrid hair cortisol concentrations showed any significant linear dependence with hybrid index score (males: $r = -0.093$, $p = 0.7064$; females: $r = 0.046$, $p = 0.876$). However the male sample did exhibit a visible trend for animals with a more hamadryas-like phenotype to have higher hair cortisol concentrations, when a phenotypically intermediate individual (ID = 10400, HI = 0.611, Cortisol = 58.8ng/mg), which had the highest hair cortisol concentration in the sample was removed from the analysis the relationship between hair cortisol concentration and external phenotype approached significance ($r = 0.448$, $p = 0.063$; see Figure 6.5).

DISCUSSION

The results suggest that the samples used in this study had preserved biologically meaningful variation in cortisol concentrations even though the samples derived from an archived collection, some 39 years old, and had been sterilized using an ethanol rinse which was likely to have leached some of the archived cortisol. The both the older treated and more recent untreated hybrid sample sets did not recover an age related decline in hair cortisol during the juvenile phase. The fact that the same pattern is preserved in both sample sets suggests that this may be a feature of hybrid populations. The 1973 samples have lower absolute cortisol concentrations than the non-drought-untreated samples (see Figure 6.1-6.3). It is not clear whether this is a result of bleaching of cortisol over time or due to leaching which occurred as a result of the ethanol rinse. Previous investigations using samples collected from archived cadavers, which had been kept frozen for several

years (Fourie and Bernstein, 2011a), in addition to samples from the same populations as is used here but that were collected more recently (~ 10 to 20 years ago – Chapter 5, and e.g. Figures 6.1-6.3), as well as studies on archaeological hair (Webb et al., 2010) and hair collected from wild bears (Macbeth et al., 2010), all suggest the long term stability of cortisol in the hair matrix. The results presented here further contribute to this growing body of evidence suggesting that cortisol archived in hair is resistant to leaching under natural conditions over time and preserves biologically relevant patterns of endocrine variation.

It was predicted that because hamadryas baboons exhibited more severe signs of malnutrition during the drought that they would also exhibit elevated hair cortisol concentrations compared to anubis and hybrid populations (H_1). The GLMM found that there were significant differences in hair cortisol concentrations among the three male populations but not the female populations. Male anubis tended to have lower hair cortisol concentrations than male hamadryas and male hybrids, a pattern of differences that did indicate greater physiological stress in hamadryas in line with observed patterns of nutritional stress. Female populations did not differ from one another as predicted by hypotheses concerning the effects of ecological stress (H_1) or hybrid dysregulation of stress (H_2), in that no differences among populations could be detected. This either suggests that female populations are not differentially affected by these factors, and that variation in female hair cortisol is sensitive to other factors, such as reproductive state, which if not controlled for may swamp evidence of ecological or hybrid effects.

Previously an association between elevated hair cortisol and a hybrid population was found among some of the same populations as is examined here (Chapter 5), it was

therefore expected that this sample would reflect a similar population level pattern of hair cortisol. It was predicted that the hybrid population and the hamadryas population (which shows signs of significant hybridization) would exhibited elevated and more variable hair cortisol concentrations compared to the anubis population (H_2). The results bear this out, but, only for males. Male anubis had significantly lower and less variable hair cortisol concentrations than male hamadryas and hybrids. These results further support earlier observations and suggest that elevated and variable basal cortisol concentrations in the male populations may be a marker of hybridity or hybrid effects on social uncertainty among ANP male baboon populations. Hybridity may result in disruption of bio-behavioral stress complexes, and behaviorally more diverse and less predictable social environments which may result in higher social stress loads. Therefore hybrid populations, and/or species populations that experience significant introgression from another species may exhibit elevated and more variable hair cortisol concentrations.

The elevated hair cortisol concentrations in hamadryas, and possibly hybrids, may be due to the effects of the drought on these populations, particularly the hamadryas population which showed the most severe signs of malnutrition. However, the ecological stress hypothesis does not predict increased variability in hair cortisol as was described in hybrids in Chapter 5. Therefore, while the elevated hair cortisol concentrations in the hybrid and hamadryas populations analyzed here is likely due to both hybrid effects (especially the hybrid population) and ecological stress (especially the hamadryas population), the more variable hair cortisol concentrations in these two populations is consistent with hypothesized hybrid effects.

Results from the biovalidation study provide further evidence of disruption of normal physiological patterns in hybrids; hybrid-age cortisol profiles differ from that of their parent species and do not exhibit the hypercortisolism typically associated with infancy and the age-related decline in cortisol concentrations throughout the juvenile period as described in other baboon species (Castracane et al., 1981, Fourie and Bernstein, 2011a; Gesquiere et al., 2005).

It was surprising that no population differences could be found among female baboons. Variation in female hair cortisol concentrations do not appear to be associated with hybridity or the effects of the drought. This may be because female baboon behavior is more generalized than that of male baboons, as suggested by the behavioral flexibility of females in adjusting to the dominant behavioral norms of their social group (Kummer et al., 1970). Females may be able to negotiate heterogeneity in the social environment with fewer stress effects. The bio-behavioral effects of hybridization appear to differentially affect female basal cortisol.

I predicted that because evidence suggests that females are more vulnerable to ecological stress than males (Bronson, 1995; Ellison, 2001; Gould et al., 1999; Hamilton, 1985; Knott, 2005; Koenig et al., 1997) that the female populations in the sample would be more acutely stressed as a result of the drought. This appears to only be true for the anubis population, though all female populations exhibit elevated hair cortisol concentrations compared to the male anubis population, but not compared to male hamadryas and hybrid populations. Female baboon stress physiology is acutely affected by various aspects of reproduction and can profoundly shape the way in which females respond to environmental and social stressors (Crockford et al., 2008). Differences in the

proportions of females in each population which are cycling, lactating or pregnant may account for the lack of inter-specific differences in hair cortisol. Further evaluation of these variables may resolve the apparent lack of differences in basal cortisol among these female populations.

Finally, the analysis of hybrid index provided the first tentative evidence showing an effect of hybrid phenotype on cortisol concentrations. Intermediate and anubis-like phenotypes were not as well represented as the hamadryas-like phenotype in this sample. A more inclusive sample may further resolve the significance of the trend described here. These results suggest that the hamadryas-like phenotype may be associated with higher hair cortisol concentrations, and that the anubis-like hybrid phenotype may be associated with the lowest hair cortisol concentrations, with intermediate hybrid phenotypes having intermediate hair cortisol concentrations (Figure 6.5).This observation is only true of males as no relationship could be found in the female sample. If these basal cortisol concentrations reflect, in part, differential social stresses associated with hybridity, then it would appear that male hamadryas-like hybrids experience elevated physiological stress in hybrid groups, whereas male anubis-like hybrids are able to negotiate the heterogeneity of social agendas in hybrid groups without similar effects on physiological stress. As previously mentioned, the hamadryas phenotype may be more acutely sensitive to violations of social expectations, which is inherent in hybrid social groups as different phenotypic variants express different socio-reproductive tendencies. Whether these differences in basal cortisol concentrations result in differential effects on fitness remains to be tested. Presently, the evidence suggests that intermediate males may have higher reproductive success than phenotypic extremes in a hybrid group (Bergman et al., 2008).

CONCLUSION

Previously it was shown that among phenotypically more homogenous male anubis and hamadryas populations no differences in hair cortisol concentrations are apparent, but that the phenotypically heterogeneous male hybrid population had significantly higher and more variable chronic cortisol concentrations (Chapter 5). It was speculated that males of the mixed hamadryas populations may experience similar dysregulation of co-adapted stress complexes. The results presented here provide further evidence for the destabilizing effect of hybridization on stress phenotypes in wild baboons. No hybrid effect on female hair cortisol concentrations was found, and only anubis female hair cortisol was elevated compared to male conspecifics suggesting, a potential sex specific effect of drought. Analyses including information on reproductive status may provide greater insights into variation in female cortisol. This study provides novel, though tentative evidence, that the hamadryas-like phenotype in the hybrid population is associated with elevated hair cortisol concentrations, suggesting a physiological cost associated with this phenotype in a heterogeneous social environment.

The results reported provide further evidence of the long term stability and resilience of biologically relevant patterns of cortisol preserved in hair and suggests future application of the technique to extract endocrine information from archival collections. However, more still needs to be done in order to evaluate the stability of cortisol in the hair matrix over time and under different storage and treatment conditions. Nonetheless, I have demonstrated the potential utility of the technique to examine population level

endocrine differences, within population variability and developmental changes in endocrine function.

Table and Figures

Table 6.1: Table of male and female adults samples along with individual identity, population, troop, sex, age, rainfall and hair cortisol information

ID	Population	Troop	Sex	Age (months)	12 Month Rainfall (mm)	Cort (ng/mg)
10001	Anubis	B	M	130	388.5	19.5
10003	Anubis	B	M	170	388.5	28
10004	Anubis	B	M	220	388.5	14
10005	Anubis	B	M	100	388.5	21.5
10006	Anubis	B	M	180	388.5	9
10008	Anubis	B	M	127	388.5	12.7
10015	Anubis	B	M	190	388.5	8.1
10016	Anubis	B	M	185	388.5	5.7
10035	Anubis	B	M	115	386.33	21.6
10085	Anubis	C	M	120	338.67	11.8
10099	Anubis	C	M	115	336.5	6.1
10109	Anubis	C	M	240	336.5	11.9
10110	Anubis	C	M	165	336.5	6
10117	Anubis	E	M	125	313.5	20.1
10119	Anubis	E	M	110	313.5	16.4
10120	Anubis	E	M	115	313.5	11.5
10121	Anubis	E	M	127	313.5	9.7
10122	Anubis	E	M	170	313.5	7.3
10124	Anubis	E	M	127	313.5	8.6
10133	Anubis	E	M	220	313.5	7.7
10139	Anubis	E	M	125	312	13.2
10149	Anubis	F	M	160	303	13.1
10162	Anubis	F	M	170	303	7.7
10177	Anubis	F	M	115	301.5	11.8
10178	Anubis	F	M	180	301.5	11
10203	Hamadryas	A	M	125	281	24
10204	Hamadryas	A	M	175	281	9
10207	Hamadryas	A	M	175	280.6	20.1
10208	Hamadryas	A	M	240	280.6	16.3
10209	Hamadryas	A	M	190	280.6	24.5
10210	Hamadryas	A	M	110	280.6	24.5
10211	Hamadryas	A	M	240	280.6	22.7
10212	Hamadryas	A	M	125	280.6	82
10213	Hamadryas	A	M	90	280.6	32.9
10216	Hamadryas	A	M	117	280.6	33.8
10217	Hamadryas	A	M	220	280.6	32.9
10218	Hamadryas	A	M	220	280.6	16.8
10231	Hamadryas	A	M	240	280.6	24.1
10232	Hamadryas	A	M	220	280.6	14.9
10244	Hamadryas	A	M	115	279.8	11.9
10252	Hamadryas	A	M	185	279	21.9
10253	Hamadryas	A	M	125	279	33.6
10269	Hamadryas	A	M	160	277.8	18.8

Table 6.1: continued…

ID	Population	Troop	Sex	Age (months)	12 Month Rainfall (mm)	Hybrid Index Score	Cort (ng/mg)
10270	Hamadryas	A	M	175	277.8		28.1
10285	Hamadryas	K	M	98	255.27		11.7
10295	Hamadryas	K	M	125	255.27		12.9
10297	Hamadryas	K	M	102	255.27		14
10300	Hamadryas	K	M	140	255.27		26.2
10301	Hamadryas	K	M	140	254		17.1
10310	Hamadryas	K	M	175	252.73		16.7
10311	Hamadryas	K	M	125	252.73		13.6
10312	Hamadryas	K	M	104	252.73		37.8
10313	Hamadryas	K	M	144	252.73		15.2
10343	Hamadryas	K	M	144	250.2		37.9
10344	Hamadryas	K	M	104	250.2		20.6
10190	Hybrid	G	M	165	284.2	0.78	26.7
10195	Hybrid	G	M	90	284.2	0.94	12.7
10196	Hybrid	G	M	180	284.2	0.56	17.7
10197	Hybrid	G	M	90	284.2	0.94	10.2
10198	Hybrid	G	M	140	283.8	0.17	17.2
10375	Hybrid	H	M	170	239.1	0.78	8.8
10378	Hybrid	H	M	100	239.1		27.6
10386	Hybrid	H	M	112	239.1	0.25	18.4
10400	Hybrid	H	M	155	238.67	0.61	58.8
10450	Hybrid	I	M	120	276.67	0.17	31.2
10461	Hybrid	I	M	140	276.67	0.33	26.1
10475	Hybrid	I	M	105	277.83	0.17	15.1
10476	Hybrid	I	M	220	277.83	0.28	22.7
10483	Hybrid	I	M	136	277.83	0.33	16
10487	Hybrid	I	M	100	284.83	0.19	15.3
10493	Hybrid	I	M	170	286	0.28	15.7
10494	Hybrid	I	M	150	286	0.17	20.4
10417	Hybrid	J	M	157	253.33	0.22	27.9
10438	Hybrid	J	M	172	253.33	0.29	22.3
10447	Hybrid	J	M	175	254.5	0.31	22.7
10011	Anubis	B	F	230	388.5		27
10017	Anubis	B	F	170	388.5		14.1
10018	Anubis	B	F	155	388.5		21.4
10019	Anubis	B	F	150	388.5		32
10027	Anubis	B	F	160	388.5		20.4
10036	Anubis	B	F	180	386.33		47.2
10045	Anubis	B	F	150	384.17		14.9
10046	Anubis	B	F	86	384.17		10
10047	Anubis	B	F	155	384.17		37.7
10060	Anubis	B	F	112	377.67		9.3
10164	Anubis	F	F	130	303		9.4
10179	Anubis	F	F	130	301.5		17.6

Table 6.1: continued…

ID	Population	Troop	Sex	Age (months)	12 Month Rainfall (mm)	Hybrid Index Score	Cort (ng/mg)
10186	Anubis	F	F	210	301.5		20.1
10188	Anubis	F	F	230	288		19.5
10220	Hamadryas	A	F	170	280.6		46.8
10234	Hamadryas	A	F	160	280.6		55.7
10236	Hamadryas	A	F	200	280.6		35.1
10255	Hamadryas	A	F	150	279		23.9
10256	Hamadryas	A	F	170	279		26.8
10257	Hamadryas	A	F	150	279		21.7
10287	Hamadryas	K	F	200	254		24.3
10291	Hamadryas	K	F	140	254		11.9
10304	Hamadryas	K	F	185	255.27		36
10305	Hamadryas	K	F	200	255.27		25.1
10306	Hamadryas	K	F	150	255.27		23.9
10314	Hamadryas	K	F	185	252.73		12.7
10315	Hamadryas	K	F	150	252.73		9.2
10316	Hamadryas	K	F	250	252.73		29.1
10318	Hamadryas	K	F	145	252.73		35
10319	Hamadryas	K	F	150	252.73		17.9
10321	Hamadryas	K	F	200	252.73		20.9
10332	Hamadryas	K	F	170	251.47		24.6
10334	Hamadryas	K	F	195	251.47		74.5
10335	Hamadryas	K	F	175	251.47		17.3
10346	Hamadryas	K	F	150	250.2		29.5
10348	Hamadryas	K	F	140	250.2		25.5
10356	Hamadryas	K	F	250	248.93		15.1
10357	Hamadryas	K	F	175	248.93		16.7
10358	Hamadryas	K	F	170	248.93		26.9
10359	Hamadryas	K	F	175	248.93		28.9
10360	Hamadryas	K	F	145	248.93		54.1
10368	Hamadryas	K	F	200	248.93		20.6
10369	Hamadryas	K	F	180	248.93		47.3
10200	Hybrid	G	F	195	283.8	0.80	16.3
10201	Hybrid	G	F	110	283.8	1.00	21.3
10379	Hybrid	H	F	110	239.1	1.00	20.7
10391	Hybrid	H	F	155	239.1	0.40	14.5
10392	Hybrid	H	F	120	239.1		9.8
10403	Hybrid	H	F	115	238.67		11.4
10413	Hybrid	H	F	155	237.37		22.1
10414	Hybrid	H	F	170	237.37	0.60	9.6
10452	Hybrid	I	F	115	276.67		16
10453	Hybrid	I	F	110	276.67	0.20	15.3
10454	Hybrid	I	F	170	276.67	0.20	14.5
10455	Hybrid	I	F	110	276.67	0.20	22.9
10469	Hybrid	I	F	210	277.83	0.50	14.4
10471	Hybrid	I	F	155	277.83	0.50	95.7
10479	Hybrid	I	F	170	277.83		26.9
10484	Hybrid	I	F	110	279		13.1
10486	Hybrid	I	F	180	280.17		14.9
10488	Hybrid	I	F	115	284.83		27.8

Table 6.1: continued…

ID	Population	Troop	Sex	Age (months)	12 Month Rainfall (mm)	Hybrid Index Score	Cort (ng/mg)
10529	Hybrid	I	F	115	325		6.8
10420	Hybrid	J	F	200	253.33	0.20	25.8
10422	Hybrid	J	F	120	253.33	0.20	11.9
10426	Hybrid	J	F	180	253.33	0.40	28
10427	Hybrid	J	F	195	253.33	0.20	19.5

Table 6.2: Table of hair samples of male infant through older juvenile animals collected during non-drought periods that were not sterilized with an ethanol rinse, which were used for demonstration of biovalidation of the use of the samples.

ID	Population	Age (months)	Cort (ng/mg)	ID	Population	Age (months)	Cort (ng/mg)
11315	Anubis	12	54.4	11245	Anubis	70	19.2
10966	Anubis	21	115.3	11340	Anubis	72	70
11252	Anubis	21	79.9	10773	Anubis	75	48.8
10941	Anubis	22	116.4	10955	Anubis	75	17.2
10938	Anubis	29	30.7	10968	Anubis	75	11.3
10942	Anubis	29	17.9	10977	Anubis	75	17.2
10951	Anubis	29	23.5	11225	Anubis	75	16.4
10964	Anubis	29	18.2	11248	Anubis	75	18.8
10998	Anubis	29	153.6	11257	Anubis	75	16
11003	Anubis	29	38.7	11258	Anubis	75	13.6
11198	Anubis	29	34.4	10920	Anubis	77	51.9
11273	Anubis	29	15.4	11231	Anubis	78	11.7
11316	Anubis	29	59.6	11051	Anubis	81	9.7
11320	Anubis	29	93	11136	Hamadryas	12	106.7
11329	Anubis	29	62.6	11151	Hamadryas	21	125.7
11336	Anubis	29	29.4	11118	Hamadryas	29	72.3
10937	Anubis	35	35.6	11131	Hamadryas	43	53.4
10980	Anubis	35	7	11146	Hamadryas	45	53.3
11240	Anubis	37	12.6	11147	Hamadryas	45	18.4
10985	Anubis	43	39.7	11161	Hamadryas	45	23.2
10961	Anubis	45	22	11343	Hamadryas	45	46.7
10963	Anubis	45	31.7	11140	Hamadryas	51	51.6
10973	Anubis	45	46.9	11163	Hamadryas	56	17.8
10984	Anubis	45	58.3	11148	Hamadryas	72	33.5
10941	Anubis	45	19.7	11162	Hamadryas	72	30.1
10999	Anubis	45	31.2	11117	Hamadryas	75	18.8
11197	Anubis	45	29.6	11120	Hamadryas	75	29.2
11239	Anubis	45	72.9	11153	Hamadryas	75	7.9
11251	Anubis	45	51.8	11348	Hamadryas	75	113
11272	Anubis	45	60.4	11216	Hybrid	12	58.7
11245	Anubis	46	21.4	11217	Hybrid	21	64.2
11241	Anubis	47	47.3	11170	Hybrid	29	47
10940	Anubis	48	43.5	11200	Hybrid	29	79.7
10863	Anubis	49	70.1	11202	Hybrid	35	41.7
10983	Anubis	51	71.7	11021	Hybrid	39	26.4
10998	Anubis	51	27.1	11168	Hybrid	45	38.9
11339	Anubis	51	81.4	11169	Hybrid	45	35.9
11015	Anubis	52	50.7	10943	Hybrid	51	43
10879	Anubis	54	27.8	11032	Hybrid	71	27.4
10936	Anubis	60	23.8	11011	Hybrid	72	79.5
10982	Anubis	65	15.5	11020	Hybrid	75	38.5
11223	Anubis	65	48.9	11027	Hybrid	75	19.4
11269	Anubis	65	24.8	11021	Hybrid	75	56
10969	Anubis	66	108.3	11177	Hybrid	75	62.3
11088	Anubis	68	31.3	11215	Hybrid	75	53.5
11241	Anubis	69	41.7	11042	Hybrid	75	28.2

Table 6.3: Table of hair samples of male infant through older juvenile animals collected during the 1973 drought period that were sterilized with an ethanol rinse, which were used for demonstration of biovalidation of the use of the samples.

ID	Population	Age (months)	Cort (ng/mg)	ID	Population	Age (months)	Cort (ng/mg)
10519	Anubis	12	14.3	10325	Hamadryas	45	14.7
10174	Anubis	12	18.2	10263	Hamadryas	45	27.2
10021	Anubis	12	18.6	10337	Hamadryas	52	9.9
10173	Anubis	12	19.7	10364	Hamadryas	59	6
10161	Anubis	12	19.8	10363	Hamadryas	59	24.1
10031	Anubis	21	11	10361	Hamadryas	62	14.4
10184	Anubis	21	20.7	10303	Hamadryas	75	7.5
10147	Anubis	21	45.8	10286	Hamadryas	75	8.9
10053	Anubis	29	5.2	10331	Hamadryas	75	12.7
10130	Anubis	29	6.9	10283	Hamadryas	75	13
10057	Anubis	29	7.8	10317	Hamadryas	75	16.7
10511	Anubis	29	11	10302	Hamadryas	75	19
10040	Anubis	29	15.1	10233	Hamadryas	75	26
11351	Anubis	29	19.6	10409	Hybrid	12	11.3
10061	Anubis	29	19.7	10459	Hybrid	12	32.7
10531	Anubis	29	20.1	10194	Hybrid	21	21.3
10054	Anubis	29	22.5	10467	Hybrid	29	10.9
10182	Anubis	29	24.4	10192	Hybrid	29	15.3
10020	Anubis	35	15.5	10395	Hybrid	29	16.1
10012	Anubis	35	18.2	10396	Hybrid	29	18.3
10039	Anubis	43	14	10248	Hybrid	29	20.8
10518	Anubis	45	12.3	10436	Hybrid	29	28.5
10030	Anubis	45	14.1	10458	Hybrid	29	33.6
10523	Anubis	45	15.1	10383	Hybrid	35	10.2
10167	Anubis	45	15.6	10485	Hybrid	35	11.7
10009	Anubis	52	7.1	10397	Hybrid	43	12.6
10159	Anubis	54	7	10407	Hybrid	45	10
10156	Anubis	54	7.1	10382	Hybrid	45	15.9
10225	Anubis	59	31.8	10442	Hybrid	45	21.8
10166	Anubis	62	7.1	10473	Hybrid	45	22.4
10104	Anubis	62	11.6	10472	Hybrid	45	23
10134	Anubis	75	8.1	10433	Hybrid	45	27.6
10152	Anubis	75	8.8	10456	Hybrid	45	38.8
10522	Anubis	75	11.9	10457	Hybrid	45	44.1
10515	Anubis	75	12.1	10191	Hybrid	51	4.9
10509	Anubis	75	12.2	10406	Hybrid	54	19.6
10153	Anubis	75	12.4	10431	Hybrid	59	17.9
10126	Anubis	75	13.9	10432	Hybrid	59	21.2
10022	Anubis	75	14.1	10381	Hybrid	65	22.7
10510	Anubis	75	15.2	10377	Hybrid	75	13

Table 6.3: continued…

ID	Population	Age (months)	Cort (ng/mg)	ID	Population	Age (months)	Cort (ng/mg)
10354	Hamadryas	12	14	10464	Hybrid	75	16.5
10374	Hamadryas	12	14.7	10390	Hybrid	75	17.6
10284	Hamadryas	12	35.7	10424	Hybrid	75	18
10373	Hamadryas	21	25.2	10466	Hybrid	75	20.3
10240	Hamadryas	29	12.2	10423	Hybrid	75	20.4
10353	Hamadryas	29	20.2	10495	Hybrid	75	21.5
10341	Hamadryas	29	42.7	10380	Hybrid	75	21.6
10264	Hamadryas	35	43.6	10478	Hybrid	75	28.6
10265	Hamadryas	35	57.8	10428	Hybrid	75	31.2
10280	Hamadryas	43	37.5	10401	Hybrid	75	37.1

Table 6.4: Table showing the mean±1standard deviation for age, rainfall and hair cortisol concentration for the male and female baboon populations.

Population	n	Mean Age (months)	± 1 Standard Deviation (months)	12 Month Rainfall (mm)	± 1 Standard Deviation (mm)	Mean Cort (ng/mg)	± 1 Standard Deviation (ng/mg)
Male Anubis	25	152.040	39.308	342.320	36.669	12.560	5.741
Male Hamadryas	30	154.933	46.921	270.300	13.220	23.883	13.649
Male Hybrids	20	142.350	35.394	269.070	18.675	21.675	10.682
Female Anubis	14	160.571	41.718	360.929	41.217	21.471	11.134
Female Hamadryas	29	175.172	28.862	257.459	11.792	28.862	14.713
Female Hybrids	23	147.174	35.510	266.122	22.272	20.835	17.424

Table 6.5: Table showing the results of the final GLMM.

GLMM	d.f.	Standardized β	F	t	p
Intercept	4	0.344		4.419	0.012
Type III Test for Fixed Effects					
Species (anubis, hamadryas, hybrid)	**2, 128**		**7.437**		**0.001**
Sex (male and female)	**1, 128**		**5.817**		**0.017**
Species x Sex	**2, 128**		**4.130**		**0.018**
Random Effect Predictions					
Troop - A	128	0.035		0.749	0.454
Troop - B	128	0.035		0.822	0.413
Troop - C	128	0.038		-0.453	0.651
Troop - E	128	0.036		-0.046	0.963
Troop - F	128	0.036		-0.278	0.781
Troop - G	128	0.036		-0.344	0.731
Troop - H	128	0.035		-0.300	0.765
Troop - I	128	0.034		0.219	0.827
Troop - J	128	0.036		0.432	0.666
Troop - A	128	0.035		-0.750	0.455

Table 6.6: Table showing the results of post hoc pair wise comparisons using a Tukey's HSD test.

		Standard Error	t	p
F*Anubis	F*Hamadryas	0.078	-2.112	0.281
	F*Hybrid	0.077	0.250	1.000
	M*Anubis	**0.070**	**3.013**	**0.031**
	M*Hamadryas	0.077	-0.780	0.971
	M*Hybrid	0.078	-0.427	0.998
F*Hamadryas	F*Hybrid	0.068	2.706	0.074
	M*Anubis	**0.066**	**5.639**	**0.000**
	M*Hamadryas	0.055	1.887	0.410
	M*Hybrid	0.069	1.885	0.412
F*Hybrid	**M*Anubis**	**0.066**	**2.901**	**0.043**
	M*Hamadryas	0.067	-1.187	0.843
	M*Hybrid	0.062	-0.852	0.958
M*Anubis	**M*Hamadryas**	**0.065**	**-4.141**	**0.000**
	M*Hybrid	**0.067**	**-3.617**	**0.004**
M*Hamadryas	M*Hybrid	0.068	0.385	0.999

Figure 6.1: Plot showing the significant negative correlation between infant and juvenile anubis hair cortisol concentrations and age in both the 1973 treated drought samples and the more recently collected non-drought samples.

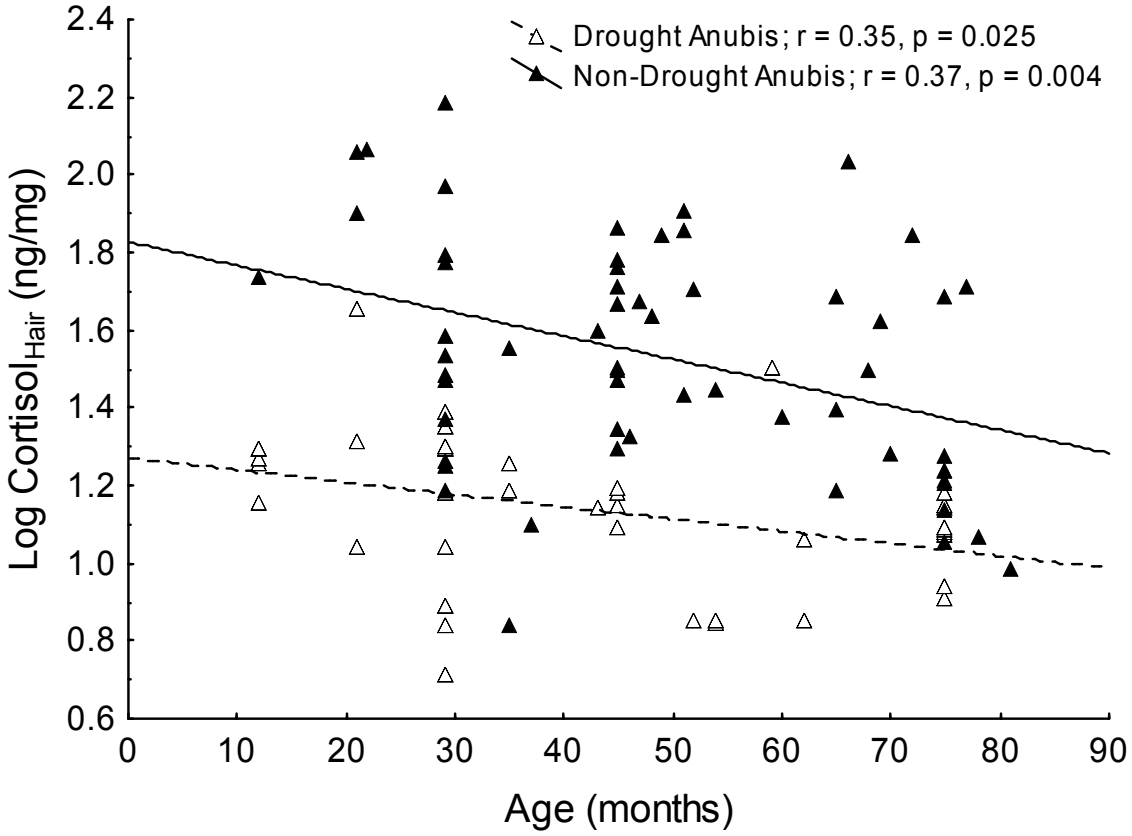


Figure 6.2: Plot showing the significant negative correlation between infant and juvenile hamadryas hair cortisol concentrations and age in both the 1973 treated drought samples and the more recently collected non-drought samples.

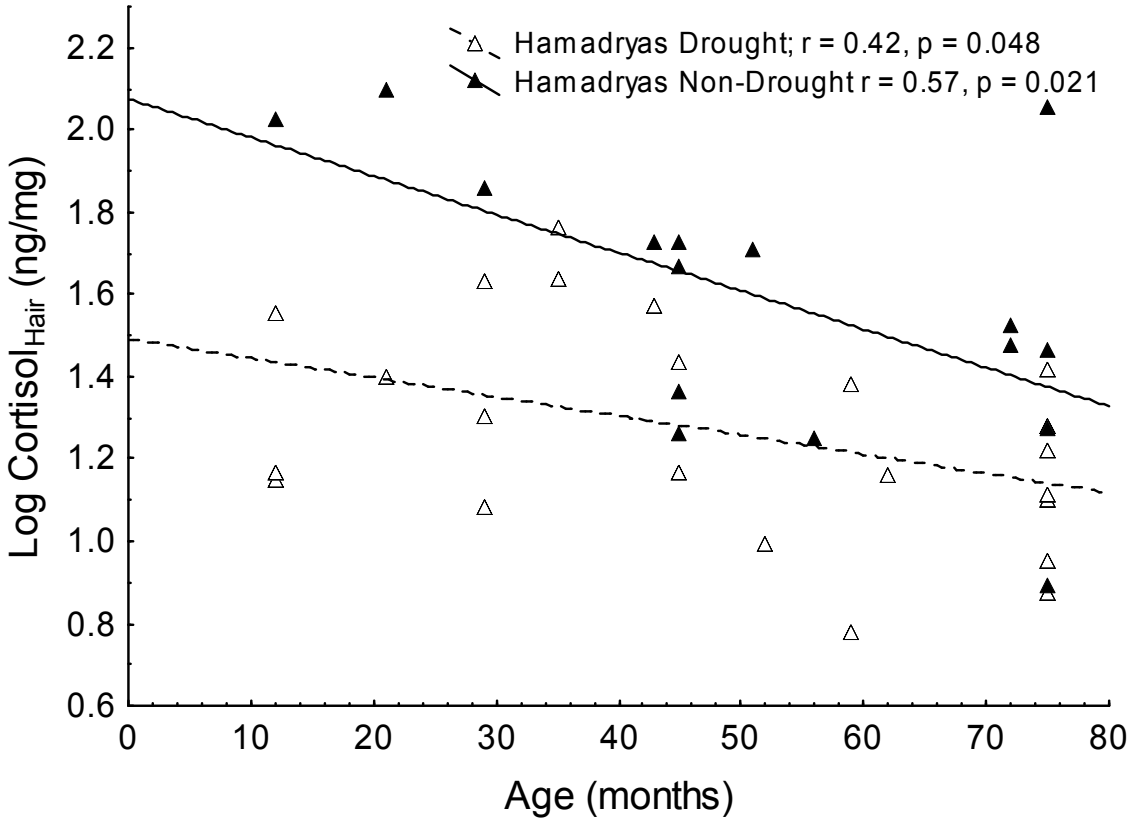


Figure 6.3: Plot showing no relationship between infant and juvenile hybrid hair cortisol concentrations and age in both the 1973 treated drought samples and the more recently collected non-drought samples.

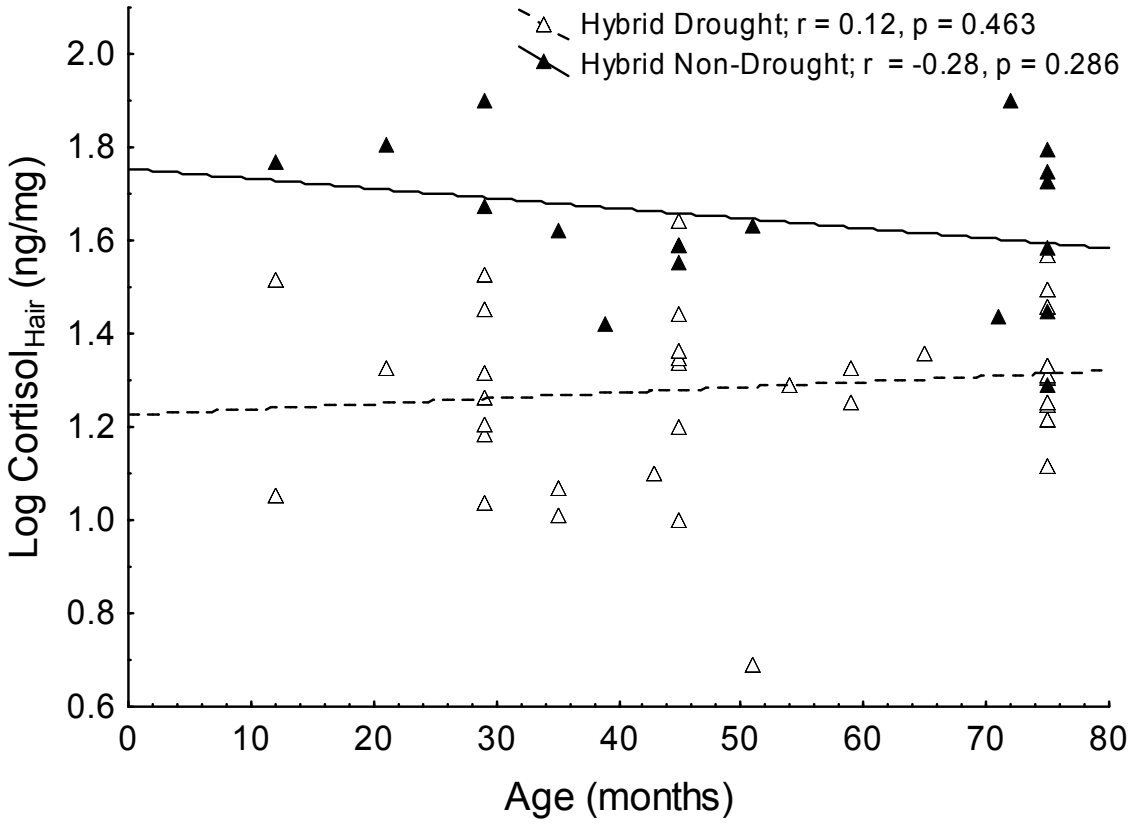


Figure 6.4: Box plot showing that both male hybrid and hamadryas population exhibit elevated hair cortisol concentrations compared to the male anubis population when pair wise comparisons are made while controlling for fixed and random effects in the GLMM. The y-axis is a log-axis to facilitate visual representation of the data.

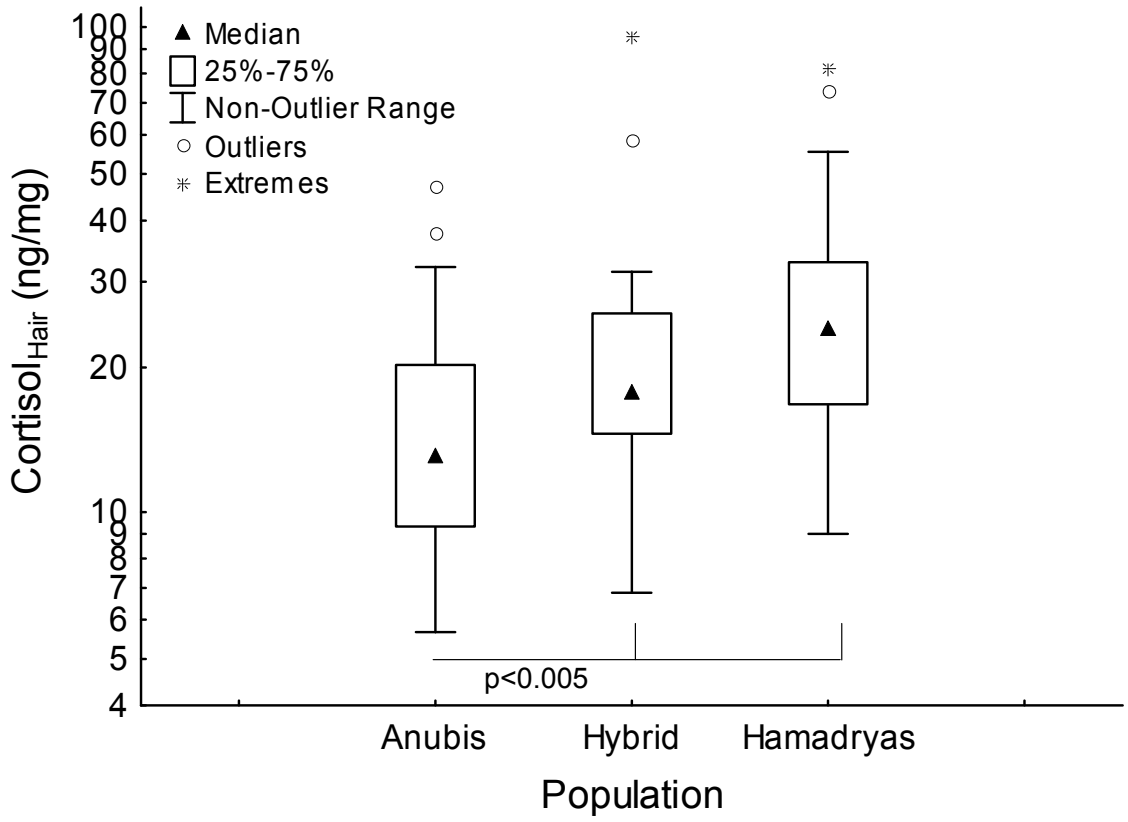
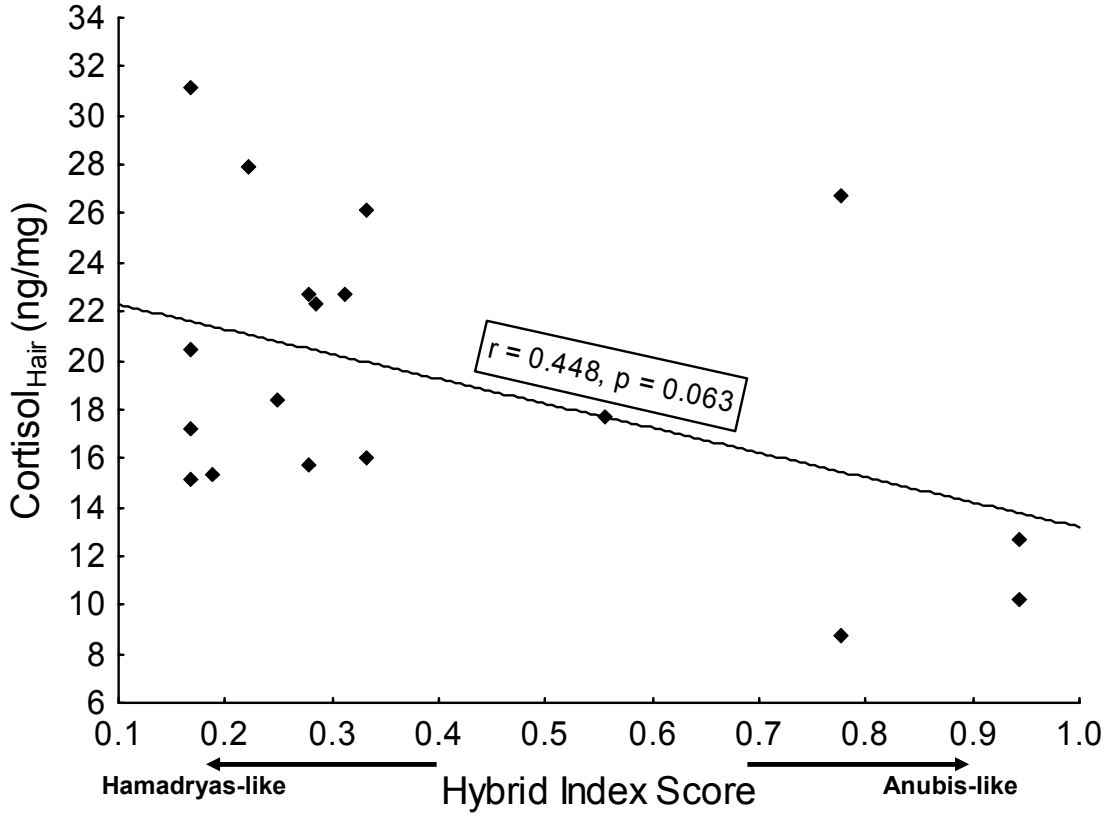


Figure 6.5: Plot showing that there is a near significant trend for more hamadryas-like males in the hybrid population to have higher hair cortisol concentrations.



Chapter 7: Variation in basal cortisol during the juvenile period in wild anubis baboons

ABSTRACT

Circulating cortisol changes during development in baboons and is sensitive to socio-ecological factors. Maternal effects on offspring fitness are well described in baboons, for example the offspring of lower ranking females tend to have low relative body mass compared to the offspring of dominant females, reflecting the effect of differential socio-ecological maternal stress loads on offspring development. This suggests a possible relationship between relative body mass and circulating cortisol in growing baboons. The main objective of this study was to examine the relationship between cortisol, age and body mass in wild juvenile male and female anubis (*Papio anubis*) baboons from the Awash National Park, Ethiopia. Cortisol was quantified in hair samples from 59 male and 53 female juveniles; associated age and weight data were collected at capture. I evaluated the hypothesis that relative juvenile body mass is an indicator of socio-ecological stress, and predicted that hair cortisol concentrations would be negatively associated with relative body mass. The relationship between hair cortisol, age and body mass (controlling for rainfall) was evaluated using multiple regression analysis. Weight-for-age residuals were calculated, which describe whether an individual is light or heavy for its age. The analyses found a significant negative relationship between hair cortisol and age in both sexes and results show a significant negative relationship between hair cortisol and relative body mass in male juveniles but not females. The association between

elevated hair cortisol and low relative body mass in juvenile baboons may be indicative of socio-ecological stress related to maternal effects.

INTRODUCTION

The juvenile period is a critical life stage in primates. External stressors affecting growth and development during this life stage can have significant fitness consequences (Altmann and Alberts 2005; Altmann et al. 1988; Bercovitch and Strum 1993; Bulger and Hamilton 1987; Cheney et al. 2004; Johnson 2003; Setchell et al. 2006; Wasser et al. 2004). Accelerated growth and sexual maturation can lead to earlier reproduction and a longer reproductive span resulting in more offspring. Growth and maturation is a resource limited trait (Altmann and Alberts, 2005; Dobson and Oli, 2001; Hofer and East, 1993; LeBlanc et al., 2001; Lochmiller et al., 2000; Lyles and Dobson, 1988; McAdam and Millar, 1999; Mori, 1979; Strum, 1991; Strum and Western, 1982). Animals that experience poor nutrition during critical stages of development may experience changes to their metabolic or physiological processes in order to cope with a poor diet (i.e. metabolic programming - Barker 1993, 2001; Godfrey and Barker, 2001; Meaney et al., 2007), which has life time consequences. In wild primates, nutrition is closely linked to habitat productivity and ecological conditions. Similarly, maternal effects impact several fitness components of offspring in primates (Alberts and Altmann, 1995b; Altmann and Alberts, 2003; 2005; Fessler et al., 2005; Setchell et al., 2006). The social status (e.g. Altmann and Alberts, 2005), physical condition (Frederick et al., 2008) and mothering style (e.g. Fairbanks et al., 1996) of reproducing females affect offspring birth weight, longevity, growth rates, relative body mass, and age at sexual maturity. Low ranking mothers tend to produce offspring with lower birth weight, and slower growth, and which

reach maturity later than conspecifics of higher ranking mothers (Alberts and Altmann, 1995b; Altmann and Alberts, 2003; 2005; Fessler et al., 2005; Setchell et al., 2006).

The exact mechanisms by which they produce these physiological phenotypes in offspring are not well understood, but in primates it is thought that low female social status results in chronically elevated hypothalamic-pituitary-adrenal (HPA) axis activity as a result of psychosocial and nutritional stress associated with subordinance (Coplan et al., 2006; Goymann and Wingfield, 2004; Walker et al., 2004). These effects may be passed on to offspring *in-utero* (fetal programming hypothesis – Barker et al., 1993; Phillips, 2007) and may persist throughout juvenile development and adulthood. The effects of such socio-ecological stressors are physiologically detectable by quantification of the stress hormone cortisol, produced by the adrenal cortex as part of the physiological stress response (Hiller-Sturmhöfel and Bartke, 1998; Sapolsky, 2000, 2004). Cortisol regulates energy metabolism, withdrawing energy resources from non-essential processes (e.g. growth, reproduction and immune function) and making energy available to processes essential for immediate survival (Nelson, 2005). Although adaptive in the short term, if chronically maintained at high concentrations, cortisol has negative impacts on health and growth (Chrousos, 2000; de Kloet et al., 1999; Sapolsky, 1992a; 2004; 2005). Chronic cortisol concentrations are therefore a useful indicator of stress load in animals. Such measures of cortisol can be obtained from hair samples, which archive time averaged concentrations of circulating cortisol (e.g. Davenport et al., 2006, also see Chapter 3).

A simple model of hair growth in adult wild baboons (Chapter 5) together with hair regrowth data from a number of captive primate species suggest that a multi-hair sample

may reflect endocrine activity averaged over an approximately 12 month period. Hair cortisol concentrations therefore reflect an average of circulating cortisol over an extended period prior to sampling. Hair cortisol in a one year old animal therefore probably reflects circulating cortisol averaged over a period starting at, or close to, birth up until the time the animal was sampled. These estimations are based on a model derived from data from adult animals and may be somewhat different for immature animals. No information are presently available to asses this speculation and the 12 month average is used as a working model.

Hair cortisol analysis is finding greater routine application in both captive and wild studies of the stress biology of a variety of mammals (e.g. baboons – Fourie and Bernstein, 2011a, Chapters 5 and 6; bears - Becshoft et al., 2010; Macbeth et al., 2010; caribou - Ashley et al., 2011; ground squirrels - Martin and Reale, 2008; humans – Dettenborn et al., 2010; hyraxes - Koren et al., 2002; macaques - Davenport et al., 2006; marmosets - Clara et al., 2008; various primates - Fourie and Bernstein, 2011a; vervet monkeys - Fairbanks et al., 2011).

In this chapter I analyzed possible sources of variation in basal cortisol during the juvenile phase, defined here as the period preceding eruption of the third molars, of a cross-sectional sample of male and female anubis baboons (*Papio anubis*) living in the Awash National Park in Ethiopia. Circulating cortisol concentrations vary with age in baboons: infants and young juveniles are typically hypercortisoloid compared to older individuals and cortisol concentrations show a rapid decline with age (captive *Papio cynocephalus*- Castracane et al., 1981, *P. hamadryas* – Crawford et al., 1997; captive *Papio papio* – Fourie and Bernstein, 2011a; wild *P. cynocephalus* - Gesquiere et al.,

2005; captive; mixed captive *P. cynocephalus* and *P. anubis* sample – Muehlenbein et al., 2002). Infants tend to exhibit a general surge in steroid hormones during the first months after parturition, during this time the adrenal glands are important sources of steroids (including androgens). The general up-regulation of adrenal function during this time may account for infant hypercortisolism although the exact mechanisms underlying this pattern remain unclear (Gesquiere et al., 2005). In yellow baboons, the only species in which ontogenetic variation in circulating and fecal cortisol concentrations in wild baboons have been characterized, males, but not females, show significant infant hypercortisolism and a rapid age related decline (Gesquiere et al., 2005). Patterns of age related cortisol variation appear to be relatively similar across the *Papio* baboons, and anubis and yellow baboons exhibit broadly similar biological and social ontogenies (Jolly and Phillips-Conroy, 2006). It is therefore predicted that the wild anubis sample examined here should closely reflect patterns of ontogenetic variation in cortisol as described in wild yellow baboons, and the first objective of this study was to characterize ontogenetic variation in anubis cortisol concentrations through the juvenile period.

The second objective of this study was to examine whether relative body mass during the juvenile period is an indicator of socio-ecological stress. It was predicted that if relative body mass in juveniles results from external stressors (e.g. maternal effects, social stress or ecological stress – e.g. Altmann and Alberts, 2005), which affect energy balance and therefore result in differences in relative body mass, then cortisol concentrations will be proportional to relative body mass. Specifically, it was predicted that animals with low relative body mass would exhibit higher cortisol concentrations than animals with higher relative body mass.

MATERIALS AND METHODS

Samples

This analysis uses samples from male and female juvenile anubis baboons (i.e. age classes 1 through 3 - Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000; see Table 7.1 for age class definitions and month age ranges). I extracted and quantified cortisol from the hair of 59 males and 53 females (see Table 7.2). The sample breakdown by age (rounded to the nearest year) and sex is given in Table 7.2. All samples used in this study were collected from animals that were trapped and sedated between 1993 and 2000. The body mass for each sedated animal was measured in kilograms using a spring scale. See Chapter 5 for details pertaining to capture, sedation and sampling. Samples were stored and shipped in paper envelopes under non-specific ambient conditions

Hair Sample Preparation

Sample preparation and cortisol extraction protocols are the same as described in Chapter 5.

Cortisol Enzyme Immunoassay (EIA)

The same assay system and protocol was followed as is described in Chapter 5.

Statistical Analysis

I described and examined the relationship between hair cortisol concentrations and age for each sex by grouping animals into year age categories in order for the description of hair cortisol with age to be comparable with that given by Gesquiere et al. (2005) for fecal glucocorticoids in yellow baboons. Age, in years, was calculated for each animal and rounded to the nearest year. No reliable age estimates (in months) are available for age class 1 animals (i.e. infants - no permanent dentition Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000). These animals were arbitrarily assigned an age of 12 months. Hair cortisol concentrations were log transformed and were plotted against year-age categories using a mean and standard error plot.

In order to test whether age, relative body mass, and rainfall significantly explained variation in hair cortisol concentrations I conducted a multiple regression analysis using log-transformed hair cortisol concentrations. Age was treated as a continuous variable, expressed in months. Relative body mass was expressed by calculating weight-for-age residuals. These residuals express whether an individual is light or heavy for their age (after Moses et al., 1992, but also see Altmann and Alberts, 2005). First a LOWESS curve is fitted to the weight (kg) and age (months) data which calculates the average weight for a predetermined number of month-age averages for males and females separately. Values were averaged over 5 iterations. Individual weight-for-age residuals (i.e. relative body mass) are then calculated by dividing an individual's actual weight by the average for its age and log transforming the result. This produces negative values

(light-for-age) and positive values (heavy-for-age). Two animals, an age class 2 male (11273) and an age class 1 female (10932) had no associated body mass data and were not included in the multiple regression analyses.

RESULTS

Males

Multiple regression analysis (Adjusted $r^2 = 0.206$, standard error of estimate = 0.269, $F_{(3,54)} = 5.9403$, $p = 0.001$) found that age (Beta-coefficient = -0.450, standard error of Beta= 0.121, $t_{(54)} = -3.741$, $p<0.001$) and relative body mass (Beta-coefficient = -0.296, standard error of Beta= 0.120, $t_{(54)} = -2.476$, $p=0.016$) but not rainfall (Beta-coefficient = -0.037, standard error of Beta= 0.119, $t_{(54)} = -0.316$, $p=0.753$) significantly predicted male hair cortisol concentrations. The model showed that hair cortisol concentrations decrease significantly with age in males (see Table 7.3 and Figure 7.1) and that hair cortisol concentrations are negatively correlated with relative body mass (see Table 7.3 and Figure 7.2). When residual analysis was used to identify and remove outliers and extremes, the relationship between age and relative body mass and hair cortisol became more significant, suggesting that the relationship is relatively robust.

Females

The multiple regression model of female cortisol was not significant (Adjusted r^2 = 0.025, standard error of estimate = 0.216, $F_{(3,49)}$=1,435, p = 0.244) when all three independent continuous predictors were included in the model, and showed that neither rainfall (Beta-coefficient = -0.052, standard error of Beta= 0.143, $t_{(49)}$ = -0.361, p=0.720) nor relative body mass (Beta-coefficient = 0.005, standard error of Beta= 0.138, $t_{(49)}$ = 0.364, p=0.971) significantly predicted female hair cortisol concentrations, but did indicate that age was a significant predictor of female hair cortisol concentrations (Beta-coefficient = -0.294, standard error of Beta= 0.142, $t_{(49)}$ = -2.064, p=0.044). When rainfall and relative body mass were removed from the model (Adjusted r^2 = 0.089, standard error of estimate = 0.213, $F_{(1,51)}$=6,078, p = 0.017), age was found to significantly predict female hair cortisol concentrations (Beta-coefficient = -0.326, standard error of Beta= 0.132, $t_{(51)}$ = -2.465, p=0.017), showing that female hair cortisol concentrations decline significantly with age.

DISCUSSION

The results show that like wild yellow baboons, anubis baboon HPA-axis ontogeny is characterized by infant hypercortisolism, and a rapid decline in circulating cortisol concentrations through the early juvenile period (Castracane et al., 1981; Gesquiere et al., 2005; also see Ginther et al., 2002; Pryce et al., 2002). Unlike descriptions of HPA axis ontogeny in wild and captive yellow baboons based on fecal and serum cortisol concentrations, it was not found that females show no or less pronounced infant hypercortisolism and age-related decline in cortisol than males (Castarcane et al., 1981;

Gesquiere et al., 2005). In this sample both males and females reach their lowest circulating cortisol concentrations around 3 years of age. Both males and females show a substantial increase in circulating cortisol concentrations around four years of age coincident with the onset of sexual maturation as documented in this population (Jolly and Phillips-Conroy, 2003). Similar increases in cortisol concentrations associated with sexual maturation have been observed in young pubescent macaques (Bercovitch and Clarke 1995). Elevation in cortisol concentrations during this period in males and females may have a psychosocial origin rather than a developmental one, as has been suggested for similar elevations in circulating cortisol during development in young cotton-top tamarins (*Saguinus oedipus* - Ginther et al., 2002). Specifically, this period of maturation may be associated with changes in relationships within and between the age cohorts resulting in elevated psychosocial stress.

I examined whether relative body mass was indicative of socio-ecological stress and found that there was a significant relationship between relative body mass and chronic cortisol concentrations in males, but not females. Rainfall did not explain any of the variation in basal cortisol concentrations. Research in yellow baboons has shown that the juveniles of low ranking mothers exhibit low relative body mass throughout development compared to the juveniles of high ranking mothers (Altmann and Alberts, 2005). It is thought that lower ranking females suffer from psychosocial and nutritional stresses related to their rank. Specifically, lower ranking females may experience harassment and displacement during foraging (Post et al., 1980; Muruthi, 1989; Samuels and Altmann, 1984; Smuts and Nicolson, 1989), decreasing foraging efficiency which would impact body condition in addition to psychosocial stresses. Lower ranking females therefore

have fewer resources to allocate to their offspring during gestation and lactation, resulting in smaller offspring (female body mass index and birth weight - Frederick et al., 2008; Rode et al., 2007). These effects are probably a combination of the direct effects of maternal resource allocation and metabolic programming. Light-for-age animals do not experience "catch-up" growth and remain small for their age throughout development (Altmann and Alberts, 2005). Maternal effects therefore appear to persist throughout development, suggesting that offspring are metabolically programmed *in utero* and therefore continue to express their metabolic phenotype even after they forage independently (Maestripieri and Mateo, 2009; Onyango et al., 2008). The sub-adult sons of low ranking females have been shown to exhibit elevated HPA axis activation compared to the sons of high ranking females (Onyango et al., 2008). In the absence of any correlation between cortisol and rainfall it is proposed that the elevated adrenocortical function in light-for-age juvenile anubis males is consistent with persistent maternal effects as have been described in yellow baboons and a number of other primate species (e.g. *Macaca mulatta* – Bercovitch and Clarke, 1995; *Mandrillus sphinx* – Setchell et al., 2006).

No relationship was found between basal cortisol concentrations and relative body mass in females. Males within this population are typically larger than females throughout development (see Table 7.1). Female baboons tend to experience relatively constant growth to full maturity, whereas males experience a pronounced growth spurt, during which growth accelerates, and the accelerated growth is maintained for a number of years (Altmann and Alberts, 2005). The larger male size combined with faster and more prolonged growth suggests that males may have higher metabolic needs than

females during this period, resulting in the observed sex difference within this population. Growth in juvenile primates appears to be principally constrained by the availability of resources (Altmann and Alberts, 2005). The resource (i.e. foraging/nutritional) stresses that result in low relative body mass may be physiologically more acutely felt by males due to their accelerated and prolonged developmental program and the elevated metabolic needs associated with a larger body size.

Relative body size in juveniles has been linked to both maternal rank and resource availability (Altmann and Alberts, 2005; Maestripieri and Mateo, 2009; Onyango et al., 2008). The results here show that, in males, low relative body mass is also associated with elevated basal cortisol concentration. This evidence suggests that light-for-age males may experience some disadvantage compared to heavy-for-age conspecifics, given the negative health effects associated with chronically elevated cortisol (Byung-Wan et al., 2010; Chrousos, 2000; Damsted et al., 2011; Das et al., 2010; de Kloet et al., 1999; Epel, 2009; Nijm and Jonasson, 2009; Sapolsky, 1992a, 2004, 2005), or that elevated basal cortisol concentrations may be part of the physiological mechanism responsible for slowed growth and reduced juvenile size. Slower growing, smaller offspring would have lower nutritional needs than larger conspecifics, allowing them to cope with the more limited allocation of resources associated with low maternal rank. Long term longitudinal approaches which integrate the various biological and physiological indices of socio-ecological stressors are needed to adequately assess the fitness consequences of low relative body mass and chronically elevated cortisol for individuals.

CONCLUSION

I report the first findings describing variation in basal cortisol concentrations in wild male and female anubis baboons during the juvenile period. It is shown that relative body size significantly explains variation in male juvenile hair cortisol concentrations and was found that rainfall (as a proxy for habitat productivity and ecological stress) did not predict hair cortisol concentrations in either sex. This suggests that factors other than immediate ecological conditions influence relative body mass and hair cortisol concentrations in juveniles. It is suggested that maternal effects, which are known to result in variation in relative body mass and circulating cortisol concentrations, likely contribute to the variation in male hair cortisol concentrations described here. I show that male animals with low relative body mass have higher basal cortisol concentrations than heavier age-cohorts. The elevated basal cortisol concentrations associated with low relative male juvenile body mass may have consequences for health and future reproduction.

Tables and Figures

Table 7.1: Definitions of age class designations and estimated age ranges for each age class.

	Age Group	Definition	Estimated Age Range (months)
Age Class 1	infant	no permanent dentition	<12
Age Class 2	young-juvenile	1 or more 1st molars visible, no erupted 2nd molars	24-50
Age Class 3	older-juvenile	1 or more 2nd molars visible, no erupted 3rd molars	50-80

Table 2: Table listing all animals that were sampled for this study, including field season, sex, and age category designations along with individual hair age, cortisol, rainfall and body mass data.

ID	Field Season	Sex	Age Category	Age (months)	Cortisol (ng/mg)	12 Month Rainfall (mm)	Body Mass (kg)
11315	2000	M	1	12	54.4	496.47	3.0
10966	1993	M	2	21	115.3	612.33	3.6
10941	1993	M	2	22	116.4	740.40	4.3
10938	1993	M	2	29	30.7	740.40	4.8
10942	1993	M	2	29	17.9	736.93	5.0
10951	1993	M	2	29	23.5	606.83	4.5
10964	1993	M	2	29	18.2	612.33	5.9
10937	1993	M	2	35	35.6	740.40	6.6
10980	1993	M	2	35	7.0	619.67	6.8
10985	1993	M	2	43	39.7	623.33	6.6
10961	1993	M	2	45	22.0	610.50	8.2
10963	1993	M	2	45	31.7	612.33	8.4
10973	1993	M	2	45	46.9	616.00	9.1
10984	1993	M	2	45	58.3	623.33	7.3
10940	1993	M	2	48	43.5	740.40	5.9
10863	1993	M	2	49	70.1	736.93	6.3
10998	1995	M	2	29	153.6	600.00	4.5
11003	1995	M	2	29	38.7	600.00	5.0
10941	1995	M	2	45	19.7	600.00	7.5
10999	1995	M	2	45	31.2	600.00	6.0
11015	1995	M	2	52	50.7	530.50	7.0
11252	1998	M	2	21	79.9	478.83	3.6
11198	1998	M	2	29	34.4	530.67	4.5
11273	1998	M	2	29	15.4	517.17	
11240	1998	M	2	37	12.6	504.60	5.9
11197	1998	M	2	45	29.6	530.67	6.8
11239	1998	M	2	45	72.9	504.60	6.8
11251	1998	M	2	45	51.8	498.47	6.8
11272	1998	M	2	45	60.4	509.50	7.3
11245	1998	M	2	46	21.4	503.07	8.6
11241	1998	M	2	47	47.3	504.60	7.7
11316	2000	M	2	29	59.6	496.47	5.0
11320	2000	M	2	29	93.0	495.20	4.1
11329	2000	M	2	29	62.6	493.93	5.0
11336	2000	M	2	29	29.4	488.87	5.5
10983	1993	M	3	51	71.7	623.33	8.2
10879	1993	M	3	54	27.8	627.00	10.9
10936	1993	M	3	60	23.8	740.40	8.2
10982	1993	M	3	65	15.5	623.33	12.3
10969	1993	M	3	66	108.3	612.33	8.0
10773	1993	M	3	75	48.8	740.40	13.0
10955	1993	M	3	75	17.2	608.67	11.8

Table 7.2: continued…

ID	Field Season	Sex	Age Category	Age (months)	Cortisol (ng/mg)	12 Month Rainfall (mm)	Body Mass (kg)
10968	1993	M	3	75	11.3	612.33	14.1
10977	1993	M	3	75	17.2	619.67	18.2
10920	1993	M	3	77	51.9	623.33	22.7
10998	1997	M	3	51	27.1	474.00	7.7
11088	1997	M	3	68	31.3	466.67	13.2
11223	1998	M	3	65	48.9	507.67	10.0
11269	1998	M	3	65	24.8	509.50	15.0
11225	1998	M	3	75	16.4	507.67	15.9
11248	1998	M	3	75	18.8	501.53	13.2
11257	1998	M	3	75	16.0	494.17	15.0
11258	1998	M	3	75	13.6	494.17	15.9
11231	1998	M	3	78	11.7	504.60	14.5
11051	1998	M	3	81	9.7	538.33	13.6
11339	2000	M	3	51	81.4	485.07	7.3
11241	2000	M	3	69	41.7	492.67	14.1
11245	2000	M	3	70	19.2	488.87	15.9
11340	2000	M	3	72	70.0	485.07	11.4
10932	1993	F	1	12	75.0	754.27	
10933	1993	F	1	12	39.5	754.27	1.9
10946	1993	F	1	12	29.8	726.53	2.3
10971	1993	F	1	12	83.5	616.00	1.1
11209	1998	F	1	12	59.4	526.07	3.2
11276	1998	F	1	12	25.0	517.17	3.2
11319	2000	F	1	12	29.8	495.20	3.6
11327	2000	F	1	12	72.5	493.93	2.1
11334	2000	F	1	12	45.1	490.13	3.2
10930	1993	F	2	29	55.9	754.27	4.7
10944	1993	F	2	47	37.3	726.53	7.3
10945	1993	F	2	26	66.4	726.53	4.5
10950	1993	F	2	51	24.8	606.83	3.9
10953	1993	F	2	29	31.5	608.67	4.5
10958	1993	F	2	45	65.0	610.50	5.9
10959	1993	F	2	29	28.1	610.50	3.9
10960	1993	F	2	20	38.2	610.50	3.2
10967	1993	F	2	20	20.2	612.33	4.3
10976	1993	F	2	66	22.0	616.00	4.8
10989	1993	F	2	45	31.6	625.17	6.4
10995	1995	F	2	35	27.8	600.33	5.5
11000	1995	F	2	45	34.3	600.00	5.0
11004	1995	F	2	20	53.1	600.00	3.0
11065	1997	F	2	37	17.2	474.67	4.8
11075	1997	F	2	45	101.3	474.00	6.4
11199	1998	F	2	45	16.7	530.67	5.0
11210	1998	F	2	20	61.8	526.07	3.2
11211	1998	F	2	20	54.5	526.07	2.7
11246	1998	F	2	29	44.4	503.07	4.1

Table 7.2: continued…

ID	Field Season	Sex	Age Category	Age (months)	Cortisol (ng/mg)	12 Month Rainfall (mm)	Body Mass (kg)
11247	1998	F	2	29	43.9	503.07	3.2
11249	1998	F	2	45	35.0	501.53	5.4
11253	1998	F	2	29	33.5	482.67	4.1
11264	1998	F	2	29	17.0	505.67	5.0
10778	1993	F	3	66	16.7	726.53	10.5
10949	1993	F	3	62	14.5	606.83	7.7
10762	1993	F	3	62	19.0	612.33	9.5
10974	1993	F	3	47	36.0	616.00	10.0
10986	1993	F	3	62	54.2	625.17	12.5
10945	1995	F	3	49	18.3	600.33	7.5
10862	1995	F	3	73	43.2	453.17	11.0
11048	1995	F	3	61	16.5	453.17	10.0
11049	1995	F	3	60	29.5	453.17	8.5
11056	1995	F	3	62	16.1	438.67	9.5
11060	1995	F	3	62	11.9	433.83	10.0
10939	1997	F	3	75	39.5	474.67	12.5
11091	1997	F	3	49	54.7	466.67	9.6
11099	1997	F	3	62	58.8	465.33	11.6
11105	1997	F	3	60	42.2	464.67	7.7
11172	1998	F	3	62	43.0	538.33	7.3
11206	1998	F	3	47	22.2	526.07	5.0
11226	1998	F	3	62	59.5	507.67	10.4
11311	2000	F	3	49	57.6	496.47	6.6
11333	2000	F	3	62	30.1	490.13	8.2

Table 7.3: Table showing the sample breakdown by age and sex and gives the mean±1standard deviation and minimum and maximum hair cortisol concentrations (ng/mg) for each year-age group. The table also shows the mean±1standard deviation hair cortisol concentrations (ng/mg) for light-for-age and heavy-for-age categories for each year-age group.

Males

age (years)	n	mean (ng/mg)	SD	min (ng/mg)	max (ng/mg)
1	1	54.40	-	-	-
2	15	59.24	43.28	15.40	153.60
3	3	18.40	15.16	7.00	35.60
4	19	46.18	19.15	19.70	81.40
5	5	28.16	12.46	15.50	48.90
6	14	34.41	27.73	11.30	108.30
7	2	10.70	1.41	9.70	11.70
Total:	59	42.70	30.58	7.00	153.60

Females

age (years)	n	mean (ng/mg)	SD	min (ng/mg)	max (ng/mg)
1	9	51.07	22.10	25.00	83.50
2	14	40.75	16.02	17.00	66.40
3	2	22.50	7.50	17.20	27.80
4	13	41.14	23.46	16.70	101.30
5	12	32.94	18.00	11.90	59.50
6	3	33.13	14.35	16.70	43.20
Total:	53	39.71	19.79	11.90	101.30

Males

	Light-for-Age			Heavy-for-Age		
age (years)	n	mean (ng/mg)	SD	n	mean (ng/mg)	SD
1	-	-	-	-	-	-
2	7	75.77	49.01	7	48.97	34.75
3	-	-	-	3	18.40	15.16
4	11	51.79	19.48	8	38.46	16.84
5	2	36.35	17.75	3	22.70	6.41
6	7	41.49	36.51	7	27.33	14.73
7	2	10.70	1.41	-	-	-
Total:	29	43.22	24.83	28	31.17	17.58

Females

	Light-for-Age			Heavy-for-Age		
age (years)	n	mean (ng/mg)	SD	n	mean (ng/mg)	SD
1	4	56.33	25.73	4	39.83	15.61
2	9	42.17	13.04	5	38.20	21.95
3	1	17.20	-	1	27.80	-
4	9	43.17	26.97	4	36.58	14.87
5	7	27.77	11.82	5	40.18	23.86
6	-	-	-	3	33.13	14.35
Total:	30	37.33	19.39	22	35.95	18.13

Figure 7.1: Mean and Error plot showing that hair cortisol concentrations decline with age in wild male and female juvenile anubis baboons.

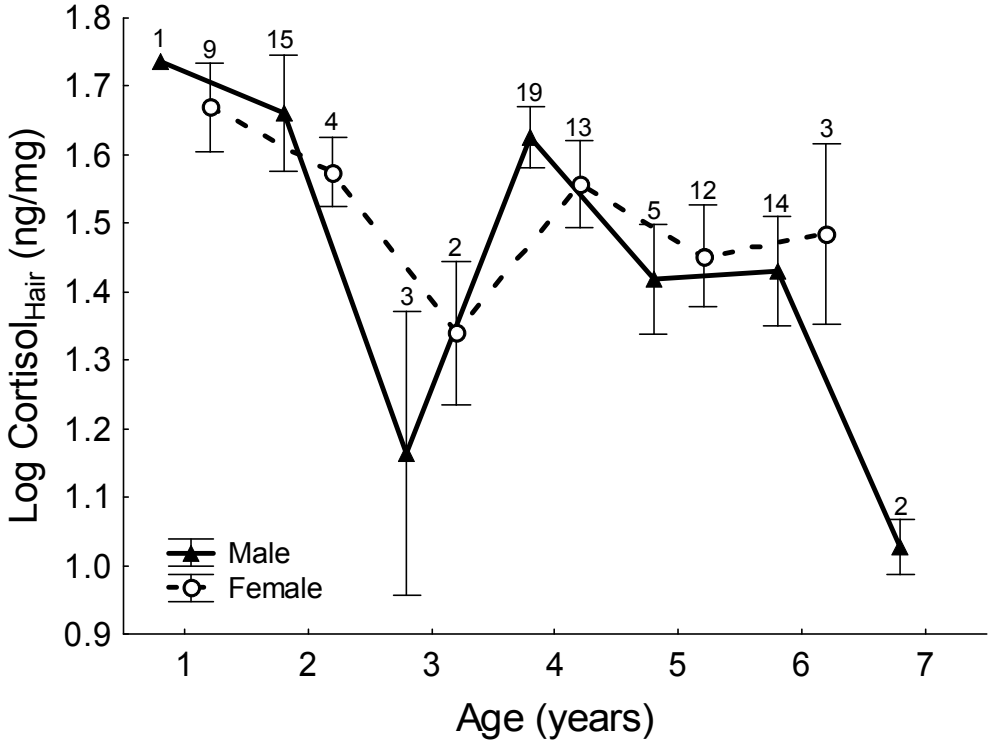
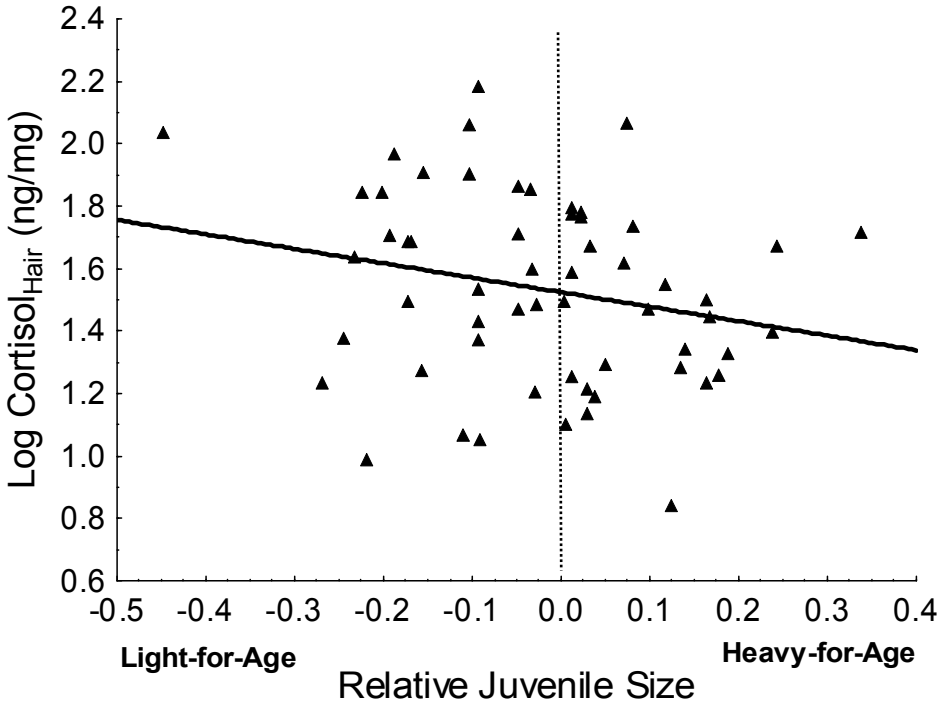


Figure 7.2: Plot showing a significant negative relationship between hair cortisol concentrations and relative body mass in males.



Summary: Hair Cortisol Variation in The Awash Baboons

The hybrid zone between anubis and hamadryas baboons in the ANP, Ethiopia, has been the focus of study since the late 1960s (e.g. Kummer, 1968a, b). Since then researchers have examined various aspects of the dynamics of the hybrid zone, and have described aspects of growth and development (e.g. Swedell and Leigh, 2006), physiology (Jolly et al., 2008), social systems and behavior (e.g. Swedell and Leigh, 2006). The research presented in Chapters 5, 6, and 7 contribute another layer of information about the adult physiology and juvenile development of the baboons of the Awash, specifically with regard to the chronic function of the endocrine axis which regulates the physiological stress response.

In Chapters 5 and 6, I describe the first evidence to suggest that hybridization may result in disruption of normal cortisol phenotypes. I found that parent populations, specifically males, have relatively narrow ranges of variation in basal cortisol concentrations, but that the hybrid male population exhibits both relatively elevated and more variable hair cortisol concentrations. This pattern is repeated in a separate sample, comprising of some of the same populations analyzed in Chapter 5, as well as other populations which show a history of hybridization. Together these results suggest that hybridization disrupts normal cortisol phenotype expression, possibly due to dysregulation of biological and behavioral stress complexes. Further analysis of hybrid morphological phenotypes and basal cortisol of animals sampled in a hybrid troop suggests that the hamadryas-like hybrid phenotype may be associated with elevated basal cortisol concentrations. It remains to be tested whether elevated and more variable

hybrid basal cortisol concentrations affect health and fitness parameters in these populations.

Basal cortisol concentrations in the Awash baboons also appear to be associated with sustained ecological stress (Chapter 6). Analysis of samples collected during a drought in the region in 1973, showed that hamadryas populations, which inhabit the most arid regions in the ANP, exhibited elevated hair cortisol concentrations compared to the anubis population. These results were consistent with observations that the hamadryas baboons sampled during this period experienced severe nutritional stress and the greatest change in body mass compared to the anubis population.

Ecological and hybrid effects on basal cortisol could not be demonstrated in females of these populations and it is speculated that hybrid and ecological differences may be masked by the effects of reproductive cycles in females. Future analyses should control for these possible confounding effects when examining cross-specific variation in basal cortisol.

Finally, analysis of juvenile anubis hair cortisol concentrations found that light-for-age juveniles males tend to have elevated basal cortisol compared to heavier for age conspecifics. These results are consistent with pervious studies on baboons finding that both relative juvenile body mass and cortisol is associated with maternal rank, specifically that low ranking females trend have light-for-age offspring (Altmann and Alberts, 2005), which exhibit elevated cortisol levels (Onyango et al., 2008).

Chapter 8: Variation in vervet (*Chlorocebus aethiops*) hair cortisol concentrations in relation to ecological disturbance by humans.

ABSTRACT

Vervet monkeys (*Chlorocebus aethiops*) often live in close proximity to humans resulting in conflict with humans. Primate groups with access to human foods experience increased intra-group aggression, males exhibit risky male foraging behaviors, but also show accelerated growth and increased body mass. Proximity to humans may result in significant behavioral stress for vervets, and males may experience greater behavioral stress than females, related to high risk male foraging strategies resulting in human aggression (H_1). It was predicted that human disturbance would be positively associated with measures of chronic stress, and that males in high human disturbance contexts would have elevated cortisol compared to females. Alternatively, proximity to humans and access to human foods reduces nutritional stress, which may mitigate behavioral stress (H_2). This hypothesis predicted a negative relationship between human disturbance and chronic cortisol. Human disturbance was quantified as a human impact score composed of indices of access to human foods and human activity for each population. Impact scores were divided into high, moderate and low human impact groups. The stress hormone cortisol was quantified in the hair of wild caught individuals from populations across South Africa. Comparisons between impact groups and sex were made using a non-parametric analysis of variance test and *post hoc* tests of pair wise comparisons. Male cortisol concentrations in moderate and high human impact groups were

significantly higher than those of low human impact groups. Female cortisol concentrations did not differ between groups. Males in the high human impact group had significantly higher hair cortisol concentrations than females.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis is the principal endocrine axis that mediates the physiological stress response, which suppresses non-essential biological functions while enhancing energy availability to biological processes essential for immediate survival (Nelson, 2005). Although adaptive in the short term chronic activation of this endocrine axis can have health consequences (Chrousos, 2000; de Kloet, Oitzel and Joels, 1999; Sapolsky, 1992a; 2004, 2005) and therefore impact fitness. Cortisol is one of the principal hormones produced by the adrenal gland as part of the HPA axis stress response and is typically used to measure HPA axis activity and stress in primates in relation to social (Sapolsky, 2000, 2004) and environmental stressors (e.g. Chapman et al., 2007; Gesquiere et al., 2008; Rangel-Negrin et al., 2009; Sapolsky, 1986).

Primates and other wild animals are increasingly impacted by human encroachment and habitat loss (Arroyo-Rodrigues et al., in press., Johns and Skorupa, 1987; Moore et al., 2010; Onderdok and Chapman, 2000). Certain primate species can even thrive in human-modified environments (Chhangani and Mohnot, 2004; Chauhan and Pirta, 2010; Singh, 1966; Waite et al., 2007). In such situations human- primate interaction increases and intensifies often resulting in conflicts (e.g. Brennan, Else and Altmann, 1985; Lee and Priston, 2005; Moore et al., 2010; Nijman and Nekaris 2010; Riley, 2007; Saj et al., 2001). Many primate species are known to raid crops, causing agricultural damage (e.g. Hill, 2000; Horrocks and Baulu 1994; 1999; MooNijman and Nekaris 2010; Saj et al.,

1999; 2001; Turner et al., 1997), whereas other primate populations, which experience human provisioning (e.g. tourists) may aggressively beg or steal from humans, sometimes resulting in property damage or injury (e.g. Baskin and Krige, 1973; Brennan et al., 1985, Chauhan and Pirta, 2010; Fa, 1992; Fuentes and Gamerl, 2005; Moore et al., 2010; Nijman and Nekaris, 2010). As a result such populations are often the target of human aggression (Chauhan and Pirta, 2010; Nijman and Nekaris, 2010). In addition to increased behavioral stress loads resulting from primate-human interactions, inter- and intra-group aggression is often elevated in populations in urban/suburban areas, or with access to human resources, compared to wild foraging groups (Brennan et al., 1985; Ciani, 1986; Southwick et al., 1976), which may constitute an additional source of psychosocial stress for animals. Alternatively, proximity to humans and access to human foods can have a positive impact on primate populations. Populations with access to human foods show accelerated growth and development, which result in higher body mass index scores (e.g. Altmann and Alberts, 2005; Turner et al., 1997; but also high cholesterol – Kemnitz et al., 2002) and high population densities (Brennan et al., 1985; Chauhan and Pirta, 2010;).

Vervet monkeys (*Chlorocebus aethiops* – Groves, 2001; Rowe, 1996) are perhaps one of the widest ranging cercopithecine monkeys in Africa (Kingdon, 1997). As a result of their extensive range and adaptability, they regularly live in close proximity to humans, exploiting agricultural and suburban resources (Basckin and Krige, 1973; Brennan et al., 1985; Boulton et al., 1996; Isbell and Truman, 1993; Ramiksoon, 2005; Saj et al., 1999, 2001). Vervets are often perceived as pests and may experience substantial harassment in human contact environments (e.g. Guy et al., 2012; Mbanjwa,

2004; Ramiksoon, 2005; Saj et al., 1999). Close proximity to humans may alleviate predator pressure (Isbell and Truman, 1993) and result in abundant easily accessible high calorie foods (Basckin and Krige, 1973; Brennan et al., 1985; Saj et al., 1999, 2001), which may result in decreased nutritional and energetic stresses. Age/sex categories more typically associated with impulsivity and risky behaviors (i.e. juvenile male) more often engage in high risk foraging behaviors (raiding, stealing and begging) than females (especially females with dependant offspring). Females are less often associated with raiding, stealing and begging compared to males (vervets – Saj et al., 1999; but also see: *Papio* sp. Forthman-Quick, 1986; Oyaro and Strum, 1984; *Macaca* sp. - Fuentes and Gamerl, 2005; O'Leary and Fa, 1993).

Based on these observations, it was predicted that human impacts on vervet ecology result in high stress loads for vervets, such that increased human impact increases chronic stress (H_1 – *Behavioral Stress Hypothesis*). It was further predicted that because males appear to preferentially engage in high risk foraging behavior in environments that experience human disturbance, that males will experience greater behavioral stress loads than females. As an alternative to H_1, it was hypothesized that vervets benefit from the proximity of humans through increased dietary quality resulting in lower energetic stress loads (H_2 – *Energetic Stress Hypothesis*), mitigating any behavioral stress that may be associated when living in proximity to humans. This hypothesis predicted that vervet populations characterized by lower levels of human disturbance and less access to human foods will exhibit higher stress loads compared to animals with regular access to human food reflecting increased nutritional and energetic stresses under natural conditions.

Hair incorporates circulating cortisol into the shaft during growth, and hair therefore archives a time averaged measure of circulating cortisol concentrations, averaged over a period of months (~ 12 months – Chapter 5, but also see Fourie and Bernstein 2011b; Appendix 1). The cortisol concentrations determined from hair are also not sensitive to the acute stress of capture, sedation and sampling. Such a measure of cortisol is especially pertinent for testing the proposed hypotheses because, as the focus is on the effects of potentially chronically stressful conditions. Further, cortisol has been extracted and measured and validated successfully in hair from both wild and captive vervet populations (Fairbanks et al. 2011; Fourie and Bernstein, 2011a).

MATERIALS AND METHODS

Samples

For the purposes of this study I only focus on sexually mature animals (sub-adults and adults – i.e. animals with erupted third molars) as younger age classes experience significant ontogenetic variation in adrenocortical function (Fourie and Bernstein, 2011a). Hair samples were collected from anesthetized sub-adult and adult animals trapped using baited box traps (see Grobler and Turner, 2010 for details of trapping procedure). Hair samples were clipped from the back between the shoulders using a pair of scissors or pulled out with forceps. Samples were collected from animals trapped in localities across South Africa (see Table 7.1 and 7.2 for sample breakdown by site and

sex). Samples were stored in paper envelopes at -20^{o}C until shipment at. Samples were shipped in cooled containers and were stored in the US at -80 ^{o}C.

Human Impact Index

Five measures of human impact on vervets were used in the current study: "Access to Human Food", "Access to Agricultural Land", "Access to Rubbish", "Provisioned" (i.e. whether animals are regularly fed by people) and "Human Activity" (the degree of access to the sites by humans, e.g. suburban sites = high human activity, site close to game lodges or in reserves = medium human activity, sites or farms or isolated rural areas = low human activity). These impact categories were devised to study variation in body size in the same populations as are used here (Pampush, 2010). The categories were assessed at each site using estimates of home range to determined any overlap with human habitation, agricultural land or rubbish dumps. Locals were also interviewed to assess whether vervets in the study sites were known or suspected to raid crops, yards or homes, or to be provisioned by humans. Categories scores as assessed by Pampush (2010) were used. The first four of these variables were scored "no" (no = 1) and "yes" (yes = 2) based on previous assessments sampling site by Pampush (2010). A "no" score in these categories implies that animals either have unreliable access, or no access to human resources; a "yes" score implies regular reliable access to the relevant human resources. "Human Activity" was scored as "light" (light = 1), "moderate" (moderate =2) and "heavy" (high =3, Pampush, 2010). The sum of these five scores were used as an index of human impact (HI) with a score of 11 reflecting a very high human impact and a

score of 5 reflecting very low human impact on the ecology of a population. Twelve sites (Blyde Resort, Futululu, Gariep, Geelhoutbos, Londolozi, Potholes, Tslowane, Reserve Lapa, Swadini) yielded a HI index score of five, the minimum possible, indicating overall low levels of human impact on their ecology (see Table 8.1). These sites were grouped as low human impact sites. The sites Maurann Farm, Oribi Gorge and Soetdoring yielded a HI index score of 6 indicating access to agricultural land, while Rooiplat yielded a HI index score of 7 indicating access to agricultural land and moderate human activity in the range of this group. These two groups were pooled as a moderate human impact group. Three sites (Loristo, Parys, and Woodhill) had a HI index score of 10 indicating regular access to human food, access to rubbish, regular provisioning and high levels of human activity in their ranges. These sites were pooled into a single high human impact group.

Sample Preparation

The same preparation and cortisol extraction protocols as described in Chapter 5 were used.

Cortisol Enzyme Immunoassay (EIA)

The same assay system and protocols were used as is described in Chapter 5.

Statistical Analysis

The relationship between hair cortisol concentrations and individual HI scores were tested using a simple linear correlation analysis. Sites were then grouped based on their human impact scores (see Table 8.2) in order to increase the statistical power of between group statistical comparisons. Southern African environments are highly seasonal, experiencing large fluctuations in rainfall and temperature between seasons (wet season and dry season) which may result in seasonal nutritional and/or thermal stresses. Vervets are seasonal breeders and differences in reproductive state can have significant effects on HPA which is known to be reflected in hair cortisol (Krischbaum et al., 2009). I therefore considered season of capture as a potential effect on hair cortisol when both wet and dry seasons were represented in samples of the same impact group. Preliminary analysis showed no difference between HI groups 6 and 7, these groups were pooled to increase statistical power. I assessed variation in hair cortisol concentrations among groups using a Kruskal-Wallis (KW) test followed by post hoc pairwise comparisons using a Dwass-Steel-Chritchlow-Fligner (DSCF) test. Two sample comparisons of hair cortisol concentrations were made using a Mann-Whitney U (MW-U) test. All tests were performed using the raw untransformed hair cortisol concentrations as the statistical tests used are non-parametric and do not make normality assumptions. This procedure was used separately for both males and females. The statistics package SYSTAT 13 (Systat, Inc., San Francisco, CA) was used for all analyses. Significance was set to $p < 0.05$.

RESULTS

Linear correlation analysis of the cortisol concentrations and the individual HI scores show that male hair cortisol concentrations increase significantly with HI score ($r = 0.531$, $p = 0.003$, Figure 8.1). No relationship between individual HI scores and female hair cortisol concentrations were found ($r = 0.273$, $p = 0.122$).

No difference (MW-U: $U = 16$, $p = 0.489$) was found in male hair cortisol concentrations of animals in the low human impact group that were sampled in the dry ($n = 11$, mean $cortisol_{hair}$±1SD = 114.372±32.798ng/mg) and wet (male: $n = 4$, mean $cortisol_{hair}$±1SD = 101.438±31.324ng/mg) seasons. Low impact group females (i.e. HI = 5) sampled during the dry season ($n = 10$, mean $cortisol_{hair}$±1SD = 140.793±49.618ng.mg) had significantly (MW-U: $U = 29$, $p = 0.041$) higher hair cortisol concentrations than females from the same impact group sampled during the wet season ($n = 12$, mean $cortisol_{hair}$±1SD = 100.652±36.290ng.mg). Low human impact females sampled during the dry season were removed from subsequent analyses; small sample sizes for different sites within this group precluded their inclusion in analyses of within group variation.

Male hair cortisol concentrations differed significantly between impact groups (KW test: $H_{2,28} = 9.522$, $p = 0.009$). Males of the low human impact category ($n = 15$, mean $cortisol_{hair}$±1SD = 110.923±34.437ng.mg) had significantly lower hair cortisol concentrations than the moderate ($n = 8$, mean $cortisol_{hair}$±1SD = 153.772±57.679ng/mg; DSCF test: $p=0.001$) and high ($n = 6$, mean $cortisol_{hair}$±1SD = 189.103±54.320ng/mg; DSCF test: $p<0.001$) human impact groups (see Table 8.3 and Figure 8.1), the moderate and high human impact groups did not differ (DXCF test: $p>0.05$).

Analyses found no differences in female hair cortisol (wet season animals only) concentrations among low (n = 12, mean $cortisol_{hair}$±1SD = 100.652±36.290ng.mg), moderate (n = 12, mean $cortisol_{hair}$±1SD = 116.675±45.950ng.mg) and high human impact groups (n = 9, mean $cortisol_{hair}$±1SD = 109.769±41.786ng.mg; KW test: $H_{2,33}$ = 0.455, p = 0.797).

Males and females in the low (MW-U test: U = 202, p = 0.867) and moderate (MW-U test: U = 27, p = 0.115) human impact groups did not differ from each other in hair cortisol concentrations. Males in the high human impact group had significantly higher hair cortisol concentrations than females (MW-U test: U = 7, p = 0.018).

DISCUSSION

Males from populations which inhabit habitats that have been intensively modified by human activity and where vervets experience high levels of interaction with humans show the highest basal cortisol concentrations. The results showed that stress in sub-adult and adult male vervets is proportional to the degree to which humans impact their physical environments and modify their ecology and suggest that males in populations which experienced moderate and high levels of human interaction and access to human foods have significantly elevated chronic cortisol concentrations compared to males from populations which experience low levels of human impact. These results support the first hypothesis that human disturbance and modification of habitats is stressful for vervets. Populations in the present sample which experience moderate levels of human impact, typically have access to agricultural land (Pampush, 2010) and may raid crops. It is not

uncommon for farmers to shoot crop raiding primates and/or to employ other deterrent devices also used to scare wild animals and birds from crop fields (Osborn and Hill, 2005; Saj et al., 1999; 2001; Warren, 2009). Males from populations that experience high levels of human impact typically inhabit suburban areas (i.e. the Loristo and Woodhill groups) or commercial rubbish dumps (i.e. the Parys group – Pampush, 2010). In these environments males may more often engage in high risk foraging behaviors such as raiding homes and gardens (Guy et al., 2012; Mbanjwa, 2004; Ramiksoon, 2005; Saj et al., 1999) which, in South Africa, are often occupied by dogs. These behaviors often lead to conflicts and animals may be chased, and intentionally harassed and injured by people and pets (Guy et al., 2012; Mbanjwa, 2004; Ramiksoon, 2005; Saj et al., 1999). The high risk foraging behaviors of males in these populations may expose them to substantial stress as is suggested by the elevated basal cortisol concentrations demonstrated in this sample.

In contrast to the findings for males, females did not exhibit differences in basal cortisol across human impact conditions and only high human impact females had significantly lower hair cortisol concentrations than males. These partially support the second prediction of the *Behavioral Stress Hypothesis* (H_1) which suggested that females avoid high risk foraging behavior and therefore would not experience the same stress loads as males. This observation needs to be confirmed with empirical behavioral data for these populations and additional sample collection across both wet and dry seasons.

The results suggest that the effects of human impact on stress loads differentially impact males and females. Specifically, males exhibit more novelty seeking behavior, are less fearful and more impulsive than females (Fairbanks and Maguire, 1993; Fairbanks et

al., 2004; Saj et al., 1999). Females may additionally avoid high risk activities as they not only risk themselves but also dependent offspring. Dependent offspring may hamper a females' ability to negotiate risky foraging strategies, increasing the inherent risks of such behavior (Saj et al., 1999). Further it also appears that females exhibited evidence of seasonal stress. Specifically, females captured during the dry season had elevated hair cortisol compared to females captured during the wet season. This may reflect nutritional and thermal stress experienced during the dry season, as well as elevated HPA activity associated with pregnancy (e.g. Kirschbaum et al., 2009). Vervets, mate during the dry season and females give birth during the wet season.

The present study also demonstrates the utility of hair hormone analysis in monitoring wildlife population stress in relation to ecologically relevant variables. Hair cortisol concentrations are not sensitive to the acute stress of trapping and cortisol in hair can remain stable for long periods of time. This means that hair samples can obtained from existing biomaterials and museum collections. Samples can be collected as part of standard wildlife monitoring protocols where animals may be immobilized or captured for conservation purposes or from hunters (e.g. subsistence hunters - Becshoft et al., 2010), or using a variety of non-invasive methods, like hair snares (e.g. Frantz et al., 2004), or even from sleeping nests (e.g. great apes). Such approaches can be useful in assessing stress at the population level in animal communities as well as examining the effects of human disturbance on animal population and developing appropriate approaches to conservation and development.

Tables and Figures

Table 8.1: Sample breakdown by sex and site. The table lists the access to human food and the degree of human activity for each site. These conditions are used to define low, moderate and high human impact categories in the sample set.

Site	Male	Female	Season	Agricultural Land	Human Food	Rubbish	Provisioned	Human Activity	HI Index	HI Group
Potholes		2	Dry	No	No	No	No	Light	5	Light
Geelhoutbos		3	Wet	No	No	No	No	Light	5	Light
Tslowane	7	3	Wet	No	No	No	No	Light	5	Light
Gariep		1	Dry	No	No	No	No	Light	5	Light
Reserve Lapa	2	4	Dry	No	No	No	No	Light	5	Light
Bird Sanctuary		2	Dry	No	No	No	No	Light	5	Light
Swadini-Office	2	1	Wet	No	No	No	No	Light	5	Light
Blyde Resort	2	2	Dry	No	No	No	No	Light	5	Light
Futululu	2	4	Wet	No	No	No	No	Light	5	Light
Soetdoring	2	5	Wet	Yes	No	No	No	Light	6	Moderate
Oribi Gorge	1	1	Wet	Yes	No	No	No	Light	6	Moderate
Maurann Farm	1	4	Wet	Yes	No	No	No	Light	6	Moderate
Rooiplatt	4	2	Wet	Yes	No	No	No	Moderate	7	Moderate
Parys	3	5	Wet	No	Yes	Yes	Yes	High	10	High
Woodhill	1	3	Wet	No	Yes	Yes	Yes	High	10	High
Loristo	2	1	Wet	No	Yes	Yes	Yes	High	10	High
Total	29	43								

Table 8.2: Table showing individual identity and field site along with associated scores for human disturbance factors, and the final human impact score, as well as season of capture, and hair cortisol concentrations.

ID	Field Site	Sex	Cortisol (ng/mg)	Access to Human Food	Access to Crops	Access to Rubbish	Human Activity	Provisioning by Humans	Human Impact Score	Season
PK 163	BirdSanctuary	F	98.96	1	1	1	1	1	5	Dry
PK 164	BirdSanctuary	F	140.76	1	1	1	1	1	5	Dry
B 022	Blyde	F	147.00	1	1	1	1	1	5	Dry
B 023	Blyde	F	247.00	1	1	1	1	1	5	Dry
SL518	Futululu	F	70.83	1	1	1	1	1	5	Wet
SL507	Futululu	F	132.20	1	1	1	1	1	5	Wet
SL505	Futululu	F	131.21	1	1	1	1	1	5	Wet
SL516	Futululu	F	63.58	1	1	1	1	1	5	Wet
G402	Gariep	F	221.66	1	1	1	1	1	5	Dry
BG497	Geelhoutbos	F	88.98	1	1	1	1	1	5	Wet
BG489	Geelhoutbos	F	155.70	1	1	1	1	1	5	Wet
BG485	Geelhoutbos	F	86.34	1	1	1	1	1	5	Wet
PK 159	Lapa	F	94.70	1	1	1	1	1	5	Dry
PK 153	Lapa	F	105.51	1	1	1	1	1	5	Dry
PK 158	Lapa	F	127.69	1	1	1	1	1	5	Dry
PK 157	Lapa	F	167.33	1	1	1	1	1	5	Dry
P 066	Loristo	F	111.97	2	1	2	3	2	10	Wet
SL513	Maurann	F	142.23	1	2	1	1	1	6	Wet
SL514	Maurann	F	118.82	1	2	1	1	1	6	Wet
SL510	Maurann	F	124.74	1	2	1	1	1	6	Wet
SL503	Maurann	F	71.02	1	2	1	1	1	6	Wet
O 137	Oribi	F	77.18	1	2	1	1	1	6	Wet
PA411	Parys	F	63.96	2	1	2	3	2	10	Wet
PA413	Parys	F	111.59	2	1	2	3	2	10	Wet
PA408	Parys	F	58.83	2	1	2	3	2	10	Wet
PA406	Parys	F	113.99	2	1	2	3	2	10	Wet
PA400	Parys	F	117.25	2	1	2	3	2	10	Wet
B 092	Potholes	F	89.96	1	1	1	1	1	5	Dry
B 090	Potholes	F	117.00	1	1	1	1	1	5	Dry
BR494	Rooiplat	F	103.77	1	2	1	2	1	7	Wet
BR491	Rooiplat	F	243.08	1	2	1	2	1	7	Wet
SO452	Soetdoring	F	102.36	1	2	1	1	1	6	Wet
SO464	Soetdoring	F	93.40	1	2	1	1	1	6	Wet
SO456	Soetdoring	F	96.48	1	2	1	1	1	6	Wet
SO451	Soetdoring	F	141.26	1	2	1	1	1	6	Wet
SO455	Soetdoring	F	85.76	1	2	1	1	1	6	Wet
B 083	Swadini	F	131.94	1	1	1	1	1	5	Dry
T482	Tslowane	F	115.40	1	1	1	1	1	5	Wet
T479	Tslowane	F	105.00	1	1	1	1	1	5	Wet
T480	Tslowane	F	57.28	1	1	1	1	1	5	Wet
P 074	Woodhill	F	170.84	2	1	2	3	2	10	Wet
P 068	Woodhill	F	68.43	2	1	2	3	2	10	Wet
P 082	Woodhill	F	171.05	2	1	2	3	2	10	Wet

Table 8.2: continued…

ID	Field Site	Sex	Cortisol (ng/mg)	Access to Human Food	Access to Crops	Access to Rubbish	Human Activity	Provisioning by Humans	Human Impact Score	Season
B 019	Blyde	M	62.07	1	1	1	1	1	5	Wet
B 018	Blyde	M	106.36	1	1	1	1	1	5	Wet
SL504	Futululu	M	81.37	1	1	1	1	1	5	Dry
SL519	Futululu	M	135.77	1	1	1	1	1	5	Dry
PK 151	Lapa	M	138.36	1	1	1	1	1	5	Wet
PK 152	Lapa	M	98.97	1	1	1	1	1	5	Wet
P 079	Loristo	M	229.99	2	1	2	3	2	10	Dry
P 076	Loristo	M	148.73	2	1	2	3	2	10	Dry
SL515	Maurann	M	257.28	1	2	1	1	1	6	Dry
O 133	Oribi	M	115.41	1	2	1	1	1	6	Dry
PA410	Parys	M	116.58	2	1	2	3	2	10	Dry
PA415	Parys	M	227.97	2	1	2	3	2	10	Dry
PA421	Parys	M	251.13	2	1	2	3	2	10	Dry
BR495	Rooiplat	M	115.51	1	2	1	2	1	7	Dry
BR493	Rooiplat	M	97.39	1	2	1	2	1	7	Dry
BR492	Rooiplat	M	209.01	1	2	1	2	1	7	Dry
BR490	Rooiplat	M	113.78	1	2	1	2	1	7	Dry
SO450	Soetdoring	M	190.10	1	2	1	1	1	6	Dry
SO457	Soetdoring	M	131.70	1	2	1	1	1	6	Dry
B 007	Swadini	M	107.06	1	1	1	1	1	5	Dry
B 013	Swadini	M	114.90	1	1	1	1	1	5	Dry
T474	Tslowane	M	167.69	1	1	1	1	1	5	Dry
T471	Tslowane	M	78.77	1	1	1	1	1	5	Dry
T481	Tslowane	M	76.56	1	1	1	1	1	5	Dry
T472	Tslowane	M	69.27	1	1	1	1	1	5	Dry
T477	Tslowane	M	158.85	1	1	1	1	1	5	Dry
T476	Tslowane	M	108.92	1	1	1	1	1	5	Dry
T473	Tslowane	M	158.95	1	1	1	1	1	5	Dry
P 070	Woodhill	M	160.23	2	1	2	3	2	10	Dry

Table 8.3: Descriptive statistics of male and female human impact categories.

		n	Mean Cortisol (ng/mg)	± 1 Standard Deviation (ng/mg)
Male	High HI	6	189.10	54.32
	Moderate HI	8	153.77	57.68
	Low HI	15	110.92	34.44
Female	Dry Season			
	High HI	9	109.77	41.79
	Moderate HI	12	116.67	45.95
	Low HI	10	100.65	32.80
	Wet Season			
	Low HI	12	140.79	49.62

Figure 8.1: Plot showing the significant positive relationship between male hair cortisol concentration and the degree of human impact. Each symbol represents the average male hair cortisol concentrations for each site.

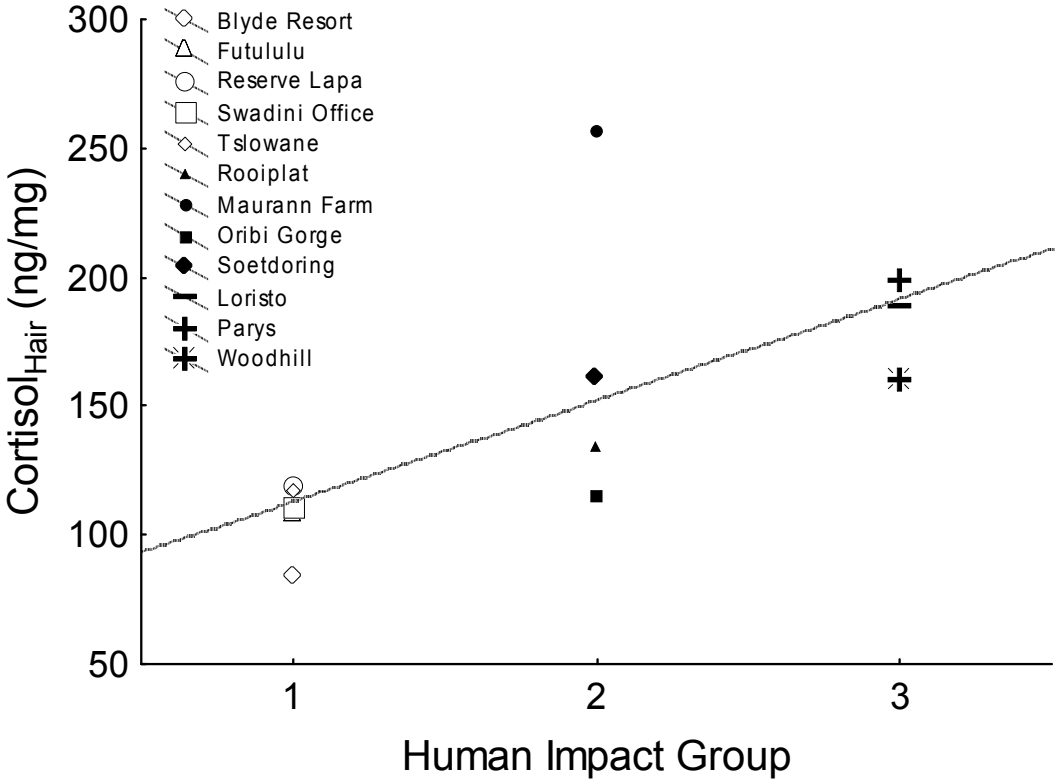
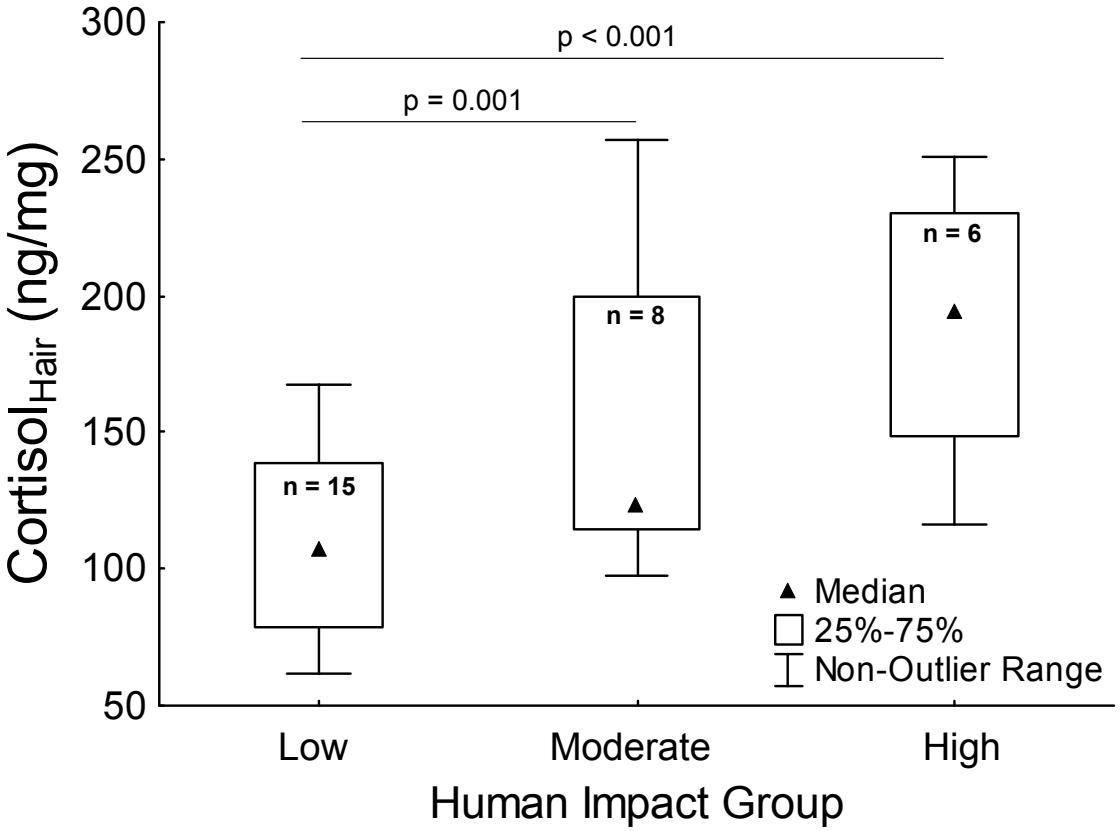


Figure 8.2: Box plot showing that low human impact males have significantly lower hair cortisol concentrations than moderate and high human impact categories.



Conclusion

Biological Validation, Hair Growth and Variation in Hair Cortisol

Although having a long history, hair hormone analysis has not found wide application in both human and animal studies until recently. Two studies by Koren et al (2002) and Davenport et al (2006) reported relatively simple hair cortisol extraction (simple alcohol extraction) and quantification (salivary ELISA) techniques to quantify cortisol in hair. These studies demonstrated the potential of the technique to investigate hormone-behavior relationships in wild and captive mammals, and seem to have been responsible for the resurgence of interest in, and wide application of, hair cortisol analysis. Since these two reports, a growing number of studies have utilized the technique to examine a large range of environmental, pathological, and behavioral issues in a growing number of mammalian species (bears - Becshoft et al. 2010; Macbeth et al. 2010; caribou - Ashley et al. 2011; ground squirrels - Martin and Reale 2008; humans – Dettenborn et al., 2010; hyraxes - Koren et al, 2002; macaques - Davenport et al. 2006; marmosets - Clara et al. 2008; various primates - Fourie and Bernstein. 2011a; vervet monkeys - Fairbanks et al. 2011).

Chapter 4 of this dissertation validated a commercially available salivary cortisol assay for use in quantifying hair cortisol in a number of different non-human primate species representing strepsirrhine, catarrhine and platyrrhine clades. I showed that cortisol concentrations archived in the hair of a number of non-human primates reflect biologically meaningful variation in circulating cortisol concentrations by demonstrating

that hair cortisol concentrations reproduce patterns of cortisol variation determined using traditional and well validated substrates. Specifically I showed that hair cortisol concentrations reproduce ontogenetic and phylogenetic patterns of variation in circulating cortisol as determined using serum and feces. These results also suggest the temporal stability of cortisol in hair as many of the samples used were derived from archived collections.

Further, novel data regarding sources of variation in hair cortisol important in considering study design were explored during the course of this investigation (Appendix 1). I report novel data which give the first rough estimates of the length of time which may be archived in individual hairs and therefore a rough estimation of the length of the period of endocrine activity recorded by non-human primate hair. The results show that non-human primate hair may archive cortisol accumulated over a 3 to 7 month period. I also provide evidence suggesting that hair growth in primates is probably asynchronous as in humans. These two findings are important to consider in project design and should inform decisions about the appropriate aliquot weight and composition that may be required to answer specific questions in future investigations (i.e. the statistical error that can be tolerated). If the focus is on inter-individual comparisons, larger aliquots, which more accurately estimate the true average cortisol concentration for a population of hair (and therefore the individual) is recommended. When the focus is broad inter-population comparisons, small samples, such as can be collected from a sleeping nest or hair traps, may be adequate to build population stress profiles. It is shown that the extraction of cortisol from hair can be improved using a sonic bath. This method is particularly relevant to small samples which may not be amenable to extensive reduction using

milling techniques. I also report that biological validation of the use of testosterone quantified from hair could not be achieved (Appendix 2).

Variation in Basal Cortisol Concentrations in Wild Baboons and Vervet Monkeys

In Chapter 5 I showed that the male hybrid populations had significantly elevated and more variable hair cortisol compared to parent populations. This result was congruent with the prediction that hybridization results in disruption of bio-behavioral and social complexes resulting in elevated basal cortisol in animals from hybrid populations. A similar association between hair cortisol and hybridity in male baboons was suggested by the results of Chapter 6, which showed that the male hybrid population, and the hamadryas population, which exhibited significant apparent anubis admixture, had elevated and more variable hair cortisol concentrations compared to the male anubis population in the environmental context of a severe drought. These results suggest a significant effect of hybridity on baboon hair cortisol concentrations. I propose that co-adapted stress complexes in the parent species are dismantled and recombined in hybrids resulting in dysregulation of the expression of physiological stress. Bio-behavioral mechanisms for coping with stress may themselves be dysfunctional, and it is likely that any inherited stress coping mechanisms in these animals (whether behavioral or biological) may simply be inadequate to deal with social stresses (unpredictability) resulting from life in a behaviorally heterogeneous social group that lacks any stable social norms. This is the first evidence to suggest a physiological cost associated with hybridity in wild baboons, although reproductive data suggest that hybrid males

reproduce well within hybrid groups and age profiles suggest that, hybrids (at least troop H) do not experience reduced lifespan. Further longitudinal studies are needed to assess hybrid fitness in order to better understand the social and biological mechanisms in hybrids that may underlie their elevated basal cortisol concentrations. The results also tentatively suggest that more hamadryas-like hybrids exhibit elevated hair cortisol concentrations compared to anubis-like and intermediate hybrids. This may be suggestive of a hamadryas disadvantage in the socially heterogeneous hybrid group. Variation in female basal cortisol concentrations did not conform to the male pattern. Female anubis and hamadryas had similar basal cortisol concentrations, whereas female hybrids had significantly lower hair cortisol concentrations than female hamadryas (Chapter 6). Female anubis exhibited significantly elevated hair cortisol concentrations under drought conditions compared to males, suggesting that females may be more acutely affected by ecological stress, however females from the hamadryas and hybrid population did not show similar tendencies.

Examination of anubis baboon basal cortisol during ontogeny showed no sex differences, and conformed to patterns of infant hypercortisolism and asymptotic age related declines in circulating cortisol (Chapter 7) as described in other *Papio* sp. (Castracane et al., 1981; Fourie and Bernstein, 2011a; Gesquiere et al., 2005). The results also show that variation in basal cortisol concentrations in juvenile male, but not female, anubis baboons is predicted by relative body mass. This relationship is suggestive of maternal effects, strongly influencing male development as described in yellow baboons (Altmann and Alberts 2005; Onyango et al. 2008). The current data is insufficient to

further explore any such relationships, but suggests the utility of hair cortisol analysis to assess maternal effects on offspring development and stress physiology.

Finally, I show that male vervet monkeys from populations which experience high levels of human interaction and habitat disturbance show elevated basal cortisol concentrations relative to animals from areas which experience moderate or low levels of human disturbance (Chapter 8). Raised cortisol within and between species in relation to hybridization, or environmental effects was primarily observed in males, but not females. Variation of hair cortisol in relation to environmental or social stressors may be obscured due to the effects of reproductive state on HPA axis activity. Reproductive state needs to be controlled for when examining variation in social and ecological stress and circulating cortisol in females. This study demonstrates the potential of hair cortisol analysis as a conservation tool to examine long term stress effects in wild animal populations.

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Appendix 1: Hair cortisol analysis in non-human primates: extraction, hair growth rates and intrinsic sources of variation

ABSTRACT

Hair cortisol analysis is finding broader application in animal studies; however there remain gaps in the current knowledge of the methodology. Increased extraction efficiency is important when using small samples. Hair growth patterns and rates affect assumptions of the period of time archived by a sample of hair. Hair cortisol is known to vary among anatomical regions in some animals; it is not known whether similar patterns of variation exist in non-human primates. The effect of sonication on the extraction of cortisol from hair was examined in samples from an adult female saki monkey (*Pithecia pithecia*). Intra-aliquot variability in hair cortisol was examined to asses whether the hair in the saki monkey exhibited mosaic growth. Hair growth rates were examined in six species (*Allenopithecus nigroviridis*, *Cercocebus chrysogaster*, *Theropithecus gelada*, *Gorilla g. gorilla*, *Symphalangus syndactylus*, and *Hylobates lar*) of non-human primates in captivity. Anatomical variation in hair cortisol was examined by comparing hair cortisol between three body regions in five female yellow baboons (*Papio cynocephalus*). Basic descriptive statistics, graphical representation and non-parametric tests for pair wise comparisons were used to assess the data. Results showed that sonication for 240 minutes significantly improves cortisol extraction, that intra-aliquot variation is inversely related to the number of hairs in the aliquot suggesting mosaic hair growth, that it takes approximately five months for hair to regrow to full length in several species and that

hair cortisol varies among anatomical regions. These findings contribute to an understanding of variation in hair cortisol and have implications for further study design.

INTRODUCTION

The use of hair as an archive of dietary information (e.g. O'Connel et al., 2001), exposure to toxins (e.g. Villain et al., 2004) and of drug use (e.g. Arnold, 1987) has been well established by biologists, toxicologists, and forensic and clinical scientists. Subsequently it has been shown that hair also archives steroid hormones (e.g. Faredin et al., 1966; Julesz et al., 1966, 1967; Lui et al., 1988; Cirimele et al., 1999; Raul et al., 2004). Interest in hair as an archive of steroid hormone concentrations stemmed from efforts to control the abuse of androgens and corticoids in sport (e.g. Cirimele et al., 1999; Bevalot et al., 2000). Subsequently in addition to its application in doping control, the technique has found some application in clinical settings as a way to monitor chronic stress in patients by measuring levels of stress hormones sequestered in the hair (e.g. Sauve et al., 2007; Stalder et al., 2010; Stuedte et al., 2011; Thomson et al., 2010). The technique has also been applied by animal researchers (e.g. Davenport et al., 2006; Accorsi et al., 2008), and archaeologists (Webb et al., 2010) as a way to asses stress in their subjects and its potential in these fields continues to be explored.

This renewed interest in hair as an archive of endocrine activity may spring from the various unique features which give hair several advantages over more traditional substrates used to determine hormone concentrations. First, hair archives the free unbound, or bioavailable fraction of steroids. There are two principal mechanisms for steroid incorporation in to the hair shaft. Steroids are incorporated into the shaft of the hair during growth through diffusion from capillaries which enmesh the follicle (Cone, 1996); steroids are also incorporated into the shaft through sweat and sebum excreted by

glands onto the skin (Anielski, 2008). This means that a hair reflects averaged steroid concentrations accumulated over the lifetime of the hair. Some evidence suggests that human hair can be analyzed serially to reveal temporal changes in circulating steroid concentrations (e.g. Webb et al., 2010; Kirschbaum et al., 2009). The time averaged nature of the steroid signal in hair has been referred to as reflecting the basal hormonal phenotype of an individual (Davenport et al., 2006). Such time averaged measures of endocrine activity is time consuming, expensive and often logistically complex to determine when using traditional substrates (e.g. blood, saliva, urine, feces) that only reflect endocrine activity over short periods of time (minutes to days). A time averaged measure of endocrine activity is especially interesting to researchers interested in chronic steroid concentrations and phenotypic variation in basal steroid profiles.

Additionally hair also has a number of practical advantages over traditional substrates. Hair appears to be an exceptionally stable environment for steroids. This means that hair can be shipped and stored at non-specific ambient conditions and that steroid concentrations in hair can remain biologically relevant for long periods of time (e.g. >1000 years; Webb et al., 2010). Hair can also be collected relatively non-invasively from both captive and wild animals (Valderama et al., 1999, Scheppers et al., 2007). The extraction of steroids from hair is relatively simple and the steroid extracted from hair can be quantified using enzyme-linked immunosorbent assay (ELISA) techniques (e.g. Koren et al., 2002; Devenport et al., 2006). These properties of hair make it an attractive steroid bearing substrate for a wide range of researchers working in controlled and natural conditions. However there remain gaps in the current knowledge of the methodology which need to be addressed for the technique to be used appropriately. These include a

paucity of information on patterns of hair growth and hair growth rates in non-human primates, both of which are important in order to understand the period of time archived by a hair sample.

Therefore I examined a) a novel extraction protocol on recovery of cortisol values, and addressed some basic questions about b) hair growth rates, patterns of hair growth and c) anatomical variation in hair cortisol.

Extraction

Wild collected samples may be small and represent a limited resource; it is therefore desirable to be able to extract a sufficient amount of hormone for analysis from small samples. Published protocols use techniques to pulverize samples which significantly increase extraction efficiency (e.g. Davenport et al., 2006). However the equipment used is expensive and may not always be readily available, furthermore such techniques may not be practical when small samples are available. Studies that pulverize samples typically pulverize large samples (50 to 100mg – D'Anna-Hernandez et al., 2011; Davenport et al., 2006; Dettenborn et al., 2010; Fairbaink et al., 2011; Hamel et al., 2011; Kirschbaum et al. 2009; Raul et al., 2004) and then take aliquots from the large homogenized pool. The reduced particle size increases extraction efficiency. It is known that sonication can significantly improve the extraction of endobiotics from substrates with similar physicochemical properties to hair (Schinor et al., 2004), and this may therefore also effect the extraction of cortisol from hair.

Hair Growth Rate and Patterns

Little is known about hair growth rates, of patterns of hair growth and anatomical variation in hair cortisol (though see Macbeth et al., 2010, Sharpley et al., 2010a, b). It is likely that hair growth patterns in most primates are asynchronous, as they are in humans (Harkey, 1993). In other words, individual hairs may be in different growth phases and may be of different ages. These observations have several implications for sampling, aliquot composition and research design. If individual hairs represent somewhat different growth periods they also record different periods of endocrine activity. Therefore a sufficient number of individual hairs must be present in the aliquot prepared for extraction so as to reduce inter-aliquot variability to a level which is acceptable to the specific research question. Additionally, the aliquot weight must be sufficient for enough hormone to be extracted for measurement by ELISA. A balance must therefore be reached between (i) the number of hairs of which an aliquot must consist to produce an acceptable estimation of the population average and (ii) the minimum weight required to produce enough hormone for accurate quantification by ELISA. No information is available on the period of time likely represented by individual hairs. Such information is crucial to estimate the period of endocrine activity likely archived in a hair sample.

Anatomical Variation

Further, hair from different anatomical regions of the body may differentially sequester cortisol and yield different raw amount of the hormone. For instance hair from

different part of the body of humans (Sharpley et al., 2010a, b) and bears (*Ursus arctos* – Macbeth et al., 2010) yield different hair cortisol concentrations. This emphasizes the need to control for body region when sampling. I examined whether such patterns of hair cortisol variability is also present in non-human primates.

MATERIALS AND METHODS

Sample Collection

Hair samples were collected from adult and sub-adult Allen's swamp monkeys (*Allenopithecus nigroviridis*, n = 2), golden bellied mangabeys (*Cercocebus chrysogaster*, n = 3), a gelada baboon (*Theropithecus gelada*, n = 1), western lowland gorillas (*Gorilla gorilla gorilla*, n = 3), a lar gibbon (*Hylobates lar*, n = 1) and a siamang (*Symphalangus syndactylus*, n = 1) by attending veterinarians at the San Diego Zoo during annual veterinary exams (see Table A1.1). Samples were cut or shaved from the intra-scapular region, as close to the skin as possible without causing injury, using hair clippers or scissors.

A large sample of hair (~1g) was obtained from an adult female white faced saki (*Pithecia pithecia*) cadaver, originating from the Santa Anna Zoo. Aliquots taken from this sample were used for investigation of the effect of sonication on cortisol extraction from hair and to examine the effects of aliquot composition on intra-aliquot variability. Hair samples taken from three anatomical body regions (shoulder, base of tail and thigh) from five adult female yellow baboons (*Papio cynocephalus*) (i.e. had erupted third

molars - Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000), from the Mikumi Game Reserve in Tanzania, were provided by collaborators. These animals were trapped and sedated using the methods described by Brett et al., (1982), see Table A1.2 for sample breakdown.

Sonication and Cortisol Extraction

In order to test the efficacy of sonication to increase the extraction of cortisol from hair, extraction by incubation with sonication (50Hz) was compared across different lengths of time. Five groups of 10mg aliquots weighed from a 500mg scissor cut homogenized hair sample of a single captive adult *Pithecia pithecia* female was incubated in a sonic bath at six different time intervals (0min, >60 min, 120min, 240min, 400min) using sonication (50Hz) at 55^{o}C.

Intra-Aliquot Variability

A large amount of hair was available for the adult white faced saki female and was used to compare the effects of aliquot weight and composition on intra-aliquot variability in hair cortisol. Three sets of aliquots of different weights (3mg, 10mg, and 20mg) were weighed out and extracted, and the cortisol concentrations were determined, as detailed below. The number of hairs required to make up a weight of 3 mg was counted in five 3mg samples. These counts were then averaged and used to estimate the number of hairs needed to make up a 1mg, hair sample which was used to estimate the number of hairs

that would be required to make up each successively larger weight (3 – 20mg) used in this analysis. The length of five hairs was also measured and averaged in order to determine the average hair length for hair from the intra-scapular region of this specimen.

Hair Regrowth

In order to assess hair growth rates among primates, collaborators at the San Diego Zoo collected hair samples from the listed animals (see Table A1.1). The animals were visually inspected by collaborators on a regular basis and the date of full regrowth was recorded for hair from the shaved patch was recorded. An estimate of time to full regrowth was thus calculated. Hair was measured for each individual received and estimates of hair growth rates for individuals were calculated.

Anatomical Variation in Hair Cortisol

10mg hair samples were weighed out for each of three body regions, the shoulder, base of tail and thigh, in each of five adult female yellow baboons (see Table A1.2). Cortisol was extracted and quantified in these samples as described below.

Sample Preparation

Aliquots were weighed out into labeled 5ml borosilicate culture tubes. Samples were twice washed in 3ml isopropanol (C_3H_8O) for 3 minutes (after Davenport et al 2006) and

let dry in a fume hood under a stream of air for 24 hours. The samples were then minced into 1-2mm pieces using a pair of surgical scissors. 4ml of methanol (CH_4O) was then added; the scissors were rinsed in the tube to ensure that no hair material was lost. Samples were incubated in an ultrasonic bath (Branson 2510) at 50Hz at 55^oC for 240min and removed and incubated on plate-shaker at room temperature (~24 ^oC) for 20 hours.

At the end of extraction the tubes were centrifuged at 4000rpm at room temperature for 5min. 3.5ml of the extract were then aliquoted into labeled polypropelene tubes and allowed to dry under a stream of air in a fume hood. The dry extract was then reconstituted in successively smaller volumes of methanol (2ml, 1ml and 500ul) and dried down under air in order to concentrate the dry extract at the bottom of the tube. Finally the dry extract was reconstituted in 500ul of phosphate buffer (0.1M $NaPO_4$, 0.149M NaCl, pH 7.0) and stored at -20^oC.

Enzyme-linked Immunosorbent Assay (ELISA)

Hair cortisol concentrations were quantified using commercially produced enzyme-linked immunosorbent assay (ELISA) kits designed to quantify the free unbound cortisol in saliva (ALPCO Diagnostics, Salem NH). Manufacturer reported cross-reactivity is as follows: cortisol = 100%, prednisolone = 13.6%, corticosterone = 7.6%, deoxycorticosterone = 7.2%, progesterone = 7.2%, cortisone = 6.2, deoxy cortisol = 5.6%, pednisone = 5.6%, dexamethasone = 1.6%. The range of detection for this kit is 1-100 ng/ml. 50ul of reconstituted sample were run in duplicate as directed by the assay protocol. Results were converted from ng/ml to ng/mg of hair. Mean intra- and inter-

assay coefficients of variation were 2.25% and 4.31%, respectively. All standards and samples were run in duplicate in accordance with the instructions from the manufacturer. The assay was independently validated for each species by showing (1) parallelism between a serial dilution of sample extract and a serial dilution of a known amount of cortisol and (2) recovery of more than 90% of a known amount of cortisol added to sample extracts before analysis (Appendix 3 and 4).

Data Analysis

Differences in the amount of cortisol extracted from hair using the different extraction conditions were tested using a Kruskal-Wallis (KW) test followed by post hoc pair wise comparison using a Dwass-Steel-Chritchlow-Fligner (DSCF) test. The use of non-parametric tests means that hair cortisol concentrations were not log transformed as no assumptions of normality are made by these tests. Intra-aliquot variability is described using basic descriptive statistics, including calculation of the coefficient of variation for each aliquot size category. Hair regrowth data were similarly described using basic descriptive statistics. Variability among the different body regions in the five yellow baboon females was tested using a KW and DSCF *post hoc* test and was visually assed for each animal using a scatter plot. Statistical significance was set to $p<0.05$ and the software package SYSTAT 13 was used to perform all statistical analyses.

RESULTS

The amount of cortisol extracted under different sonication extraction conditions differed significantly (KW test: $H_{4,35} = 16.541$, $p = 0.002$). All extraction conditions except sonication for less than one hour yielded significantly more cortisol than incubation without sonication (DSCF test: all $p<0.001$). Four hours of sonication yielded the most cortisol compared to all other sonication incubation times (DSCF test: all $p<0.001$, see Table A1.3 and Figure A1.1)

For the white faced saki female hair sampled from the intra-scapular region measured on average 6.13±0.37cm (n=5). Approximately 7.1±4.3 hairs (n=5) have a weight of 1mg, a 3mg sample therefore contains approximately 21 hairs, a 10mg sample contains approximately 71 hairs and a 20mg sample contains approximately 142 hairs. The inter-aliquot coefficient of variation decreased from 40% for 3 mg aliquots (n=7), to 13% in 10mg samples (n=13) and 9% in 20mg samples (n=5) (Figure A1.2).

Hair growth rates varied between 0.7±0.1cm per month in golden bellied mangabeys (n=3) to 3.5 cm per month in the gelada baboon (n=1). Time to full regrowth varied between 2.8 months in western lowland gorillas (n=3) to 7.1 months in golden bellied mangabeys. See Table A1.4 for growth rates and time to regrowth for each species.

For four out of the five baboons examined, hair from the thigh region yielded higher hair cortisol concentrations compared to hair from the base of the tail or the shoulder (see Figure A1.3), though overall differences in hair cortisol among body regions were not significant (KW test: $H_{2,15} = 4.500$, $p = 0.105$). No consistent differences in hair cortisol could be observed between hair from the base of the tail and the shoulder.

DISCUSSION

Most recently published extraction protocols for the extraction of cortisol from hair requires the reduction of the substrate to small particles, either through cutting (e.g. Sauve et al., 2007) or milling (e.g. Davenport et al., 2006; Kirschbuam et al., 2009). Incubation is usually done at relatively elevated temperatures (e.g. ~50^{o}C Sauve et al., 2007) for between 16 (e.g. Sauve et al., 2007; Koren et al., 2002) and 24 hours (e.g. Davenport et al., 2006; Kirschbaum et al., 2009). The results presented here show that it may be possible to further increase extraction efficiency through incubation in a sonic bath for a prolonged period of time (~240min, though see 30min step Anielski, 2008; Kalra et al., 2007; Koren et al. 2002). Ultimately, incubation using sonication and high temperatures may present a viable alternative for increasing extraction efficiency in the absence of access to expensive, cumbersome milling equipment, or when very small samples are used.

Examination of intra-aliquot variability shows that samples containing more hairs have lower intra-aliquot variability. This suggests that as in humans, hair growth in this primate species (white faced saki) is likely asynchronous, meaning that a population of hair consists of individual hairs of different ages and in different growth phases, each recording different, and sometimes overlapping periods of endocrine activity. Therefore the number of hairs in an aliquot needs to estimate the mean for the population of hair with accuracy acceptable to the project design. For instance, if the focus of the study is to make inter-individual comparisons, larger (i.e. greater number of hairs) aliquots may be necessary, whereas when comparing two populations a larger error in the estimation of individual hair cortisol may be tolerated. The relationship between the number of hairs

and aliquot weight will depend on the length of the hair. Therefore, in large primates with longer and thicker hair a larger sample weight may be required, as only a few individual hairs may yield enough cortisol for quantification. Small primates, like marmosets or squirrel monkeys, have short dense hairs and a sample with the lowest weight necessary to yield enough cortisol for quantification could contain hundreds of hairs.

Hair growth rates vary considerably within and between primate species (Table A1.1 and A1.4), but suggest that individual hairs take several (~ 5) months to reach mature length in captive animals. The methods for collecting hair regrowth data is crude but provides the first rough estimates of the period of endocrine activity that non-human primate hair may archive. It should be kept in mind that the primates used in this study are well provisioned zoo kept animals. Hair growth rates may be overestimated compared to wild conspecifics with more variable ecologies. Nonetheless the results suggest that, depending on the length of the hair and the rate of growth, primate hair records endocrine activity over several months. The results show that primate hair grows somewhat faster than reported in humans (~1cm/month, Harkey, 1993). Non-human primate hair also has a relatively short minimum age as the maximum hair length is reached quickly and is much shorter than the maximum age for human scalp hair (2 to 6 years to reach maximum length - Price, 1999; Robbins, 2002). It remains unknown how long individual hairs remain on the animal before shedding and whether some species, especially primates in temperate zones, experience seasonal hair growth and molts. In the case of the captive catarrhine non-human primates for which data were available for this study, it appears that the minimum period represented by a full grown hair is between 5 and 8

months, meaning that a mature hair in these taxa records endocrine activity for at least that length of time.

Further, cortisol concentrations measured in hair from different anatomical regions suggest variation in the hair cortisol concentrations between body regions. These results are preliminary and would benefit from larger sample sizes and multiple aliquots per individual per body region to confirm these initial observations. However, the variability across body regions in hair cortisol described here is consistent with reports on anatomical hair cortisol variation in humans (Sharpley et al., 2010a, b) and grizzly bears (Macbeth et al., 2010), and emphasizes the importance of controlling for anatomical region when sampling hair. This may not always be possible, especially when hair is collected opportunistically or using non-invasive methods. In such cases, anatomical variability in hair cortisol should be examined for the study species and considered in the study design; again, depending on the nature of the questions examined it may be possible to accommodate the error resulting from differences in anatomical region in the study design.

CONCLUSIONS

This paper reports a novel technique which increases the amount of cortisol extracted from hair and may be especially salient to the application of small samples, such as may those that be collected non-invasively, which do not lend themselves to reduction by milling. Results from an examination of intra-aliquot variability suggest that hair growth in at least in non-human primate species is asynchronous, and that aliquot composition is

important, specifically that more hair per aliquot reduces inter-aliquot variability. These results and data give the first indication of the length of the period of endocrine activity that may be archived in a sample of primate hair. Further, it appears that primate hair from different body regions archive different concentrations of hair cortisol. It is therefore important to consider these three factors (hair growth pattern, growth rate and across body region variation) in study design.

Tables and Figures

Table A1.1: List of all animals that for which hair regrowth estimates were collected along with individual hair identity, age, sex and hair regrowth data.

ID	Species	sex	Age	Time to Regrowth (months)	Mean Length (cm)	±1 Standard Deviation (cm)	Growth Rate (cm/month)
699527	*Allenopithecus nigroviridis*	F	Adult	4.3	6.50	0.34	1.52
504208	*Allenopithecus nigroviridis*	F	Adult	2.6	5.00	0.00	1.95
505053	*Cercocebus chrysogaster*	F	Adult	7.6	5.25	0.71	0.69
505054	*Cercocebus chrysogaster*	F	Sub-Adult	7.6	5.50	1.20	0.72
505052	*Cercocebus chrysogaster*	F	Adult	7.6	4.38	0.44	0.57
598276	*Theropithecus gelada*	F	Adult	3.4	12.00	1.20	3.50
696080	*Gorilla gorilla gorilla*	F	Adult	4.0	4.63	0.56	1.15
689008	*Gorilla gorilla gorilla*	M	Adult	2.4	13.88	1.28	5.78
699538	*Hylobates gabriellae*	F	Adult	5.7	4.63	1.51	0.81
31857	*Symphalangus syndactylus*	F	Adult	4.9	5.13	0.52	1.04

Table A1.2: List of yellow baboon females and associated hair cortisol concentrations for hair from each sampled body region.

ID	Body Region	Cort (ng/mg)
2010	Base of Tail	0.74
2010	Shoulder	0.97
2010	Thigh	0.99
2022	Base of Tail	0.88
2022	Shoulder	1.00
2022	Thigh	0.93
2023	Base of Tail	0.94
2023	Shoulder	1.04
2023	Thigh	1.17
5012	Base of Tail	0.60
5012	Shoulder	0.70
5012	Thigh	0.96
5020	Base of Tail	0.93
5020	Shoulder	0.63
5020	Thigh	1.59

Table A1.3: Sonication extraction results for multiple 10mg samples.

Time in Sonication (min)	n	Mean (ng/mg)	± 1 Standard Deviation (ng/mg)
0	6	2.26	0.10
<60	4	2.77	0.29
120	4	2.81	0.37
240	17	3.16	0.69
>360	4	3.04	0.33

Table A1.4: Table showing the hair growth rates and time to full regrowth of hair for six zoo-kept primate species.

Species	n	Average Growth Rate (cm/month)	±1 Standard Deviation (cm/month)	Average Length (cm)	±1 Standard Deviation (cm)	Length of Time Archived (months)
A. nigroviridis	2	1.7	0.3	5.8	1.1	3.4
C. chrysogaster	3	0.7	0.1	5.0	0.6	7.1
T. gelada	1	3.5		12.0		3.4
G. g. gorilla	3	2.7	2.7	7.5	6.5	2.8
H. gabriellae	1	0.8		4.6		5.8
S. syndactylus	1	1.0		5.1		5.1

Figure A1.1: Mean and standard error plot showing the effect of sonication on the extraction of cortisol from hair.

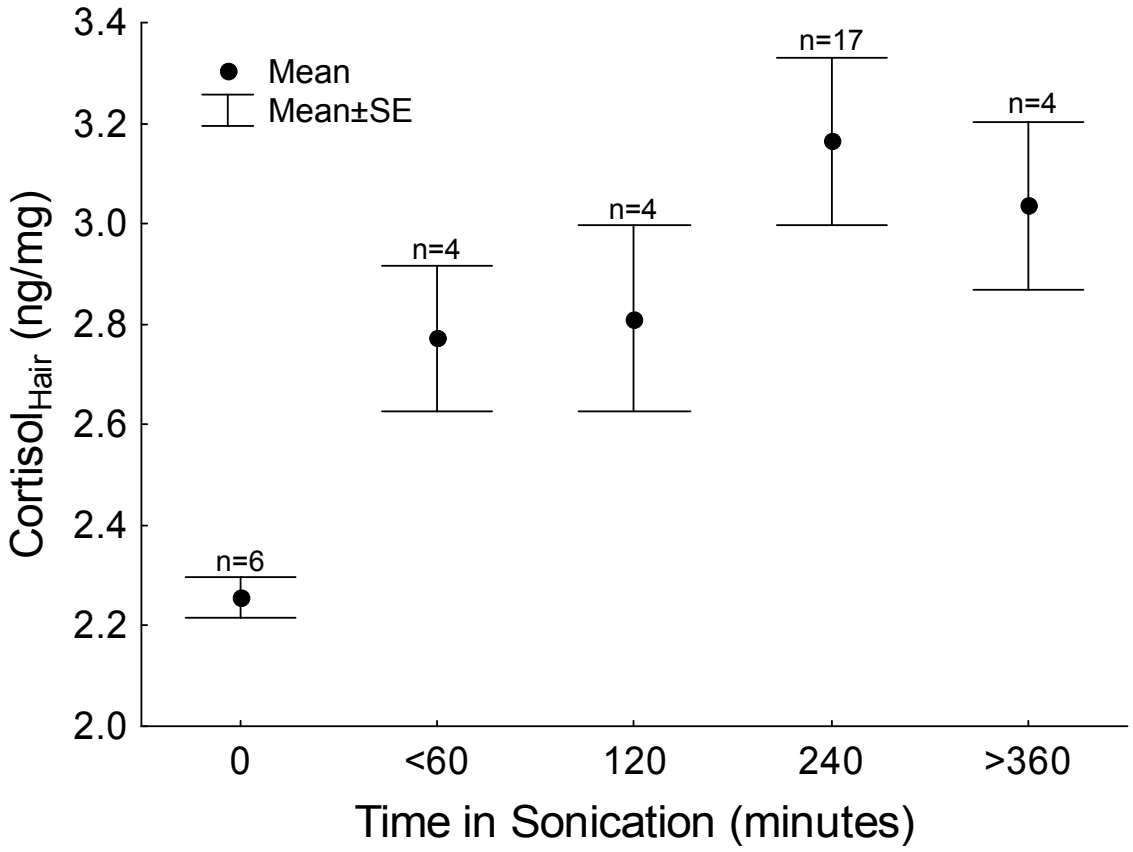


Figure A1.2: Inter-aliquot variance in whole-hair (i.e. not homogenized) samples of a *P. pithecia* at different weights and therefore a different number of hairs.

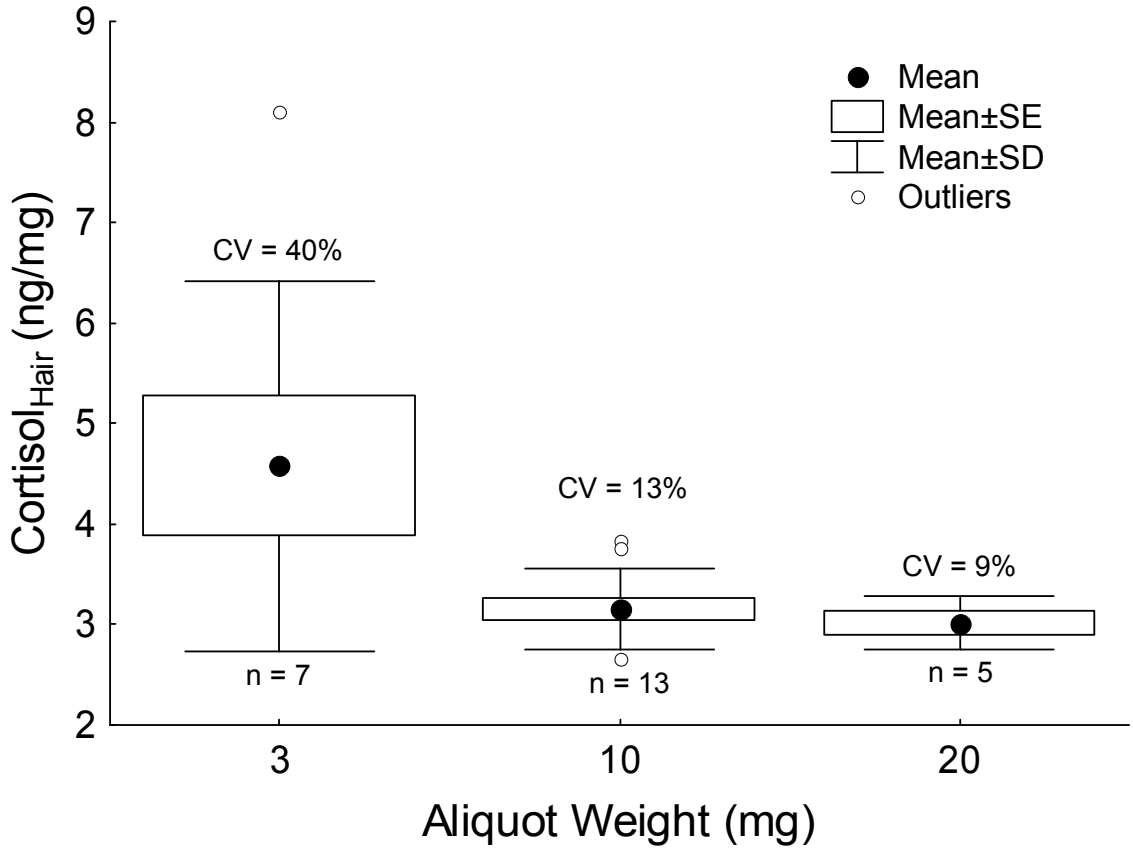
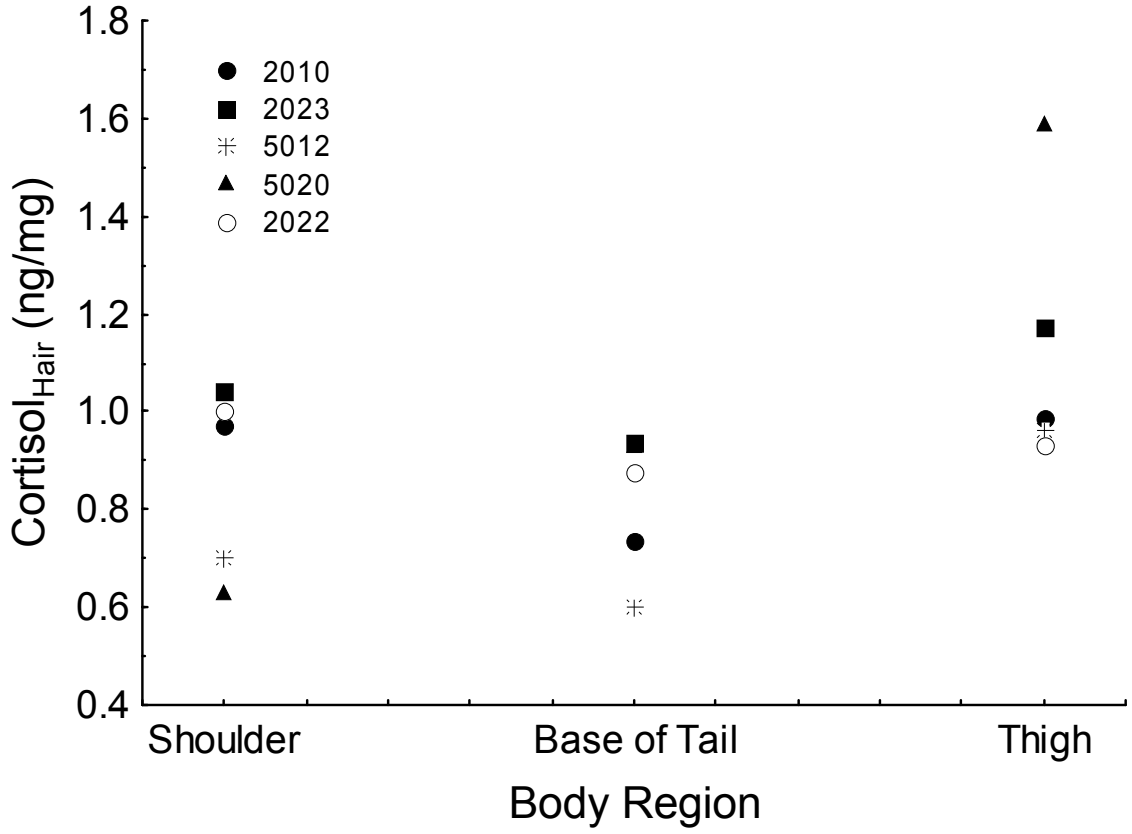


Figure A1.3: Plot showing a trend for higher cortisol concentrations to be sequestered in hair from the thigh region compared to hair from the base of the tail and should regions in five adult female yellow baboons.



Appendix 2: Hair testosterone concentrations in wild juvenile and adult baboons

ABSTRACT

Hair hormone analysis is a novel technique in endocrinology. Currently only hair cortisol is routinely extracted and quantified using enzyme immunosorbent assay (EIA) techniques. A small number of studies have shown that it may be possible, using similar protocols as is used in the quantification of cortisol from hair, to extract and quantify testosterone. Here I attempted to biologically validate hair testosterone concentrations in wild baboons from wild populations in the Awash National Park, Ethiopia. If hair testosterone concentrations reflect time averaged circulating levels of the hormone then hair testosterone concentrations should retrieve similar patterns of variation as have been demonstrated in circulating testosterone concentrations using traditionally analyzed substrates. Specifically, adult male baboons have significantly elevated circulating testosterone concentrations compared to juveniles, and hair testosterone concentrations should replicate this pattern. Testosterone was extracted from approximately 10mg hair samples representing juvenile and adult anubis (*Papio anubis*, 59 juveniles, 39 adults), hamadryas (*P. hamadryas*, 14 juveniles, 18 adults) and hybrid baboons (16 juveniles, 21 adults). Testosterone was quantified using a free testosterone enzyme-linked immunosorbent assay. I compared hair testosterone concentrations in juvenile and adult conspecifics using a Mann-Whitney U Test. Juvenile anubis had significantly higher hair testosterone concentrations than adult conspecifics. Juvenile hamadryas and hybrids did not differ in hair testosterone concentrations from adult conspecifics. The results do not

show that testosterone concentrations archived in the hair of these samples reflect biologically meaningful variation.

INTRODUCTION

Hormones are often used as biomarkers in the analyses of specific life history patterns and behaviors; variation in hormone levels among species, and individuals within species, have implications for understanding the fitness tradeoffs inherent in- and the adaptive significance of different behavioral and life history strategies (e.g., Wingfield et al., 1990; Gray, 2003; Gray et al., 2007; Beehner et al., 2009).

Androgens are known to be important for development and expression of physical and behavioral characteristics associated with reproduction and reproductive competition (specifically testosterone - Nelson, 2005), however if maintained at chronically high levels androgens are associated with negative health effects (e.g., compromised immune function – Dorner et al., 1980; Ahmed et al., 1985, increased risk of injury/mortality associated with competitive and dominance behavior – Wingfield et al., 1987; 1990). It is thought that animals should only invest in the production of high levels of testosterone when the benefits of these (e.g. in competition for reproductively relevant resources) outweigh the costs (e.g. compromised immune function). This idea is known as the "Challenge Hypothesis" (Wingfield et al., 1990) and has been demonstrated in various vertebrates (e.g., birds - Wingfield et al., 1987; mongoose - Creel et al., 1993), including non-human primates (lemurs – Cavigelli and Pereira, 2000; chimpanzees - Muller and Wrangham, 2004; baboons - Beehner et al., 2009). Paradigms such as these help us to describe hormone-behavior relationships in adaptive and evolutionary terms.

Typically, studies of the bidirectional relationship between androgens and behavior tend to employ substrates sensitive to acute stimuli. For example, salivary testosterone levels rise in male victors of chess games but diminish in the losers, demonstrating a clear relationship of hormone with mental state following the outcome of a game that simulates dyadic aggression (Mazur et al., 1992). Similarly, urine and fecal cortisol levels reflect acute endocrine responses to stress within 12 to 48 hours of an individual's exposure to a social stressor (e.g. Clarke and Boinski, 1995; Whitten et al., 1998b). However, when the desired foci of study are social trends, stable behavioral patterns, personality, or group level behavioral profiles, the acute sensitivity of such substrates to short-term temporal changes presents logistical and financial challenges for the researcher wishing to obtain an overall "average" hormone profile. Daily samples over weeks/months are needed to derive average trends, and this requirement poses challenges in terms of sample collection, storage, and transport.

An alternative technique involves retrieving hormonal information from hair and shows much potential as a hormone bearing substrate for investigating allostatic load and male reproductive strategies within and between taxa and populations. Hair as a hormone bearing substrate has several advantages over traditional substrates and archives a uniquely averaged hormone signal which may extend the reach of endocrine research (Davenport et al., 2006).

The present study tests the possibility of using testosterone concentrations quantified from the hair of wild primates, using recently published protocols for quantification by enzyme linked immunosorbent assay (ELISA), to study natural biologically relevant variation in chronic circulating testosterone concentrations. To date hair cortisol analysis

has been repeatedly biologically validated in a number of mammalian species (e.g. bears - Becshoft et al., 2010; Macbeth et al., 2010; caribou - Ashley et al., 2011; ground squirrels - Martin and Reale, 2008; humans – Dettenborn et al., 2010; hyraxes - Koren et al., 2002; macaques - Davenport et al., 2006; marmosets - Clara et al., 2008; various primates - Fourie and Bernstein, 2011a; vervet monkeys - Fairbanks et al., 2011) and the use of hair cortisol analysis in endocrine research in wild (e.g. Becshoft et al., 2010; Macbeth et al., 2010), captive (e.g. Davenport et al., 2006) and clinical (e.g. Dettenborn et al., 2010) animal and human research is becoming more widespread. Hair testosterone analysis is much less commonly used and biological validation of the method using hair cortisol-like extraction protocols and using ELISA to quantify hair testosterone concentrations has not yielded consistent results (e.g. Koren et al., 2002, Koren and Geffen, 2006; Koren et al., 2009; Thomson et al., 2009).

A previous study on baboons used a simple dichotomy, namely that adult male baboons have elevated circulating testosterone concentrations compared to juveniles, biologically validate the use of testosterone extracted from the fecal samples of three baboon species (Beenher et al., 2009). Here I used the same paradigm to validate the use of testosterone extracted from the hair of anubis (*Papio anubis*) and hamadryas baboons (*P. hamadryas*) and their natural hybrids from populations in the Awash National Park, Ethiopia. If hair testosterone concentrations reflect circulating levels of the hormone adult male animals (i.e. $\geq$ 8 years old) of the three populations should exhibited significantly elevated hair testosterone concentrations compared to juvenile conspecifics (i.e. 2-6 years old). A positive result would provide strong evidence that hair testosterone concentrations faithfully reflect biologically relevant concentrations and patterns of variation in

circulating testosterone and can be used to examine hormone-behavior relationships in these taxa.

MATERIALS AND METHODS

Samples

The present study used dental juvenile (i.e. males with no erupted third molars, Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000) and dental adult male animals (i.e. males with third molars and canines in full occlusion - Phillips-Conroy and Jolly, 1988; Phillips-Conroy et al., 2000). I extracted and measured testosterone accumulated in hair to determine time averaged measures of circulating testosterone.

Samples were derived from anubis (juvenile n = 59, adult n = 39; troops C, D, E, F) and hybrid (juvenile n = 16, adult n = 21, troop H) troops inhabiting the margins of the Awash River (Phillips-Conroy and Jolly, 1986); hamadryas samples (juvenile n = 14, adult n = 18, group Filoha) derive from a group which inhabit the Filoha hot-springs region (Swedell, 2002). Animals were captured using manually operated cage traps and anesthetized (as described by Brett et al. 1982). Approximately 50 to 100mg of hair was clipped from between the shoulder blades as close to the skin as possible without causing injury. Samples were stored and shipped in paper envelopes under non-specific ambient conditions

Hair Sample Preparation

The sample preparation protocol was modified from the basic version generally used for cortisol extraction (e.g. Davenport et al. 2006; Sauve et al., 2007; Yamada et al., 2007), but which has also been successfully employed for extraction of testosterone from hair for quantification by enzyme immunoassay (Koren et al., 2002, Koren and Geffen, 2006; Koren et al., 2009; Thomson et al., 2009). Hair samples (10mg) were washed twice in 3mL isopropanol (C_3H_8O) and dried in a fume hood under a stream of air for approximately 16hrs until incipient dryness (after Davenport et al., 2006). The wash procedure removed exogenous contaminants and sebum. Samples were weighed out into ~10mg aliquots, placed in labeled borosilicate tubes and cut into approximately one to three millimeter pieces using a pair of fine surgical scissors (after Sauve et al., 2007, Yamada et al., 2007). Four milliliters of methanol (CH_4O) were then added to each tube, which was then incubated at room temperature on a reciprocating shaker at 100rpm for 16hrs. The samples were centrifuged for 10min at 4000rpm and 3.5mL of the resulting supernatant was pipetted into labeled polypropylene tubes. The supernatant was dried under a stream of air in a hot water bath at 60^{o}C in a fume hood. The dried mass was then reconstituted in 500µL of assay dilution buffer (0.1M $NaPO_4$, 0.149M NaCl, pH 7.0) and stored at -20^{o}C.

Testosterone Enzyme Immunoassay (EIA)

A testosterone EIA was used to measure testosterone extracted from hair samples and relied on a polyclonal testosterone antibody (R156/7, 1:7, 5000; C. Munro, University of

California, Davis; Munro and Lasley, 1988). The antibody cross-reacts with testosterone 100%, 5α-Dihydrotestosterone 57.37% (DHT), Androstenedione 0.27%, Androsterone 0.04%, DHEA 0.04%, Cholesterol 0.03%, B-Estradiol 0.02%, and less than 0.02% with Progesterone, Pregnenolone, Hydrocortisone, Cholic Acid, Chenodeoxycholic Acid; Cholic Acid Methyl Ester, Dehydrocholic Acid, Lithocholic Acid; Glycholic Acid, Tauodeoxycholic Acid, Taurocholic Acid, Taurochenodeoxycholic Acid, and Glycochenodeoxycholic Acid (C.J. Munro, pers. comm.).

Assay sensitivity was 2.3 pg/well. Intra-assay and inter-assay coefficients of variation were less than 10% (n = 30) and 15% (n = 20) respectively. The assay was independently validated for anubis, hamadryas and hybrid baboons by showing (1) parallelism between a serial dilution of sample extract and a serial dilution of a known amount of testosterone and (2) recovery of more than 90% of a known amount of cortisol added to sample extracts before analysis (Appendix 3 and 4).

Statistical Analysis

Juvenile and adult hair testosterone concentrations were compared using a Mann-Whitney U test. Significance was set to the 95% level. This non-parametric test does not require normalization of the data by log-transformation. The raw untransformed data was therefore used. All statistical testing was done using the Statistica 7.0 statistical software package.

RESULTS

Juvenile male anubis (n = 59, mean ± one standard deviation = 78.413±57.297pg/mg) had significantly (Mann–Whitney U = 869.000, p = 0.041 two-tailed, see Figure A2.1) higher hair testosterone concentrations than adult male anubis (n = 39 , mean ± one standard deviation = 57.065±52.443pg/mg).

Juvenile (n = 14, mean ± one standard deviation = 60.960±40.644pg/mg) and adult (n = 18, mean ± one standard deviation =46.406±21.409pg/mg) male hamadryas did not differ significantly (Mann–Whitney U = 102.000 , p = 0.362 two-tailed, see Figure A2.1) in hair testosterone concentrations.

Juvenile (n = 16, mean ± one standard deviation = 40.807±14.299pg/mg) and adult (n = 21, mean ± one standard deviation = 57.088±44.256pg/mg) hybrid males did not differ significantly (Mann–Whitney U = 113.000, p = 0.092 two-tailed, see Figure A2.1) in hair testosterone concentrations.

DISCUSSION

If hair testosterone concentrations reflect biologically relevant concentrations and variation of the hormone then it is expected that hair testosterone concentrations should replicate known differences in circulating testosterone concentrations. The results do not satisfy this expectation and find no evidence in the present sample set that adult male baboon hair testosterone concentrations are significantly elevated compared to those of juveniles. Previous studies using this methodology for the extraction and quantification of testosterone is limited to the rock hyrax (*Procavia capensis* - Koren et al., 2002, Koren

and Geffen, 2006; Koren et al., 2009), and humans (Thomson et al., 2009). Koren et al. (2009) do not find any sex differences in hyrax (*Procavia capensis*) hair testosterone concentrations but do demonstrate rank related differences in hair testosterone in males (Koren et al., 2002), although later work found no relationship between male rank and hair testosterone concentrations (Koren et al., 2009). In humans no difference in circulating testosterone concentrations in eugonadal and hypogonadal men could be detected using hair, whereas serum did yield the expected differences in circulating testosterone (Thomson et al., 2009). Hair testosterone analysis did however detect the clinical administration of testosterone in hypogonadal men.

Typically, studies where hair has successfully been used to quantify testosterone are studies using the method to identify exogenous administration of usually illegal performance enhancing androgens (e.g. Anielski et al., 2005; 2008). These studies use chromatographic and/or mass-spectrometric methods of hormone quantification (e.g. Anielski et al., 2005; Shen et al., 2009b), which are more sensitive than ELISA. In addition these methods use extraction protocols during which the whole hair is often digested and the resulting extractions are purified and relevant fractions may be isolated (e.g. Anielski, 2008; Stolker et sl., 2009; Wheeler et al., 1998). In two studies Radioimmuno Assays (RIA) were used in combination with these extraction and extract purification methodologies and yielded positive results (Gleixner and Meyer, 1997; Wheeler et al., 1998). These observations suggest that the negative results reported by this investigation may be because these extracts contain androgens and other substances with similar immunoreactivity to testosterone resulting in cross-reactivity with the specific antibody used in this study (the antibody used here has approximately 60%

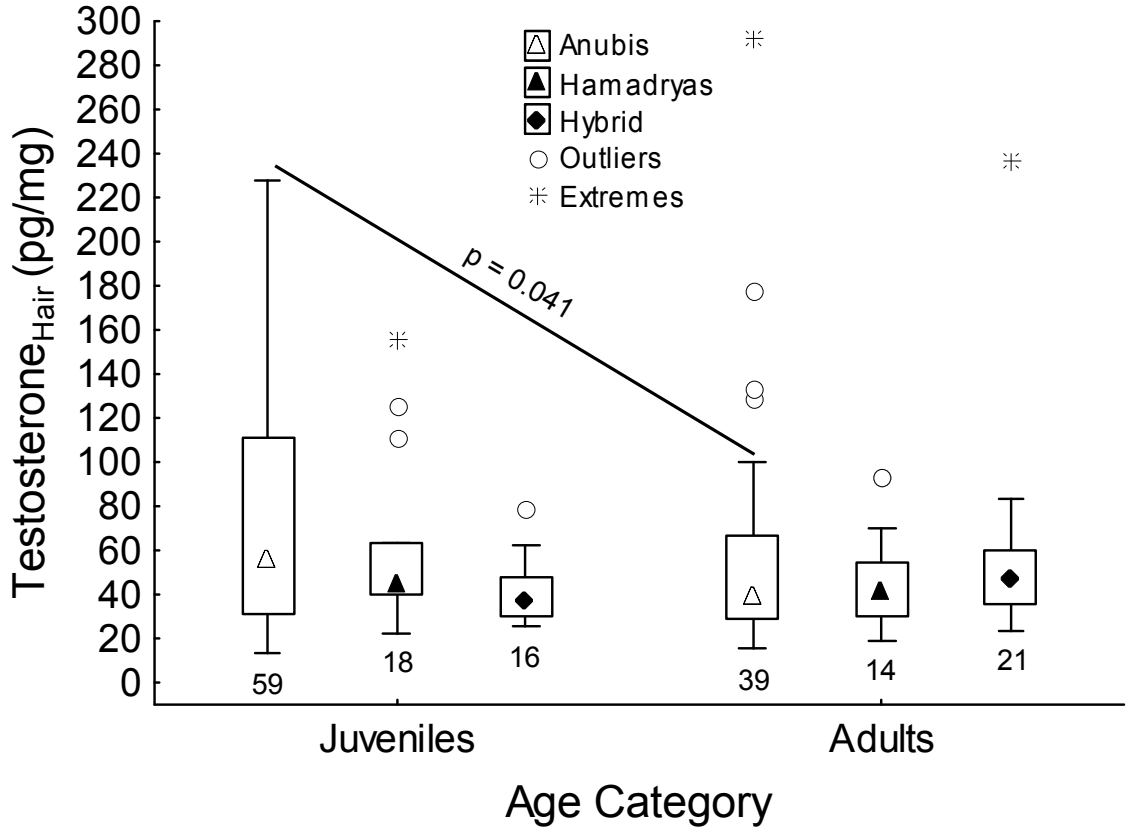
cross-reactivity with DHT). Using an antibody with greater specificity for testosterone (e.g. as in RIA techniques) or non-immuno reactive techniques (e.g. chromatography or mass-spectrometry) may resolve this issue; additionally, different extraction methodologies and greater purification of resulting extracts may enhance the testosterone signal. Further, it is known that testosterone undergoes significant conversion to DHT in the hair follicle (e.g. Takayasu and Adachi, 1972). DHT and not testosterone may be a more appropriate target in hair in order to characterize circulating androgen concentrations. Finally, cortisol is known to be very stable in the hair matrix and shows little degradation along the shaft (Bennet and Hayssen, 2010; Davenport et al., 2006) and over time (e.g. Web et al., 2010), thereby preserving biologically meaningful variation. The same may not be true of testosterone in hair. The samples used in this investigation were collected between 12 and 22 years ago and consistently produce biologically meaningful cortisol concentrations and no evidence of temporal degradation. It may be that hair testosterone concentrations in these samples have been degraded to the point where they no longer record biologically meaningful variation.

Hair hormone analysis is a new tool in endocrine research which, as has been shown using hair cortisol analysis, has the possibility of significantly extending the reach of endocrine research in wild and captive animal populations. It is a technique which is of specific interest to those interested in long term time averaged concentrations of hormones as may be relevant to the study of stable behavioral profiles, personality, temperament or environmental trends. It is therefore worthwhile to continue to expand the number of hormones which can be measured in hair. Future research should attempt to identify the appropriate extraction and quantification methodologies for hair

testosterone analysis while expanding our knowledge of the potential and limitations of the technique.

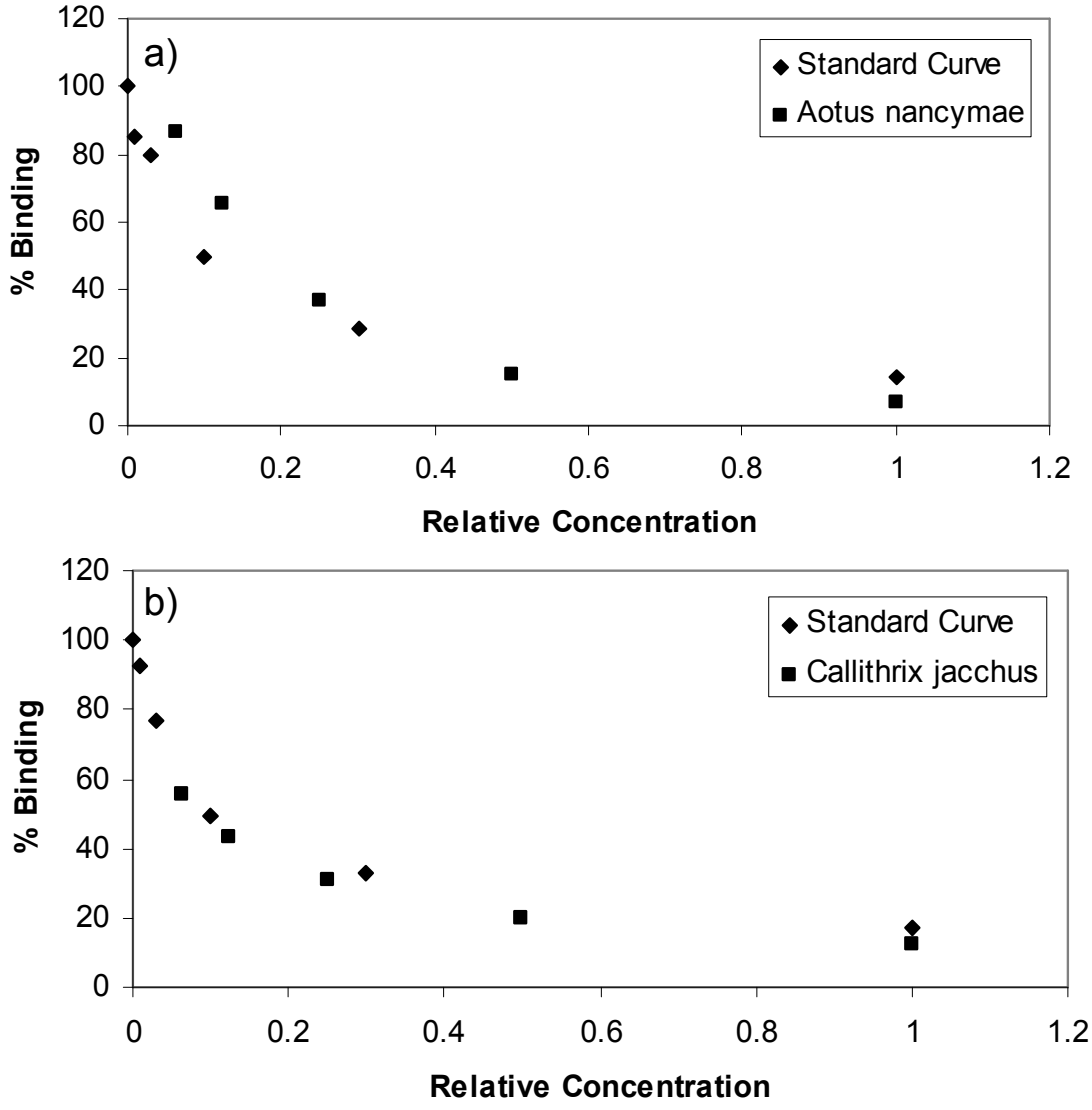
Tables and Figures

Figure A2.1: Box and whisker plot showing median (symbol) and 25th and 75th percentile ranges of hair testosterone concentrations of juvenile and adult male anubis, hamadryas and hybrid baboons from wild populations in the Awash National Park, Ethiopia. The whiskers represent the non-outlier range and the numbers below each box indicate the sample size.



Appendix 3

Figure A3.1a to m: Graphs showing parallel displacement of the dilution curve of for 13 primate species to that of the standard dilution curve in the ALPCO Salivary Cortisol assay, showing similar immunoreactivity between cortisol extracted from hair and cortisol of known concentration.



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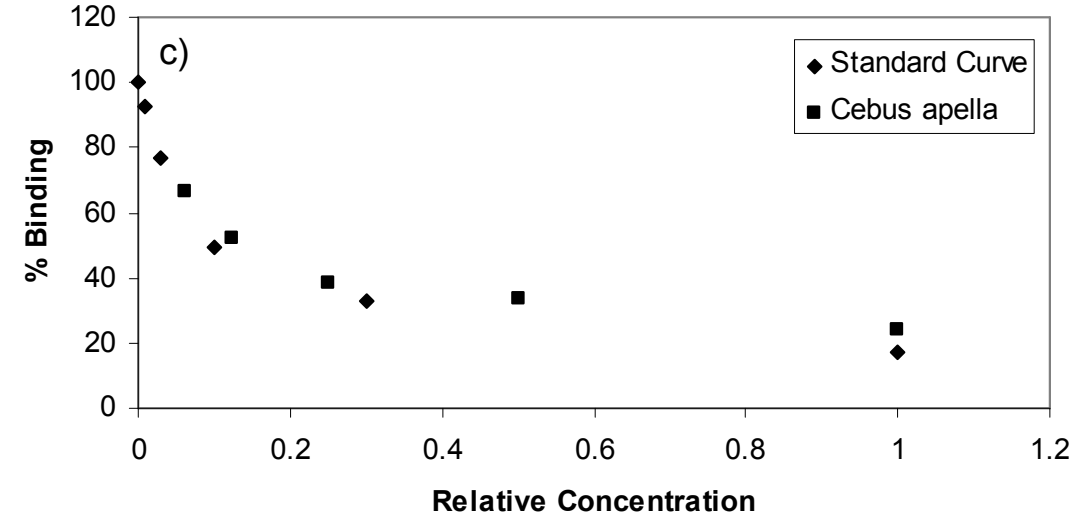

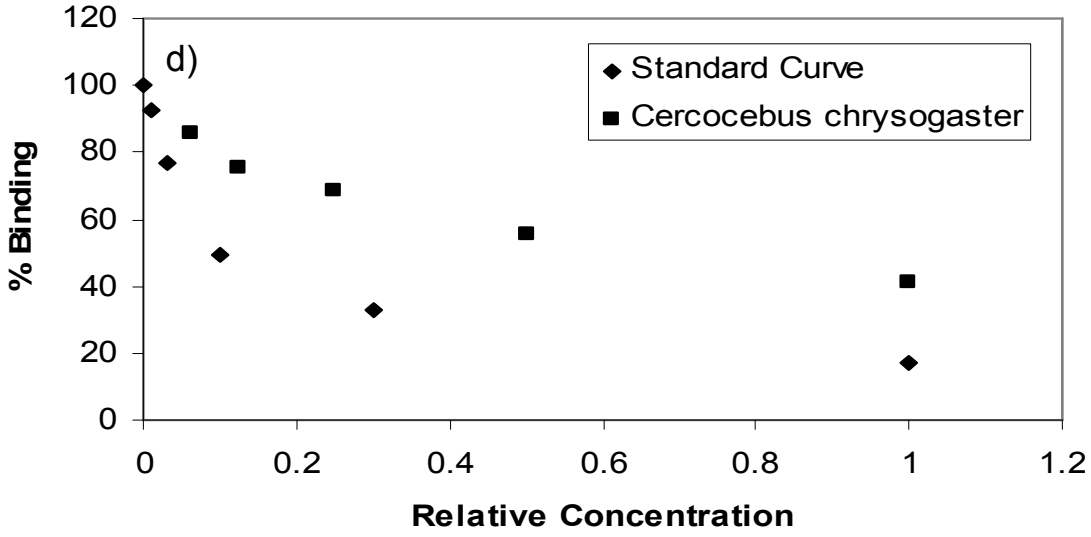

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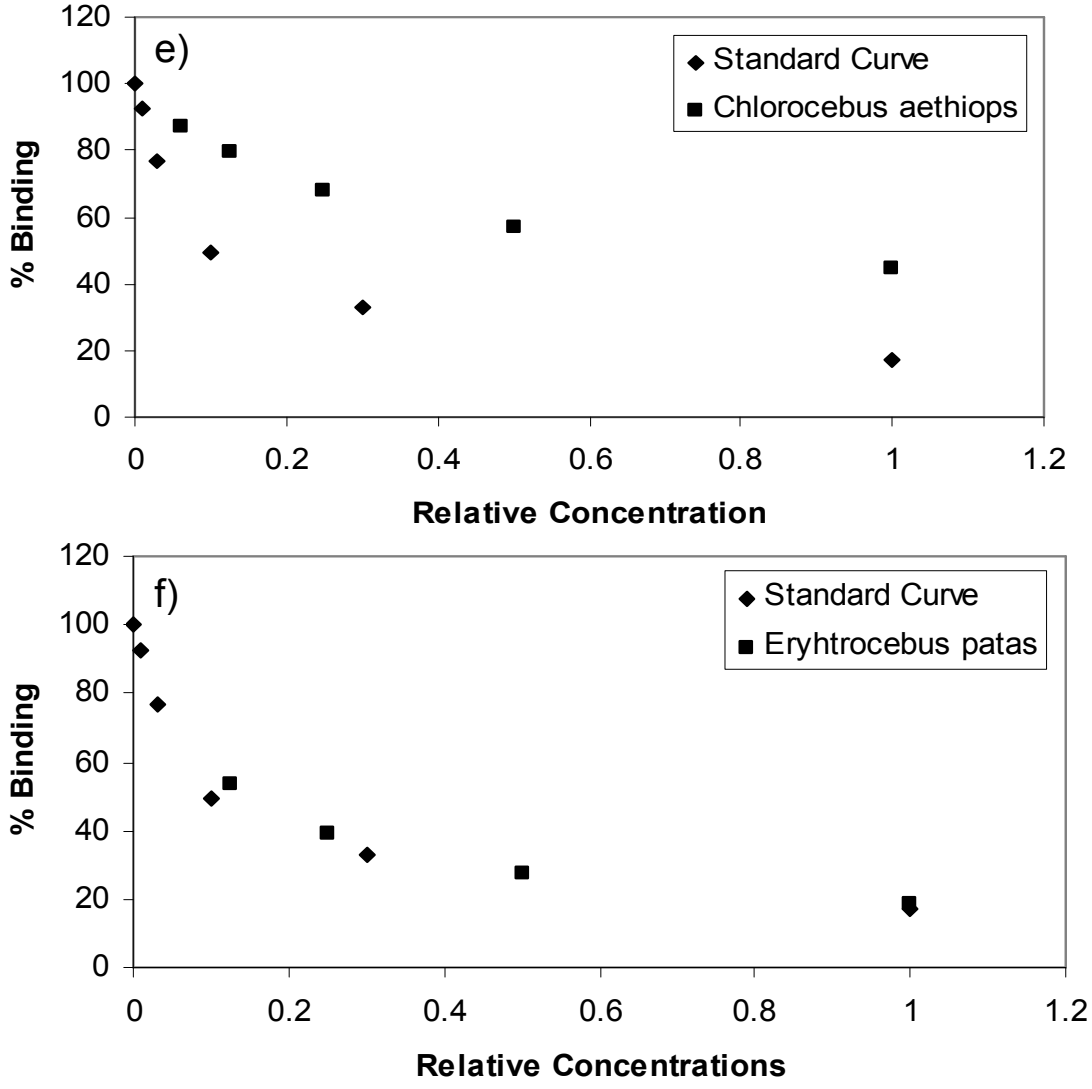

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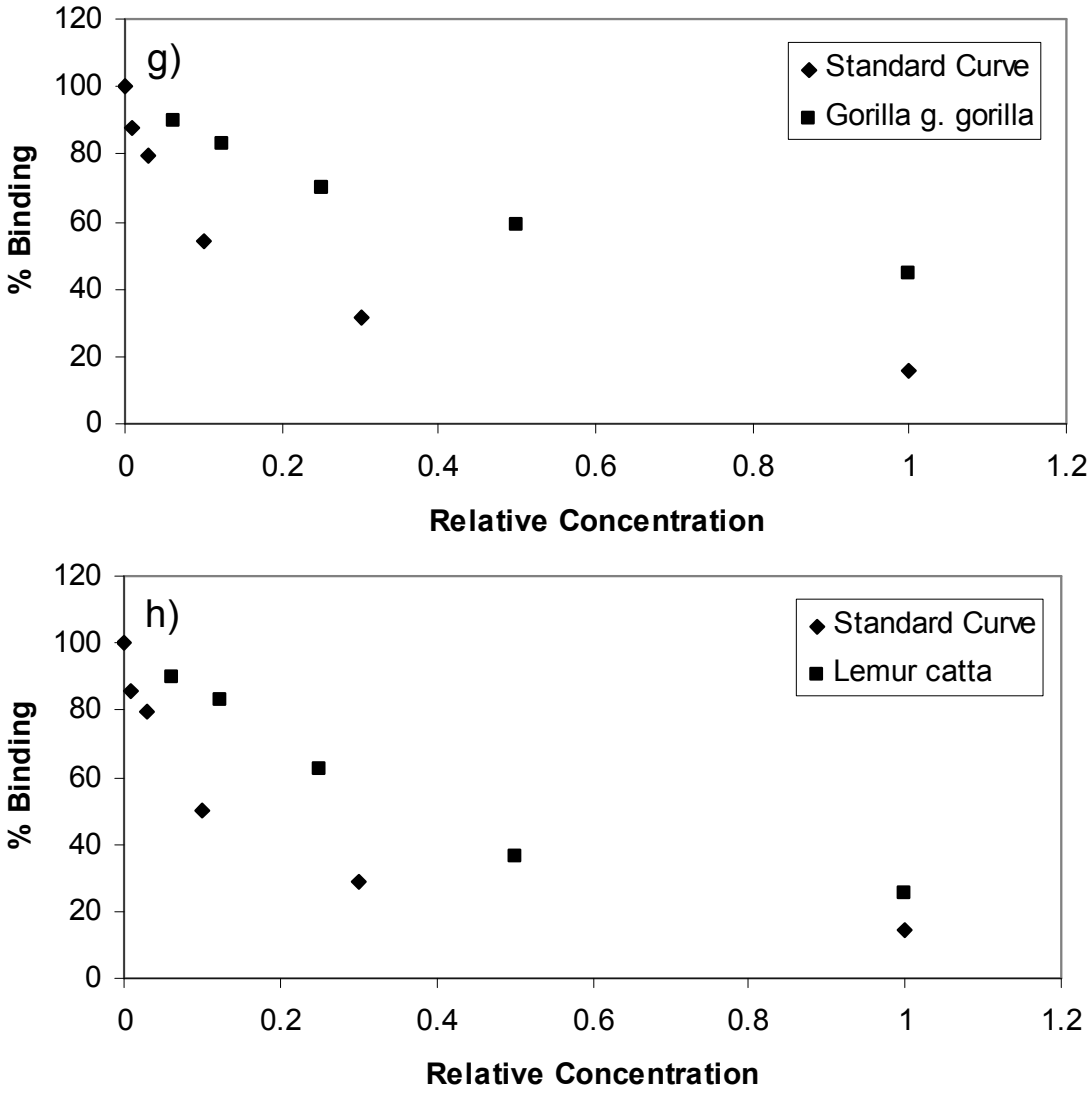

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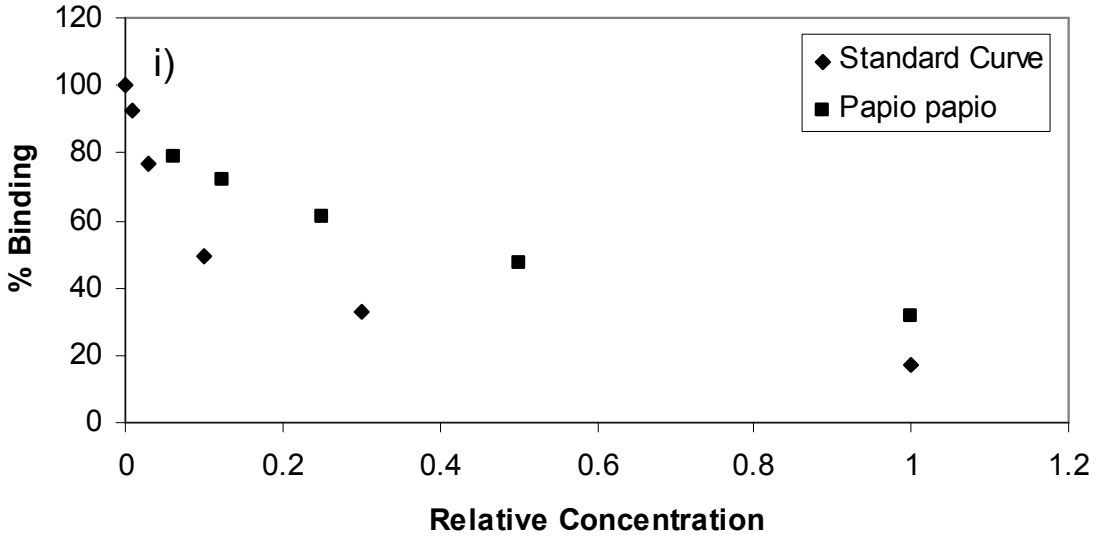

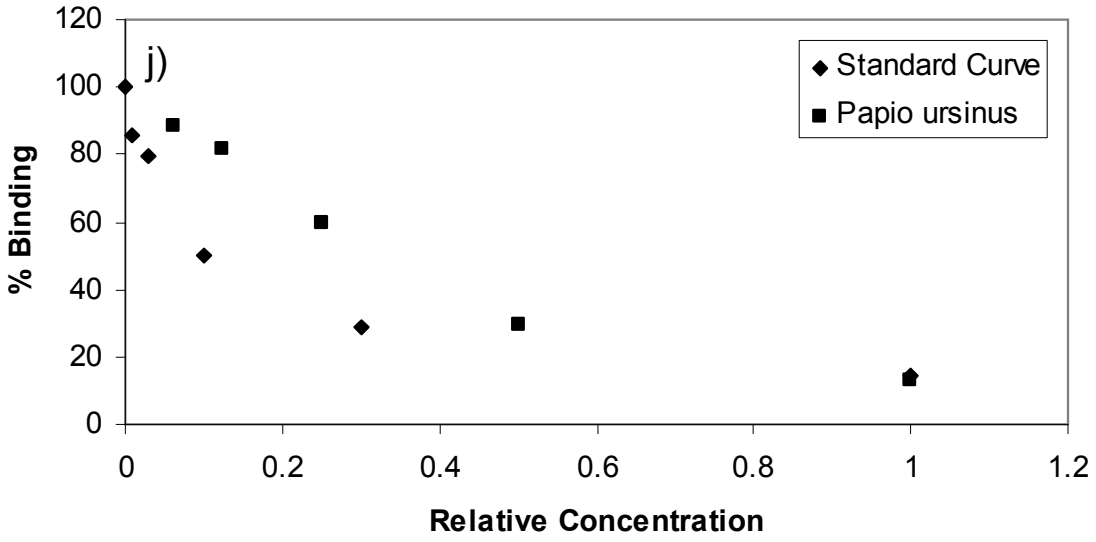

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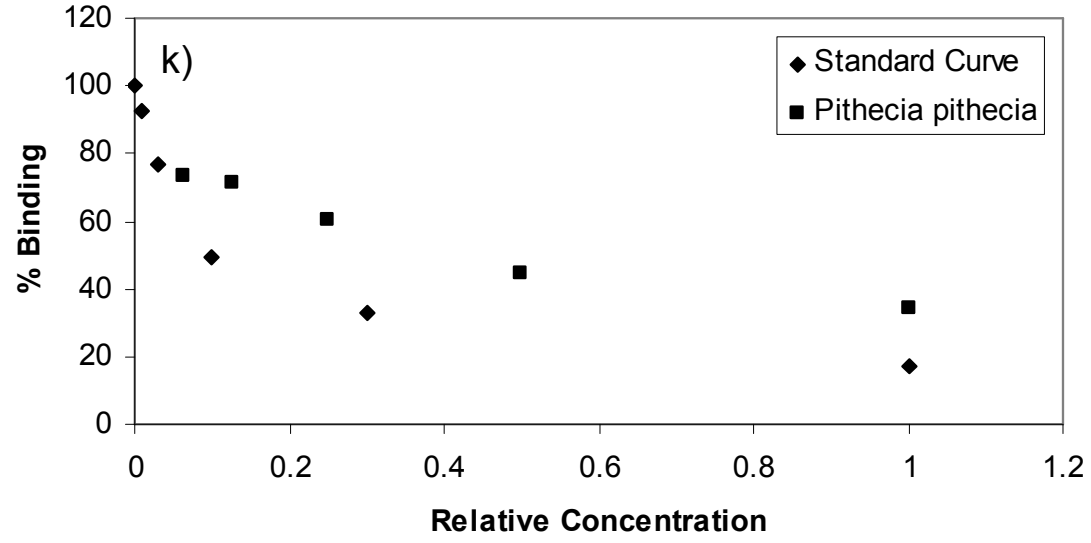

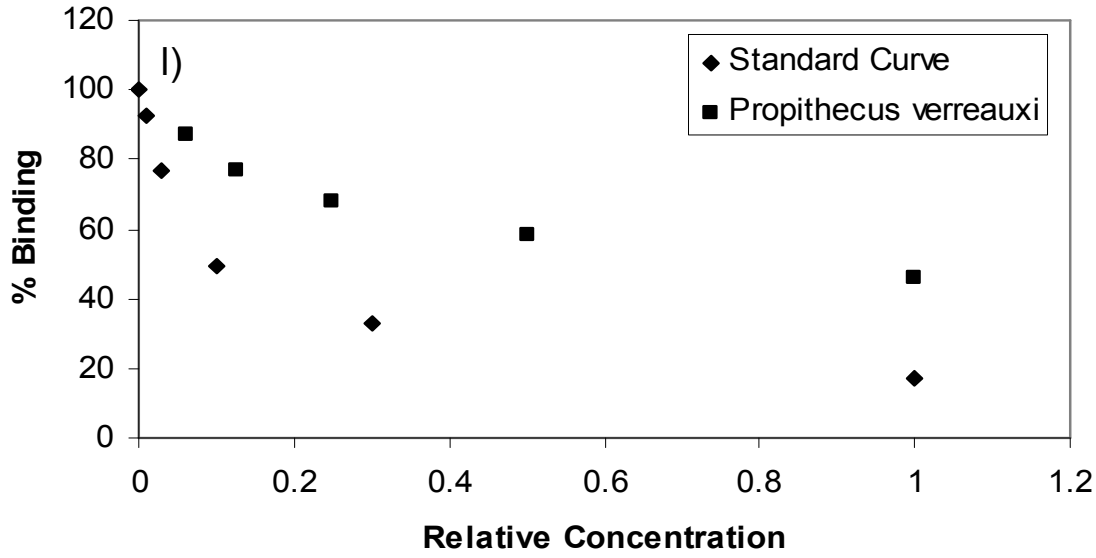

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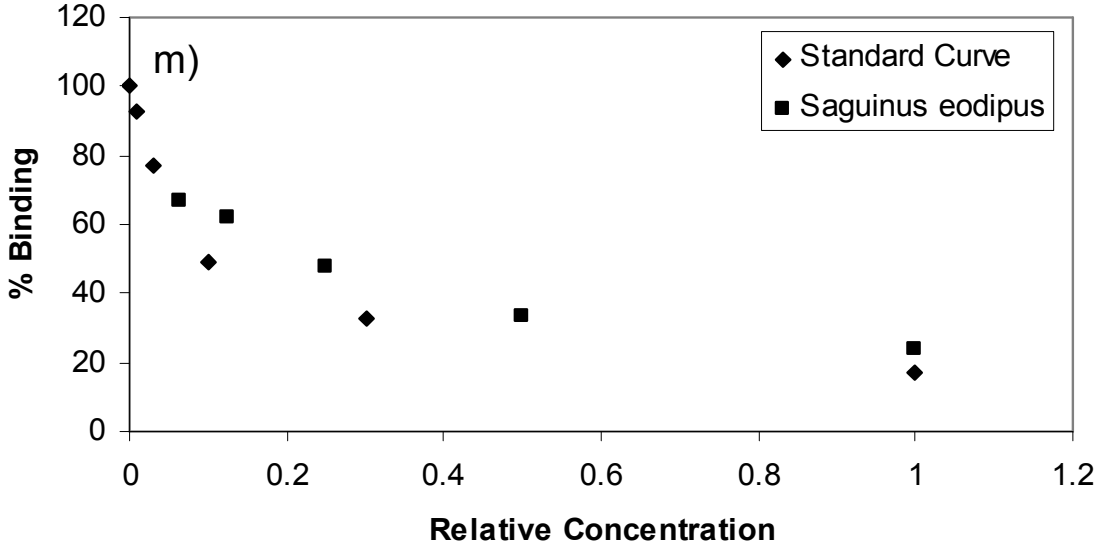


Figure A3.2a to e: Graphs showing parallel displacement of the dilution curve of for anubis, hamadryas and hybrid (anubis x hamadryas) baboons and vervet monkeys to that of the standard dilution curve for the assays performed at the Conservation Biology Institute in Front Royal, Virginia, showing similar immunoreactivity between cortisol extracted from hair and cortisol of known concentration.

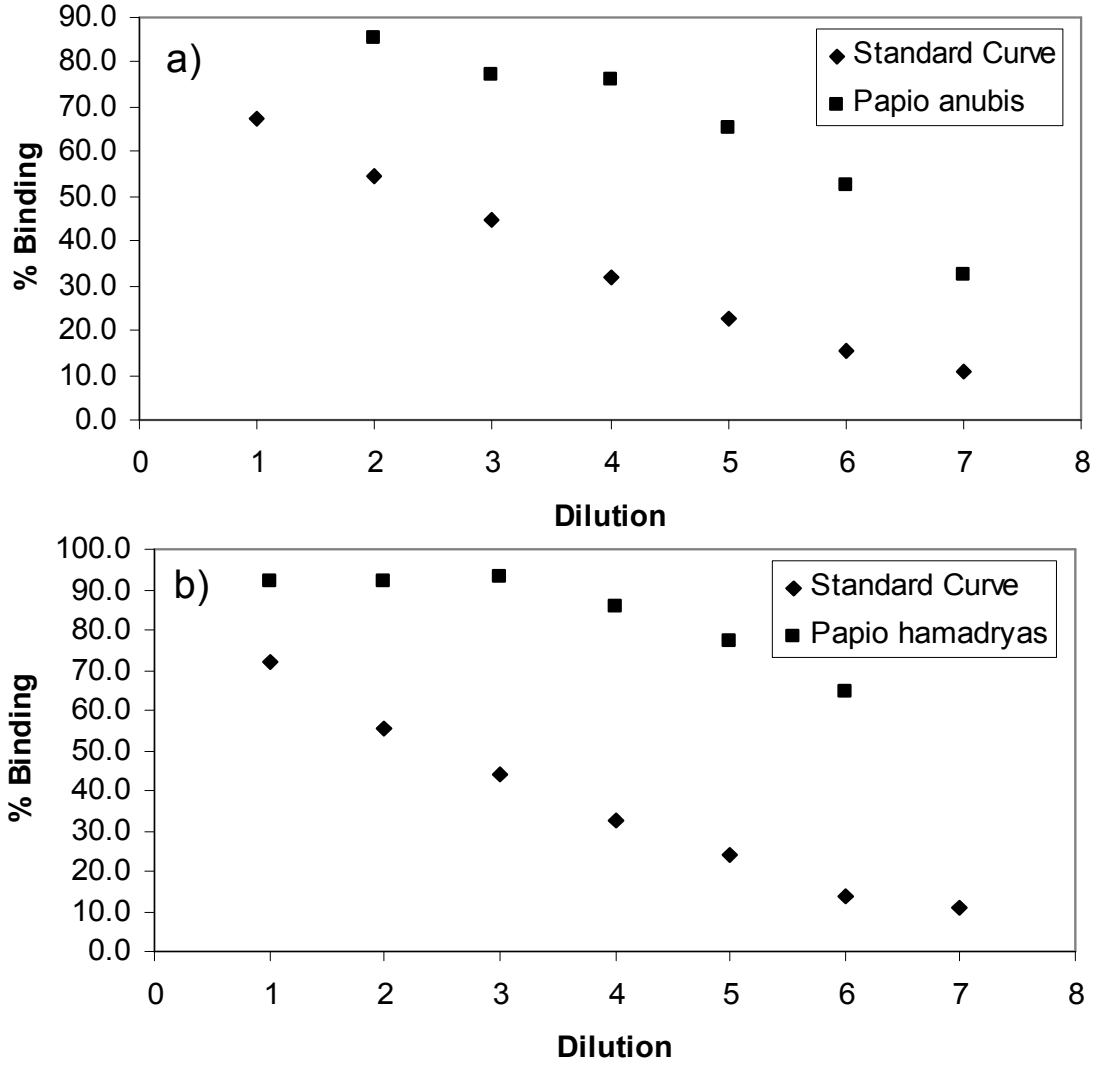


Figure A3.2: continued…

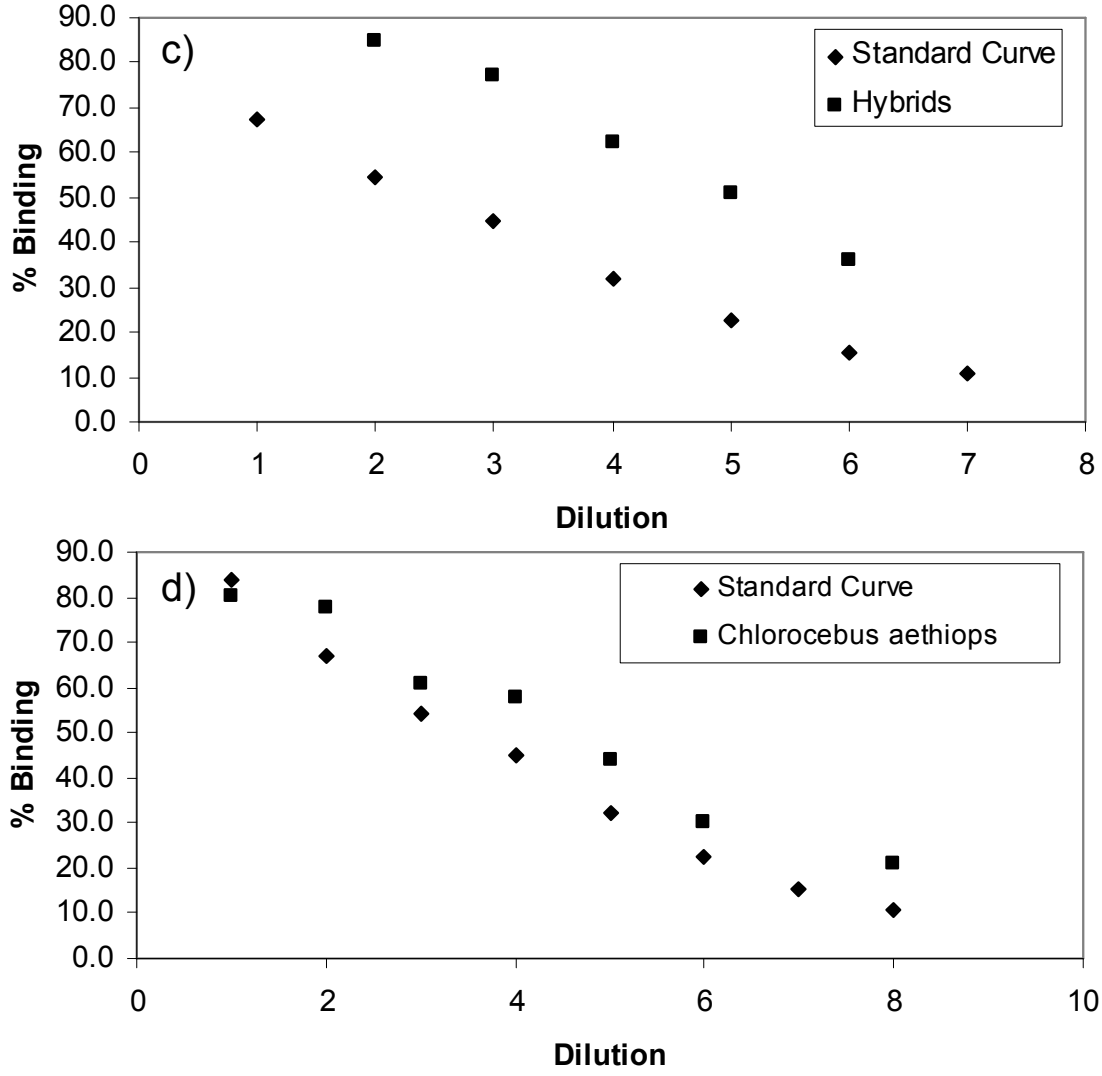
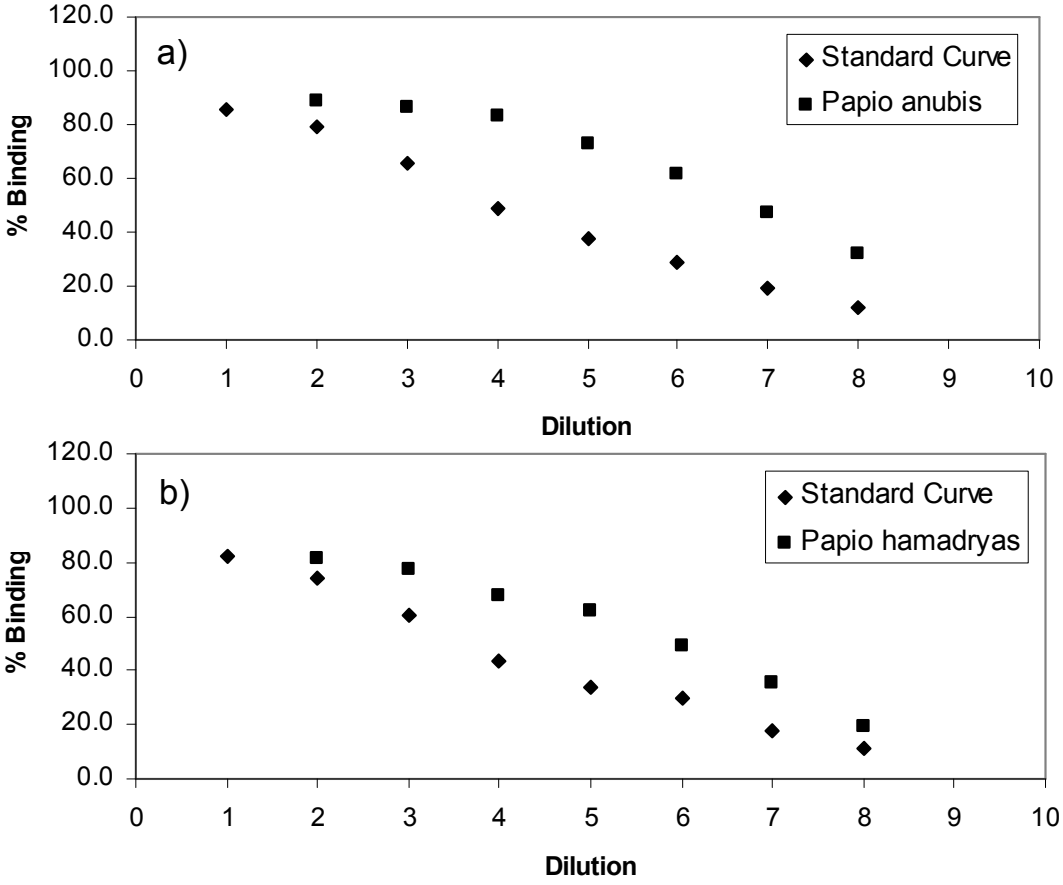


Figure A3.3a, b and c: Graphs showing parallel displacement of the dilution curve of for anubis, hamadryas and hybrid (anubis x hamadryas) baboons to that of the standard dilution curve for the assays performed at the Conservation Biology Institute in Front Royal, Virginia, showing similar immunoreactivity between testosterone extracted from hair and testosterone of known concentration.



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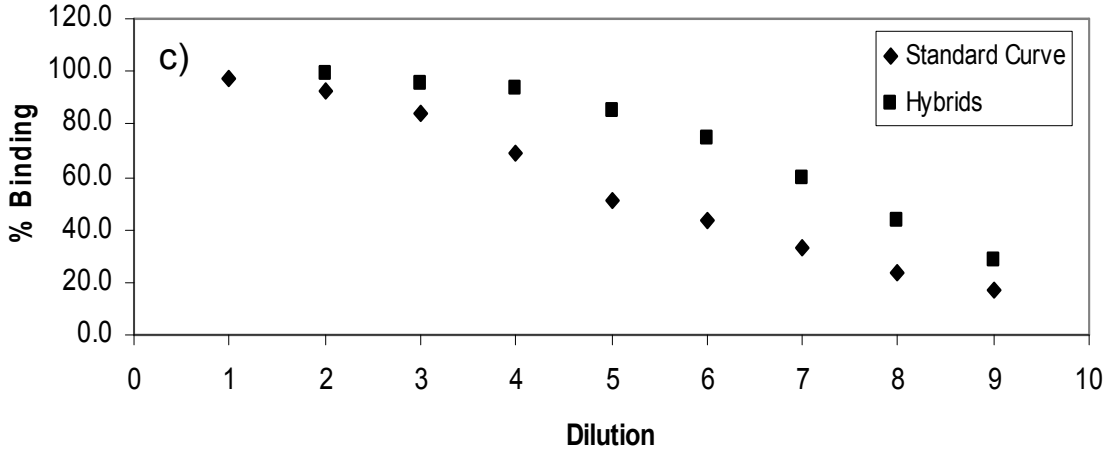
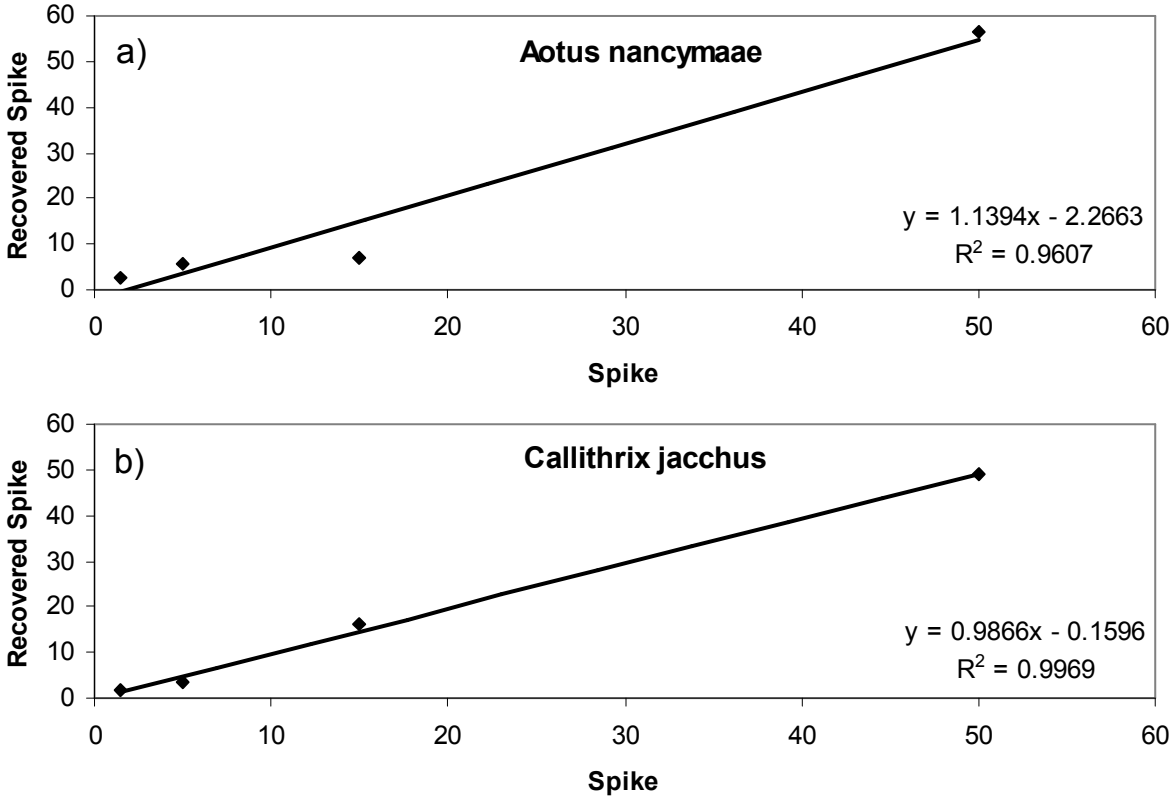


Figure A3.4a to m: Graphs showing correlation of recovery of cortisol spikes added to sample extracts prior to assaying the samples, showing that substances contained in the extracts did not significantly interfere with the assay.



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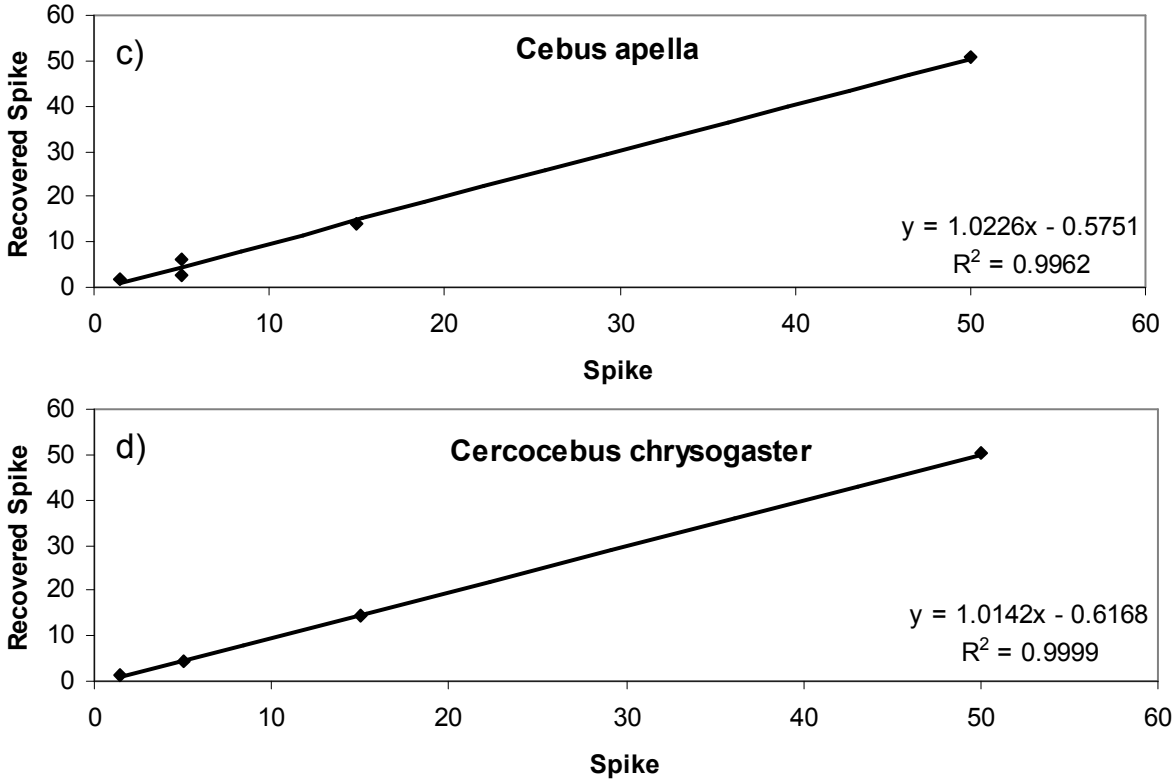

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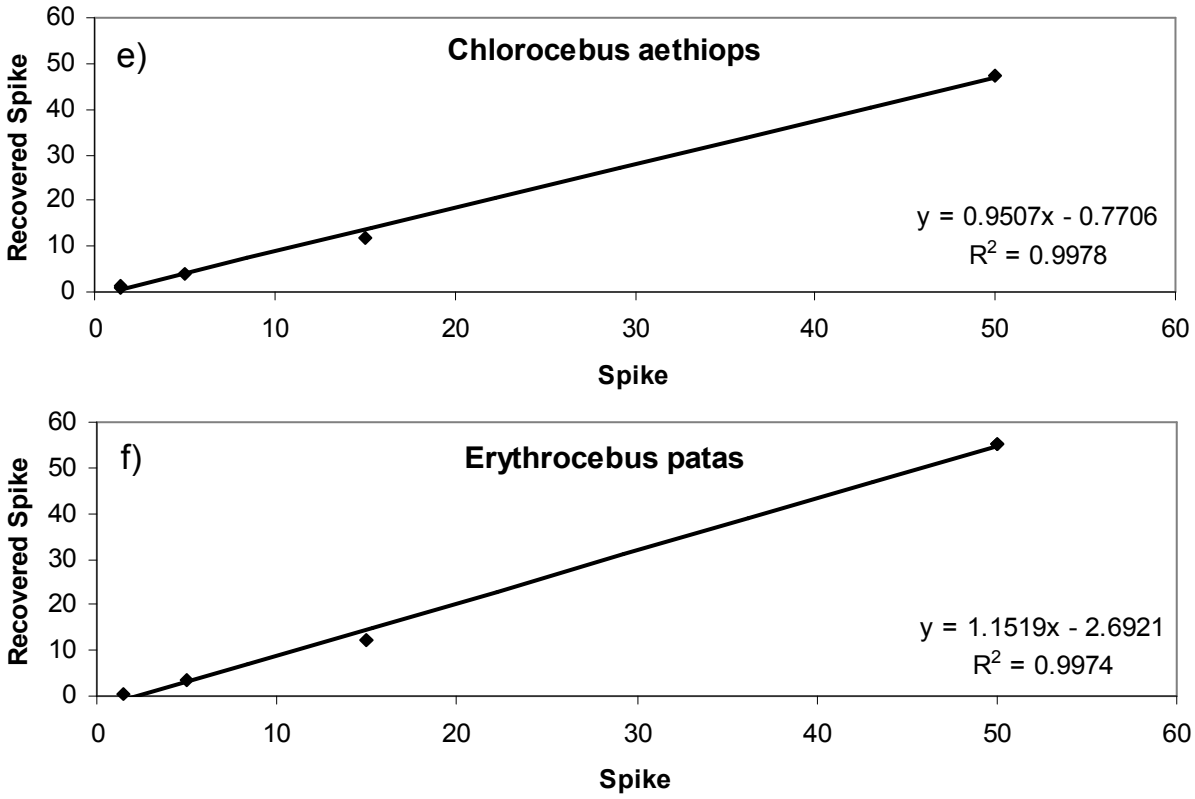

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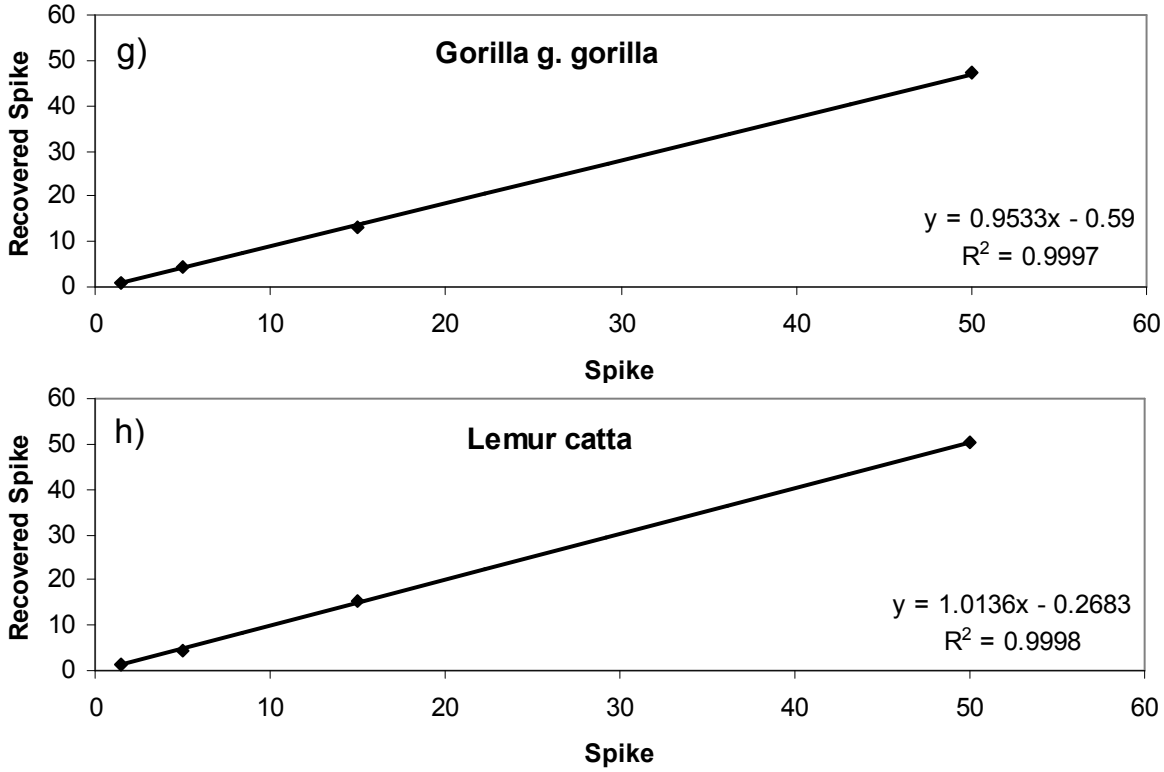

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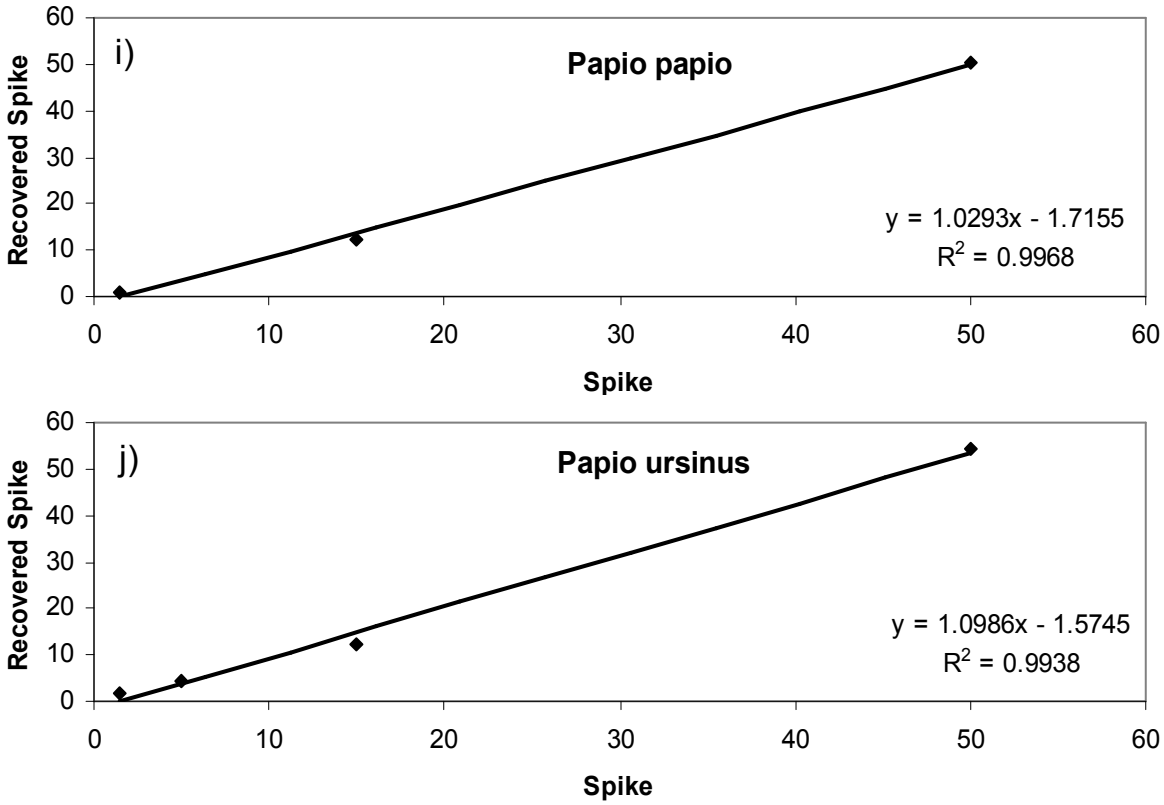

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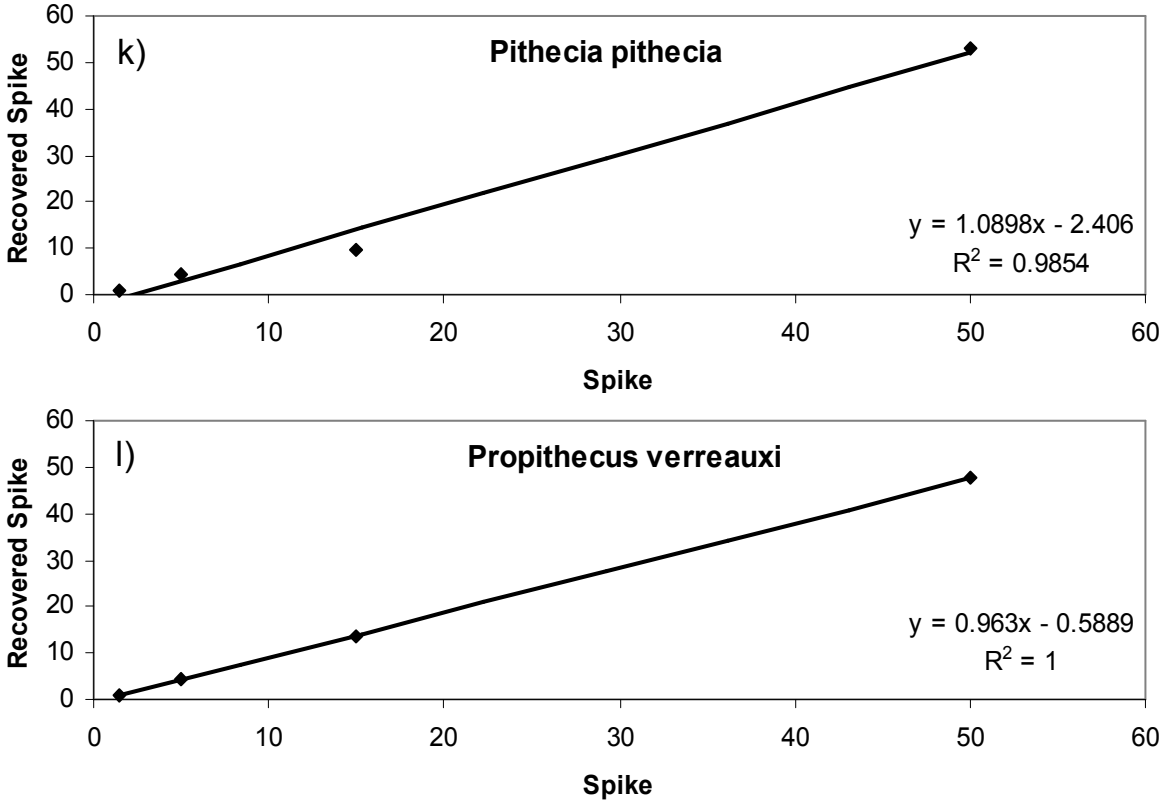

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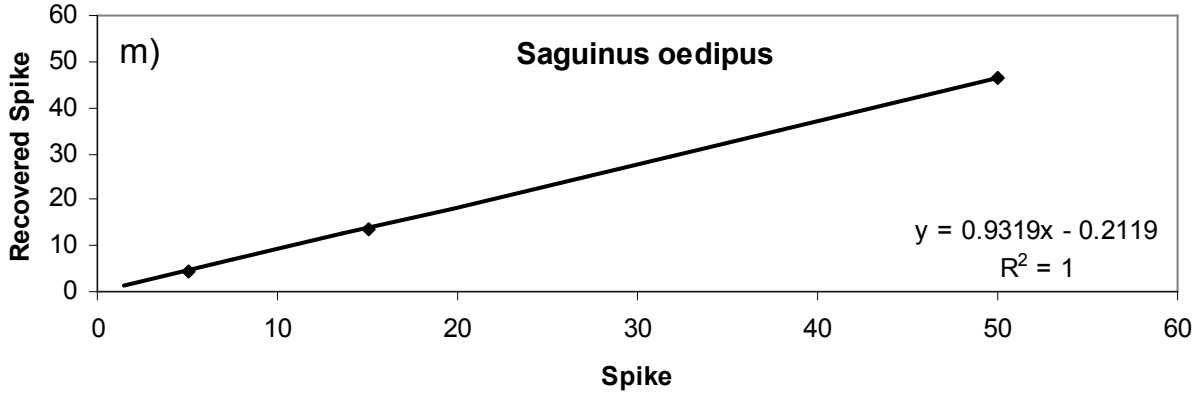
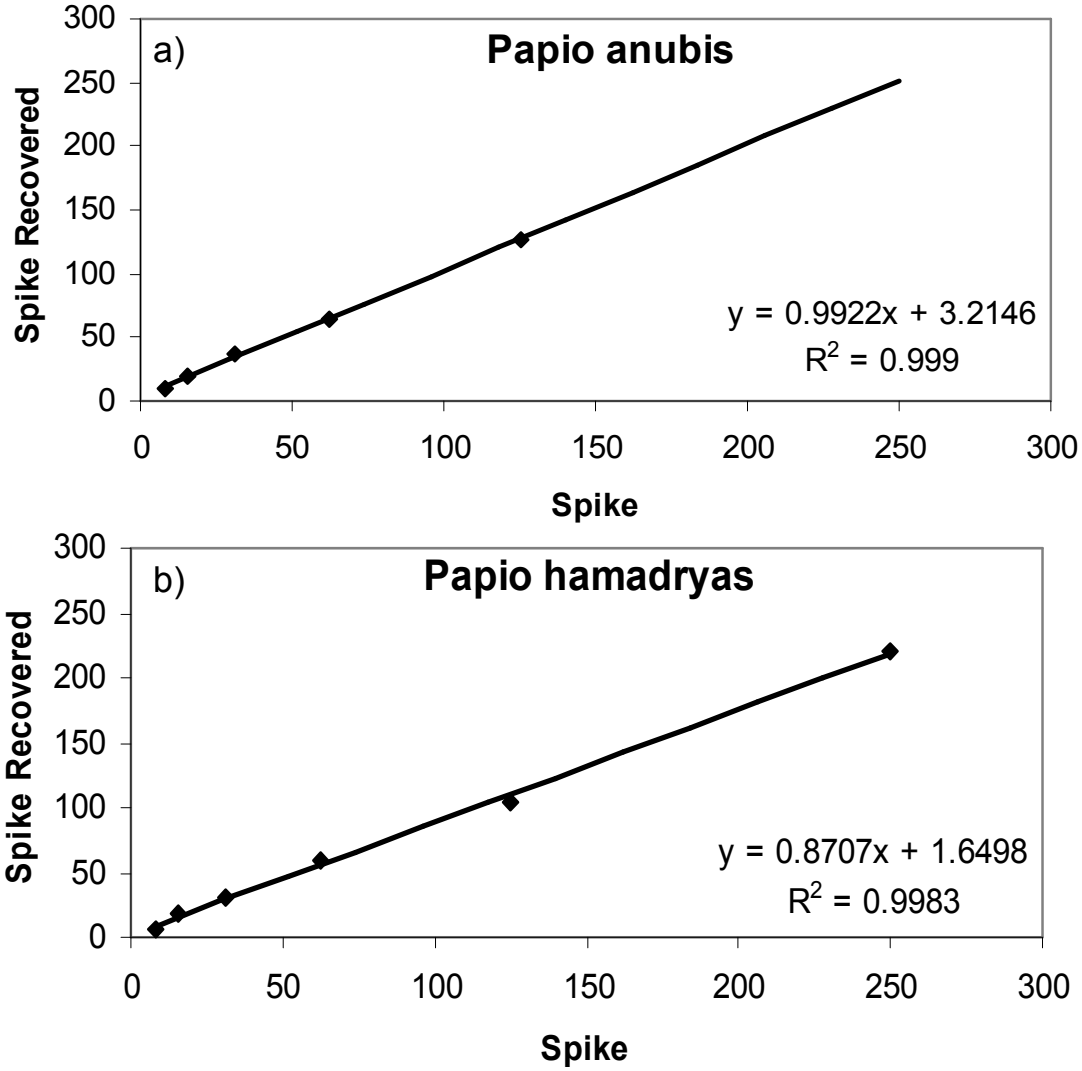


Figure A3.5a to e: Graphs showing correlation of recovery of cortisol spikes added to sample extracts prior to assaying the samples, showing that substances contained in the extracts did not significantly interfere with the assay.



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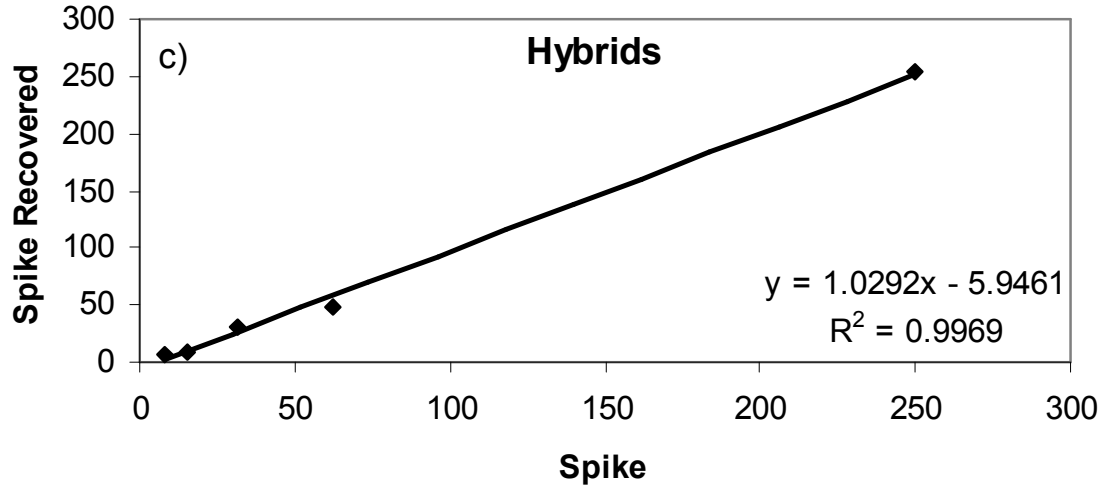


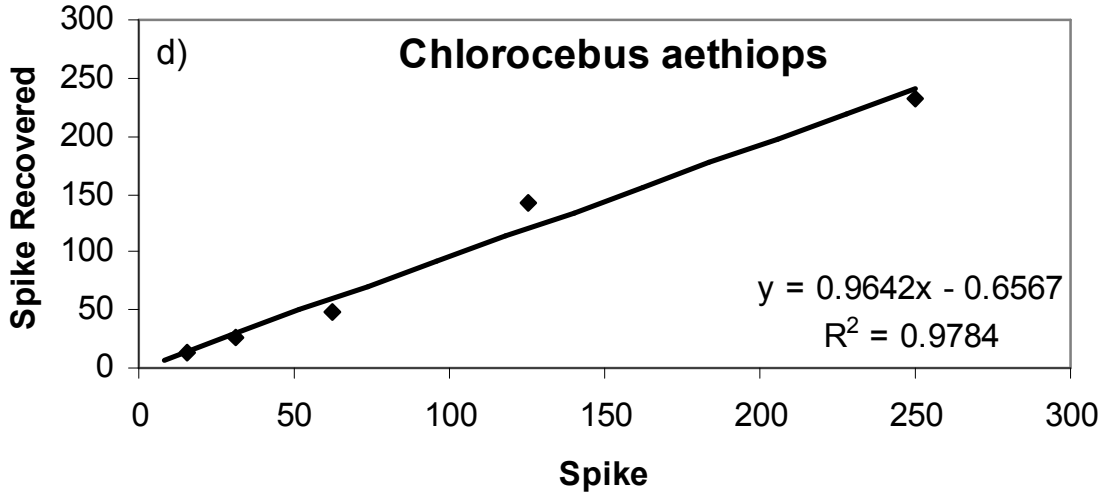
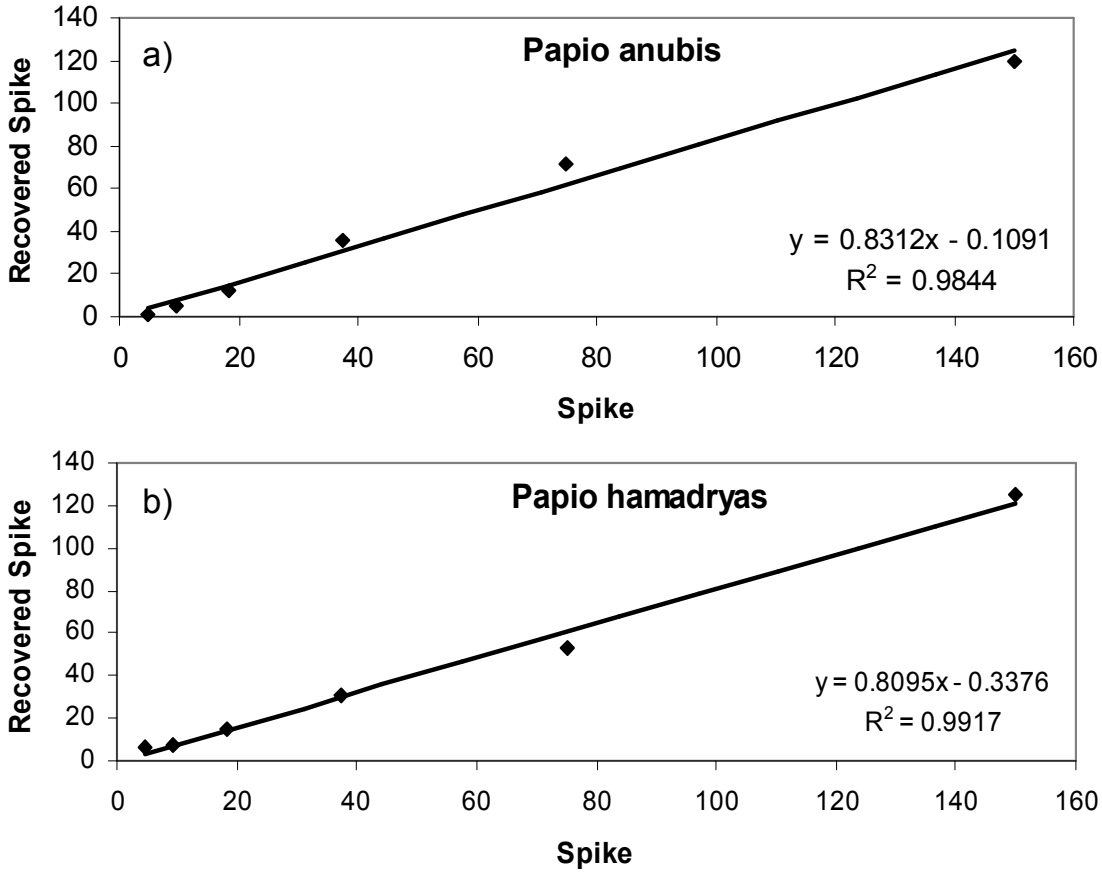


Figure A3.6a, b and c: Graphs showing correlation of recovery of testosterone spikes added to sample extracts prior to assaying the samples, showing that substances contained in the extracts did not significantly interfere with the assay.



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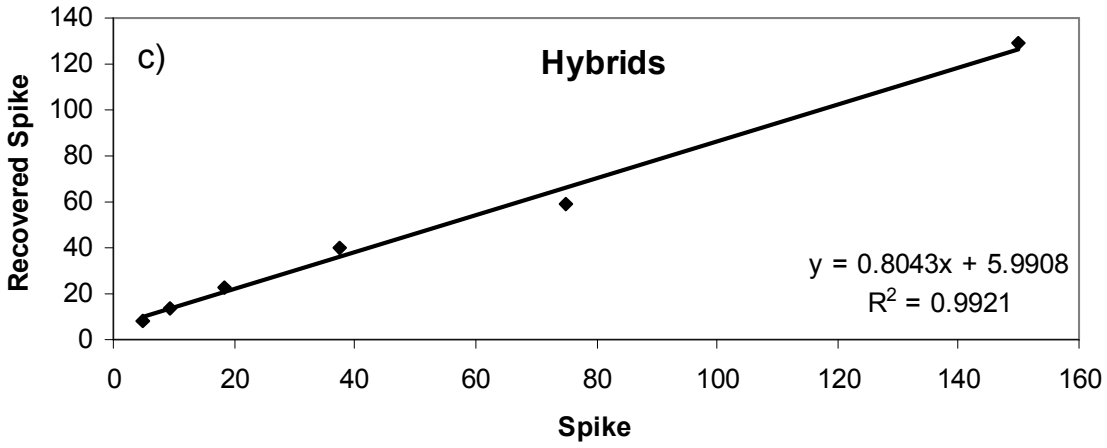

Appendix 4

Table A4.1: Quality control table for ALPCO Salivary Cortisol Assay Kits. Plates were rerun when control or standards exceeded ± 2 standard deviations around the mean.

Date	Plate Number	Control 1	Control 1	1.00	3.00	10.00	30.00	100.00
		%B	Conc (ng/ml)	%B	%B	%B	%B	%B
7/2/2010	12:17	49.3	10.8	89.1	79.0	50.5	30.5	15.1
7/27/2010	08:30	51.8	11.7	92.5	80.2	55.0	31.6	15.4
4/7/2010	21:06	47.6	11.5	85.4	75.9	50.2	28.3	14.4
4/9/2010	14:44	47.5	11.4	85.7	74.9	49.5	28.8	13.5
4/10/2010	15:09	47.9	11.5	85.4	76.3	50.0	28.8	14.1
4/19/2010	12:58	50.7	12.0	87.8	79.3	54.1	31.5	15.5
5/11/2011	16:31	48.0	10.3	89.2	71.9	48.3	29.9	15.2
6/28/2010	17:29	54.6	11.3	94.4	86.7	55.6	34.4	17.5
6/28/2010	11:35	52.9	11.3	92.8	84.3	54.4	31.8	15.7
7/15/2010	20:12	53.1	11.1	90.9	82.5	54.3	32.1	16.2
12/8/2010	15:17	39.3	13.7	74.9	62.3	42.7	28.9	12.8
12/10/2010	14:01	44.9	11.8	79.2	66.8	47.1	31.7	18.4
1/12/2011	18:45	44.9	11.3	87.4	70.8	47.3	27.8	14.3
1/13/2011	16:21	47.6	10.5	86.4	70.4	48.2	30.0	15.5
1/13/2011	12:58	46.2	10.6	91.3	74.2	46.3	28.3	14.5
1/14/2011	18:32	45.2	10.6	82.3	70.1	45.7	27.1	14.4
1/17/2011	19:14	45.0	12.8	85.3	73.4	49.5	30.5	15.7
1/21/2011	19:51	44.7	10.8	81.1	68.3	46.3	26.7	13.8
1/24/2011	20:12	44.4	10.4	88.1	72.4	29.7	26.4	13.7
1/25/2011	15:17	47.7	11.3	90.8	75.2	48.3	32.3	16.5
1/25/2011	15:15	47.8	11.3	90.9	75.4	48.3	32.3	16.5
11/14/2011	12:23	45.3	10.3	87.9	72.3	45.1	27.0	14.2
11/22/2011	13:28	40.4	11.8	76.1	63.3	42.9	26.0	12.0
	Mean	**47.2**	**11.3**	**86.7**	**74.2**	**48.2**	**29.7**	**15.0**
	Count	23	23	23	23	23	23	23
	Std Dev	3.7	0.8	5.2	6.1	5.5	2.3	1.5
	CV	**7.9**	**7.1**	**5.9**	**8.3**	**11.3**	**7.7**	**9.8**
	Mn +2SD	54.7	12.9	97.0	86.4	59.1	34.3	17.9
	Mn-2SD	39.7	9.7	76.4	61.9	37.3	25.1	12.1
	Max	54.6	13.7	94.4	86.7	55.6	34.4	18.4
	Min	39.3	10.3	74.9	62.3	29.7	26.0	12.0

Table A4.2: Quality control table for the CBI Cortisol Assay. Plates were rerun when control or standards exceeded ± 2 standard deviations around the mean.

Date	Plate Number	Control 1	Control 1	Control 2	Control 2	3.90	7.80	15.60	31.25	62.50	125.00	250.00	500.00	1000.00
		%B	Conc (ng/ml)	%B	Conc (ng/ml)	%B	%B	%B	%B	%B	%B	%B	%B	%B
4/23/2011	974	23.8	237.4	57.4	41.0	88.6	84.3	74.1	63.6	49.1	32.8	22.8	17.7	10.5
4/23/2011	971	26.9	326.1	65.1	46.1	96.1	91.6	85.3	73.6	57.3	42.2	32.6	22.7	14.1
4/22/2011	969	31.3	240.1	65.5	41.2	93.6	89.2	84.1	69.5	55.7	43.7	31.3	21.2	12.1
4/22/2011	967	27.8	227.9	68.4	32.6	88.4	85.4	82.3	69.2	52.8	38.7	27.8	19.2	11.6
4/22/1201	963	29.3	275.7	67.3	38.0	97.4	89.1	83.9	72.0	55.8	43.1	30.9	22.5	12.8
4/22/2011	907	24.4	258.4	62.0	30.1	91.0	81.1	75.6	62.4	45.3	35.5	26.8	16.2	11.7
4/22/2011	909	26.1	293.5	63.9	39.3	90.7	86.1	79.8	69.6	51.9	40.7	30.3	19.0	13.3
4/22/2011	913	24.1	211.6	60.7	29.0	84.7	80.2	74.8	57.5	43.3	33.2	22.5	17.0	10.8
4/22/2011	915	26.8	239.1	62.7	36.5	87.5	84.2	79.2	65.4	48.7	37.8	28.7	18.7	12.0
4/22/2011	916	29.6	260.7	68.6	33.5	94.1	89.6	84.4	68.8	54.1	42.5	32.2	21.3	12.8
4/22/2011	945	26.6	285.5	65.4	33.7	92.3	89.7	78.5	66.4	52.9	39.3	30.2	20.3	10.1
4/22/2011	948	26.7	326.7	62.5	46.4	87.3	85.0	83.0	69.9	47.4	45.0	35.5	21.7	10.6
4/22/2011	951	24.8	264.3	61.9	37.3	86.9	82.1	77.2	64.2	50.2		27.3	17.4	10.5
4/22/2011	952	28.5	255.6	63.3	45.5	91.8	87.1	84.1	69.4	55.4		30.2	19.2	12.6
4/22/2011	955	30.1	277.1	66.0	39.8	96.2	91.5	82.9	69.3	55.0	48.0	30.3	21.5	11.8
4/22/2011	961	20.0	277.2	54.9	33.4	82.3	77.1	70.4	55.5	40.7	30.9	23.8	14.2	9.6
4/22/2011	962	28.1	202.5	61.7	36.7	85.7	82.3	78.7	64.1	49.2	35.2	28.2	16.7	10.7
4/23/2011	976	27.0	247.1	59.5	44.5	93.7	84.9	79.1	66.3	54.5	35.7	27.2	20.0	11.1
4/23/2011	978	28.8	259.3	61.5	46.9	93.4	88.0	80.5	70.7	52.6	43.3	30.0	19.2	12.9
4/23/2011	980	21.7	315.0	57.7	46.5	92.4	87.6	81.9	66.7	47.2	36.9	27.4	17.3	10.8
4/23/2011	981	27.4	312.4	67.0	34.4	90.2	85.8	80.9	67.6	54.5	43.8	33.1	19.0	13.9
	Mean	**26.6**	**266.3**	**63.0**	**38.7**	**90.7**	**85.8**	**80.0**	**66.7**	**51.1**	**39.4**	**29.0**	**19.1**	**11.7**
	Count	21	21	21	21	21	21	21	21	21	19	21	21	21
	Std Dev	2.8	35.1	3.7	5.7	4.0	3.8	3.9	4.5	4.5	4.7	3.3	2.2	1.3
	CV	**10.4**	**13.2**	**5.8**	**14.7**	**4.4**	**4.4**	**4.9**	**6.7**	**8.8**	**12.0**	**11.5**	**11.6**	**10.8**
	Mn +2SD	32.2	336.4	70.3	50.1	98.7	93.4	87.9	75.7	60.1	48.8	35.7	23.6	14.2
	Mn-2SD	21.1	196.2	55.6	27.3	82.6	78.2	72.1	57.8	42.1	29.9	22.3	14.7	9.2
	Max	31.3	326.7	68.6	46.9	97.4	91.6	85.3	73.6	57.3	48.0	35.5	22.7	14.1
	Min	20.0	202.5	54.9	29.0	82.3	77.1	70.4	55.5	40.7	30.9	22.5	14.2	9.6

Table A4.3: Quality control table for the CBI Testosterone Assay. Plates were rerun when control or standards exceeded ± 2 standard deviations around the mean.

Date	Plate Number	Control 1	Control 1	Control 2	Control 2	2.30	4.70	9.40	18.80	37.50	75.00	150.00	300.00	600.00
		% B	Conc (ng/ml)	% B	Conc (ng/ml)	% B	% B	% B	% B	% B	% B	% B	% B	% B
3/7/2011	153	31.2	148.0	65.3	22.4	96.8	92.6	83.9	68.8	50.0	43.3	33.4	24.0	16.6
3/8/2011	180	27.9	148.0	58.9	24.9	89.3	85.1	78.7	65.4	46.1	38.3	30.5	22.5	15.0
3/10/2011	230	30.4	139.3	70.6	20.4	95.3	88.2	70.8	55.9	40.4	31.7	31.7	21.8	13.9
3/15/2011	341	27.2	159.6	66.3	21.3	96.1	90.6	83.8	67.7	52.7	40.3	29.4	19.7	13.1
3/15/2011	347	29.9	143.3	73.9	17.6	98.8	94.9	87.1	72.9	54.7	41.3	31.3	21.5	13.7
3/16/2011	369	29.3	137.8	71.3	17.3	95.6	89.8	83.9	70.0	52.9	39.1	30.1	20.9	13.6
3/18/2011	423	25.6	166.6	60.7	20.2	89.3	84.5	76.5	61.2	46.0	37.6	28.7	20.4	14.5
3/18/2011	430	31.2	151.2	63.3	23.9	91.5	83.7	81.9	67.5	53.0	40.6	33.6	24.5	17.1
3/26/2011	545	26.5	160.4	60.2	20.3	86.1	81.2	74.9	60.6	48.0	36.5	28.7	21.3	15.1
3/26/2011	547	28.7	170.5	63.4	21.9	90.3	84.8	78.5	66.9	51.3	40.0	31.9	24.1	16.9
3/26/2011	551	29.2	143.9	62.3	20.4	88.3	82.0	75.4	65.2	48.5	38.7	30.2	21.7	15.5
3/26/2011	555	30.2	177.1	64.2	22.3	91.1	85.6	78.2	67.8	53.7	42.2	33.8	24.9	17.5
3/26/2011	560	30.1	167.1	63.3	24.8	92.2	86.2	79.5	69.6	53.8	42.3	32.6	23.8	16.6
3/26/2011	562	32.4	170.6	67.4	23.7	93.7	88.7	83.5	72.5	56.0	45.9	35.8	25.9	18.3
3/26/2011	567	30.0	174.0	67.1	20.6	92.0	87.3	79.5	69.7	54.4	43.2	32.8	24.1	17.4
3/26/2011	571	28.7	197.2	63.7	25.1	90.3	85.7	80.7	68.8	54.2	43.7	34.1	24.2	16.7
3/29/2011	602	28.3	157.9	62.3	17.7	89.8	81.9	75.4	59.5	48.8	38.1	30.3	22.0	15.8
4/22/2011	893	23.5	208.9	60.4	27.6	86.0	83.9	78.1	68.6	51.7	40.6	28.9	20.1	13.5
4/22/2011	894	27.3	174.5	68.1	19.1	88.3	80.8	76.0	70.9	52.5	41.2	31.7	21.2	12.9
4/22/2011	900	25.2	166.3	59.4	22.8	89.6	77.3	74.7	64.9	49.0	37.5	27.4	17.4	11.5
	Mean	**28.6**	**163.1**	**64.6**	**21.7**	**91.5**	**85.7**	**79.0**	**66.7**	**50.9**	**40.1**	**31.3**	**22.3**	**15.3**
	Count	20	20	20	20	20	20	20	20	20	20	20	20	20
	Std Dev	2.2	18.4	4.1	2.8	3.5	4.2	4.1	4.4	3.8	3.1	2.2	2.1	1.9
	CV	**7.8**	**11.3**	**6.4**	**12.7**	**3.9**	**5.0**	**5.1**	**6.7**	**7.5**	**7.8**	**7.0**	**9.4**	**12.2**
	Mn +2SD	33.1	200.0	72.9	27.2	98.6	94.2	87.2	75.6	58.5	46.4	35.7	26.5	19.0
	Mn-2SD	24.2	126.2	56.3	16.2	84.4	77.2	70.9	57.8	43.3	33.8	27.0	18.1	11.5
	Max	32.4	208.9	73.9	27.6	98.8	94.9	87.1	72.9	56.0	45.9	35.8	25.9	18.3
	Min	23.5	137.8	58.9	17.3	86.0	77.3	70.8	55.9	40.4	31.7	27.4	17.4	11.5