ABSTRACT

WESTON, EMILY IRIS. Evaluation of Cortisol in Saliva Relative to Serum in Lactating Cows, Heifer Calves and Piglets in Response to Applied Stress. (Under the direction of Dr. Scott Whisnant.)

The objective was to examine the efficacy of saliva collection techniques and salivary cortisol assay to determine potential stress response in dairy heifers post challenge with adrenocorticotropin (ACTH) (Experiment 1), lactating cows without experimental stressors (Experiment 2), and gilt piglets versus boar piglets within two hours post castration stress (Experiment 3). Data were from the Holstein and Jersey dairy herd maintained by the Dairy Educational Unit at North Carolina State University and the crossbred swine herd maintained by the Swine Educational Unit also at North Carolina State University. In cattle (Experiments 1 & 2), saliva was collected using gauze squares secured by hemostats and introduced to each animal's mouth until soaked. The same procedure was followed for the piglets (Experiment 3) with the addition of Sprite™ to the gauze before introduction into the animals' mouth. Serum and saliva cortisol concentrations show a similar pattern after a stimulus to the adrenal gland with ACTH in Experiment 1. Salivary cortisol concentrations were lower than that found in serum and ranged from 27.40% to 59.94% of serum concentrations. In Experiment 2 the lack of an induced stress in lactating cows did not provide sufficient circulating cortisol for the sensitivity of the radioimmunoassay procedure utilized in this project. Data from these collections do illustrate the lack of a measureable stress response in animals from all three cow groups studied: Blood only, blood and saliva, or saliva only collection suggesting that neither sampling method elicits more stress response than the other. Sufficient saliva and circulating cortisol were obtained from the boar and gilt piglets in Experiment 3 to support our hypothesis that saliva is a viable tool for assessing stress in piglets. Mean concentrations

for gilts were significantly lower than that of the castrated boars (1.36±0.301 versus 2.3074± 0.22 ng/mL).

Evaluation of Cortisol in Saliva Relative to Serum in Lactating Cows, Heifer Calves and Piglets in Response to Applied Stress

by
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DEDICATION

This thesis is dedicated to the people who have been there for me in my most formative years and without whom this thesis would never have been written.

To my loving parents Ken and Stephany, who have offered strong words of encouragement and sound advice when I needed it and just as eagerly offered a comforting smile and a shoulder to cry on when that was all I needed. I know that I am fortunate to have had you there for me every step of the way. I dedicate my thesis to you for being the people who took the time to teach me how to be the person I am today. I love you both.

To my fiancé, Clayton Thomas Hearne, for the years you have given me and the unending support you so willingly offer me every day of our lives together. You are the single most constant beacon in my life. I will forever be indebted to you for your never ending patience and encouragement on this wild ride. I love you.

And last but not least… To my faithful four-pawed companion, River.

BIOGRAPHY

Emily Iris Weston was born to Ken and Stephany Weston on April 17, 1985 in Winter Park, Florida. Her family later relocated to North Carolina where she and her mother started a small scale family farm to raise livestock including poultry, goats, and sheep. Her passion for animal husbandry and livestock handling developed with her experience. She showed her first chickens at the Florida State Fair show as a five year old. Once in middle and high school she was active in her local 4-H and FFA programs in Orange County and later worked for Lemola Dairy Farm in Chapel Hill North Carolina with Everett and Michael Cheek. These influential experiences and those who led her along the way helped her realize her passions and chose her career path. She graduated from Orange High School in 2003 and earned her Bachelor's degree at North Carolina State University in Poultry Science with a minor in Animal Science in 2007 during which time she met and became engaged to Mr. Clayton Hearne. After graduation, Emily began working on her Master's of Science degree in Animal Science at North Carolina State University under the direction of Dr. Scott Whisnant.

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Finally I would like to thank my entire family for their never ending support of my dreams! To my big brother, Eli, you have no idea how much your love and listening has

meant to me. I know that one day we will look back on these stressful times and realize how fortunate we were to have had each other, even if we are hundreds of miles apart!

To my parents, I could never have made it this far without their love and never-ending support! They have always encouraged me to follow my dreams and pursue my passions. I am eternally grateful to them.

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Literature Review

A problem in assessing animal welfare is that collecting data in itself may be stressful to the animals. Therefore, less invasive methods for collecting data have to be devised and tested. Cortisol has long been and is still frequently used to measure the stress response. Increasing levels of cortisol in an animal's plasma indicate a stress response by that animal. Cortisol is secreted at higher levels during the 'fight or flight' response and could be used as an animal welfare or management tool when evaluations of the level of stress or comparisons between different treatments must be made.

The use of saliva for determining circulating concentrations of cortisol has been reported for various species including guinea pigs (Fenske, 1961), elephants (Danthe et al.1992), goats (Greenwood, 1992), sheep (Fell et al. 1985a; Fell and Shutt, 1986b), calves (Fell et al. 1986a; Fell and Shutt, 1986b), horses (Lebelt et al. 1996), dogs (Vincent and Michell, 1992; Beerda et al. 1996), and humans (Walker et al. 1978; Vining et al. 1983 a, b; Tunn et a1. 1992; Bonnin et a1. 1993). In the studies where salivary cortisol was compared to blood cortisol, a strong correlation was found between salivary and blood cortisol, with salivary cortisol being at lower concentrations than blood cortisol and usually following the same time course as blood cortisol in humans (Tunn et a1. 1992; Bonnin et al. 1993), goats (Greenwood and Shutt, 1992), and dogs (Vincent and Michell 1992; Beerda et a1.1996). This review will cover research testing the idea that salivary levels of cortisol can be used in addition to or in place of blood plasma levels in determining the stress response of various

species. Furthermore, a wide range of information covering the ideas of stress as a measure of animal welfare, cortisol as a measure of stress, and salivary levels of cortisol as an applicable and noninvasive measure of cortisol will be reviewed.

Stress as a Response

Stress research conducted in the last decade indicates that the endocrine, immune, and central nervous systems network together and respond to stressful stimuli in a coordinated approach to regain or maintain homeostasis. Because hormones and their receptors, as well as neurotransmitters, exist in all three systems, we can assume that there is a common communication structure between these integral parts of the stress response. Both physical and psychological stressors are perceived by these systems as equally important. Historical research supports the idea that situations of insecurity, social pressure, and fright are compelling stressors with significance for the well-being of animals.

To appreciate the effects of stress on any body system, the term stress must be defined. There are as many ways to define stress as there are stressors; however, several definitions have survived years of review and include the earliest known explanation of Claude Bernard in 1878 to the classical explanation of Hans Selye in 1936. Bernard's explanation of stress response was stated as "the effort of the body to maintain a stable internal environment to challenges from widely variable environments (von Borell, 2001)." This was later described by Walter Cannon in 1932 as homeostasis. This concept of stress and stressors was later described by Cannon and Bernard in 1914 and 1936 respectively and is as follows: physiological stressors such as pain, hunger, etc. can, if allowed to become chronic, become pathological in effect later in the stress response (von Borell, 2001). Selye's

definition of stress came about as a description of the General Adaptation Syndrome (GAS) in 1946 (von Borell, 2001). He hypothesized that the acute (emergency reaction) to stress leads to the chronic response to stress, and he divided the response to stress into three stages: alarm, resistance and exhaustion. The acute or alarm response is adaptive and even beneficial, but if the stress response continues, it becomes damaging to health--as the term exhaustion implies. Stress is generally defined as the response, and factors that cause stress are called stressors. Today, stress is used as a very broad term that implies a threat to which the body needs to adjust or a condition in an animal that results from the action of one or more stressors that may be of external or internal origin (von Borell, 2001). Common to most stressors, however, is the fact that the animals' adaptive responses to threatened homeostasis include an activation of the hypothalamic-pituitary adrenal (HPA) axis as discussed previously. The result is a rise in blood levels of glucocorticoids which leads to a variety of other adaptive responses at the neuroendocrine, autonomic, and behavioral levels (Angelucci, 2000). Animals (beef cattle) with higher cortisol levels showed more anxiety-related behaviors: less time ruminating, more vocalizations, and distance from other cows (Bristow and Holmes, 2007).

That said, cortisol can be increased by positive or non-stressful excitement. Data support the suggestion that specific events during natural mating activity can alter endocrine secretions of cortisol and growth hormone in bulls and cortisol and testosterone in boars (Borg et al, 1991). During sexual encounters, boars experienced an acute increase in cortisol, followed closely by increased testosterone concentrations (Liptrap and Raeside, 1978). Similar adrenal-testicular responses were observed after administration of exogenous

adrenocorticotropin (ACTH) (Liptrap and Raeside, 1975) and cortisol (Liptrap and Raeside, 1983), whereas adrenalectomized boars given ACTH failed to show increased testosterone secretion (Liptrap and Raeside, 1968). The boars in Borg's research, however, produced concomitant increases in serum cortisol and testosterone concentrations rather than a sequential enhanced cortisol and testosterone secretion as seen in previous research (Borg et al, 1991). Acute elevations in cortisol also enhanced the sensitivity of the pituitary to exogenous gonadatropin releasing hormone or GnRH (Liptrap and Raeside, 1983). The analyses from the pooled data indicated that cortisol enhancement during sexual activity in boars was most attributable to achieving a service and (or) fewer than five mounts. Correlations of mounting and intromission to cortisol concentrations were significant and nearly significant, respectively.

Methods for Stress Assessment

Noninvasive methods for measuring stress-indicating variables have been developed in addition to classical descriptive behavioral observations, allowing an evaluation of stress by multiple criteria (von Borell, 2001). The stress an animal might experience during the routine procedures such as blood sampling may interfere with the applied stress condition used in the research situation. Because blood sampling techniques could be stressful, less invasive techniques have been studied including sampling of urine (Hultgren, 1988) and saliva (Cooper et al., 1989). This review will focus on the use of salivary concentrations of cortisol for assay rather than plasma concentrations. It has been shown that salivary cortisol concentrations are positively correlated with plasma concentrations (Negrao et al., 2004).

Vocalization analysis has also been useful to obtain information on stressful procedures such as the castration of young pigs (White et al., 1995; Weary et al., 1998; Horn et al., 1999). Certain housing environments are not conducive to continuous blood sampling, such as in group pens, as other animals may interfere with catheters. Group housing is usually a necessity for consideration of the naturally occurring circadian release of hormones (Ladewig and Smidt, 1989) and certain behaviors. Remotely controlled blood sampling devices (Carragher et al., 1997) and wireless heart-rate monitors (Hopster and Blokhuis, 1994; Marchant et al., 1997; Hansen and von Borell, 1998) can help to overcome some of those problems. Elevated hormone concentrations as well as an increase in heart rate do not necessarily relate to a stressful situation with negative consequences for the well-being of the animal; however, equipment damage from other animals or other failures may occur.

The theory of habituation is also important when using stressors in research efforts or in measuring response to stress. Habituation to novel stressors/fear inducers can decrease stress response in some animals (rats, cattle) but not in others (sheep) depending upon treatment and outcome during the first experience with the stressor (e.g. electrified alley for rats, inversion, or shearing for sheep) (Hutson, 1995; Hargreaves and Hutson, 1990; Fell and Shutt, 1986b). In mice the idea of habituation is explored using an elevated plus maze in which time spent on open arms relative to open and closed arms is used as an index of anxiety and, hence, of the anxiolytic or anxiogenic effects of selected pharmaceutical agents. When rats are confined to the open arms rather than the closed ones, they exhibit significantly more fear-related behaviors and significantly higher plasma corticosterone concentrations (Pellow et al., 1985). Further evidence that habituation is important when

conducting stress research is the fact that cattle trained and habituated to a squeeze chute have baseline cortisol levels while those extensively reared will have higher cortisol levels in the same squeeze chute. (Grandin, 1997)

Hypothalamic-Pituitary-Adrenal Axis and the Stress Response

In order to appreciate the method in which the various body systems interact as a coping mechanism for stressors, whether positive or negative, one must first understand the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA axis, which ultimately controls the secretion of glucocorticoids from the adrenal cortex, is one arm of a central system that appears to be responsible for coordinated behavioral, neuroendocrine, autonomic, and immune responses to alterations in homeostasis (Dallman et al., 2007). Corticotrophin releasing hormone, CRH, is a hormone produced by the hypothalamus that stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). The HPA axis is activated during stress, resulting in the release of glucocorticoids from the adrenal cortex in blood and subsequently the appearance in saliva (Korte, 2001). There are marked circadian rhythms in the HPA axis, well-integrated with other circadian rhythms to optimize the daily release of CRH and arginine vasopressin, AVP, and its primary function is to stimulate steroidogenesis by the adrenal cortex leading to release of cortisol (Buckley and Schatzberg, 2005). In addition, the HPA axis is responsive to stimuli (stressors) that threaten homeostasis, either psychologically or physically (Pacák and Palkovits, 2001). These responses are a necessary survival tool utilized by animals when facing danger or any other stress.

One of the most common methods for evaluating stress in livestock species is through the measurement of hormones of the hypothalamic-pituitary axis (HPA). Serum cortisol

concentrations have been proven to be a useful indicator of stress (Broom and Johnson, 1993; Terlouw et al., 1997). Cortisol is released into the blood via a complex cascade that begins as the cognitive brain centers, such as the cerebral cortex, recognize a threat and initiate a response mechanism via nerves. These nerve signals activate CRH producing neurons in the paraventricular neurons of the hypothalamus (Johnson et al., 1992). CRH is then secreted by axon terminals that project into the median eminence then secrete CRH which is transported via the hypophyseal portal system into the anterior pituitary. It is here that CRH increases the synthesis and secretion of ACTH and other proopiomelanocortin metabolites (Axelrod and Reisine, 1984). The action of ACTH is to increase the secretion of glucocorticoids from the adrenal cortex. This mechanism is maintained until ample amounts of glucocorticoids (i.e., cortisol) in the blood act on the anterior pituitary to inhibit further ACTH secretion, thereby exhibiting the powerful negative feedback control. Corticotropin releasing hormone receptors have also been identified in areas other than the cognitive centers such as the limbic areas (DeSouza et al., 1991).

What is Cortisol?

When working with cortisol, it is important to understand the biology behind its actions. Corticosteroids do not control emotional behavior directly; instead, they stimulate chemical changes in particular sets of neurons, making certain behaviors more likely to occur in a particular context as a result of the strengthening or weakening of particular neural pathways (Dhabhar et al., 1993). Adrenal steroids such as cortisol can influence many behavioral actions because the central nervous system not only organizes the hormonal response, but also serves as a major target organ for the released corticosteroids (McEwen,

1986). Due to their lipophilic nature, corticosteroids can readily cross the blood brain barrier and enter the brain. They can either bind to membrane receptors (Orchinik, 1992) or freely cross neuronal cell membranes to bind to specific cytoplasmic receptors (McEwen, 1968). Therefore, corticosteroids may alter neural activity rapidly by modulation of ion channels and second-messenger systems (Orchinik, 1992) and by receptor-mediated protein-protein interactions (Reichardt and Schutz, 1998) or more slowly by receptor-mediated, long-lasting genomic actions (McEwen, 1979) Through the latter mechanism, corticosteroids may lead to altered transcription of specific genes resulting in changes in protein synthesis and, consequently, in the regulation of enzymes, neurotransmitters, and receptors (McEwen, 1979; Axelrod and Reisine, 1984).

Salivary Cortisol versus Plasma Cortisol

Many studies have shown that one can mimic a stressful situation hormonally by administering a common stress hormone such as cortisol or ACTH. One such study showed that increasing corticosteroid levels in non-pregnant sows mimics the prenatal stress of pregnant sows (Kranendonk G., et al., 2005). That study illustrates how one can replicate the normal prenatal stress response when hydrocortisone acetate (HCA) is administered to non-pregnant sows. Both plasma and salivary cortisol concentrations were increased in sows that received the higher doses of HCA comparable to concentrations found in the pregnant sows. Other research showed that when plasma and salivary concentrations of cortisol were measured following an ACTH challenge, both profiles were similar and there was significant and positive correlation between the salivary and plasma concentrations in dairy cattle, (Negrao et al., 2004). A similar study was conducted in swine (Bushong et al., 2000). That

research measured total cortisol concentrations for concurrent saliva and blood samples after various techniques for administering ACTH. Correlations between salivary and plasma cortisol within treatments were: r = 0.60; r=0.58; and r=0.79, depending upon the treatment applied. This illustrates the clear correlation between the concentrations of cortisol derived from the two sampling techniques (Bushong et al., 2000). Another study that used meat quality pigs as a model and measured the efficacy of supplemental tryptophan to decrease incidence of stress reported significant correlations between salivary and plasma cortisol concentrations (Guzik, 2006). Further research with weanling piglets produced similar results (Koopmans, 2006). That group used the stress caused by weaning and mixing piglets to test the effectiveness of the supplemental tryptophan (Trp) in reducing stress. In that study, salivary but not plasma cortisol concentrations were reduced approximately 50% before and at mixing in Trp-supplemented pigs compared with control pigs. The reason the authors suggest for this discrepancy between salivary and plasma cortisol concentrations is the fact that the cortisol concentration in saliva is mainly in a free (unbound) biologically active form and reflects only 5 to 10% of the total (free and bound) cortisol concentration in plasma (Kirschbaum and Hellhammer, 1989; Parrott et al., 1989). It is possible that any changes in the free cortisol concentration in plasma could be masked by the surplus total concentration of cortisol in plasma.

Several issues arise when using measures of cortisol derived from a salivary sample rather than from a blood plasma sample. However, several studies suggest that collection of salivary samples and determination of hormone concentrations can prove to be a less invasive and perhaps more accurate method for determining the well-being of an animal

based on the presence or absence of stress. A number of studies in humans (Riad-Fahmy et al., 1982; Vining et al., 1983a) as well as ruminants (Fell et al., 1986a; Shutt et al., 1987) have shown that salivary cortisol levels correspond to free serum levels; however, this may not hold true in swine. Blackshaw and Blackshaw reported that due to the lack of continuous salivary flow, much lower secretion rate, and overall difficulty of collection, swine present many limitations for the use of salivary techniques in measuring hormones (1989). They concluded that prolonged collection of saliva required pilocarpine (naturally occurring cholinomimetic alkaloid) and reported that after an intramuscular injection of pilocarpine, the pig produced large amounts of saliva for ~15 minutes beginning 5-10 minutes post injection (Blackshaw and Blackshaw, 1989). However, others have collected repeated saliva samples from pigs without administration of pilocarpine or other reagents to increase saliva production (Read et al., 1982; Vining et al., 1983b; Fell et al., 1985a; Dathe et al., 1992; Greenwood and Shutt, 1992; Vincent and Michell, 1992; Malmud and Tabak, 1993).

In recent years, interest in measurement of stress in commercially produced swine has dramatically increased. Identification of the causes of the stress and the quantification of their biological effects has also increased. An essential practice in stress research is an evaluation of the status of the HPA axis. Increased activity of the HPA axis suggests a physiological response to stressors; as a result, measurement of cortisol is a common component of stress research. Typically, total cortisol concentrations are measured by immunoassay procedures in serum or plasma and, rarely, in urine. Blood and urine sampling methods have several intrinsic problems. Obtaining a blood sample cannot be achieved without restraining the animal, thereby initiating a confounding activation of the HPA axis (Nyberg et al., 1988).

The use of venous catheters overcomes this problem; however, there are several constraints on their utility: The insertion of a cannula is a skilled technique, but they are often difficult to preserve over extended periods of time, their use is limited to studies involving small numbers of animals, and they can be extremely difficult to use in situations outside the environment of the animal's pen or barn (Cook et al., 1996).

In addition to the problems of obtaining blood samples, the measurement of cortisol in blood has physiological limitations. Cortisol in circulation is predominantly bound (90%) to cortisol-binding globulin (CBG) and albumin. The remaining 10% is in a "free" form, and it is this fraction that is available for uptake by target tissues. Under stress conditions the binding capacity of CBG becomes increasingly saturated, with a resulting disproportional increase in free cortisol. Thus, measurement of the total cortisol concentration in blood does not necessarily reflect the biologically active fraction of the hormone (Cook et al., 1996). Mechanical difficulties involved in the measurement of the free steroid hormone in blood makes this approach impractical for most routine purposes (Brien, 1980).

Cortisol concentrations in urine consist of the free hormone. However, urine is very difficult to collect, requiring special collection devices, severely limiting the number of animals that can be used in an experiment at any one time. Furthermore, cortisol levels in urine are dependent on urine volume. The concentration is often expressed as units of cortisol over a defined period (e.g., nanomoles per 24 h); consequently, the acute response of the HPA axis cannot be assessed (Cook et al., 1996).

Limitations of sampling and assay accuracy can be largely overcome by measuring cortisol in saliva. This sampling procedure is relatively easy to perform, does not require

restraint of the animal, and can be achieved under conditions that are incompatible with blood and urine sampling, (e.g., during transport as in the study by Cook et al. (1996). Cortisol enters saliva by a process of passive diffusion; the concentration is independent of flow rate and is a direct reflection of the free fraction in blood (Riad Fahmy et al., 1982). Numerous studies have testified to the utility of salivary cortisol measurement for assessment of HPA axis status in various species (Read et al., 1982; Vining et al., 1983a; Fell et al., 1985b; Dathe et al., 1992; Greenwood and Shutt, 1992; Vincent and Michell, 1992; Malmud and Tabak, 1993). The utility of the measure in pigs is less well-documented. Nevertheless, Cooper et al. (1989), Parrott et al. (1989), and Parrott and Misson (1989) have demonstrated good agreement between blood and saliva concentrations of cortisol in swine. In contrast, Blackshaw and Blackshaw (1989) reported "severe limitations" in the use of salivary cortisol due to the lack of a significant correlation between salivary and plasma concentrations.

Research by Cook et al. (1996) used ACTH to stimulate the HPA axis in swine and then measured both serum and salivary cortisol to determine response. Saliva samples were collected before and after handling and transport. There were significant correlations between serum and salivary cortisol values following ACTH stimulation ($r = 0.8813$, $P < 0.025$) and snaring ($r = 0.7964$, $P < 0.05$). The overall ratio of saliva to serum cortisol was 9%. The saliva:serum cortisol ratio was concentration dependent. In pre-stimulation samples, the ratio was 8.6% and at maximal concentrations was 13.3%. Handling and transport stress stimulated increases in salivary cortisol concentrations. Differences between pre and post-transport concentrations were significant ($P < 0.0001$). Variation in the concentration of cortisol-binding globulin (CBG) is an important factor for the interpretation of adrenal

response. Because unbound cortisol is the biologically active fraction of plasma cortisol (Ballard ,1979; Milgrom, 1990), a more accurate image of effective HPA activity might be obtained through salivary cortisol measurements.

A study by Bushong et al. in 2000 also found high correlations between salivary and plasma cortisol, and the fit of the regression equation for the salivary to plasma cortisol relationship confirmed the efficacy of salivary cortisol as a measure of plasma cortisol concentrations in swine. This research supports the results of Cook et al. (1996) and Parrott et al. (1989) that salivary cortisol does respond to ACTH stimulation, which challenges data from Blackshaw and Blackshaw (1989). However, in the Bushong study and the studies of Cook et al. (1996) and Parrott et al. (1989), multiple synchronized blood and saliva samples were taken from each animal over the time course of ACTH stimulation. Blackshaw and Blackshaw (1989), however, reported single blood and saliva samples obtained after restraint by rope, restraint by nose-rope, or exercise, and multiple samples from two sows collected over a 14-day period with no apparent stimulus to the sows. Blackshaw and Blackshaw (1989) also used an aspiration tube connected to a vacuum pump to collect saliva and stated that the rate of saliva secretion was low and the collection of adequate saliva samples took approximately 5 minutes. In the Bushong study, saliva was collected using a cotton swab that was chewed by the animals, all saliva samples were obtained concurrent with blood sampling, and both samples took 1 minute or less to obtain. Both Cook et al. (1996) and Parrott et al. (1989) also used cotton swabs chewed by the subjects to obtain saliva samples and reported either less than 2 minutes or about 30 seconds, respectively, for sampling to occur. It is possible that the time lag which may have occurred between the saliva and blood

samples taken in the Blackshaw and Blackshaw (1989) study influenced their finding that salivary cortisol was poorly correlated with plasma cortisol in pigs.

Is Saliva a Viable Alternative to Serum?

Recent research has shown that salivary cortisol is a viable tool in many species for several reasons. Corticosteroids enter the saliva by passive diffusion; therefore, concentrations are unaffected by salivary flow rate (Riad-Fahmy et al., 1982). Corticosteroid concentrations in saliva have been found to be directly related to those in plasma in humans, dogs, pigs, and domestic ruminants (Cook et al., 1996 and 2002). Rate of delivery of hormones to the human salivary glands may depend in part on bound hormone levels. Cortisol may enter the saliva via intracellular diffusion of unbound cortisol and possibly by diffusion between acinar cells, allowing some bound hormone to enter (Albertini, 1982). Comparison of cortisol concentrations in plasma, parotid saliva, and whole saliva in persons undergoing investigations for assessing adrenal function, including stimulation with cosyntropin (Synacthen™, synthetic ACTH) and suppression with dexamethasone, indicated that changes in plasma cortisol concentration were accurately and immediately reflected in saliva from either the parotid gland or whole saliva (Walker et al., 1978).

Basal total plasma, plasma free, and salivary cortisol levels were compared in patients with panic disorder (n = 47) and in healthy individuals (n = 23). Correlations between those fractions were calculated. All three basal cortisol fractions were significantly elevated in patients compared to controls. There were significant correlations among all three cortisol fractions (Wedikind et al., 2000). Another experiment in which saliva, plasma, and urine cortisol levels were measured in dogs provides strong support for using saliva sampling and

urine collection as noninvasive methods to establish stress-induced cortisol responses (Beerda, et al., 2000). This study found positive correlations between salivary and urine outputs of cortisol. Saliva levels were consistently lower, but that is due to the fact that only about 15% of the total cortisol fraction in blood, namely the unbound fraction, transfers into the saliva. No significant delay was found in the change in cortisol levels in saliva compared to blood because the unbound portion is very small and highly lipid-soluble, thereby crossing cell membranes and entering saliva with ease as compared to the bound fraction.

Determination of Cortisol Levels

The use of assays to measure cortisol levels in saliva was validated by Chacon-Perez and colleagues in 2004. This research showed that commercially available radioimmunoassay kits for human plasma (detection range: 10-100 ng/ml) were not sensitive enough for animals with low concentrations of salivary cortisol (< 4 ng/ml). Thus, enzyme immunoassay EIA was used in the study. Sensitivity, specificity, precision, and accuracy of the EIA tests showed this method to be suitable and reliable. The detection limit was found to be 0.024 ng/ml, representing an improvement on previously described techniques. Intra-assay and inter-assay variation coefficients were 1.47 to 7.30% and 2.40 to 9.78%, respectively. The recovery rates for cortisol added to saliva samples were 91.36 to 126.5%. Parallelism tests showed that saliva cortisol levels can be determined in cattle samples without extraction. The correlation between saliva and plasma cortisol was positive ($r = 0.75$) and the saliva:plasma cortisol ratio was around 10%. Therefore, saliva samples were found to be a suitable alternative to plasma samples in bovine HPA (hypothalamic-pituitary-adrenal) axis evaluation (Chacon-Perez et al., 2004).

With the increasing public concern over animal welfare in agriculture, methods for the assessment of stress in domestic livestock are needed. Collection of blood samples can be a stressor if the animal is not catheterized. Maintaining intravenous catheters is very difficult if not impossible in group housed pigs and perhaps other species as well. Urine collection can be done, but animals may not urinate at the desired times and could be very labor intensive. Fecal corticosteroid concentrations reflect events that happened some hours previously; thus, timing of sample collection may prove difficult. However, in pasture based animals or wildlife, this may be the optimal method. If saliva samples can be collected without causing stress to the animal based on previous research showing the correlation between blood and plasma, this could be the best method for intensively raised livestock. The objectives of the current research were to develop methods for effective collection of saliva in pigs and cattle, to validate assay techniques for salivary cortisol, to assess the relationship between serum cortisol and salivary cortisol in cattle where less research has been done, and to evaluate the stressfulness of saliva collection in cattle and piglets.

CHAPTER 1

Introduction

A problem in assessing animal welfare is that collecting data in itself may be stressful to the animals. Therefore, less invasive methods for collecting data have to be devised and tested. Cortisol has long been and is still frequently used to measure stress response. Increasing levels of cortisol, a corticosteroid secreted from the adrenal gland, in an animal's plasma indicate an increase in hypothalamic-pituitary axis activity, also referred to as a "stress response" by that animal. Cortisol is secreted at higher levels during the 'fight or flight' response and could be used as an animal welfare or management tool when the presence of stress must be determined. There are as many ways to define stress as there are stressors; however, several definitions have survived years of review and include the earliest known explanation of Claude Bernard in 1878 to the classical explanation of Hans Selye in 1936. Today, stress is a very broad term that implies a threat to which the body needs to adjust or a condition in an animal that results from the action of one or more stressors that may be of external or internal origin (von Borell, 2001).

Stress can be assessed utilizing several methods including but not limited to cortisol assays of salivary secretions (Korte, 2001), blood plasma (Broom and Johnson, 1993; Terlouw et al., 1997), urine, and feces. While corticosteroids can easily be measured in urine and feces, the concentrations and time course show large variability among animals (Palme et al., 1996).

Corticosteroids enter the saliva by passive diffusion; therefore, concentrations are unaffected by salivary flow rate (Riad-Fahmy et al., 1982). Corticosteroid concentrations

have been found to be directly related to those in plasma in humans, dogs, pigs, and domestic ruminants (Cook et al., 1996 and 2002). Saliva samples have been shown to be a suitable alternative to plasma samples in evaluating the bovine hypothalamic-pituitary-adrenal, HPA, axis evaluation (Chacon-Perez, 2004). In studies where salivary cortisol was compared to blood cortisol, a strong correlation was found between salivary and blood cortisol, with salivary cortisol being at lower concentrations than blood cortisol and usually following the same time course as blood cortisol in humans (Tunn et a1., 1992; Bonnin et al., 1993), goats (Greenwood and Shutt, 1992), and dogs (Vincent and Michell, 1992; Beerda et a1.,1996). Other research has shown that saliva collection and assay in swine is inconclusive due to many factors including the variation of salivary flow rate in swine (Blackshaw and Blackshaw, 1989). This research used adult pigs and required the use of both pilocarpine nitrate to elicit sufficient salivary flow as well as restraint. In comparison to the plasma collection in the same animals, saliva collection proved much more difficult and yielded inconsistent results (r=.260). One reason for such inconsistencies was the use of the pilocarpine. High correlations (r=.883) between salivary and plasma concentrations were found in the absence of pilocarpine. Riad-Fahmy (1982) suggested that salivary flow is of little importance in swine due to the fact that steroid hormones enter the saliva by passive diffusion and are therefore unaffected by salivary flow rate. The objective of the current study is to determine if the collection of saliva from piglets and dairy cattle is a viable tool for evaluation of cortisol and monitoring of stress.

Materials and Methods

This project consists of 3 main experiments: Experiments 1 and 2 were carried out at the Dairy Education Unit, North Carolina State University, Raleigh, North Carolina, and Experiment 3 was carried out at the Swine Education Unit, North Carolina State University, Raleigh, North Carolina. Experimental procedures were approved by the Institutional Animal Care and Use Committee of North Carolina State University before beginning the experiment (Cattle: 06-137-A and Swine: 06-109-A).

Experiment 1: ACTH Challenge in Dairy Heifer Calves

Six dairy heifers (3 Holsteins and 3 Jerseys) ranging in age from 5 to 6 months (167.3±6.2 d) and in weight from 159 to 216 kg (193.5±21.6 kg) were utilized for this experiment. The animals were housed in a group pasture setting for the time leading up to the experiment and then trained to a halter and lead rope once per day for nine days before sampling to reduce stress caused by handling. On the day of collection, each heifer was non-surgically fitted with a jugular cannula and allowed to recover for at least 3 hours before sampling. The animals remained tied and were allowed ad libitum access to feed and water throughout the duration of the collections. At Time 0, adrenocorticotropin (porcine ACTH, Sigma, Saint Louis, MO) was administered (0.6 IU/kg of body weight) via the cannula.

Blood and saliva samples were collected at -15, 0 minutes before, and 15, 30, 45, 60, 90, 120, 150, 180, and 210 minutes after the ACTH injection. Each saliva sample (up to 1mL) was collected immediately following each blood collection (3-5 mL). Blood samples required approximately 0.25 minute, and saliva samples required up to 1 minute per

collection. Blood samples were placed on ice until transported to the lab where they were centrifuged and serum stored at -20°C. Saliva samples were collected using 4x4 inch squares of gauze rolled and clamped into hemostats and manually placed in the animals' mouths, moved around, and removed once soaked. The soaked gauze was then placed into a funnel-shaped tube within a 10mL tube for later centrifugation and placed on ice until transport to a -20°C freezer where the samples were stored until assay.

Experiment 2: Salivary and Blood Sampling Effects in Lactating Dairy Cows

17 Holstein dairy cows between the ages of 3 to 5 years from the high lactation group (were housed in a free stall barn and milked two times daily) were utilized in this experiment. The feeding and milking schedules were not affected throughout this project. Samples were collected in the afternoon just before afternoon milking which occurred at 1630 hours. Three different groups were sampled: tail vein blood samples were collected from Group A (n=6), salivary samples were collected as described in Experiment 1 from Group B (n=6), and both saliva and blood samples were collected from Group C (n=6). All collections were performed before milking while animals were in the free stall barn, using the existing head lock system. Saliva samples were collected immediately following tail vein blood collection at 0, 15, 30, 45, 60, 75, and 90 minutes, and all samples were placed on ice until transport to a -20°C freezer where samples were stored until assay. Blood samples were handled as described for experiment one.

Experiment 3: Effect of Salivary Collections Pre and Post Castration in Boar Piglets and in Gilt Piglets

Piglets utilized were housed in farrowing crates with the mother sow at the Swine Education Unit. Yorkshire cross piglets approximately seven days of age, boars (n=7) and gilts (n=5), were randomly chosen and assigned a number prior to the beginning of castration procedures. All piglets came from two litters. Castration served as time 0 for both groups even though only males were castrated. Gilts were held in a prone position against the body of the sampling technician to minimize stress caused by sampling. Saliva samples were taken at 0, 15, 30, 60, and 120 minutes post castration. Saliva was collected using 2x2 inch squares of dry gauze, soaked in Sprite™ soda, excess soda expressed manually until almost dry, clamped with a hemostat, and manually placed in each piglet's mouth for approximately 0.5-1 minute. In the current study, Sprite™ was utilized instead of pilocarpine (Blackshaw and Blackshaw, 1989) to stimulate ample saliva secretion in the piglets. Samples were placed on ice until transport to a -20°C freezer where samples were stored until assay.

Cortisol Assay

Serum and salivary cortisol concentrations were determined using the Coat-a-Count cortisol assay (Siemens Medical Diagnostics, Wallingford, CT). Instructions from the manufacturer were followed with the following exceptions. For serum, the volume added to tubes was increased from 25 uL to 100 uL to reflect the generally lower circulating cortisol

concentrations in cattle compared to humans. Additional points of 0.0625, 0.125, 0.25, and 0.50 were added to the low end of the standard curve by diluting the manufacturer supplied standards with the zero sample reagent from the kit. For saliva samples, 100 uL was added to the tubes and the same additional points were added to the standard curve. Sprite™ (100 uL) was added to some tubes to determine if there was anything in it that would interfere with binding, and all tubes containing Sprite™ alone had binding equivalent to the non-specific binding tubes, indicating no interference in the assay.

Statistical Analysis

Analysis was conducted using SAS 9.1 (Cary, NC). Cortisol concentrations were compared among times and or between groups using PROC GLM. Groups consisted of: Experiment 1, none; Experiment 2, Group A, B, C; and Experiment 3, gilts and boars. The model statement included the effects of time, group and the two way interaction. Tukey's test was used to compare means. Correlations between serum and saliva cortisol concentrations were determined using PROC CORR . Significance was denoted at $P < 0.05$.

Results

The results of the ACTH Challenge / Experiment One indicate (Figure 1) that serum and saliva cortisol concentrations show a significant and positive correlation ($r = 0.255$, $n=6$) after a stimulus to the adrenal gland with adrenocorticotropin. Salivary cortisol concentrations were lower than that found in serum and ranged from 27.40% to 59.94% of serum concentrations (average for all animals =35.71%). Several of the collections in both serum and saliva were found to be below assay sensitivity and were removed from the average so as not to interfere with the actual comparisons.

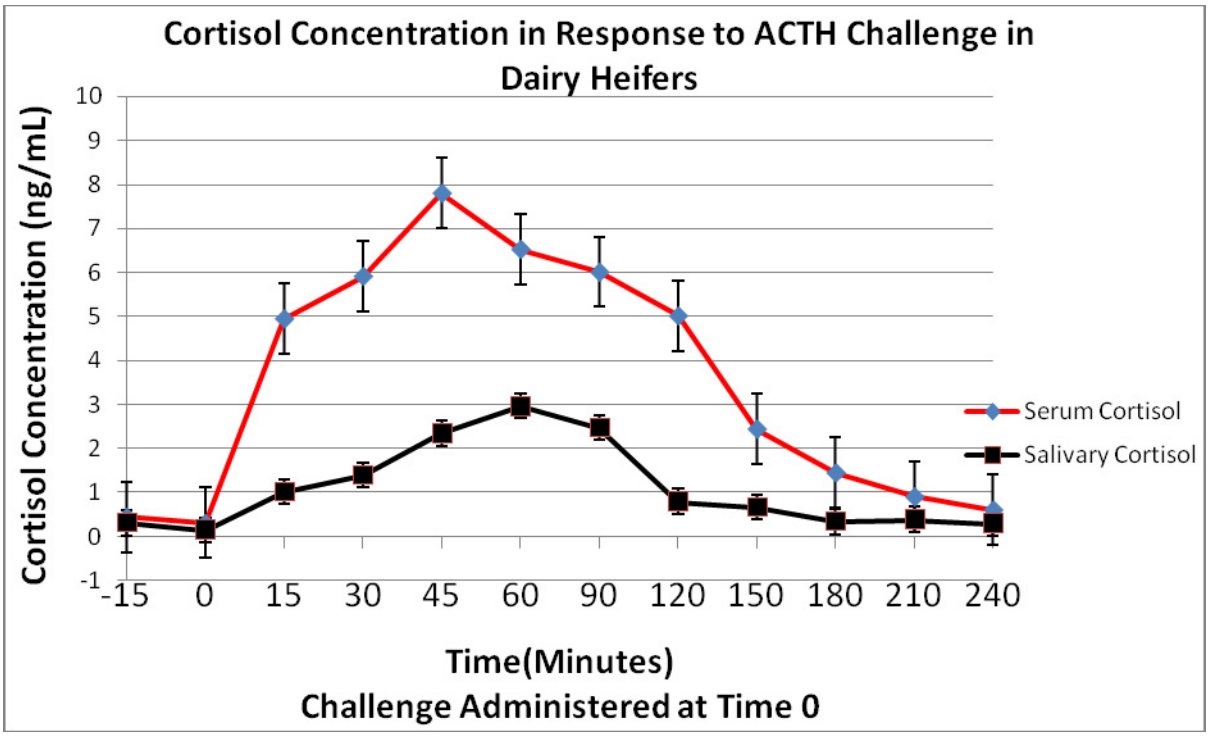

Figure 1

In experiment two, saliva cortisol concentrations were below the sensitivity of the assay for most samples. Many of the serum samples from these cows also had non-detectable levels of cortisol. Again, saliva concentrations were lower than those found in serum. There was no difference in cortisol concentrations between groups of cows that had blood samples collected and those that had both saliva and blood samples (Figure 2) collected or in saliva samples from those that had both types of sample collected and those that had saliva samples only.

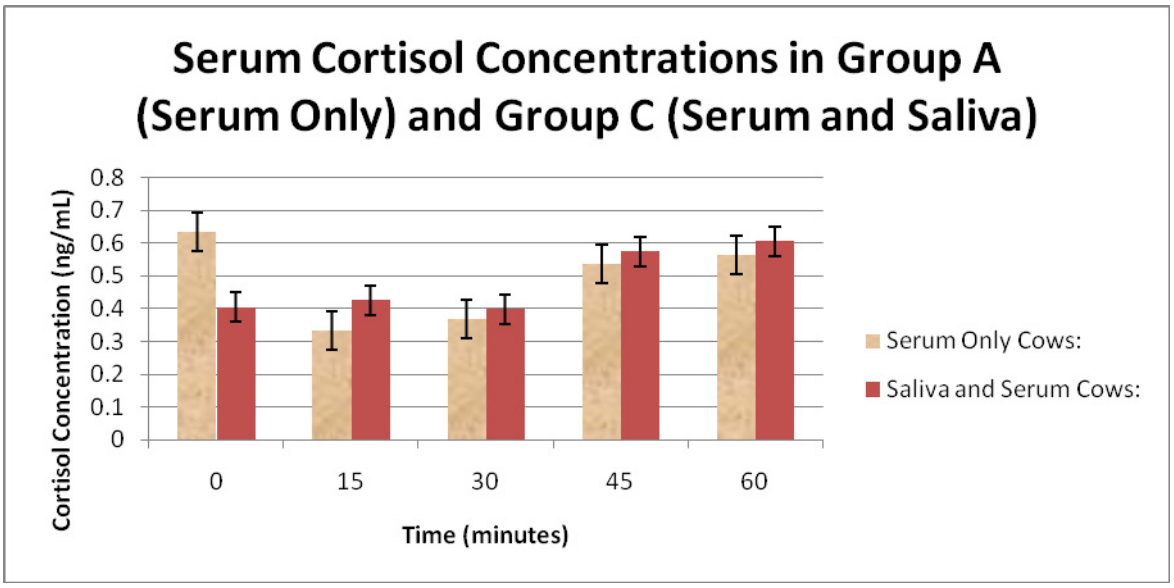

Figure 2

Saliva cortisol concentrations were collected from male piglets that had recently been castrated and from some of their female littermates. We found differences in cortisol concentrations at several time points, indicating a handling effect in both sexes. With time as the only variable, collection #3 (3.92±0.36), 30 min post castration, was significantly different from collection #1 (1.03±0.08) and #5 (1.42±.1129), 0 and 120 minutes post castration, respectively ($P > 0.05$). Figure 3 shows a significant increase in cortisol concentration in male piglets post-castration as would be expected. This same figure represents the lack of increase in cortisol in the gilts not subjected to the extra stress of castration (only handling for collection). Mean concentrations for gilts (1.36±0.301 ng/mL) were significantly lower than that of the castrated boars (2.3±0.22 ng/mL) when analyzed using Tukey's Studentized Range Test.

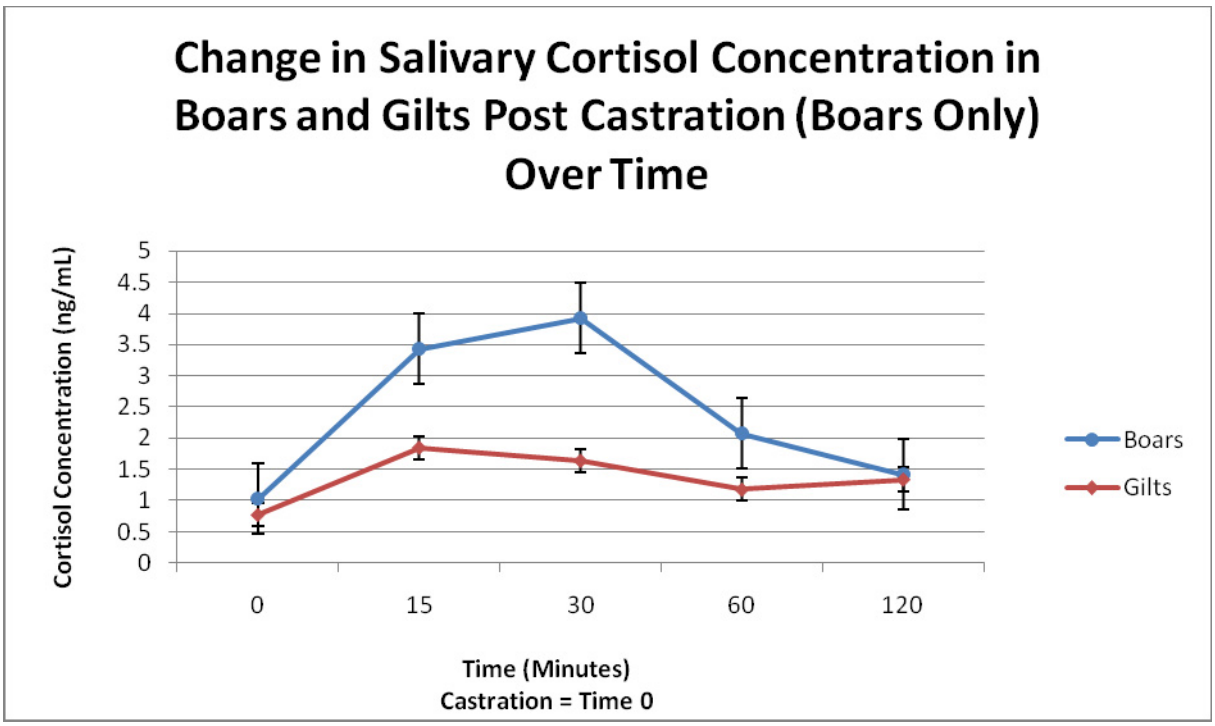

Figure 3

Discussion

Our results agree with previous research in cattle (Negrao et al., 2004) that an ACTH challenge can be used to elicit a cortisol response and that serum and saliva concentrations of cortisol change in parallel after the ACTH challenge (Figure 1). Serum and saliva results from the current study are also significant and positively correlated (r = 0.255) whereas the correlation found in the Negrao et al. study reported an r value of 0.71 with n=192. We hypothesize that with a larger number of experimental units, we may have found results more similar to those of the previous study (Negrao et al., 2004).

The rise in cortisol after ACTH was slightly delayed in saliva samples compared to serum, and this may reflect the fact that cortisol is first released into the serum and then travels to the salivary glands where it diffuses into the saliva. These data support the potential

of saliva is a viable tool for assessment of cortisol concentration in dairy heifer calves ranging in age from 5 to 6 months. This assessment can be used in the future as a means of determining animal welfare based on stress responses in the calf. Samples may be collected by a minimally trained technician, removing the necessity for an individual who is trained to take tail vein blood samples. Furthermore, our saliva collection techniques and handling methods as described previously do not seem to elicit enough stress response in those animals to alter data collected if the animals are properly acclimated to the procedure.

Cortisol concentrations were higher in calves after the ACTH challenge than in the lactating cows that were not challenged with ACTH. This may indicate that those cows were not stressed by their environment or by the collection of the samples (neither blood nor saliva). Our hypothesis was that saliva sampling would be more stressful to the cows than tail vein blood sample collection because saliva collections required forcing open the mouth of the cows. The data do not support this hypothesis. Neither method of sample collection caused stress to the cows as measured by cortisol concentrations, as we saw no increase in cortisol concentration or significant differences in cortisol concentration between groups A (blood only), B (saliva only), or C (blood and saliva) (Figure 2). Either method could be used to collect samples to measure cortisol, but if the cows are not exposed to a stressor expected to increase cortisol concentration, then the saliva samples will likely have to lyophilized and concentrated in order to detect the hormone. Further research with beef animals is needed to determine if intensive rearing practices employed with dairy animals accounts for the lack of stress response to sampling procedures. If this same experiment were to be performed using minimally handled beef animals, results might show a much greater response to handling

required to attain both saliva and tail vein blood samples. One study conducted using beef heifers was able to collect saliva for cortisol assay without restraint because the animals were "very tame," thereby supporting the claim that intensively reared animals will not exhibit an increased stress response during saliva collection (Gonzalez et al., 2009).

In piglets, saliva cortisol concentrations were sufficiently high to measure in all samples. Our hypothesis was that the handling of the piglets necessary to collect saliva samples at this age would cause stress and result in increased cortisol concentrations such that female piglets would have concentrations similar to the males that were castrated. We found that the females, once accustomed to the sampling procedures (one to two collections), maintained constant cortisol concentrations throughout sampling. Boars showed a significant increase in stress response post-castration; however, these levels returned to baseline concentrations at the last collection, 120 minutes post-castration (Figure 3).

In conclusion, saliva samples can be used to measure cortisol in cattle, but unless a stimulus is applied, the concentrations will be below the sensitivity of the commercial assay used in this study. This would necessitate lyophilization and concentration of the saliva samples. In contrast, piglets' cortisol concentrations can be detected with normal assay procedures, but the handling required to collect the samples for this age piglet may in itself be a stressor, at least initially.

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APPENDIX

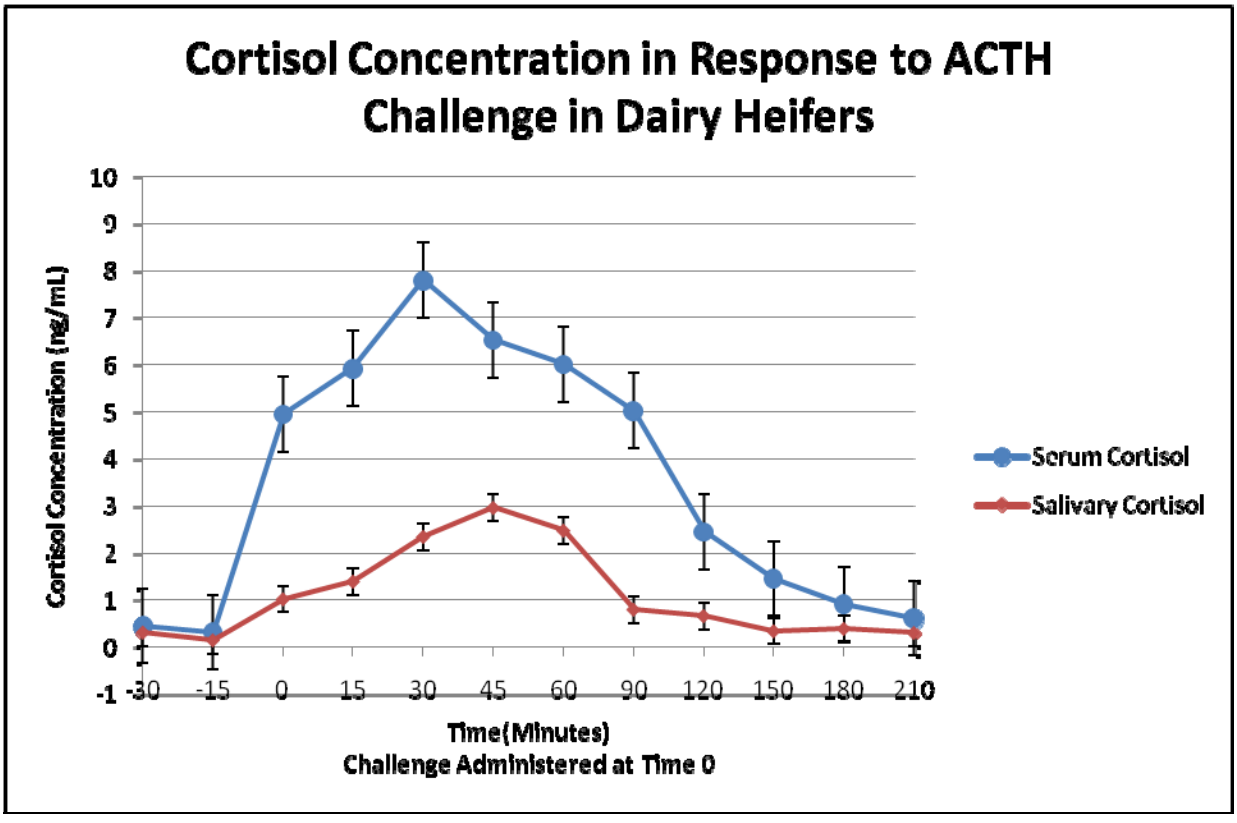
Cortisol Concentration in Response to ACTH Challenge in Dairy Heifers
Cortisol Concentration (ng/mL)
10
9
8
7
6
5
4
3
2
1
0
-1
-30
-15
0
15
30
45
60
90
120
150
180
210
Time(Minutes)
Challenge Administered at Time 0
Serum Cortisol
Salivary Cortisol

Figure 1: Cortisol concentration in response to ACTH challenge administered at time 0. Research conducted at the Dairy Education Unit at North Carolina State University in February of 2009.

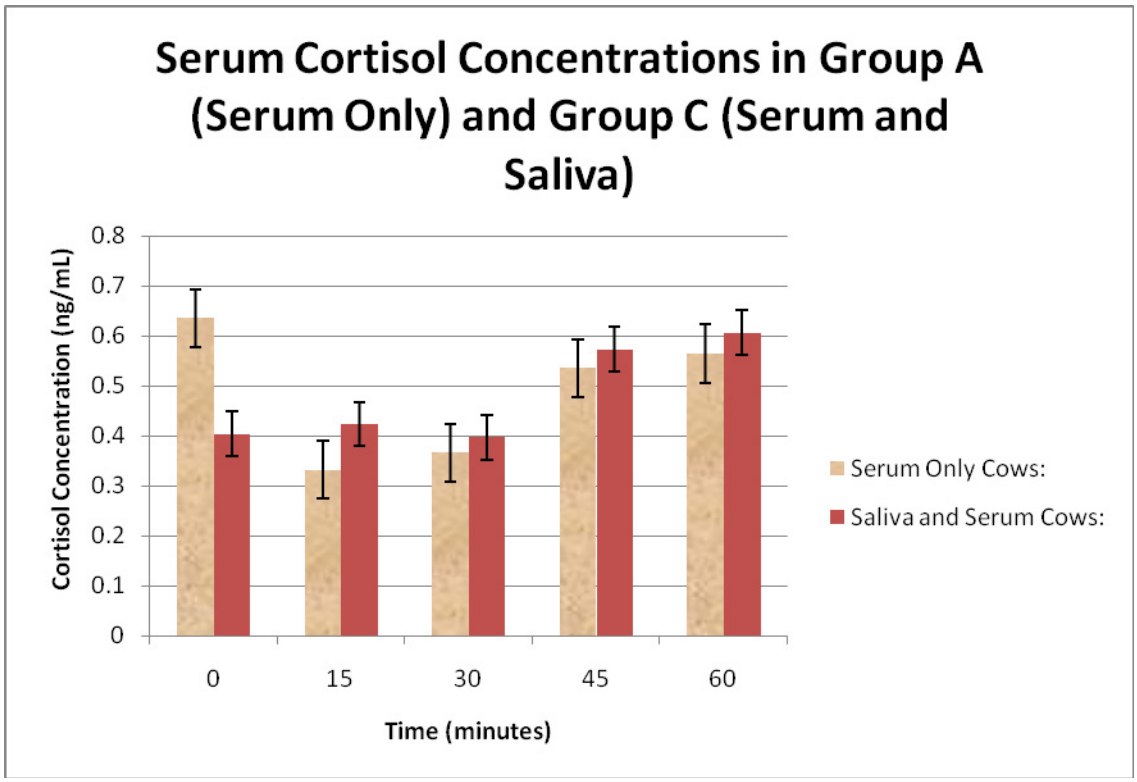

Figure 2: Cortisol concentrations as found in serum collected from lactating cows in 2 groups: Group A, only blood collected and Group C, both blood and saliva collected. Research conducted at the Dairy Education Unit at North Carolina State University in March of 2009.

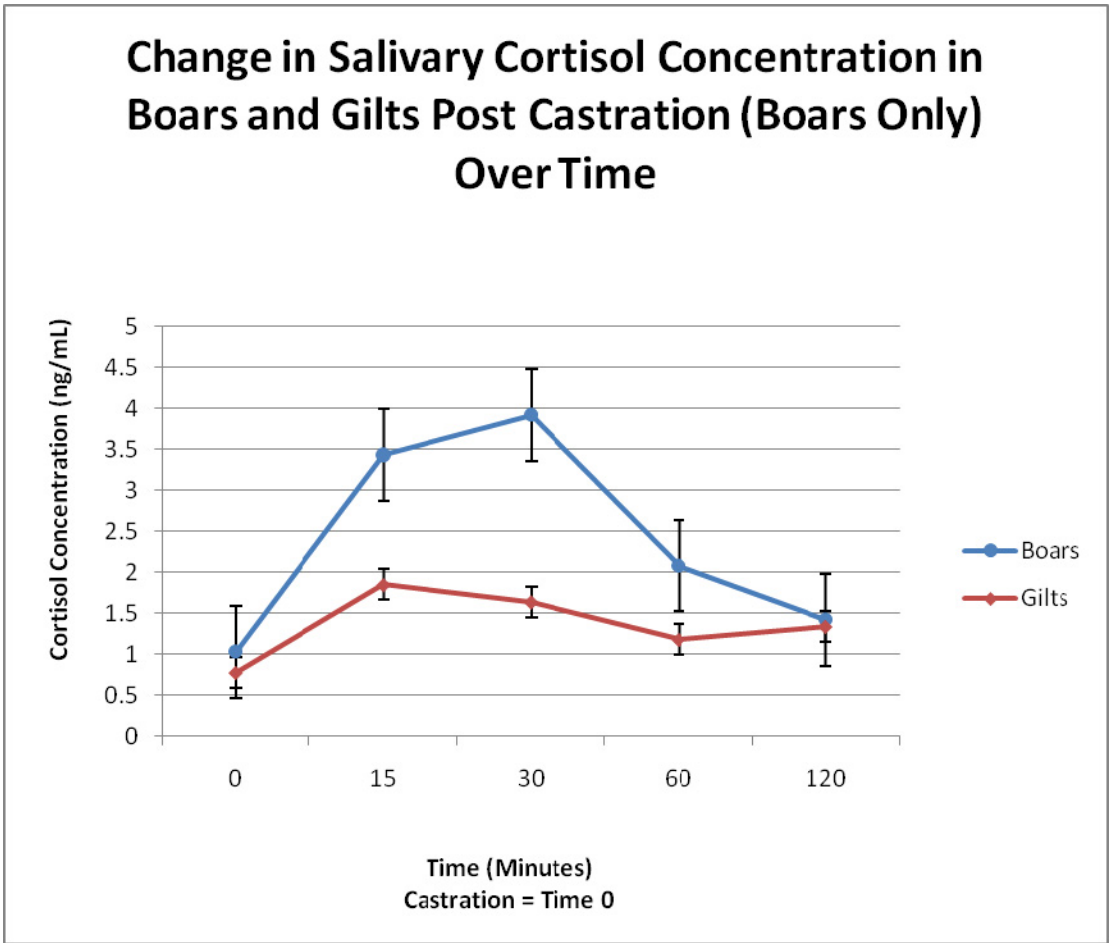

Figure 3: Cortisol concentrations as measured from saliva collected from 2 groups of piglets: Boars, with castration at time 0 and Gilts, without stress of castration. Research conducted at the Swine Education Unit at North Carolina State University in March of 2009.