CpG MOTIF-BASED ADJUVANT ENHANCES IMMUNOGENICITY OF A RECOMBINANT LHRH VACCINE AND NONINVASIVE MONITORING OF ADRENAL AND GONADAL FUNCTION IN THE JAGUAR (*Panthera onca*)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of VALÉRIA AMORIM CONFORTI find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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A recombinant ovalbumin-luteinizing hormone-releasing hormone (ova-LHRH) antigen has been developed for immunocontraception. In the first study, a novel immunostimulant for ova-LHRH immunization, CpG Oligodeoxynucleotide (ODN) 2006, was compared against *Mycobacterium butyricum* in female rats. Also, the immunogenicity of ova-LHRH after lyophilization and exposure to organic solvents was assessed. Rats received either ova-LHRH solubilized in urea; lyophilized ova-LHRH; lyophilized ova-LHRH exposed to methylene chloride; or lyophilized ova-LHRH exposed to ethyl acetate. After lyophilization, there was a decrease in immunogenicity of ova-LHRH. Exposure to ethyl acetate further decreased immunogenicity of ova-LHRH. CpG ODN 2006 was a more effective immunostimulant than *M. butyricum* for LHRH immunization. In the second study, ova-LHRH fusion protein was used for immunosterilization of heifers. Two adjuvants were compared for the dose of 3.4 mg ova-LHRH: Freund's complete adjuvant (FCA) and a novel CpG motif-based oligodeoxynucleotide (CpG ODN 2006). Additionally, increasing doses of ova-LHRH in CpG ODN in a water-in-oil (w-o) emulsion were tested. Seven treatment groups (n = 8 heifers per group) were used in this

study: 1) untreated control; 2) 1.5 mg ova-LHRH with CpG ODN in w-o; 3) 2.3 mg ova-LHRH with CpG ODN in w-o; 4) 3.4 mg ova-LHRH with CpG ODN in w-o; 5) 5.1 mg ova-LHRH with CpG ODN in w-o; 6) 7.6 mg ova-LHRH with CpG ODN in w-o; and 7) 3.4 mg ova-LHRH in FCA. Animals received two immunizations at weeks 0 and 14. Treatment with 3.4 mg CpG ODN 2006 resulted in higher production of LHRH antibodies compared to the same dose of ova-LHRH in FCA. Among all treatment groups that received CpG ODN/w-o as an adjuvant, the treatment group that resulted in optimal production of LHRH antibodies was that receiving a dose of 3.4 mg ova-LHRH. Compared to the untreated control group, all treatment combinations resulted in decreased reproductive tract weight at slaughter and decreased proportions of cyclic heifers as measured by serum progesterone concentrations. All heifers in the control group were cycling at the end of the study (week 27). Treatment groups 2, 4, 7 had 8 (out of 8) acyclic heifers at week 27. Treatment groups 3, 5, and 6 had one cyclic heifer at week 27. In summary, treatment with 3.4 mg ova-LHRH in CpG ODN 2006/w-o per immunization is recommended for LHRH immunization in heifers. A third study, independent study was conduct with the following objectives: 1) to validate a protocol for noninvasive assessment of changes in corticoid concentrations due to acute stress in jaguars by measuring fecal corticoid metabolite concentrations in males and females before and after ACTH injection; and 2) to investigate the relationship between fecal corticoid and androgen metabolite concentrations in male jaguars. Eight adult jaguars were used in this study: 3 intact males, 1 vasectomized male, 3 intact females, and 1 ovariectomized female. All animals, with the exception of the vasectomized male, which was used as an untreated control, were chemically restrained and treated with 500 IU/animal of ACTH gel to simulate the effects of acute stress (day 0). Fecal samples were collected for 20 consecutive days (days – 10 through 10). Samples were frozen, lyophilized, and

extracted in 90% ethanol. Extracts were assayed for corticoid (males and females) and androgen metabolites (males) by radioimmunoassay. Overall, there was a significant ($P < 0.01$) increase in fecal corticoid metabolite concentrations after ACTH injection (pre-ACTH: 924.79 ± 157.67 ng/g dry feces; post-ACTH: 2613.37 ± 443.54 ng/g). No significant effect of sex on corticoid metabolite concentrations was detected ($P > 0.05$). Androgen metabolite concentrations increased in males 1 and 2 in the post-ACTH injection period. In male 2 only, a (positive) correlation between corticoid and androgen metabolite concentrations was found (0.60; $P < 0.01$). Results from this study suggest the existence of individual differences in corticoid and androgen production associated with acute stress in male jaguars. To our knowledge, this is the first study that investigated the effects of acute stress after chemical restraint and ACTH injection on gonadal function in males and adrenal function in male and female jaguars. In conclusion, this study presents a simplified and efficient protocol for extracting and measuring steroid hormone metabolites in fecal samples of jaguars. This protocol allows for detection of changes in corticoid metabolites concentrations in fecal samples due to acute stress, and can be used by zoos interested in measuring the physiological effects of different handling procedures and other potential stressors on adrenal function in their jaguars. Ultimately, this protocol can be used as a tool to monitor the welfare of jaguars.

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Dedication

This dissertation is dedicated to my parents, Sérgio and Léa, who have always supported me in all my endeavors.

CHAPTER ONE

Literature Review

1. The Immune System

The immune system in vertebrates is a dynamic, sophisticated mechanism of protection, whose primary functions are self/non-self recognition and neutralization of antigens [Roitt et al., 2001]. Any molecule able to stimulate an immune response is considered an antigen. The immune system is constantly working for the body is regularly being exposed to antigens, usually in the form of biological infectious agents, or pathogens. In relatively rare cases, the immune system mistakenly identifies non-foreign components as foreign, which results in autoimmune conditions. Healthy immune systems, however, effectively distinguish and attack only what is foreign and have efficient mechanisms to prevent hypersensitivity (e.g. allergy) to antigens, which could cause unnecessary tissue damage. Various organs, tissues, types of cells and their different protein products must interact for efficient immunity. The basic components of the immune system and their major roles are discussed below.

a. Innate and Adaptive Immune Responses

Basically, the immune system responds to antigens, including pathogens, vaccine antigens, and other immunostimulatory agents such as adjuvant components, through two different, yet interacting, mechanisms: the innate and the adaptive immune responses. Both types of response have cell-mediated and humoral components, but only the adaptive immune response operates in an antigen-specific manner. Unlike the innate immune response, which has maximum reaction upon exposure to a threat, in the

adaptive immune response there is a period of time between exposure and maximum reaction. Also, the antigen-specific nature of the adaptive immune response allows for the creation of immunological memory that will enable the immune system to react more efficiently in case a second encounter with the antigen occurs.

i. The Cells of the Immune System

The cells of the immune system have their origin from stem cells in the bone marrow. These stem cells (hemocytoblasts) give rise to myeloblasts, lymphoblasts, monoblasts, which will in turn originate white blood cells (leukocytes). Hemocytoblasts can also differentiate into proerythroblasts and megakaryoblasts, which will originate red blood cells (erythrocytes) and platelets, respectively.

A myeloid precursor (myeloblast) originates the granulocytes, which are the leukocytes that contain cytoplasmic granules: neutrophils, basophils, and eosinophils. A lymphoid precursor (lymphoblast) gives rise to lymphocytes (B and T cells, and Natural Killer (NK) cells). Monoblasts originate monocytes/macrophages. Lymphocytes and monocytes do not contain granules; thus they are collectively called agranulocytes. Another class of immune cells – the dendritic cells, can originate from both myeloid and lymphoid precursors. Mast cells also contain granules, but are thought to have a precursor different than that of granulocytes.

Macrophages and dendritic cells are usually found in tissues – the latter type is particularly found in the interface between tissues and the external environment, such as the skin, nose, lungs, and digestive tract. Neutrophils, basophils, and eosinophils are

found in the blood stream. Mast cells are involved in allergic reactions and are present in mucous membranes and connective tissue.

The innate immune cells include leukocytes that can kill pathogens through contact or by engulfing them. These leukocytes include phagocytes (i.e. macrophages, dendritic cells, and neutrophils), as well as basophils, eosinophils, mast cells, and NK cells. Phagocytes are cells that are able to perform phagocytosis, which is the engulfment and digestion of pathogens and cellular debris. Cells from the innate immune response, such as macrophages, release cytokines to attract additional immune cells (including other phagocytes) to the site of infection. Cytokines also attract cells that will participate in the adaptive immune response.

The adaptive immune response involves production of antigen-specific antibodies by plasma cells as a result of B cell differentiation, which is stimulated by T cells. T cells have receptors named TcRs that distinguish them from other cell types of the immune system. T cells mature in the thymus before going to other organs and tissues and express either CD4 or CD8 markers. $CD4^+$ T cells will later differentiate into either type 1 helper T cell (Th1) or type 2 helper T cell (Th2). Th2 cells stimulate differentiation of B cells into plasma cells, which are able to produce antibodies. Th1 cells help $CD8^+$ T cells to differentiate into cytotoxic T cells.

ii. Cell-Mediated Response

The cell-mediated component of an immune response involves activation of cells that are able to recognize and attack infected cells. This action is performed by cell types such as macrophages, natural killer (NK) cells, and antigen-specific cytotoxic T cells.

NK cells have two types of surface receptors – killer activating receptor (KAR) and killer inhibiting receptor (KIR). Normal cells express surface proteins known as major histocompatibility complex (MHC) class I molecules, which are the ligands for KIR. Viruses- and bacteria-infected cells as well as tumorous target cells fail to express enough levels of MHC class I molecules and therefore, become a target to NK cells.

Some cells, named antigen-presenting cells (or APCs), specialize in phagocytosis of antigens and subsequent display of antigen parts on their surface as an antigen-MHC complex. Macrophages, as well as dendritic and B cells, can act as APCs. The displaying of antigen-MHC complexes by APCs is an important part of the interaction between cell-mediated and humoral responses given that it stimulates proliferation of helper T cells (Th). Also, an antigen-MHC complex simultaneously presents the antigen and indicates that the macrophage is not a pathogen. Antigen parts (epitopes) on infected cells are recognized by antigen-specific cytotoxic T cells, which initiate their lysis.

iii. Humoral Response

The Complement System

The humoral component of the innate immune response is characterized by the production of several types of proteins produced by macrophages, called complement

proteins. These proteins bind directly to the surface of microbes or to antibodies bound the microbe and activate the innate immune response. Complement proteins release proteases that start a biochemical cascade of events that signals the presence of a pathogen and attract additional immune cells. These proteases also directly damage the plasma membrane of the target cell contributing to its lysis. They are called complement proteins because their action is thought to be "complementary" to that of antibodies from the adaptive immune response.

Antibodies

The humoral component of the adaptive immune response involves production of Y-shaped glycoproteins known as immunoglobulins (IgGs) or antibodies. B cell differentiation into plasma cells can happen through different mechanisms.

One of the mechanisms involves the priming of naïve helper T (Th) cells by contact with antigen epitopes presented by APCs. Once primed, Th cells will stimulate differentiation of B cells into plasma cells upon contact with B cells presenting the same antigen that primed the Th cell. Differentiation of B cells into plasma cells is stimulated by cytokines released by the primed Th cell. Plasma cells will then produce antibodies that are specific for the antigen.

Th1/Th2 Model

Immune responses are characterized by a bias in the helper T cell differentiation. Basically, Th cells will differentiate into Th-1 or Th-2 types and the immune response will follow accordingly. Macrophages are stimulated by Th-1 cells and inhibited by Th-2 cells. Cytotoxic T-lymphocyte (CTL) stimulation is strong in Th-1, but not Th-2, immune responses.The bias in the response is characterized by a different cytokine and IgG profiles.

A Th1-biased response typically results in the release of the cytokines, such as interferon-gamma (IFN-γ), tumor necrosis factor-beta (TNF-β), and interleukin-12 (IL-12). Macrophages secrete IL-12 and NK cells or T cells secrete IFN-γ. Interferons downregulate protein synthesis in infected cells to suppress viral activity. TNF induces apoptosis. IL-12 and IFN-γ stimulate differentiation of naïve T cells into Th1 cells. Th1 cells in turn secrete cytokines (e.g. IFN-γ, TNF-β, IL-2, and lymphotoxin).

A Th2-biased response is characterized by the release of interleukins (IL) IL-4, IL-5, IL-13, and others. IL-4 stimulates differentiation of Th2 cells; Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13.

The types of immunoglobulins G (IgG) released differ between Th-1 and Th-2 responses – Th-1 cells stimulate production of antigen-specific IgG2a by B cells; IFN-γ induces switching to IgG2a, which is characteristic of a Th1 response [Finkelman et al., 1990]. A Th-2 type response favors switching to IgG1 and IgE. Thus, Th-1 and Th-2 responses differ in the ratio IgG1:IgG2a released in the event of a mixed response (i.e. release of both immunoglobulins). A Th-1 response releases more IgG2a than IgG1, and the opposite is true for a Th-2 response.

Th-1 responses are associated with strong cell-mediated immunity, and therefore it is important in case of intracellular pathogens. Humoral immunity is important in case of extracellular pathogens, and can be conferred by both Th-1 and Th-2 types of response. There are also undesirable side effects related to each type of response, for example, a Th-1 response can result in autoimmune conditions [Yip et al., 1999], whereas allergies and asthma might result from a Th-2 response [Kline et al., 1998; Klinman et al., 1999; Krieg, 1998[b]].

b. Vaccines

Edward Jenner, an English doctor, developed the first successful vaccine in the late 1700's. The vaccine was against *Vaccinia virus*, which caused smallpox in humans. Jenner actually used *Cowpox virus* in the vaccine preparation, which as the name indicates, causes pox in cattle. *Cowpox virus* and *Vaccinia virus* are related and both cause disease in humans, but cowpox is a much milder condition compared to smallpox. Jenner noticed that milkmaids that had contracted cowpox had become immune to further cowpox cases and to smallpox as well. He collected fluid from skin lesions of individuals recovering from cowpox and used it to immunize people against smallpox. The word vaccine comes from vacca, meaning cow in Latin.

The principle of vaccination is to introduce into a healthy organism an antigen that is either the inactivated or attenuated form of the pathogen that causes the disease, or an antigen that is less harmful but similar enough to that which causes the disease so that immunity against the target antigen is conferred.

Vaccines are usually administered before the exposure to the antigen occurs, but can also attenuate conditions or increase chances of survival when administered post-exposure. Pre-exposure prophylaxis uses vaccines to induce active immunity (i.e. production of antibodies by the patient). In cases of deadly diseases with rapid progression such as rabies, post-exposure prophylaxis includes both vaccination (active immunity) and administration of antibodies (passive imnunity) as a means to give fast immunity to the patient who already has the pathogen in their body. The rabies vaccine, originally developed by Louis Pasteur and Émile Roux in the late 1800's, contained inactivated rabies viruses harvested from affected rabbits and is an example of post-exposure vaccination. Since Jenner's discovery, vaccines against many different types of pathogens have been developed.

i. LHRH Vaccines

Autoimmune conditions have been induced by vaccination in experimental animals for the purpose of studying autoimmune diseases, such as rheumatoid arthritis. Autoimmunity occurs when the mechanisms of self/non-self recognition of the immune system mistakenly identify a self-component as foreign. Antibodies are raised against this component and self-tissue damage processes begin to take place. In the case of rheumatoid arthritis, it is thought that a pathogen that has enough structural similarity with joint proteins start an immune response in which the antibodies, for their relatively low specificity to the pathogen, are able to bind joint proteins as well. Vaccines can

mimic the triggering factors of autoimmune conditions by introducing an antigen that is similar to the target self-component.

Vaccines against reproductive hormones have been used to induce sterilization in animals. Luteinizing hormone-releasing hormone (LHRH) is a hypothalamic decapeptide that is essential for reproductive function in both sexes. LHRH is released from hypothalamic neurons and through hypothalamic-hypophyseal portal vessels reaches the pituitary, where it binds to receptors on gonadotrophs to stimulate secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Through circulation, gonadotropins reach the gonads (ovaries/testes) and stimulate secretion of reproductive hormones progesterone, estradiol, and testosterone.

There are many different types of LHRH vaccines being tested. Originally, LHRH vaccines had LHRH chemically conjugated to a carrier protein, which augments immunogenicity of the vaccine by increasing the size of the antigen molecule and by making the antigen appear foreign to the immune system. Antibodies raised against these antigen molecules will be structurally able to bind the endogenous hormone as well. These vaccines are still produced today for experimental purposes but they have the disadvantage of unpredictability of antigen chemical structure. Chemical conjugation results in the desirable antigen-carrier protein molecule but also results in antigen-antigen and carrier protein-carrier protein molecules as well. Thus, batches of vaccine produced by chemical conjugation are not consistent in antigen chemical structure, and therefore may not be approved by the FDA for commercialization. As an alternative, recombinant antigens have been produced with consistent chemical structure. A recombinant LHRH-ovalbumin (ova-LHRH) fusion protein has been expressed in *E. coli* cells transformed by

a plasmid containing LHRH inserts into a fragment of ovalbumin. This vaccine has successfully sterilized laboratory and domestic species, including cattle, sheep, and goats. The typical ova-LHRH immunization protocol is given in Freund's adjuvant.

ii. Adjuvants

Adjuvants increase the efficacy of vaccines by augmenting the immune response to an antigen. There are several mechanisms by which adjuvants act. For example, an adjuvant may increase the immune response by extending the time of antigen release in the body, distributing the antigen, presenting the antigen to immune cells, or by directly activating the immune system, among other actions. The first can be accomplished by entrapping the antigen in some reservoir (e.g. water-in-oil emulsions, antigen microencapsulation). The second is usually achieved through the presence of components that are normally recognized by the vertebrate immune system as parts of another organism, such as bacterial cell wall components or bacterial DNA. Some adjuvants are able to exert multiple actions.

The 'depot' effect of antigen entrapment results in the release of low antigen doses prolonged periods of time, which contributes to selective stimulation of B cells with high-affinity receptors and production of high-affinity antibodies [Siskind and Benacerraf, 1969].

At the injection site, dendritic cells and macrophages engulf and digest (endophagocytosis) the antigen/adjuvant material. Dendritic cells, macrophages, and phagocytes are able to detect presence of microorganisms through recognition of

pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs). These cells then migrate to lymph nodes and spleen, where they will interact with other immune cells for activation of antigen-specific antibody production by plasma cells.

To date, 10 TLRs have been identified [Akira, 2003]. Different adjuvants may activate different TLRs leading to distinct biological actions.

Adjuvants also play a role in directing the type of immnune response that a vaccine triggers. Some adjuvants, such as Cytosine Guanine oligodeoxynucleotide (CpG ODN) can be used to treat allergic reactions by turning a Th-2 into a Th-1 response [Choudhury et al., 1999; Metzger et al., 1999]. In cases where an autoimmune condition is intended, such as immunoneutralization of endogenous hormones (e.g. LHRH immunization), a Th-1 response might be more desirable than a Th-2 response. Below are the descriptions of three adjuvants of interest for LHRH immunization: CpG ODN, Freund's complete adjuvant (FCA), and Freund's incomplete adjuvant (FIA).

CpG ODN

Bacterial DNA is rich in motifs that contain unmethylated cytosines followed by guanines as dinucleotides, which are flanked by particular base sequences. These CpG motifs are usually differentiated by a hexanucleotide sequence that contains at least one CG dinucleotide. In vertebrates, the frequency of CG dinucleotides in DNA is about 3 to 4 times lower compared to bacterial DNA, a phenomenon known as CG suppression [Bird AP, 1986; Bird AP, 1987]. Also, cytosines are usually methylated on the 5' position in vertebrate DNA. The vertebrate system recognizes DNA containing CpG motifs (CpG

DNA) as non-self DNA [Bird AP, 1987], which triggers an immune response against the invading pathogens.

Because CpG DNA warns the immune system of danger [Krieg, 1996], it can act as an immunostimulant in vaccines. In fact, synthetic CpG oligodeoxynucleotides (ODNs), which can mimic the immunostimulatory effects of bacterial DNA [Krieg et al., 1995], have been successfully used as adjuvants.

Besides serving as adjuvants for protein or nucleic acid vaccines, CpG ODNs have several potential applications in prophylaxis, including immunization against bacterial, viral, fungal, and parasitic pathogens. Additionally, the effects of CpG motifs are being tested in cancer immunotherapy [Biragyn and Kwak,1999].

The initial (innate) response to the presence of CpG ODN is fast and not antigen-specific. During the innate response, there is proliferation of B cells [Hartmann et al 2000; Krieg 1996], activation of NK cells [Ballas et al., 1996], and release of cytokines [Krieg et al., 1999]. Cytokines attract additional immune cells that will then start an adaptive (antigen-specific) immune response.

In vitro, CpG ODN stimulates splenocytes to produce IL-12 and IFN-γ, characterizing a Th-1 response, and also production of IL-6, typical of a Th-2 response [Klinmann et al., 1996]. Phosphorothioate CpG ODN 2006 stimulated proliferation of spleen and lymph node cells of dogs and cats in vitro [Wernette et al., 2002]. Maximum stimulation depended on both oligonucleotide concentration and CpG motif – the GpC variant of the same oligonucleotide also stimulated cell proliferation in those tissues but to a lesser degree [Wernette et al., 2002].

In mice, CpG motifs stimulate macrophages to secrete cytokines (e.g. IL-12, TNF-α, and IFN-α/β) [Stacey et al., 1996]. IL-12 in turn stimulates NK cells to secrete IFN-γ [Ballas et al., 1996; Halpern et al., 1996]. CpG motifs also stimulate B cells and dendritic cells in the mouse.

CpG ODN acts through Toll-like receptor-9 (TLR-9) [Takeshita et al., 2001]. CpG motifs directly stimulate B cell proliferation and T cell-independent secretion of immunoglobulins, and increases their resistance to apoptosis [Messina et al., 1991; Halpern et al., 1996; Yi and Krieg, 1998]. IL-12, interferons (α/β), and tumor necrosis factor alpha (TNF-α) are examples of cytokines whose release is stimulated by CpG motifs. CpG-induced secretion of IL-12 by macrophages stimulates NK cells to mediate the rapid production of IFN-γ, independently of T cells [Chace et al., 1997, Halper et al., 1996].

Interestingly, if the CpG dinucleotides are eliminated from bacterial DNA sequence or if the cytosines become methylated, the immunomodulatory effect of bacterial DNA or ODN is reduced [Stacey et al., 1996; Ballas et al, 1996; Krieg et al., 1995].

Sensitivity to CpG motifs might be species-specific, as suggested by the poorer stimulation of dog and cat cells when another variant of CpG ODN was used, which is thought to be the best CpG ODN for stimulation of lymphocyte proliferation in the mouse [Wernette et al., 2002].

It appears that endophagocytosis of CpG DNA by immune cells is necessary for it to exert its immunostimulatory effects [Stacey et al., 1996]. Yi et al [1998] proposed a mechanism of action by which CpG motifs regulate cytokine and proto-oncogene

expression in B cells and monocytic cells. It starts with endocytosis of CpG DNA or CpG ODN into endosomal compartments where acidification takes place. Then, a pH-dependent, sequence specific generation of reactive oxygen species (ROS) would occur leading to phosphorilation and degradation of Inhibitor kappa B alpha (IκBα) and inhibitor kappa B beta (IκBβ) and subsequent activation of the transcription factor nuclear factor-kappa B (NF-κB), which is involved in cytokine and proto-oncogen expression.

Freund's Complete and Incomplete Adjuvants

Freund's adjuvants have been the adjuvant of choice for several experimental immunization protocols for decades [Broderson, 1989; Billiau and Matthys, 2001; Stills, 2005]. Both Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) are a combination of 85% light mineral oil and 15% mannide monooleate, which acts as a surfactant. The only difference between them is that FCA contains heat-killed and dried *Mycobacterial* cells as immunostimulants. Originally, *Mycobacterium tuberculosis* was the species used in FCA; *M. avium* and *M. butyricum* are used in more recent preparations.

Since the early and mid 20th century, when Freund tested this adjuvant [Freund et al., 1937; Freund J, 1951], the quality of mineral oil has improved by purification. Contaminates present in the original crude mineral oil, such as paraffins, olefins, and aromatic hydrocarbons are not present in mineral oil used currently in FCA or IFA, which reduces toxicity of these adjuvants [Stills, 2005]. Nonetheless, both adjuvants are

still known for causing several undesirable side effects, including skin lesions [Gendimenico and Mezick, 1995], granuloma formation and ulceration [Broderson, 1989], arthritis [Haak et al., 1996], and pneumonia [Broderson, 1989], which make them unacceptable for therapeutic use in humans and animals. Freund's adjuvants are still frequently used in laboratory animals for experimental and polyclonal antibody production purposes, but there is significant concern about pain and distress caused by these adjuvants, leading to testing of alternative adjuvants.

The oil component of Freund's adjuvants has other roles besides creating the 'depot' effect in water-in-oil emulsions: it contributes to distributing of antigen through the lymphatic system to lymph nodes and spleen and it interacts with antigen-presenting cells [Stills, 2005]. Oil can still be found in the body one-year post-injection of Freund's adjuvants [Bollinger, 1970]. Administration of Freund's adjuvant using multiple injection sites is recommended as a means of increasing the immune response while decreasing the injection volume and lesion [Stills and Bailey, 1991].

In susceptible strains of rodents, FIA alone (without autoantigens) is able to induce arthritis in an acute manner [Holmdahl and Kvick, 1992], while FCA (without autoantigens) can induce chronic arthritis [Pearson, 1956]. These adjuvants cause arthritis and skin lesions, including granulomas, through a systemic process mediated by the immune system. Granuloma formation is more severe after FCA injection compared to FIA [Billiau and Matthys, 2001].

Delayed-type hypersensitivity reactions that accompany a second injection of FCA, but not of IFA, depend on the presence of *Mycobacteria* [Raffel, 1948]. It has been speculated that heat shock protein (HSP)65 present in *Mycobaterial* cells would lead to

generation of HSP65-specific T cells and antibodies that cross-reacted with host HSP causing autoimmune disease. However, studies have shown that HSP immunization reduces success of subsequent autoimmunity-inducting procedures [van Eeden et al., 1998]. Besides HSPs, several other components of *Mycobacterial* cells are thought to contribute to the immune response characteristic of FCA's. Bacterial cell wall components, such as muramyl dipeptide (MDP) are known to have haematopoietic properties [Galelli and Chedid, 1983]. Trehalose dimycolate (TDM) and lipoarabinomannan (LAM) are glycolipids found in *Mycobateria* and also thought to have immunostimulant effects [reviewed by Billiau and Matthys, 2001]. CpG motifs are also present in mycobacterial cells and may contribute to the immunostimulatory effects of FCA. Unlike CpG ODNs, which activate TLR-9, the mycobacterial components of FCA activate TLR-2, -4, and -6 [reviewed by Stills, 2005]. Species of *Mycobacteria* differ in cell wall composition, leading to activation of different TLRs [Quesniaux, 2004].

Upon injection, both FCA and FIA trigger the innate immune response and undergo phagocytosis by local dendritic cells and phagocytes. Local cells containing engulfed antigen/adjuvant material travel to lymph nodes where they mature into APCs and present antigen epitopes to T cells [Billiau and Matthys, 2001]. During the innate immune response, there is release of cytokines and activation of the adaptive immune response, resulting in stimulation and proliferation of $CD4^+$ lymphocytes.

FIA directs helper T cells towards a Th2 type of response. The *Mycobaterial* cells and the oil component in FCA stimulate Th1 and Th2 cells, respectively [Billiau and Matthys, 2001], resulting in a Th1/Th2 mixed response [Weeratna et al., 2000]. FCA, but

not FIA, induces delayed-type hypersensitivity to autoantigens [Billiau and Matthys, 2001]. Unlike FIA, FCA triggers a 'danger' signal due to the presence of Mycobacterial cells, which are recognized by PAMP receptors. This 'danger' signal directs the immune system towards a Th1 type of response.

A simplification of the proposed model [Billiau and Matthys, 2001] for the activation of the cytokine network by FCA is as follows: mononuclear phagocytic cells (MPCs) and dendritic cells (DCs) start secreting TNF-α, IL-12, and IL-6 after detecting the presence of *Mycobacteria* through TLRs. IL-12 induces secretion of IFN-γ by NK cells. In a positive feedback, IFN-γ stimulates further production of IL-12, which stimulates helper T cells to differentiate into Th1 cells.

CpG ODN vs. Freund's Complete Adjuvant

The oil component in Freund's adjuvant stimulates a Th-2 response, while the presence of *Mycobacteria* contributes to a Th-1 type of response to antigens. The presence of both components in Freund's complete adjuvant results in a mixed Th-1/Th-2 type of response. The ratio of IgG2a:IgG1 (< 1) release in response to FCA indicates a Th-2 biased response [Weeratna et al., 2000]. CpG ODN alone also stimulates a mixed (IgG2a/IgG1) response, but it is Th-1 biased [Sato et al., 1996; Raz et al., 1996; Weeratna et al., 2000].

Administration of hen egg lysozyme (HEL) in IFA resulted in a Th-2 response to lysozyme. However, when HEL in IFA plus CpG ODN was administrated, the response to lysozyme switched to production of IgG2a, which is typical of a Th-1 response [Chu et

al., 1997]. These authors reported that immunization against HEL with IFA-CpG ODN (1826) resulted in secretion of antigen-specific IFN-γ that was 2- to 4-fold greater compared to HEL-CFA. Secretion of antigen-specific IFN-γ I creased with increased amounts of CpG ODN. Production of antigen-specific antibody was greater after immunization with HEL-IFA-CpG ODN (1826) compared to HEL-CFA.

In another study in mice [Weeratna et al., 2000], the use of CpG ODN along with FIA in a vaccine preparation against a hepatitis B surface antigen showed sinergistic effects resulting in higher antibody production than each of the adjuvants alone. Moreover, although FIA is known to stimulate Th-2 type responses, the combination of CpG ODN plus FIA increased the Th-1 bias of the response compared to CpG ODN alone. The combination of CpG ODN and FIA resulted in higher antibody production than FCA. However, the combination CpG ODN plus FCA was less immunostimulatory and less Th-1 biased than CpG ODN plus FIA. The authors suggest that CpG ODN and *Mycobacteria* might have similar mechanisms of action, which might be the reason for a lack of synergism. In fact, one should expect some similarity in their actions given that bacterial DNA with CpG motifs are probably present in the mycobacterial component of FCA's as well. Interestingly, synthetic CpG ODNs alone seem to be more immunostimulatory than whole *mycobacteria* cells, which likely present CpG motifs present in their DNA along with other bacterial components known to be immunostimulatory, such as bacterial cell walls.

Unlike FCA, which usually causes severe and sometimes lethal hypersensitivity reactions after a second administration [Broderson, 1989], CpG ODN in FIA has been

admnistered multiple times with no apparent increase in skin lesions incidence [Chapters 2 and 3].

Side effects of adjuvants, such as muscle damage at the injection site, seem to be more severe in the more immunostimulatory adjuvants. In mice, the combination of CpG ODN plus FIA caused more muscle damage than each one of these immunostimulants alone [Weeratna et al., 2000]. However, the combination of CpG ODN plus an aluminum hydroxide gel adjuvant (alum) was as immunostimulatory as CpG ODN plus FIA, but caused significantly less muscle damage. Alum alone did not cause muscle damage. Aluminum compounds also create a 'depot' effect for slow release of antigen through binding to antigen by hydrophobic interactions, hydrogen bonding, and van der Waals forces [Gupta and Rost, 2000].

The use of CpG ODN plus alum seems promising for it has the advantage of having a potent, strongly Th-1 biased immunostimulory action, with mild or no muscle damage.

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CHAPTER TWO

The effectiveness of a CpG motif-based adjuvant (CpG ODN 2006) for LHRH immunization

The effectiveness of a CpG motif-based adjuvant (CpG ODN 2006) for LHRH immunization

ABSTRACT: A recombinant ovalbumin-luteinizing hormone-releasing hormone (ova-LHRH) antigen has been developed for immunocontraception. In this study, a novel immunostimulant for ova-LHRH immunization, CpG Oligodeoxynucleotide (ODN) 2006, was compared against *Mycobacterium butyricum*. Also, the immunogenicity of ova-LHRH after lyophilization and exposure to organic solvents was assessed. Rats received either ova-LHRH solubilized in urea; lyophilized ova-LHRH; lyophilized ova-LHRH exposed to methylene chloride; or lyophilized ova-LHRH exposed to ethyl acetate. Immunogenicity of lyophilized ova-LHRH was reduced compared with solubilized ova-LHRH. Exposure to ethyl acetate further decreased immunogenicity of ova-LHRH. CpG ODN 2006 was a more effective immunostimulant than *M. butyricum* for LHRH immunization. This project was supported by National Research Initiative Competitive Grant no. 2003-35203-13514 from the USDA Cooperative State Research, Education, and Extension Service.

1. Introduction

Luteinizing Hormone-Releasing Hormone (LHRH) is a hypothalamic decapeptide essential for reproduction in both sexes. A recombinant vaccine against LHRH has been developed for sterilization of animals as an alternative to surgical castration [1]. The vaccine antigen is an LHRH fusion protein. It is expressed in *E. coli* cells containing a plasmid composed of ovalbumin (carrier protein) with seven LHRH inserts. Immunized animals produce antibodies that bind endogenous LHRH, preventing it from binding its receptors in the pituitary. As a result, gonadotropin secretion decreases and normal reproductive function is impaired.

The typical ovalbumin-LHRH (ova-LHRH) immunization protocol consists of a primary injection of antigen in a water-in-oil emulsion of modified Freund's complete adjuvant, followed by two booster injections of antigen in Freund's incomplete adjuvant. Modified Freund's complete adjuvant contains the immunostimulant *Mycobacterium butyricum*, instead of *M. tuberculosis*, present in Freund's complete adjuvant.

This vaccine produced satisfactory results in domestic and laboratory species in terms of anti-LHRH antibody production and sterilization [1,2,3]. However, the inflammatory process induced by Freund's adjuvant can cause skin lesions in rats [4] as well as arthritis in dogs [5]. Thus, acceptance for commercialization in pets will require an adjuvant other than Freund's.

Like bacterial DNA, synthetic cytosine guanine oligodeoxynucleotides (CpG ODN) are rich in CpG motifs, which are unmethylated CG dinucleotides flanked by specific bases. In mammalian DNA, however, cytosines are usually methylated and the

frequency of CG dinucleotides is relatively low due to a phenomenon called CpG suppression. Thus, mammals recognize CpG motifs as non-self DNA, which elicits an immune response. One potential advantage of CpG ODN over modified Freund's is that CpG might cause less tissue damage.

The second focus of this study was to investigate the effect of certain processes on the immunogenicity of ova-LHRH. Future trials for the preparation of this vaccine might include insertion of ova-LHRH into polymers. The idea would be to produce a vaccine where the antigen could be slowly released so that its concentrations would stay relatively high for a longer period of time. A slower release of the antigen might eliminate the need for booster injections.

A mix of polymers, such as lactide:glycolide, can entrap the antigen slowing down its rate of release. However, the placing of the antigen into polymers requires the antigen to be exposed to further processing. Lyophilization of the antigen may be desirable since its solid form may be more easily placed into the polymer. Additionally, the antigen may need to be exposed to polymer solvents during the mixing of polymer and antigen. It is therefore desirable to evaluate the effects of these processes (lyophilization and exposure to solvents) on the immunogenicity of ova-LHRH fusion protein.

The objectives of this study were: 1) to evaluate a CpG oligonucleotide (CpG ODN 2006) as an immunostimulant compared against *M. butyricum*; 2) to assess the immunogenicity of ova-LHRH after lyophilization; and 3) to assess the immunogenicity of ova-LHRH after being exposed to polymer solvents (methylene chloride or ethyl acetate).

2. Materials and methods

2.1. Animals

Fifty-four Sprague Dawley mature female rats were used in this study. At the start of the study, rats were between 3 and 10 months of age. They were stratified according to age and randomly assigned to one of the vaccine treatments. Rats were caged in pairs. Food (rat chow) and water were provided *ad libitum*. Each pair received the same vaccine treatment. All procedures conducted in this study were approved by the Washington State University Institutional Animal Care and Use Committee (LARC protocol #1780).

2.2. Production of the Antigen

The plasmid used to transform *E. coli* cells contained a fragment of the carrier protein ovalbumin with seven inserts of the LHRH sequence [1]. The resulting fusion protein, ova-LHRH, is approximately 55 KDa in size and is insoluble. Ova-LHRH contains a His-Tag at the C-terminus for protein purification purposes.

Ova-LHRH was solubilized in 6.5M guanidine and purified using nickel chelation chromatography. The purified protein was then dialyzed against 6M urea buffer for removal of guanidine. Briefly, the solution of ova-LHRH in guanidine buffer was inserted into dialysis cassettes (Slide-A-Lyzer®, Pierce) and immersed in urea buffer (4°C) overnight in a stirrer. During dialysis, urea buffer was changed several times. A

protein assay (BCA™ Protein Assay Kit, Pierce) was performed to determine the concentration of ova-LHRH in urea.

2.3. Lyophilization of the Antigen

Some treatment groups received lyophilized ova-LHRH. For those groups, before lyophilization, the solution containing ova-LHRH in urea buffer was dialyzed against water for removal of urea. The solution was inserted into dialysis cassettes (Slide-A-Lyzer®, Pierce) and immersed in double-distilled water (4°C) overnight in a stirrer. During dialysis, water was changed several times. Removal of urea caused the ova-LHRH to precipitate. The protein suspension in water was then removed from the cassettes and transferred to 50-mL conical plastic tubes and frozen. The openings of the tubes were covered with Kimwipe® and placed in a lyophilizer (-20°C) until dry.

2.4. Exposure of the Antigen to Polymer Solvents

Lyophilized ova-LHRH was exposed to: no solvent; solvent 1 (methylene chloride); or solvent 2 (ethyl acetate). Solvent was added to microcentrifuge tubes containing the dried ova-LHRH. The protein was exposed to solvent 1 overnight in a hood, which was enough time for complete evaporation of the solvent. Exposure of ova-LHRH to solvent 2 was done under air until complete evaporation of the solvent, which took less than 30 min.

2.5. Mycobacterium butyricum immunization protocol

The treatment groups that received *M. butyricum* as an immunostimulant received the primary injection of ova-LHRH (either in solution in urea or lyophilized) in modified Freund's complete adjuvant. The two booster injections had ova-LHRH in Freund's incomplete adjuvant. Modified Freund's complete adjuvant was composed of 0.1% dry cells of inactivated *M. butyricum* (Difco Laboratories, Detroit, MI) in 85% light mineral oil NF (Drakeol® 5; Penreco, Dickinson, TX) and 15% mannide monooleate (Sigma, St. Louis, MO). Freund's incomplete adjuvant was composed only of drakeol (85%) and mannide monooleate (15%).

2.6. CpG ODN 2006 immunization protocol

The treatment groups that received CpG ODN as an adjuvant received the primary injection and the two booster injections with a 2:1 ratio of ova-LHRH:CpG (25 μg CpG per dose) in Freund's incomplete adjuvant. The CpG ODN used in this study was the nuclease resistant phosphorothioate CpG ODN 2006 (Oligos Etc) presenting the following nucleotide sequence: TCGTCGTTTTGTCGTTTTGTCGTT [6].

2.7. Vaccine Preparations

The vaccine dose in all treatment groups was 50 μg ova-LHRH/100 μl/rat/injection. All vaccine preparations, including those with CpG ODN, contained Freund's incomplete adjuvant and were repeatedly mixed between two syringes until the consistency became

indicative of a water-in-oil emulsion. Vaccine preparations that contained ova-LHRH in urea buffer received an additional amount of phosphate saline buffer so that each dose had 50 µl of buffer. Freund's incomplete adjuvant was added so that each dose contained 50 µl of the oil. Vaccine preparations that contained lyophilized ova-LHRH also received buffer and oil so that each dose of 50 µg ova-LHRH contained 50 µl of buffer and 50 µl of oil. The water-in-oil emulsion was produced as described above.

2.8. Treatment Groups

Nine treatment groups (n = 6 rats per group) were used in this study: 1) ova-LHRH in urea with modified Freund's adjuvant; 2) lyophilized ova-LHRH with modified Freund's adjuvant; 3) lyophilized ova-LHRH, exposed to solvent 1, with modified Freund's adjuvant; 4) lyophilized ova-LHRH, exposed to solvent 2, with modified Freund's adjuvant; 5) ova-LHRH in urea with CpG ODN; 6) lyophilized ova-LHRH with CpG ODN; 7) lyophilized ova-LHRH, exposed to solvent 1, with CpG ODN; 8) lyophilized ova-LHRH, exposed to solvent 2, with CpG ODN; and 9) untreated control.

The ova-LHRH fusion proteins used in all immunizations were originated from the same batch. All treatment groups received a primary injection (week 0) followed by two booster injections (weeks 6 and 12). The primary injection and the first booster were given (IM) on alternate sides at the base of the tail; the second booster was given (SC) at the nape of the neck.

2.9. Sample Collection

Monthly, rats were weighed and blood samples were collected. Approximately 0.5 mL of blood was collected either by withdrawal from the ventral tail artery or by saphenous vein puncture. Blood samples were centrifuged (16,000 x g, 20 min, 4°C), and sera were separated and kept frozen (-20°C) until analysis.

In order to evaluate the longevity of the response to ova-LHRH immnunization, rats were kept until week 50. Then, rats were weighed and euthanized by decapitation after being anesthetized with a mixture of isoflurane and oxygen [7]. Blood samples were collected and the uteri, ovaries, and spleens were weighed. Ovaries were kept in Bouin's solution for histological evaluation.

2.10. Assessment of Anti-LHRH Antibody Production

Anti-LHRH antibody production was assessed by radioimmunoassay. Serum antibody binding activity was measured by the amount of ^{125}I-LHRH bound in 1:1000 diluted sera.

2.11. Ovarian Histology

At week 50, animals were classified according to ovarian activity as either cycling (presence of a corpus luteum); or non-cycling. Ovaries were dissected and fixed in Bouin's fluid. Tissue was dehydrated by immersion in increasing percentages of alcohol (70-100%). Xylene was used as a clearing agent. Ovaries were embedded in paraffin (58.6°C). Blocks of paraffin were sliced at 5 μm by a rotatory microtome. Five to twelve

sections were obtained from each ovary for slide preparation. Slides were stained by eosin/hematoxylin, and analyzed under a microscope.

2.12. Statistical Analysis

The effects of protein and adjuvant on the percentage of anti-LHRH antibody binding activity (^{125}I-LHRH % binding) were analyzed by Analysis of Variance (ANOVA) using the general linear models procedure (Proc GLM) of SAS Software (Version 9.1). The experimental design was completely randomized (CRD) with a two-way treatment structure (protein at four levels and adjuvant at two levels) with repeated measures (time). The factor "protein" refers to: ova-LHRH solubilized in urea; lyophilized ova-LHRH; lyophilized ova-LHRH exposed to solvent 1; or lyophilized ova-LHRH exposed to solvent 2. The factor "adjuvant" refers to either modified Freund's or CpG ODN. The control group was removed from the analysis of effects of protein and adjuvant on ^{125}I-LHRH % binding. Values are expressed as mean ± S.E.M. The level of significance was set at $P < 0.05$.

The effects of protein and adjuvant on the ^{125}I-LHRH % binding were also analyzed as a CRD with a two-way treatment structure (protein, adjuvant) at each observation time via ANOVA. In addition, Fisher's least significant difference (LSD) was used for all multiple comparisons when the ANOVA F test was significant. Normal distribution of data was verified by normality plots and by the Anderson-Darling and Wilk-Shapiro tests. Equality of variances was assessed by residual plots and by Levene's

test. When the assumptions of ANOVA were violated, nonparametric ANOVA was performed [8].

The correlation between ^{125}I-LHRH % binding and body weight was assessed using Pearson's bivariate correlation as implemented by SAS Proc Corr. The effects of protein and adjuvant on body weight at each observation time were assessed by ANOVA using Proc GLM. The correlations between ^{125}I-LHRH % binding and uterine and ovarian weights collected at week 50 were assessed using Proc Corr. The effects of protein and adjuvant on uterine and ovarian weights collected at week 50 were analyzed by ANOVA using Proc GLM, with both raw and ranked data.

The difference between treatment groups for the presence/absence of corpus luteum (binary response data) from ovarian histology were analyzed by logistic regression using Proc Logistic. If the overall likelihood ratio statistic was significant for differences between treatment proportions, pairwise comparisons were subsequently made between the control group and each treatment group using the likelihood ratio statistic.

3. Results

Sera from blood samples collected at weeks -1, 2, 10, 14, 19, 23, 27, 31, and 50 were assayed for LHRH antibody activity. There was a protein effect on the LHRH % binding. Immunization with ova-LHRH solubilized in urea resulted in higher ($P < 0.01$) LHRH % binding compared to lyophilized ova-LHRH.

The mean LHRH % binding of the two groups of ova-LHRH solubilized in urea combined (group 1, with modified Freund's as an adjuvant; and group 5, with CpG ODN as an adjuvant) was 30.9% ± 2.6 (Figure 1). When the results of two groups of lyophilized ova-LHRH (not exposed to solvents) were combined (group 2, with modified Freund's; and group 6, with CpG ODN), the mean LHRH % binding was 11.7% ± 2.6. There was also an effect of exposure of ova-LHRH to solvent 2 on LHRH % binding. Sera of rats immunized with lyophilized ova-LHRH that had been exposed to solvent 2 (groups 4 and 8), had lower ($P < 0.05$) LHRH % binding (3.8% ± 2.6) compared to lyophilized ova-LHRH that had not been exposed to any solvent (groups 2 and 6; 11.7% ± 2.6). Exposure of lyophilized ova-LHRH to solvent 1 (groups 3 and 7) did not affect ($P > 0.05$) LHRH % binding when compared to lyophilized ova-LHRH not exposed to solvents (groups 2 and 6) (8.8% ± 2.6 vs. 11.7% ± 2.6, respectively).

An adjuvant effect was observed (Figure 2). The vaccine treatments containing CpG ODN as an adjuvant resulted in higher ($P = 0.01$) LHRH % binding compared to those receiving modified Freund's as the adjuvant (17.1% ± 1.8 vs. 10.5% ± 1.8, respectively). There was no significant interaction between protein and adjuvant ($P > 0.1$).

Among the eight treated groups, the two individual treatment groups with the highest anti-LHRH antibody activity both contained ova-LHRH solubilized in urea (groups 1 and 5) (Figure 3); however, the treatment group that had CpG ODN 2006 as an adjuvant (group 5) had higher ($P < 0.05$) anti-LHRH antibody activity than the treatment group containing modified Freund's adjuvant (group 1). Also, the initial immune response, as measured by the LHRH % binding at week 2, was higher ($P < 0.01$) in the

group receiving ova-LHRH solubilized in urea with CpG ODN (7.8% ± 1.3) compared to ova-LHRH solubilized in urea with modified Freund's (1.5% ± 1.3).

There was a positive correlation ($P < 0.05$) between peak LHRH percent binding and body weight measured at the time of peak percent binding. However, neither treatment group, nor protein or adjuvant (separately) had an effect on body weight at any time point.

Before week 50, four rats died spontaneously, each one from a different treatment group, including the control group. One rat had to be euthanized separately due to poor health conditions.

At week 50, there was still a protein effect ($P < 0.01$) on the LHRH percent binding activity in serum. The treatment with ova-LHRH solubilized in urea resulted in the highest ($P < 0.01$) LHRH percent binding (16.4 % ± 1.9) compared to lyophilized ova-LHRH (5.7 % ± 1.8), lyophilized ova-LHRH exposed to methylene chloride (1.3 % ± 1.9), or lyophilized ova-LHRH exposed to ethyl acetate (1.1 % ± 1.9). At week 50, no adjuvant effect was detected ($P > 0.05$) by ANOVA, although the non-parametric analysis indicated an adjuvant effect ($P < 0.05$) in which CpG ODN resulted in higher anti-LHRH binding.

At the end of the study (week 50), the biological response to ova-LHRH immunization was evaluated by differences in ovarian and uterine weights as compared to the control group (Figure 4). There was a protein effect on both uterine and ovarian weights ($P < 0.05$). Rats with abnormal uterine contents (fluid or pus; n = 2) were eliminated from the uterine weight analysis. Rats that had been immunized with ova-LHRH solubilized in urea had smaller ($P < 0.05$) ovaries (0.03g ± 0.01) compared to the

control group (0.07g ± 0.01) and to those immunized with lyophilized ova-LHRH exposed to ethyl acetate (solvent 2) (0.08g ± 0.01). Uterine weight of rats immunized with ova-LHRH solubilized in urea (0.39g ± 0.09) did not differ ($P > 0.05$) from control rats (0.54g ± 0.10), but had smaller ($P < 0.05$) uteri compared to those immunized with lyophilized ova-LHRH exposed to no solvents (0.70g ± 0.10), exposed to methylene chloride (solvent 1) (0.64g ± 0.10), or exposed to ethyl acetate (solvent 2) (0.65g ± 0.09).

Across all treatments, there was a negative correlation between peak LHRH percent binding and uterine ($P < 0.05$) and ovarian ($P < 0.01$) weights. No correlation ($P > 0.5$) was observed between spleen weights and peak LHRH percent binding.

The results from the analysis of the binary data (presence/absence of a corpus luteum) from histological evaluations of ovaries using logistic regression are shown in Table 1. Overall, there was a significant effect ($P = 0.01$) of vaccine treatment on the probability of finding at least one corpus luteum on the ovaries of treated rats. With the exception of lyophilized ova-LHRH exposed to solvent 2 in modified Freund's ($P > 0.05$), all other vaccine treatments reduced the probability of presence of a corpus luteum ($P < 0.05$).

4. Discussion

In this study, the immunogenicity of a recombinant ovalbumin-LHRH fusion protein was tested in female rats using two types of adjuvants, modified Freund's and CpG ODN. Additionally, the effects of lyophilization and exposure of the protein to different organic solvents were investigated.

Lyophilization decreased the immunogenicity of the ova-LHRH antigen in the present study. The process of lyophilization exposes the protein to the stresses of both the freezing and drying processes [9] and can cause changes in the secondary structure of the protein [10]. Addition of stabilizing compounds seems to be necessary to maintain the native protein structure. Polyethylene glycol (PEG) is an example of a protein stabilizing agent that protects the protein structure during freezing [9]. The process of drying removes the hydration shell of the protein, making it unstable. Certain compounds, such as sugars, are able to act as water replacements for the hydration shell during drying by hydrogen-binding to the protein [11]. These compounds can stabilize proteins during dehydration. Studies have shown that a combination of PEG and sugars stabilizes enzymes during freezing and drying [9]. In this study, ova-LHRH was lyophilized without stabilizing agents, which might have resulted in destabilization of the protein and consequent decrease in its immunogenicity.

Another possible reason for the reduced immunogenicity of lyophilized ova-LHRH is that proteins in solid state may form aggregates [10], which might reduce the release of antigen from the injection site into the body system. Although formation of aggregates has been shown to increase the antigenicity of certain protein antigens [12], in the present study aggregation might have reduced the release of antigen. In the present study, although lyophilization could have potentially contributed to protein aggregation, the process of dialysis itself might have facilitated formation of protein aggregates. During dialysis against water, before lyophilization, we observed formation of clumps of protein. Thus, the absence of urea after dialysis against water might have contributed to

protein aggregation. Urea was removed before lyophilization and the presence of urea might be necessary for proper folding and epitope presentation of ova-LHRH.

Another possible reason for the decreased immunogenicity of lyophilized ova-LHRH is the fact that urea was removed before lyophilization and maybe the presence of urea is necessary for proper folding and epitope presentation of ova-LHRH.

Our future goals are to focus on the production of single dose LHRH vaccines with ova-LHRH encapsulated in poly(lactide-co-glycolide)-based microspheres. However, it is important to consider that encapsulation in microspheres might add factors that could affect the release of antigen, such as protein-polymer interactions [13]. This would further compromise the efficiency of the vaccine by increasing the amount of antigen that is unable to be released from the polymer [14].

In the preparation of single dose vaccines with microsphere-encapsulation of protein antigens, the protein is typically exposed to organic solvents in the protein loading step [13]. The choice of organic solvent is also an important factor to consider. Previous studies have shown that organic solvents, such as methylene chloride can cause denaturation of proteins [15,16,17]. Moreover, with the exception of protein C, most proteins are more sensitive to emulsification-induced denaturation after exposure to methylene chloride than to ethyl acetate [18]. In this study, the immunogenicity of lyophilized ova-LHRH was reduced by exposing it to ethyl acetate (solvent 2), but not to methylene chloride (solvent 1).

The choice of adjuvant is a critical factor in the making of an effective vaccine. CpG ODN activates the toll-like receptor 9 (TLR9) pathway [19], and causes a Th1-biased immune response [20]. However, the immune response to Freund's complete

adjuvant is Th2-biased [20]. This difference might explain why the immunostimulant CpG ODN was superior to *M. butyricum*. In this study, rats immunized against ova-LHRH with CpG ODN in Freund's incomplete adjuvant had significantly higher anti-LHRH antibody activity compared to those immunized with ova-LHRH with modified Freund's complete adjuvant. Moreover, following primary immunization, the comparison between the two groups immunized with ova-LHRH solubilized in urea showed that LHRH % binding was higher in the sera of rats receiving CpG ODN as the immunostimulant compared to those receiving *M. butyricum*. The superiority of another CpG ODN over *Mycobacteria* as an immunostimulant has also been reported in a study where mice were immunized against hepatitis B surface antigen in different adjuvants, including FCA (with *M. tuberculosis*) and CpG ODN (1826) plus FIA [21].

It is not known whether rats immunized against ova-LHRH with modified Freund's complete adjuvant required a booster injection to start showing anti-LHRH antibody activity in their sera, because we had only one blood sample collection between the primary and the first booster injections. However, rats immunized against ova-LHRH with CpG ODN had significant antibody activity before the first booster injection (Figure 3).

Throughout the whole study, the two treatment groups that showed the highest LHRH binding were those where ova-LHRH was solubilized in urea as opposed to lyophilized ova-LHRH. The results suggest that the water-in-oil emulsion with ova-LHRH solubilized in urea constitute a better delivery system of the antigen than the water-in-oil emulsion with lyophilized ova-LHRH.

A positive correlation between LHRH percent binding and body weight was observed. Although Christoffersen and colleagues [22] did not observe an increase in the body weight of castrated Sprague-Dawley male rats, an increase in the amount subcutaneous fat was reported. Thus, a possible reason for the positive correlation between peak LHRH percent binding and body weight observed in the present study is an increase in subcutaneous fat in treated females, although we did not assess this parameter.

Immunization with ova-LHRH solubilized in urea resulted in a biological response as measured by a decrease in ovarian weights as compared to the control animals. This is in agreement with previous studies in which LHRH immunization caused gonadal regression in the bovine [3,23]. The biological response to ova-LHRH immunization was also reflected in the reduced probability of presence of a corpus luteum in most treatment groups.

In summary, lyophilization and exposure to ethyl acetate reduced the immunogenicity of recombinant ova-LHRH. Moreover, modified Freund's complete adjuvant, which has been typically used as an immunostimulant in protein vaccines, was less efficient than CpG ODN 2006 in Freund's incomplete adjuvant. In this study, the immunization treatment against LHRH that resulted in the highest immune response was composed of ova-LHRH solubilized in urea plus CpG ODN 2006 in an oil emulsion adjuvant. Future studies with this treatment should address the tissue integrity at the injection site in food and pet species.

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Table 1. Effect of vaccine preparation on ovarian histology of rats immunized with ova-LHRH as measured by the probability of presence of a corpus luteum at week 50.

Group[†]	Ovarian Cyclicity[‡] (%)
Solubilized in urea (mFreund's)	20[b]
Lyophilized (mFreund's)	33[a]
Lyophilized solv. 1 (mFreund's)	20[b]
Lyophilized solv. 2 (mFreund's)	80
Solubilized in urea (CpG ODN)	0[c]
Lyophilized (CpG ODN)	20[b]
Lyophilized solv. 1 (CpG ODN)	20[b]
Lyophilized solv. 2 (CpG ODN)	33[a]
Control	100

[†]Group indicates the immunization treatment according to type of ova-LHRH fusion protein used as an antigen and type of adjuvant (in parenthesis).

[‡]Ovarian cyclicity indicates the probability of finding a corpus luteum.

Superscripts indicate differences ([a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$) compared to control group.

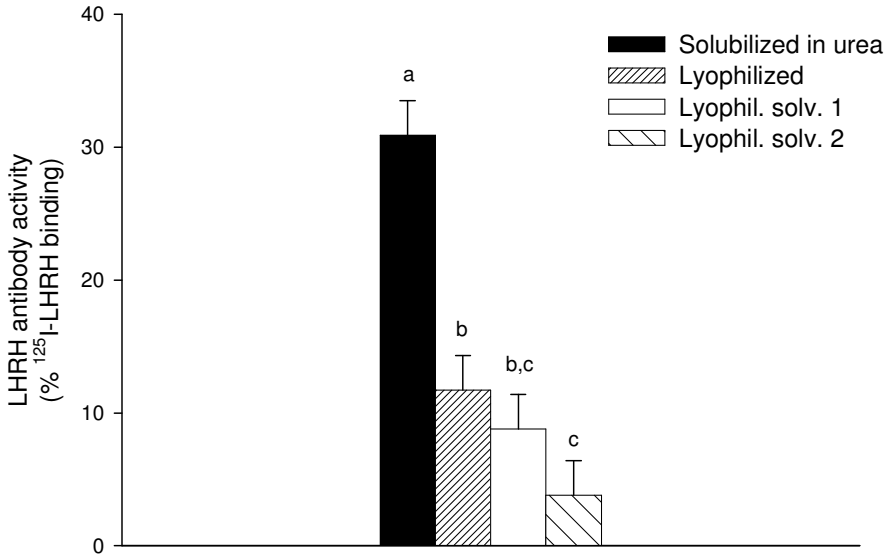

Figure 1. Mean activity of anti-LHRH antibodies in serum of rats immunized with: ova-LHRH solubilized in urea, lyophilized ova-LHRH, lyophilized ova-LHRH exposed to solvent 1 – methylene chloride, or lyophilized ova-LHRH exposed to solvent 2 – ethyl acetate. N = 12. Error bars indicate S.E.M. Means without a common letter differ ($^{a,b}P < 0.01$; $^{b,c}P < 0.05$).

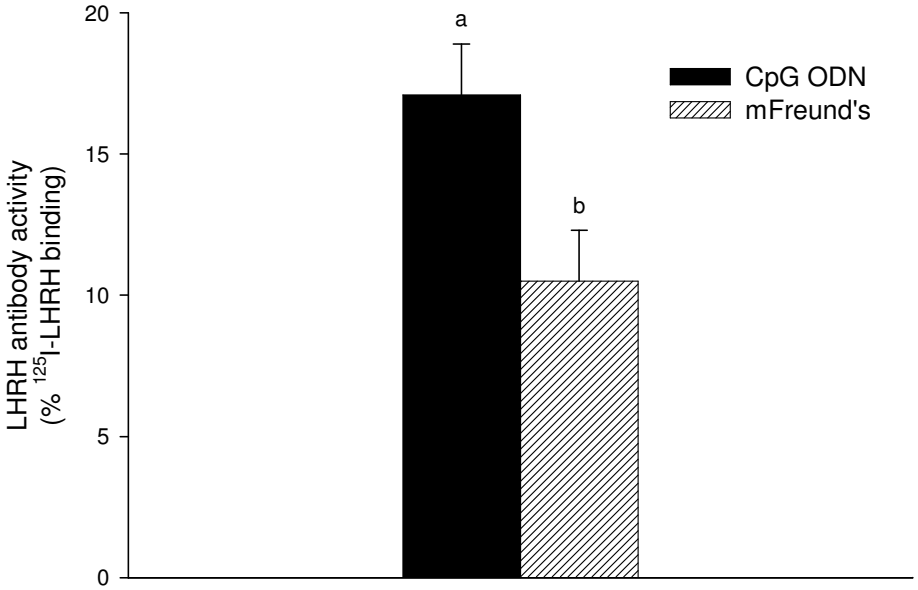

Figure 2. Mean activity of anti-LHRH antibodies in serum of rats immunized with ova-LHRH with CpG ODN 2006 in Freund's Incomplete Adjuvant or ova-LHRH in modified Freund's Complete Adjuvant. N = 24. Error bars indicate S.E.M. Different letters indicate difference ($^{a,b}P = 0.01$).

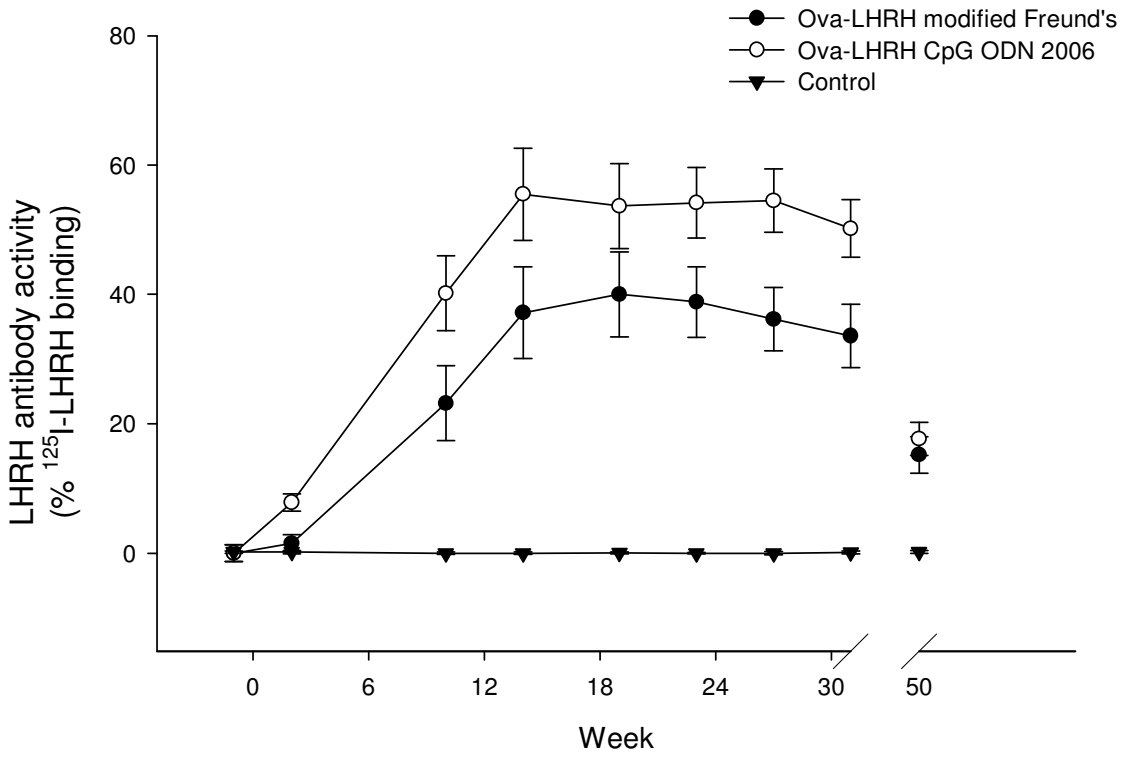

Figure 3. Percentage of binding of ^{125}I-LHRH in serum of rats immunized against ova-LHRH with modified Freund's adjuvant (●-●), ova-LHRH with CpG ODN 2006 (○-○), or untreated control (▼-▼). Rats were immunized at weeks 0, 6, and 12. N = 6. Values are mean ± S.E.M.

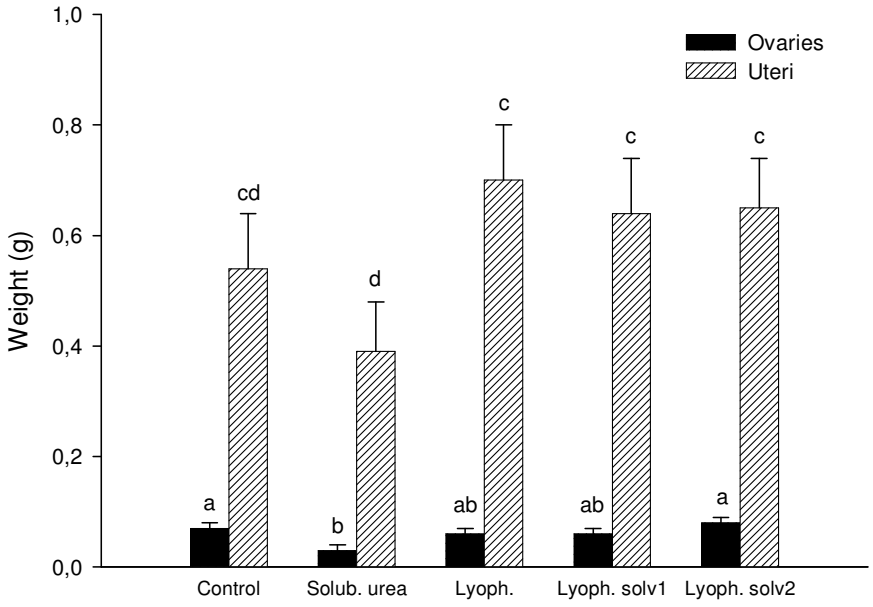

Figure 4. Ovarian and uterine weights at week 50 of untreated rats (control; n = 5); rats immunized with ova-LHRH solubilized in urea (n = 11); or lyophilized ova-LHRH (n = 12); lyophilized ova-LHRH exposed to methylene chloride (solvent 1; n = 11); or lyophilized ova-LHRH exposed to ethyl acetate (solvent 2; n = 11). Bars indicate S.E.M. Differences ($P < 0.05$) in organ weight among treatment groups are indicated by different superscripts (^{a,b}ovaries; ^{c,d}uteri).

CHAPTER THREE

CpG Motif-based Adjuvant Enhances Immunogenicity of a Recombinant LHRH Vaccine

CpG Motif-based Adjuvant Enhances Immunogenicity

of a Recombinant LHRH Vaccine

ABSTRACT: A recombinant ovalbumin-LHRH (ova-LHRH) fusion protein was used in this study for immunosterilization of heifers. Two adjuvants were compared for the dose of 3.4 mg ova-LHRH: Freund's complete adjuvant (FCA) and a novel CpG motif-based oligodeoxynucleotide (CpG ODN 2006). Additionally, increasing doses of ova-LHRH in CpG ODN in a water-in-oil (w-o) emulsion were tested. Seven treatment groups (n = 8 heifers per group) were used in this study: 1) untreated control; 2) 1.5 mg ova-LHRH with CpG ODN in w-o; 3) 2.3 mg ova-LHRH with CpG ODN in w-o; 4) 3.4 mg ova-LHRH with CpG ODN in w-o; 5) 5.1 mg ova-LHRH with CpG ODN in w-o; 6) 7.6 mg ova-LHRH with CpG ODN in w-o; and 7) 3.4 mg ova-LHRH in FCA. Animals received two immunizations at weeks 0 and 14. Treatment with 3.4 mg CpG ODN 2006 resulted in higher production of LHRH antibodies compared to the same dose of ova-LHRH in FCA. Among all treatment groups that received CpG ODN/w-o as an adjuvant, the treatment group that resulted in optimal production of LHRH antibodies was that receiving a dose of 3.4 mg ova-LHRH. Compared to the untreated control group, all treatment combinations resulted in decreased reproductive tract weight at slaughter and decreased proportions of cyclic heifers as measured by serum progesterone concentrations. All heifers in the control group were cycling at the end of the study (week 27). Treatment groups 2, 4, 7 had 8 (out of 8) acyclic heifers at week 27. Treatment groups 3, 5, and 6 had one cyclic heifer at week 27. In summary, treatment with 3.4 mg

ova-LHRH in CpG ODN 2006/w-o per immunization is recommended for LHRH immunization in heifers.

Introduction

Pregnancy in feedlot heifers is undesirable for economic and humane reasons. Each year, twelve million heifers enter the feedlots in the U.S. [USDA, 2000]; three to 20% of them are pregnant [Edwards and Laudert, 1984]. The economic value of open heifers is approximately $66.00 more than pregnant heifers [Jim et al., 1991]. Palpation and administration of abortifacients might be used in feedlot heifers; however, induction of abortion and other pregnancy-related conditions, such as dystocias and cesarean-sections, increase both health risks and production costs. Estrous cyclicity is also unwanted in the feedlots for it leads to behaviors, such as mounting, that may increase risk of injuries. Thus, suppression of estrus is desirable in feedlot heifers.

Traditional methods for suppression of estrus in heifers include daily addition of the synthetic progestin melengestrol acetate (MGA) to feed [Bloss et al., 1966]. MGA can suppress estrus in nearly 100% of treated animals [Cook et al., 2001], but has been associated with increased susceptibility to pneumonia in cattle [McAllister et al., 2002]. Permanent sterilization by spaying is used in heifers [Horstman et al., 1982]. Costs associated with this procedure are not high, but there is a certain amount of death loss.

Alternatively, immunization against luteinizing hormone-releasing hormone (LHRH) has been tested as a method of sterilization in several laboratory [Kumar et al., 2000; Ferro et al., 2004], domestic [Ferro et al., 2004], and other species [Miller et al., 2000; Miller et al., 2004]. Those studies used LHRH chemically conjugated to a carrier protein for immunogenic purposes.

LHRH is a decapeptide hormone produced by the hypothalamus. It is responsible for stimulating the synthesis and secretion of LH and FSH in the pituitary and therefore, essential for reproduction in both sexes. LHRH immunization results in production of antibodies that neutralize the endogenous hormone, preventing it from binding to its receptors in the pituitary. Consequently, serum gonadotropin concentrations become insufficient for proper gonad stimulation and reproductive function is impaired.

A vaccine using a recombinant ovalbumin-LHRH (ova-LHRH) fusion protein has been developed [Zhang et al., 1999]. Ova-LHRH is expressed in E. coli cells and is synthesized as a 55-kDa protein. Immunization against ova-LHRH has resulted in gonadal suppression in several species, including mice [Zhang et al., 1999]; rats [Conforti et al., 2007]; rams [Ülker et al., 2005], and cattle [Sosa et al., 2000; Hernandez et al., 2005; Stevens et al., 2005; Geary et al., 2006]. Unlike chemically conjugated LHRH antigens, recombinant LHRH fusion proteins are produced with predictable chemical structure, which would facilitate future approval of the vaccine for commercialization.

The typical ova-LHRH immunization protocol includes a primary injection in modified Freund's complete adjuvant (FCA) followed by two booster injections of the antigen in Freund's incomplete adjuvant (FIA). Modified FCA contains inactivated *Mycobacterium butyricum*, instead of *M. tuberculosis*. FIA, which is the basis of all Freund's adjuvants, is simply a combination of paraffin oil and mannide mono-oleate. Freund's adjuvants have been the adjuvant of choice for immunization in laboratory animals. However, these adjuvants have been associated with several local and systemic pathologies, including skin lesions (e.g. necrosis and ulceration), pneumonia [reviewed by Broderson, 1989] and arthritis [Haak et al., 1996]. FIA alone is able to induce arthritis

in an acute manner [Holmdahl and Kvick, 1992], while FCA can induce chronic arthritis [Pearson, 1956].

Recently, synthetic cytosine guanine oligodeoxynucleotides (CpG ODNs) adjuvants have shown to produce satisfactory immunostimulation [Zhang et al., 2003; Conforti et al., 2007]. These adjuvants contain nucleotide sequences with a relatively high frequency of unmethylated CG dinucleotides, which mimics the immunostimulatory effects of bacterial DNA, which is identifiable as non-self DNA by the mammalian immune system. Unlike FCA, CpG ODN's stimulate a Th-1 biased immune response [Vollmer et al., 2004]. Additionally, CpG ODN's might cause less tissue damage than FCA. Thus, CpG ODN may be an alternative to Freund's adjuvants as immunostimulants in animals.

The objectives of this study were: 1) to compare CpG ODN against modified Freund's adjuvant as an immunostimulant; and 2) to compare different doses of ova-LHRH as an antigen plus CpG ODN for LHRH immunization in heifers.

Material and Methods

Animals

Fifty-six crossbred heifers were used in this study. At the start of the study, heifers were between 11 and 12 months of age. They were stratified according to body weight and randomly assigned to one of the vaccine treatments. Heifers were housed in groups of 5 to 6 individuals per pen with mixed treatment groups. The diet consisted of 70% barley,

4% supplement, 2% blue grass straw, 4% alfalfa, and 20% potato byproduct on an as-fed basis. Water was provided *ad libitum*. All procedures conducted in this study were approved by the Washington State University Institutional Animal Care and Use Committee (LARC protocol #1780).

Production of the Antigen

E. coli cells were transformed by a plasmid containing seven inserts of the LHRH sequence in a fragment of the carrier protein ovalbumin [Zhang et al., 1999]. The resulting recombinant ovalbumin-LHRH (ova-LHRH) fusion protein is approximately 55 KDa in size and contains a His-Tag at the C-terminus for protein purification purposes.

Briefly, ova-LHRH was solubilized in 6.5M guanidine buffer, purified by nickel chelation chromatography, and then dialyzed against 6M urea buffer for removal of guanidine. Ova-LHRH in guanidine buffer was placed in dialysis cassettes (Slide-A-Lyzer®, Pierce) and immersed in urea buffer (4°C) overnight in a stirrer. During dialysis, urea buffer was changed several times. The concentration of ova-LHRH in urea was assessed by protein assay (BCA™ Protein Assay Kit, Pierce).

Mycobacterium butyricum immunization protocol

The treatment group that received *M. butyricum* as an immunostimulant had the primary injection of ova-LHRH in Freund's complete adjuvant. The booster injection had ova-

LHRH in Freund's incomplete adjuvant. Freund's complete adjuvant was composed of 1.0 mg dry cells of inactivated *M. butyricum* (Difco Laboratories, Detroit, MI) in 85% light mineral oil NF (Drakeol® 5; Penreco, Dickinson, TX) and 15% mannide monooleate (Sigma, St. Louis, MO). The difference between modified Freund's complete adjuvant and Freund's incomplete adjuvant is that the latter does not contain *Mycobacteria*.

CpG ODN 2006 immunization protocol

The treatment groups that received CpG ODN as an immunostimulant had both the primary and the booster injections with 1.0 mg CpG ODN 2006 in 85% light mineral oil NF and 15% mannide monooleate, which produced a water-in-oil (w-o) emulsion. CpG ODN 2006 (Oligos Etc) is a nuclease resistant phosphorothioate with the following nucleotide sequence: TCGTCGTTTTGTCGTTTTGTCGTT [Zhang et al., 2003].

Treatment Groups, Vaccine Preparations, and Immunizations

Seven treatment groups (n = 8 heifers per group) were used in this study: 1) untreated control; 2) 1.5 mg ova-LHRH with CpG ODN in w-o; 3) 2.3 mg ova-LHRH with CpG ODN in w-o; 4) 3.4 mg ova-LHRH with CpG ODN in w-o; 5) 5.1 mg ova-LHRH with CpG ODN in w-o; 6) 7.6 mg ova-LHRH with CpG ODN in w-o; and 7) 3.4 mg ova-LHRH in FCA.

Vaccine doses in all treatment groups contained 2.6 ml of urea buffer with the proper amount of ova-LHRH, 4.2 ml FIA, 1.6 ml double-distilled water, and 100 µl hemaglutin buffer (used to dissolve CpG ODN in the treatment groups that received CpG ODN). Vaccine preparations were repeatedly mixed between two syringes with a double hub connector until a water-in-oil emulsion was obtained.

All treatment groups received a primary injection (week 0) followed by one booster injection (week 14). Both immunizations were given (SC) on alternate sides of the neck and divided into 3 injection sites. The volume of each dose was approximately 8.5 ml. Ova-LHRH protein used in each immunization was originated from the same batch for all treatment groups.

Sample Collection

Heifers were weighed every 28 days. Weekly blood samples were collected by withdrawal from the coccygeal vein from weeks –5 through 27. Blood samples were centrifuged (2,600 x g, 30 min, 4°C), and sera were separated and kept frozen (-20°C) until analysis. At week 28, heifers were slaughtered and carcass data were collected. The ovaries and uteri were collected, refrigerated, transported to the laboratory and weighed.

LHRH Antibody and Progesterone Assays

LHRH antibody production, as measured by ^{125}I-LHRH % binding in sera at 1:10^3 and 1:10^4 serum dilutions, was assessed by radioimmunoassay (RIA).

A commercial double antibody RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX) was used for assessment of serum progesterone levels.

Evaluation of Biological Response to the Vaccine and Determination of Cyclicity Status

The biological response to the vaccine was measured by assessment of serum progesterone concentrations, weight of the reproductive tract (uteri and ovaries) at slaughter, and evaluation of the ovaries for presence of a corpus luteum.

A heifer was considered to be cycling at an observation time if the progesterone concentration at the observation time or at any of the two consecutive times (before or after) was equal or greater than 1 ng/ml.

Statistical Analysis

The effects of vaccine treatment on the percentage of LHRH antibody binding (^{125}I-LHRH % binding) were analyzed by Analysis of Variance (ANOVA) using the general linear models procedure (Proc GLM) of SAS Software (Version 9.1). The experimental design was completely randomized (CRD) with a one-way treatment structure (at six levels) with repeated measures (time). The factor treatment (antigen (adjuvant)) refers to: 1.5 mg ova-LHRH (CpG ODN/w-o); 2.3 mg ova-LHRH (CpG ODN/w-o); 3.4 mg ova-LHRH (CpG ODN/w-o); 5.1 mg ova-LHRH (CpG ODN/w-o); 7.6 mg ova-LHRH (CpG ODN/w-o); or 3.4 mg ova-LHRH (FCA). The control group was removed from the

analysis of effects of vaccine treatment on ^{125}I-LHRH % binding. Values are expressed as Least Squares Means ± S.E.M. Level of significance was set at 0.05.

The effects of vaccine treatment on the ^{125}I-LHRH % binding were also analyzed as a CRD with a one-way treatment structure (treatment) at each observation time via ANOVA. In addition, Fisher's least significant difference (LSD) was used for all multiple comparisons when the ANOVA F test was significant. Normal distribution of data was verified by normality plots and by the Anderson-Darling and Wilk-Shapiro tests. Equality of variances was assessed by residual plots and by Levene's test. When the assumptions of ANOVA were violated, nonparametric ANOVA was performed [Zar, 2006].

The effects of treatment on serum progesterone levels were also analyzed by ANOVA with Repeated Measures, ANOVA by time, and non-parametric ANOVA.

The difference between treatment groups for cyclicity status (binary response data: cycling or not) from assessment of progesterone levels at week 27 were analyzed by logistic regression using Proc Logistic. If the overall likelihood ratio statistic was significant for differences between treatment proportions, pairwise comparisons would subsequently be made between the control group and each treatment group using the likelihood ratio statistic. The same analysis was used to assess differences between treatment groups and the control group for the presence/absence of corpus luteum (binary response data) from ovarian evaluation at slaughter (week 28).

Means of reproductive tract (uteri and ovaries) weight, ovarian weight, body weight (BW), and average daily gain (ADG) were compared among treatment groups by parametric and non-parametric ANOVA. Correlations between ^{125}I-LHRH % binding,

reproductive tract and ovarian weights, start body weight, BW, and ADG were assessed using Pearson's bivariate correlation as implemented by SAS Proc Corr.

Results

Results from the analysis of % LHRH antibody binding in sera diluted at $1:10^3$ show an interaction between treatment and time ($P < 0.0001$). When % LHRH antibody binding was analyzed at each observation time, LSD's indicated differences between treatment groups towards the end of the study (weeks 25-27). At week 27, the % LHRH antibody binding for each treatment group were: 27.5 % ± 6.5 (1.5 mg ova-LHRH in CpG ODN/FIA); 10.4 % ± 6.5 (2.3 mg ova-LHRH in CpG ODN/FIA); 36.4 % ± 6.5 (3.4 mg ova-LHRH in CpG ODN/FIA); 14.8 % ± 6.5 (5.1 mg ova-LHRH in CpG ODN/FIA; 15.8 % ± 6.5 (7.6 mg ova-LHRH in CpG ODN/FIA); and 14.6 % ± 6.5 (3.4 mg ova-LHRH in FCA). Sera of heifers immunized with 3.4 mg ova-LHRH with CpG ODN/FIA had higher ($P < 0.05$) LHRH % binding compared to all other treatment groups, except group 2 (1.5 mg ova-LHRH with CpG/FIA) ($P > 0.05$) Table 1.

A comparison between adjuvants for the dose of 3.4 mg ova-LHRH showed that the use of CpG ODN in FIA resulted in higher ($P < 0.05$) LHRH antibody production than FCA (Figures 1).

Serum progesterone levels decreased ($P < 0.05$) in all treatment groups compared to the control group. At week 27, the percentages of cycling heifers in treatment groups were highly reduced ($P < 0.001$) by treatment. Also, results from the analysis of the binary data (presence/absence of a corpus luteum) from physical evaluations of ovaries at

week 28 using logistic regression showed that all vaccine treatments reduced the probability of presence of a corpus luteum ($P < 0.0001$).

Overall, there was a treatment effect ($P < 0.0001$) on the weights of the reproductive tracts (uterus and ovaries combined) and ovaries at week 28.

Data from one reproductive tract (group 3) were missing. Mean values of reproductive tract and ovarian weights at slaughter (week 28) for each treatment group are shown in Table 1.

There was no effect ($P > 0.1$) of treatment group on BW or ADG. There was a negative correlation between LHRH % binding (at weeks 10-27) and reproductive tract weights (-0.27 to -0.59; $P < 0.05$) and between LHRH % binding (at weeks 12-27) and ovarian weights. (-0.28 to -0.48; $P < 0.05$).

Discussion

This study compared the use of increasing doses of a recombinant ovalbumin-LHRH fusion protein (ova-LHRH) for LHRH immunization in heifers. Additionally, two adjuvants – Freund's Complete Adjuvant (FCA) and CpG ODN in water-in-oil (w-o) emulsion were compared. The biological response to ova-LHRH immunization in this study was evidenced by estrus suppression as measured by serum progesterone concentrations, and by regression of the reproductive tract and ovarian weights.

The optimal combination of antigen dose and type of adjuvant for maximal LHRH antibody production in this study was 3.4 mg ova-LHRH in CpG ODN/w-o. The superiority of CpG ODN/w-o to FCA as an adjuvant for ova-LHRH immunization shown

in this study is in agreement with a previous study that compared CpG ODN/w-o and FCA as adjuvants for ova-LHRH immunization in female rats [Conforti et al., 2007].

Previous studies in cattle used ova-LHRH in FCA. Hernandez et al [2005] observed 31 ± 1.8% ^{125}I-LHRH binding (at 1:10^3 serum dilution) 100 days after a third immunization with 1.5 mg of a combination of ova-LHRH and thioredoxin-LHRH fusion proteins in FCA in *Bos indicus* bulls. Stevens et al [2005] observed approximately 30% maximal ^{125}I-LHRH binding (at 1:10^3 serum dilution) with 3 injections of 0.4nmol ova-LHRH (per injection) in FCA in *B. taurus* heifers. The similarity in intensity of immune responses between *B. taurus* and *B. indicus* animals may be coincidental but can be compared with the present results. In the present study, at week 16, ^{125}I-LHRH % binding (at 1:10^3 serum dilution) was 39.8 ± 7.3% after two injections of 3.4 mg ova-LHRH in CpG ODN/w-o in *B. taurus* heifers. Percentages of ^{125}I-LHRH binding remain high (> 36%) until the end of the study (week 27). Treatment groups receiving 1.5 mg ova-LHRH in CpG ODN/w-o and 3.4 mg ova-LHRH in FCA had peak values of ^{125}I-LHRH binding percent of 32.8 ± 7.3% and 31.0 ± 7.3%, respectively. In this study, there was prolonged interval (14 weeks) between primary and secondary immunizations. Stevens et al [2005] administered the vaccine at weeks 0, 4, and 9. Hernandez et al [2005] administered the vaccine at weeks 0, 7, 41, and 55.

Geary et al. [2006] immunized *B. taurus* heifers with 3 injections of 0.5 mg ova-LHRH at a 6-week interval and observed a maximal ^{125}I-LHRH % binding below 20% after the first booster and below 30% after the second booster, which suggests that the of 0.5 mg ova-LHRH was suboptimal. Sosa et al [2000] immunized heifers with 3 injections (7 weeks apart) of 1 mg ova-LHRH in Z-Max adjuvant and observed a peak value of 37.0

± 11.0% ^{125}I-LHRH between boosters, but duration of estrus suppression as measured by serum progesterone concentrations ranged from 60 to 238 d. The choice of adjuvant in Sosa's study might have contributed to the short duration of vaccine effect observed in some heifers. In the present study, longevity of the response to the vaccine was not evaluated since heifers were sent to slaughter 3 months post-booster injection. However, in the 45 (out of 48 treated) heifers that showed estrus suppression, none of them returned to cycling after estrus suppression started – a period that lasted over 180 d in some individuals (until the end of the study). Although other individuals responded to the vaccine and remained acyclic throughout the study, their observed vaccine effect was shorter because they had a relative delay in the response to the vaccine.

The overall percentage of responders in terms of biological response (estrus suppression) in this study was 94% (after 2 injections), which is in agreement with the biological response (decreased serum testosterone concentrations) observed in immunized bulls in Hernandez's study [Hernandez et al., 2005] (92% and 93%, after 3 and 4 injections, respectively). Our results are also in agreement with Stevens et al [2005], where 97% of treated heifers did not have estrous activity after 3 immunizations with ova-LHRH. In the present study, only 3 out of 48 treated heifers did not show considerable estrus suppression during the experimental period; one of them had a two-week period of estrus suppression. The other two heifers did not stop cycling once estrous activity started.

Individual analysis of treatment groups showed 100% estrous suppression in three treatment groups: 1.5mg ova-LHRH in CpG ODN/w-o, 3.4 mg ova-LHRH in CpG ODN/w-o, and 3.4 mg ova-LHRH in FCA.

Evaluation of responders relative to production of LHRH antibodies showed that all 48 immunized heifers produced detectable LHRH antibodies. Only two treated heifers had low (< 5%) values of peak ^{125}I-LHRH % binding. Another study with ova-LHRH immunization in bulls showed a relatively lower percentage of responders in terms of production of detectable LHRH antibodies: 75% after 3 immunizations [Wells, 2006].

An ideal antigen:antibody ratio is thought to be necessary for optimal immune response. In vitro, it has been shown that if minute amounts of antigen are introduced in the system, a phenomenon known as antibody-mediated feedback inhibition is likely to take place, whereas the immunological tolerance occurs when excess antigen is introduced [Diener and Feldmann, 1970]. In both cases, the immune response is sub-optimal.

Amounts of ova-LHRH in treatment groups 5 and 6 (5.1 mg and 7.6 mg, respectively) might have been above the optimal dose, possibly leading to immunological tolerance to a certain degree. The reason why treatment group 2 (1.5 mg ova-LHRH) did not differ significantly from treatment group 4 (3.4 mg) in terms of ^{125}I-LHRH % binding at 1:10^3 serum dilution, while treatment group 3 (2.3 mg) was inferior to treatment group 4 is not known. It is possible that with increasing sample sizes the superiority of treatment group 4 over the other groups would become more evident. At serum dilution 1:10^4 however, the difference in ^{125}I-LHRH % binding between treatment groups 2 and 4 at week 27 approached significance (4.8 ± 1.7% vs 9.1 ± 1.7%, respectively; $P = 0.07$).

The apparent advantage in extending the interval between primary and secondary immunizations from 4-7 to 14 weeks may be related to increased production of specific

antibodies, which tends to occur when the booster is given when the response to the primary immunization is already declining (Palmer, G., personal communication).

In summary, although longevity of vaccine effect was not evaluated in this study, percentage of estrus suppression and LHRH antibody production after a primary and one booster injections of the vaccine were comparable to those of previous studies where animals were immunized at least 3-4 times. The choice of adjuvant (CpG ODN/w-o vs. FCA), the higher ova-LHRH dose (3.4 mg vs. 1.5 mg), and the prolonged period of time between primary and booster immunizations (14 weeks vs. 4-7 weeks) might have contributed to the apparent higher efficiency of the ova-LHRH immunization protocol used in this study.

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Table 1. Mean values (lsmeans ± S.E.M.[†]) of ^{125}I-LHRH % binding throughout the study (weeks –5 to 27), at week 27, and reproductive tract and ovarian weights (week 28).

Treatment Group ova-LHRH (mg)	^{125}I-LHRH binding (%) (weeks –5 to 27)	^{125}I-LHRH binding (%) (week 27)	Reproductive Tract Weight (g)	Ovarian Weight (g)
0.0	$0 \pm 0^{‡}$	$0 \pm 0^{‡}$	287.0 ± 19.1^{a}	17.6 ± 1.8^{a}
1.5	10.9 ± 2.7^{ab}	27.5 ± 6.5^{ab}	$156.8 \pm 19.1^{b,c}$	11.0 ± 1.8^{b}
2.3	5.5 ± 2.7^{a}	10.4 ± 6.5^{a}	$160.7 \pm 20.4^{b,c}$	$9.4 \pm 1.8^{b,c}$
3.4	15.4 ± 2.7^{b}	36.4 ± 6.5^{b}	$113.0 \pm 19.1^{c,d}$	$8.0 \pm 1.9^{c,d}$
5.1	7.2 ± 2.7^{a}	14.8 ± 6.5^{a}	170.4 ± 19.1^{b}	11.0 ± 1.8^{b}
7.6	7.7 ± 2.7^{ab}	15.8 ± 6.5^{a}	$136.5 \pm 19.1^{b,c,d}$	$8.9 \pm 1.8^{b,c,d}$
3.4[§]	8.8 ± 2.7^{ab}	14.6 ± 6.5^{a}	102.1 ± 19.1^{d}	5.4 ± 1.8^{d}

[†] Lsmeans and S.E.M. from the ANOVA model.

[‡] Not included in the comparison among treatment groups by ANOVA.

[§] This treatment group received Freund's Complete Adjuvant. All other treatment groups received CpG ODN/w-o as an adjuvant.

Values without a common superscript within columns differ (^{a,b,c,d} $P < 0.05$).

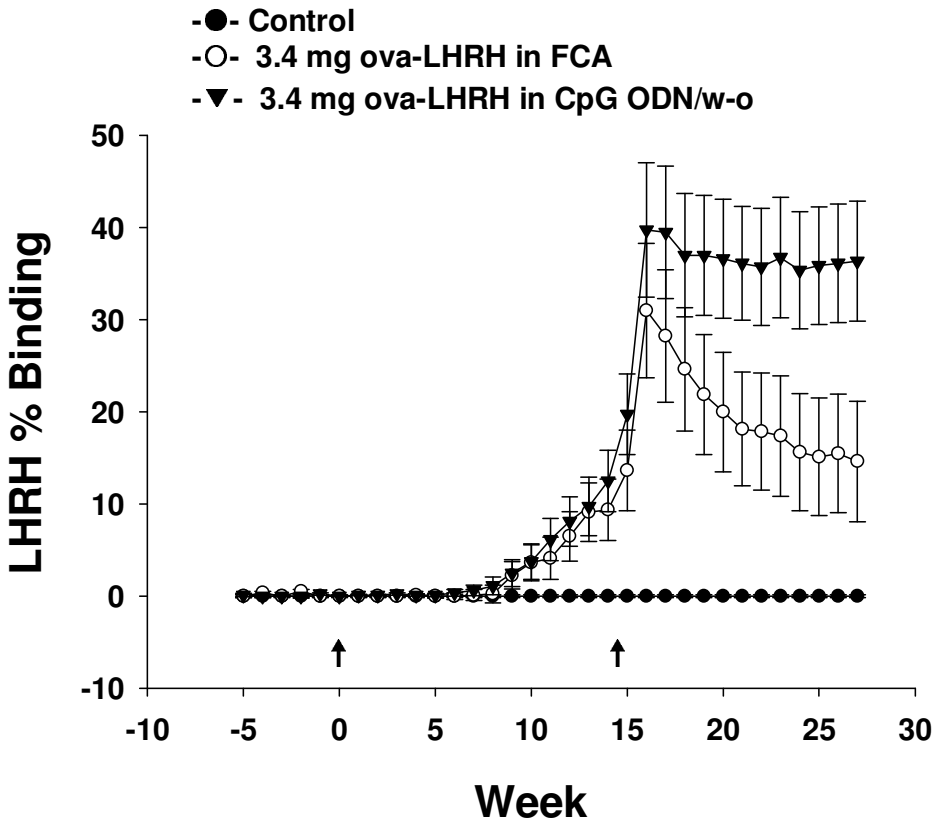

Figure 1. Comparison between CpG ODN adjuvant and Freund's Complete Adjuvant. ^{125}I-LHRH % binding in serum of heifers immunized with 3.4 mg ova-LHRH with FCA (○-○), 3.4 mg ova-LHRH with CpG ODN 2006 adjuvant (▼-▼), or untreated control (●-●). Arrows indicate immunization times (weeks 0 and 14). N = 8. Values are mean ± S.E.M.

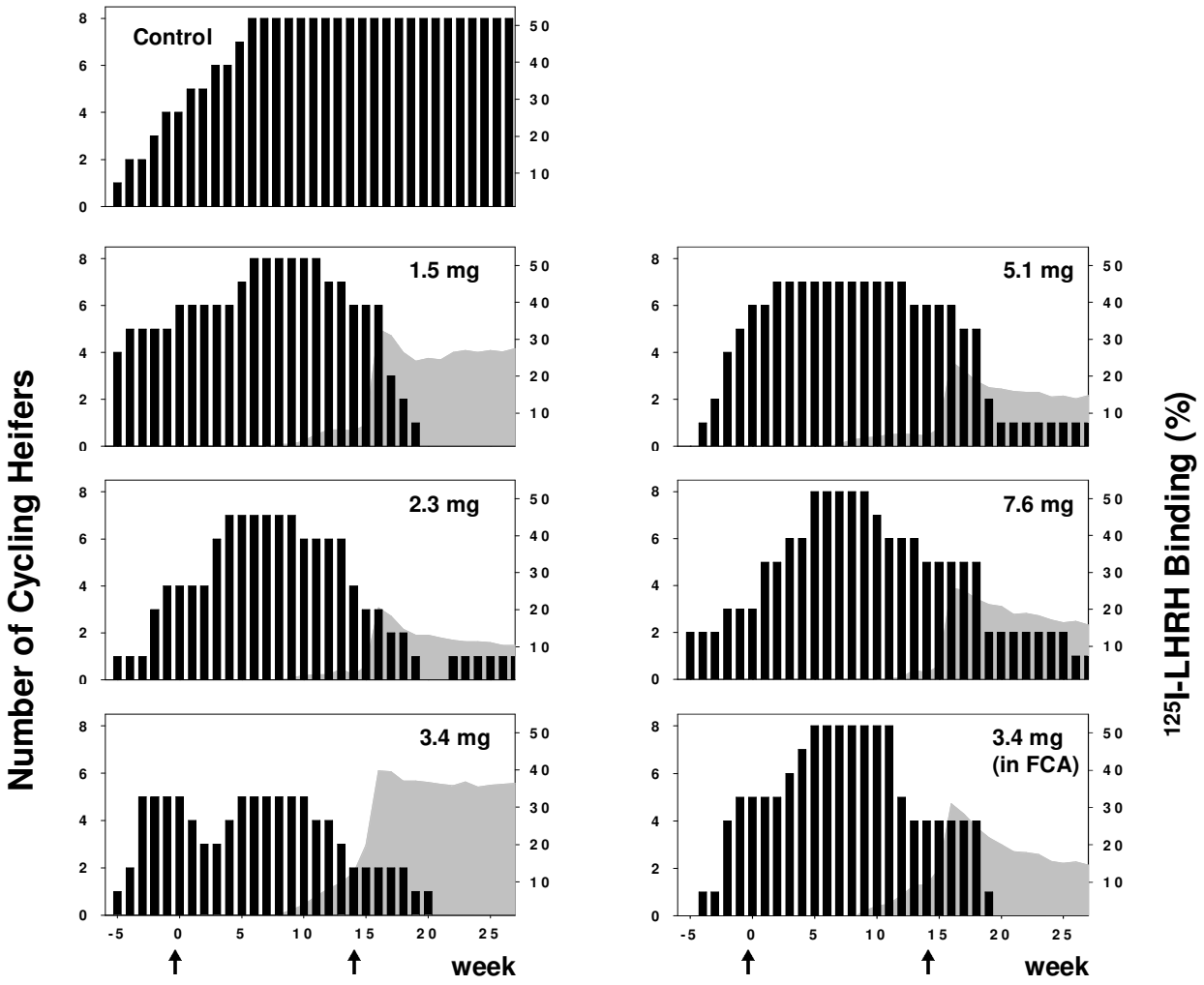

Figure 2. Number of heifers displaying estrous activity (bars) and ^{125}I-LHRH % binding (grey area) with respective amounts of ova-LHRH (mg) for each treatment group. All treatment groups received ova-LHRH in CpG ODN/w-o, except the treatment group receiving 3.4 mg ova-LHRH in FCA (last on the right). Arrows indicate immunization. N = 8.

CHAPTER FOUR

Noninvasive monitoring of adrenal and gonadal function in the jaguar (*Panthera onca*)

Noninvasive monitoring of adrenal and gonadal function

in the jaguar (*Panthera onca*)

ABSTRACT: The objectives of this study are: 1) to validate a protocol for noninvasive assessment of changes in corticoid concentrations due to acute stress in jaguars by measuring fecal corticoid metabolite concentrations in males and females before and after ACTH injection; and 2) to investigate the relationship between fecal corticoid and androgen metabolite concentrations in male jaguars. Eight adult jaguars were used in this study: 3 intact males, 1 vasectomized male, 3 intact females, and 1 ovariectomized female. All animals, with the exception of the vasectomized male, which was used as an untreated control, were chemically restrained and treated with 500 IU/animal of ACTH gel to simulate the effects of acute stress (day 0). Fecal samples were collected for 20 consecutive days (days – 10 through 10). Samples were frozen, lyophilized, and extracted in 90% ethanol. Extracts were assayed for corticoid (males and females) and androgen metabolites (males) by radioimmunoassay. Overall, there was a significant ($P < 0.01$) increase in fecal corticoid metabolite concentrations after ACTH injection (pre-ACTH: 924.79 ± 157.67 ng/g dry feces; post-ACTH: 2613.37 ± 443.54 ng/g). No significant effect of sex on corticoid metabolite concentrations was detected ($P > 0.05$). Androgen metabolite concentrations increased in males 1 and 2 in the post-ACTH injection period. In male 2 only, a (positive) correlation between corticoid and androgen metabolite concentrations was found (0.60; $P < 0.01$). Results from this study suggest the existence of individual differences in corticoid and androgen production associated with acute stress in male jaguars. To our knowledge, this is the first study that investigated the effects of acute stress after chemical restraint and ACTH injection on gonadal function in males and adrenal function in

male and female jaguars. In conclusion, this study presents a simplified and efficient protocol for extracting and measuring steroid hormone metabolites in fecal samples of jaguars. This protocol allows for detection of changes in corticoid metabolites concentrations in fecal samples due to acute stress, and can be used by zoos interested in measuring the physiological effects of different handling procedures and other potential stressors on adrenal function in their jaguars. Ultimately, this protocol can be used as a tool to monitor the welfare of jaguars.

Introduction

The increasing destruction of natural habitats is one of the major threats to large carnivore populations, such as the jaguar (*Panthera onca*). Maintenance of captive populations of endangered species plays an important role in environmental education programs by increasing public's awareness about the species and their ecological importance. Additionally, captive populations represent a valuable genetic resource, which may be used for conservation purposes of critically endangered species. However, captive animals are exposed to factors that may cause stress, which theoretically could affect their health, welfare, and reproductive activity. Husbandry factors, enclosure characteristics, and interaction with humans are potential stressors.

Stress levels of an individual can be monitored by assessing activity of the adrenal glands, which are responsible for production of corticoid hormones, such as cortisol. In some species, it has been shown that corticoid profile can be monitored by measuring corticoid metabolites in fecal samples. This non-invasive technique is ideal for monitoring steroid production in wild species given that it does not require physical restraint for collection of samples, which could be an additional stressor.

Metabolite concentrations found in feces represent a pool of metabolites from the period of time between excretions of feces; in carnivores, it usually represents the result of hormone production and metabolism over a 24- to a 48-hour period. This is particularly advantageous when assessing the endocrine profile of hormones that follow circadian fluctuations, such as glucocorticoids, given that levels of hormone metabolite in a fecal sample will reflect the average hormone production over a certain period of time.

Because what is actually measured in fecal samples is the metabolite(s) of the original hormone, the differences in chemical structure between them may prevent antibodies raised against the hormone to bind to its metabolite(s). Thus a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) commercial kit for a specific hormone may not have a satisfactory performance when measuring the metabolites of this hormone in fecal samples. Moreover, the metabolism of a particular hormone may vary between species resulting in different metabolites. Thus, hormone assays must be validated for each species.

Stress stimulates interactions between the hypothalamus, the pituitary gland, and the adrenal glands, known as hypothalamic-pituitary-adrenal (HPA) axis. The actions of the HPA axis begin with secretion of corticotrophin releasing hormone (CRH) by the hypothalamus, which acts on the pituitary gland causing the release of adrenocorticotrophic hormone (ACTH). Through circulation, ACTH reaches the cortex of the adrenal glands, where it stimulates production of steroid hormones [Broom & Johnson, 1993]. Cortisol and corticosterone are examples of corticoids, also known as stress hormones. Among other actions, corticoids stimulate hyperglycemia and increase blood flow to muscles. In the context of stress, such actions prepare the body for the 'fight-or-flight' response.

The relationship between stress and adrenal gland activity has been studied for many years [Selye, 1946, 1956; Sapolsky, 1992]. Corticoid production follows a daily cyclic pattern so that plasma levels vary throughout the day [Broom & Johnson, 1993]. For that reason, a single daily measurement of corticoid plasma levels may not be a valuable piece of information. However, analysis of hormone metabolite concentrations

in fecal samples allows for the assessment of levels of hormones excreted over time, avoiding, therefore, variation due to circadian cycles [Bonier, 2001]. Chronic stressors are also associated with increase in corticoid levels [Sakellaris & Vernikos-Danellis, 1975; Gamallo et al., 1986; Craig et al., 1986; Pitman et al., 1990]. However, continuous exposure to a stressor may lead to refractoriness of the adrenal glands [Ladewig, 2000]. In this case, chronic stressors may become incapable of causing an increase in corticoid levels. Therefore, low corticoid levels do not necessarily mean that the individual is not suffering from stress.

Recent studies have focused on the effects of stress on reproduction in different species. Data on fish and mammals support the idea that stress can reduce the reproductive potential of an individual. White et al. [2002] suggested that changes in stress levels in certain species of fish could affect the genetic expression of luteinizing hormone-releasing hormone (LHRH), which controls reproduction. In mammals, it was observed that stress can reduce both frequency and amplitude of luteinizing hormone (LH) secretion, which is fundamental in the process of ovulation [Smith & Dobson, 2002]. Thus, stress can result in decreased production of LHRH and LH. As a consequence, ovarian follicles require longer periods of time to develop and estradiol secretion decreases [Dobson & Smith, 2000], which in turn can reduce the intensity of estrous behavior [Dobson et al., 2001]. These findings indicate the existence of an interaction between the hypothalamic-pituitary-ovarian (HPO) axis and the HPA axis [Phogat et al., 1997].

Previous studies have measured fecal steroid metabolite concentrations in cat species [Brown et al., 1996a; Terio et al., 1999]. Methods of hormone metabolite extraction vary

considerably between laboratories. Apparently, most studies were conducted using an extraction protocol where a solvent, usually diluted ethanol, is added to the fecal samples and taken to boiling. More recently, some laboratories started to use a vortexing procedure instead of the boiling procedure to extract steroid metabolites from fecal samples [Wasser et al., 2000].

Little is known about reproductive physiology or adrenal function in the jaguar. Knowledge on the effects of stressors on adrenal and reproductive physiology in jaguars is an important step in planning management and conservation strategies for the species. Thus, the objectives of this study are: 1) to validate a protocol for noninvasive assessment of changes in corticoid concentrations due to acute stress in jaguars by measuring fecal corticoid metabolite concentrations in males and females before and after ACTH injection; and 2) to investigate the relationship between fecal corticoid and androgen metabolite concentrations in male jaguars.

Materials and Methods

Animals

Eight jaguars were included in this study (Table 1). Average age was 10 ± 7 years (mean ± S.D.) for males and 7 ± 6 years for females. Housing of jaguars at RioZoo was as follows: females # 5 and 6 were housed together and alternated use of the enclosure for public display with male # 1. When animals were not in the enclosure for public display, they were in a smaller back enclosure. Male # 4 was housed in another enclosure and occasionally shared it with an adult female (not included in this study). Housing of jaguars in Zoológico de Ilha Solteira was as follows: jaguars # 2, 3, and 8 were individually housed, and jaguar # 7 shared the enclosure for public display during visitation hours with an adult (reproductive status) male (not included in the study).

ACTH Challenge

Three males (# 1, 2, and 3) and four females (# 5, 6, 7, and 8) were treated with ACTH. All ACTH-treated animals were first anesthetized with Zoletil® (Virbac do Brasil, Jurubatuba, SP) i.m. at a dose of 10 mg/kg BW. Anesthesia was given with either a CO_2 pistol or a blow dart. Male # 4 did not receive any treatment (anesthesia or ACTH) and was used as an untreated control. Within 20 min of anesthesia, animals were treated with 500 IU ACTH/animal i.m. as a gel. Males #2 and 3 were electroejaculated for semen sample collection for another research project after chemical restraint and before ACTH injection for this study.

ACTH Gel Preparation

Products followed by a catalog number were purchased from Sigma-Aldrich (St. Louis, MO). ACTH gel was prepared using sterile technique, as follows: 0.625 g sodium carboxymethylcellulose (#C4888, medium viscosity) was slowly added to 40 ml double-distilled water swirling on a non-heated magnetic stirrer until particles were dissolved (~20-30 min). The solution was then autoclaved and allowed to cool to room temperature. Separately, 0.3125 g of liquid phenol (#P9346, for antimicrobial activity, VSP grade), equivalent to 0.25 g phenol, was added to a beaker followed by addition of 2.565 g dextrose (#D9434, VSP grade) and 10 ml double-distilled water and mixed until dextrose was completely dissolved. Dextrose/phenol solution was poured into the ACTH vial (#A6303-5000IU, amino acids 1-39, 5,000 IU powder porcine ACTH) and mixed

until completely dissolved. ACTH was withdrawn into a syringe and slowly filtered through a 0.22 μm diameter filter while poured into the swirling solution of carboxymethylcellulose. The solution was then mixed until homogeneous (~5 min) and the pH was checked (pH range should be between 4.5-6.5 and adjusted with 10% HCl if necessary). Using a glass pipette, ACTH gel was transferred to brown glass vials, sealed with rubber stoppers and a crimping tool, and stored at 4°C until use. Approximately 46 ml of ACTH gel at 113 IU/ml were prepared with this protocol.

Fecal Sample Collection

Fecal samples were collected daily from all jaguars starting 10 days before ACTH injection through 10 days after injection. Samples were collected in the morning and the entire bolus was placed in a plastic bag, labeled and stored frozen (-20°C) until processing. On days where there were more than two samples from an individual, data were combined for analysis and presentation. Peak values of corticoid metabolites were expected to be found in the first fecal samples collected after chemical restraint and ACTH injection procedures. Therefore, the first two days where samples were found after these procedures were considered peak days.

Fecal Hormone Metabolite Extraction

Extraction of fecal hormone metabolites was performed using a vortexing method adapted from Wasser et al [2000]. Briefly, 3 ml of 90% ethanol (10% double-distilled water) were added to glass tubes containing approximately 0.2 g of fine powered of

freeze-dried and homogenized feces. Samples were covered with parafilm® and vortexed for 30 min in a multi-tube vortexer (VX-2500, VWR™). Samples were then centrifuged (500 x g, 20 min, 4°C) and the supernatants collected into new set of glass tubes. Pellets were re-suspended in 2 ml 90% ethanol and samples were covered with parafilm® and vortexed individually for 15 sec. Re-suspended pellets were centrifuged (500 x g, 20 min, 4°C) and both supernatants were combined. An additional centrifugation step (500 x g, 10 min, 4°C) was performed to precipitate remaining fecal particles. Extracts were transferred to labeled polypropylene tubes, capped, and stored frozen (-20°C) until assayed.

Extraction Efficiency Assessment

Before extraction, 100 µl (~8,000 cpm) of ^{3}H-cortisol in phosphate buffered saline gel (PBS-Gel; 2.76 g monobasic phosphate, 11.36 g dibasic phosphate, 1 g sodium azide, 8.76 g NaCl, 1 g gelatin in double-distilled water for a final volume of 1 L of buffer; pH 7.4) were added to additional samples and to a pair of scintillation vials to determine extraction efficiency. Tritiated cortisol-spiked samples were extracted as previously described. From each spiked sample extracted, 200 µl were transferred to scintillation vials. Two ml of scintillation liquid (ScintiVerse®, Fisher Scientific) were added to each vial containing spiked extracts, to both vials containing tritiated cortisol solution, and to a pair of blank vials, and individually vortexed (15 sec). Vials were left in the dark for at least 1 h before counting in a beta counter (LS 6500 multi purpose scintillation counter, Beckman Coulter™). Blank vials were used to calculate background values. Extraction

efficiency was calculated as percentage of tritiated cortisol added that was present in extracts after subtraction of background values.

Fecal Hormone Metabolite Assessment

Corticoid metabolite concentrations in fecal samples were measured using a commercial corticosterone double antibody ^{125}I radioimmunoassay (RIA) kit (#07-120103, MP Biomedicals, Solon, OH). Fecal extracts (5 ml) were diluted at 1:100 (1:500 final dilution) in kit steroid diluent for corticoid metabolite assays. Androgen metabolite concentrations were measured using a commercial Coat-A-Count® Total Testosterone RIA kit (#TKTT2, Diagnostic Products Corporation, Los Angeles, CA). Fecal extracts were diluted at 1:5 (1:25 final dilution) in PBS-Gel. Fecal metabolite concentrations were calculated as ng/g on a dry feces basis.

Assays were performed following manufactures' directions and were validated by assessing recovery efficiency of exogenous tritiated hormone (> 80%; as previously described) and by assessing parallelism between binding of serial dilutions of jaguar fecal extracts and that of the standard curve.

Statistical Analysis

Baseline mean values were calculated for all pre-treatment samples for each individual jaguar. These values were averaged to obtain an overall pre-ACTH mean for males versus females, and for all cats combined. Values from post-ACTH peak days were also averaged for all individuals

and across gender. Analysis of Variance (ANOVA) and non-parametric ANOVA were used to assess differences between mean values of the pre-ACTH period and the post-ACTH peak days and between genders using the Proc Glm procedure of SAS (version 9.1). Within each individual, Proc Autoreg was used to perform autoregression analysis to verify if samples from pre- and post-ACTH periods were auto-correlated. Proc Glm was used to compare mean values of hormone metabolite concentrations in the pre- and post-ACTH periods within individual. In males, correlation between corticoid metabolite concentrations and androgen metabolite concentrations was verified by Regression Analysis using Proc Reg and by Pearson's Coefficient of Correlation using Proc Corr. Values are expressed as mean ± S.E.M or S.D. Level of significance was set at $P < 0.05$.

Results

Intra- and inter-assay coefficients of variation for hormone metabolite assays were less than 3% and 8%, respectively.

Females 5 and 6 were mother and daughter that were housed together and received ACTH treatment within 1 h from one another. On some days during the ACTH challenge, food dye was given in the feed of one these females for sample identification. However, on some days, the feed given to both females had no dye, thus sample identification was not possible. Therefore, hormone metabolite concentrations of these individuals were averaged.

Overall, there was a significant ($P < 0.01$) increase in fecal corticoid metabolite concentrations after ACTH injection (pre-ACTH: 924.79 ± 157.67 ng/g dry feces; post-ACTH: 2613.37 ± 443.54 ng/g). There was no interaction between period (pre- or post-ACTH injection)

and sex ($P > 0.05$). No significant effect of sex on corticoid metabolite concentrations was detected ($P > 0.05$). Mean baseline concentrations of fecal corticoid metabolites from samples collected during the 10 days before ACTH injection were calculated for each individual jaguar. Percentages of mean baseline concentration of corticoid metabolites at each observation time were plotted for each individual jaguar (Figures 1 and 2). Androgen metabolite concentrations (ng/g dry feces) at different observation times throughout the ACTH challenge in individual males are also shown in Figure 1.

Table 2 shows individual differences in hormone metabolite concentrations during the ACTH challenge. As expected, there was no difference in concentrations of corticoid and androgen metabolites in male 4 (untreated control). Males 1 and 2, as well as females 5 and 6 (combined) and 7 had increased concentrations of corticoid metabolites after ACTH treatment. Male 3 and female 8 had no significant increase in corticoid metabolites concentrations on the expected peak days (first two days of sample collection after ACTH treatment). However, when samples collected after expected peak days were included in the analysis, male 3 had a significant increase in corticoid, but not androgen, metabolite concentrations (pre-ACTH: 623.48 ± 289.18 ng/g; post-ACTH from days 1 through 8: 1705.73 ± 327.90 ng/g; $P < 0.05$).

Androgen metabolite concentrations increased in males 1 and 2 in the post-ACTH injection period. In male 2 only, a (positive) correlation between corticoid and androgen metabolite concentrations was found (0.60; $P < 0.05$) (Figure 3).

Discussion

In this study, steroid hormone metabolites were extracted from fecal samples and measured using a simplified protocol compared to previous studies. In this protocol, hormone metabolite extraction was done by vortexing, instead of boiling, the samples. This change simplifies the protocol because it does not require heating of the solvent, thus there is no need to adjust for loss of solvent volume, which usually occurs during boiling. Typically, adjustment for loss of solvent volume is done by completely evaporating the extract and re-suspending it in methanol. By eliminating the need of evaporating the extract, the sample extract used for the assays is obtained directly from the supernatants produced after vortexing and centrifuging the samples.

Monitoring endocrine profiles through hormone metabolite concentrations in fecal samples is a technique that has been widely used in several species, including wildlife, for its non-invasive nature [Brown et al., 1996a; Terio et al., 1999; Morato et al., 2004a]. Collection of feces, unlike that of blood, does not disturb the animal, nor does it put the animal, and staff, under the typical risks of physical and chemical restraining for sample collection. Moreover, the sample collection process itself does not bring about additional stress to the animals sampled, so that it is ideal for adrenal function assessment.

Fecal hormone metabolite analysis has been used as a non-invasive method to assess adrenal activity in feline species. Terio et al. [1999] observed an increase in cortisol levels due to changes in management in captive cheetahs. Moreira [2001] investigated the effects of transferring animals from a large, environmentally enriched enclosure to a small, non-enriched enclosure on gonadal and adrenal activity in female oncillas and margays. Oncillas showed a decrease in estrogen levels and a concomitant increase in corticoid levels after being transferred to the smaller, non-enriched enclosure. Margays also showed an increase in corticoid levels; however, unlike oncillas, margays showed an increase in estrogen levels. Management factors

significantly influenced the number of litters produced by small-sized felid species in captivity [Mellen, 1991]. Carlstead et al. [1993b] observed a decrease in LH secretion in laboratory cats after modifying a controlled environment into a more 'stressful' environment. Similarly, placing the small-sized leopard cat (*Prionailurus bengalensis*) in a new environment causes persistently elevated glucocorticoid excretion while reducing exploratory behaviors [Carlstead et al., 1993a]. A negative relationship also has been measured between adrenal and ovarian activity cheetahs [Jurke et al., 1997]. Female cheetahs with high circulating cortisol concentrations experienced less follicular activity and prolonged periods of anestrus.

This study shows that changes in steroid hormone concentrations in jaguars can be measured noninvasively by measuring hormone metabolite concentrations in fecal samples. Thus, the protocol described in this study can be a useful tool for zoos interested in the welfare of their jaguars. Changes in adrenal function due to handling, restraining, and other procedures commonly used in zoos can be monitored through this protocol and may contribute to improve handling and other techniques.

Data on reproductive and adrenal activity in feline species have been collected through fecal steroid metabolite analysis. In females, assessment of estradiol and progesterone metabolite levels in feces allows for monitoring ovarian activity. Thus, information on duration of estrous cycle and gestation can be collected. In cheetahs, duration of the estrous cycle, gestation, and anestrous periods were determined through fecal corticoid metabolite analysis in captive individuals [Brown et al., 1996b]. Data on reproductive activity in captive females of ocelots (*Leopardus pardalis*), margays (*L. wiedii*), and oncillas (*L. tigrinus*) were also collected through corticoid metabolite analysis in fecal samples [Moreira, 2001]. In male jaguars, data on testicular activity and

seasonal variation in testosterone production were collected using fecal samples [Morato et al., 2004b].

In the clouded leopard (*Neofelis nebulosa*), analysis of fecal corticoid metabolite levels revealed that the internal height of the enclosure could influence the adrenal activity of the animals; individuals kept in enclosures with lower height showed higher cortisol levels [Wielebnowski et al., 2002]. In the wild, this species has arboreal habits so that the possibility of having a high refuge in the enclosure might serve as a means to relieve stress. Confinement of any predator under intensive management conditions and the inevitable exposure to conspecifics (and often heterospecifics) is likely to impose a stress [Moberg, 1985]. Thus, it should not be surprising that in the few solitary species studied that sensitivity to a captive environment appears to contribute to low reproductive success [Jurke et al., 1997; Wielebnowski et al., 2002].

These studies indicate that management and enclosure factors can influence the activity of the gonads and adrenal glands in felines in a species-specific manner. Fecal steroid analyses can serve as an effective tool in identifying environmental factors that affect production of gonadal and adrenal steroids. Thus, this methodology can contribute to improving welfare and reproductive potential in captive individuals. Apparently, there are no data on how environmental factors influence adrenal activity in captive jaguars. Moreover, the relationship between adrenal and gonadal activity is not known.

To our knowledge, this is the first study that investigated the effects of acute stress after chemical restraint and ACTH injection on gonadal function in males and adrenal function in male and female jaguars. Morato et al. [2004a] studied the effects of chemical restraint and electroejaculation in male jaguars and measured corticoid and androgen metabolite

concentrations. They investigated changes in hormone metabolite concentrations by averaging samples collected during the 5 days preceding restraint and electroejaculation procedures and detected peaks that were considered significant when values were above 2 SD. Despite the fact that peaks of corticoid metabolite concentrations were observed, Morato et al. did not detect changes in androgen metabolite concentrations; thus no evidence of an effect of corticoids on androgen secretion could be found. Results from the present study suggest the existence of individual differences in corticoid and androgen production as a result of acute stress in male jaguars. Two (out of 3 treated) males (# 1 and 2) had an increase in corticoid metabolite concentrations that was detected in the expected peak days post-treatment; the other male (# 3) did not show increased corticoid metabolite concentrations during the expected peak days; however, when subsequent days (within the 10-day post-ACTH period) were included in the analysis, this male showed a significant increase in corticoid metabolite concentrations (data not shown). Although this finding suggests the existence of individual variations in the timing of increased corticoid production in response to stress, one should not rule out the possibility that increased corticoid metabolites concentrations observed in male # 3 after the expected peak days post-ACTH treatment were a result of another stress-inducing event.

Another difference observed between males # 1 and 2 and male # 3 was that the first two males had a significant increase in fecal androgen metabolite concentrations in the post-ACTH injection period. In jaguars # 1 and 2, both corticoid and androgen metabolite concentrations during the ACTH challenge show that both hormone metabolites had a similar pattern of fluctuation.

A study on wild baboons showed a relationship between testosterone concentrations after acute stress and social ranking: high-ranking males (as determined by reproductive criteria)

responded to acute stress from rapid capture and immobilization with an increase in testosterone concentrations in blood, while subordinate males showed a decrease in testosterone concentrations under the same stressors [Sapolsky, 1986]. One could speculate that an increase in testosterone during acute stress might contribute to increase aggressiveness and consequently an individual's position in the social ranking. In solitary species, such as the jaguar, an increase in testosterone might contribute to a male's success in defending his territory against an invading male.

In conclusion, this study presents a simplified and efficient protocol for extracting and measuring steroid hormone metabolites in fecal samples of jaguars. This protocol allows for detection of changes in corticoid metabolites concentrations in fecal samples due to acute stress, and can be used by zoos interested in measuring the physiological effects of different handling procedures and other potential stressors on adrenal function in their jaguars. Ultimately, this protocol can be used as a tool to monitor welfare in jaguars.

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Table 1. Jaguar individual data.

Institutional ID #/Name	Study ID #	Sex	Age (years)	Reproductive Status	Origin	Institution
Alexandre	1	M	13	Intact	Wild	R.Z.*
1443	2	M	3	Intact	Wild	I.S.‡
1232	3	M	5	Intact	Wild	I.S.
Cabeça	4	M	18	Vasectomized	Captivity	R.Z.
Preta	5	F	16	Intact	Wild	R.Z.
Gabi	6	F	4	Intact	Captivity	R.Z.
1442	7	F	2	Intact	Wild	I.S.
1313	8	F	5	Ovariectomized	Wild	I.S.

* Fundação RioZoo – Rio de Janeiro State.

‡ Centro de Conservação da Fauna Silvestre – CESP – Parque Zoológico de Ilha Solteira – São Paulo State.

Table 2. Mean values of corticoid metabolite concentrations ± S.E.M. (males and females) and androgen metabolite concentrations (males) in individual jaguars during pre- and post-ACTH injection periods.

Animal	Corticoid Metabolites (ng/g dry feces)		Androgen Metabolites (ng/g dry feces)	
	Pre-ACTH	Post-ACTH	Pre-ACTH	Post-ACTH
Male 1	1412.27 ± 272.03	3803.74 ± 577.06**	58.68 ± 48.68	345.32 ± 103.28*
Male 2	1000.81 ± 234.73	3715.14 ± 621.03**	124.78 ± 18.62	588.11 ± 49.25**
Male 3	968.94 ± 260.62	1993.14 ± 689.54	43.78 ± 15.73	78.27 ± 41.61
Male 4 ‡	1501.31 ± 601.08	2797.30 ± 1202.15	108.01 ± 21.17	68.09 ± 42.34
Females 5 and 6§	1067.86 ± 259.30	3178.96 ± 710.13*	-	-
Female 7	326.37 ± 38.83	629.40 ± 98.99*	-	-
Female 8	933.16 ± 170.31	1794.28 ± 450.61	-	-

‡ Untreated control.

§ Values of females 5 and 6 were averaged.

Stars indicate differences (*$P < 0.05$; **$P < 0.01$) within type of metabolite and individual.

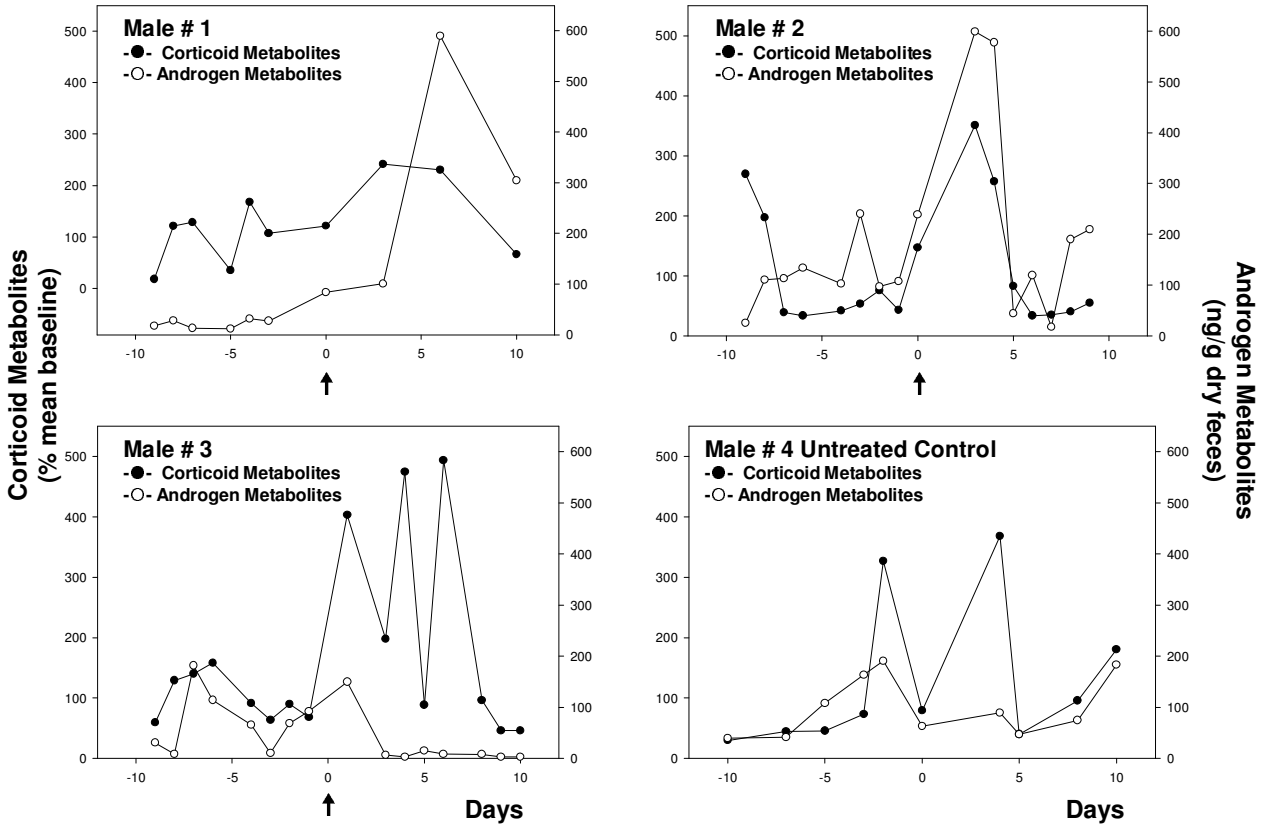

Figure 1. Percentage of mean baseline concentration of corticoid metabolites (-●-) and androgen metabolite concentrations (ng/g dry feces) (-○-) in individual males throughout the ACTH challenge. Arrow indicates ACTH injection (500 IU/jaguar).

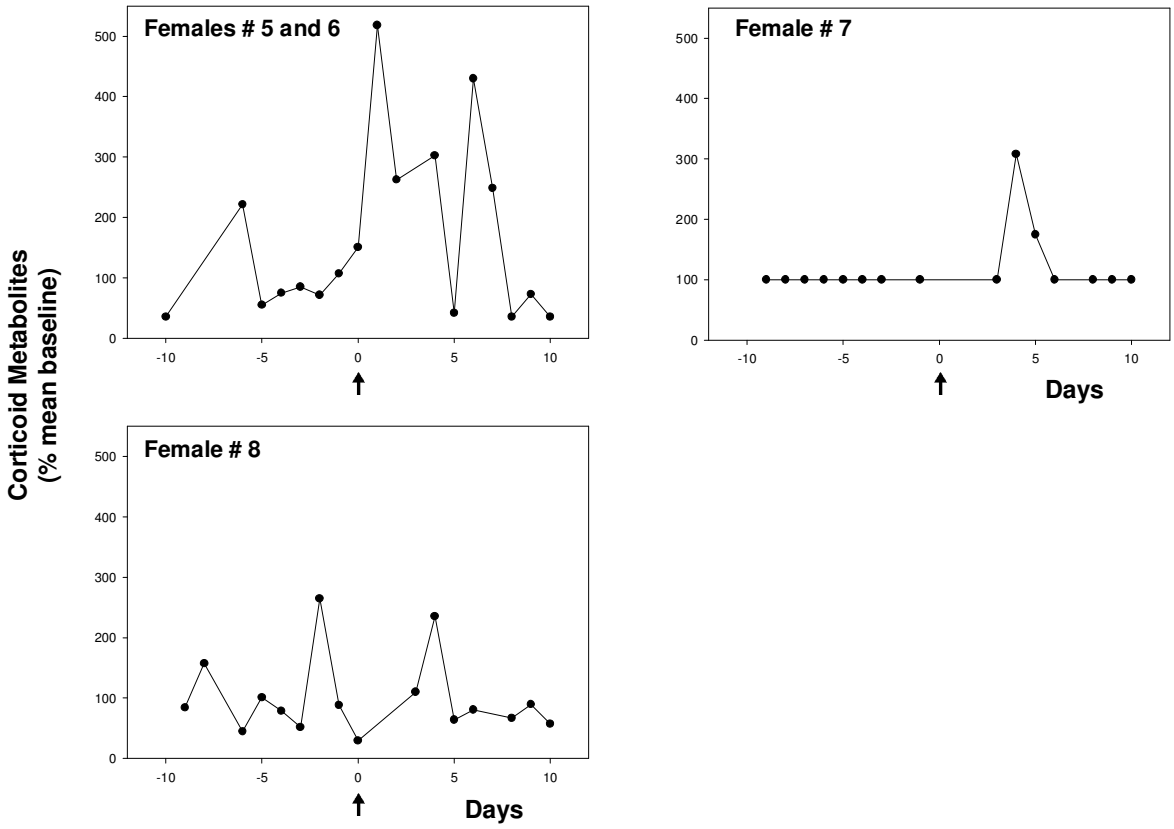

Figure 2. Percentage of mean baseline concentration of corticoid metabolites in individual females throughout the ACTH challenge. Arrow indicates ACTH injection (500 IU/jaguar).

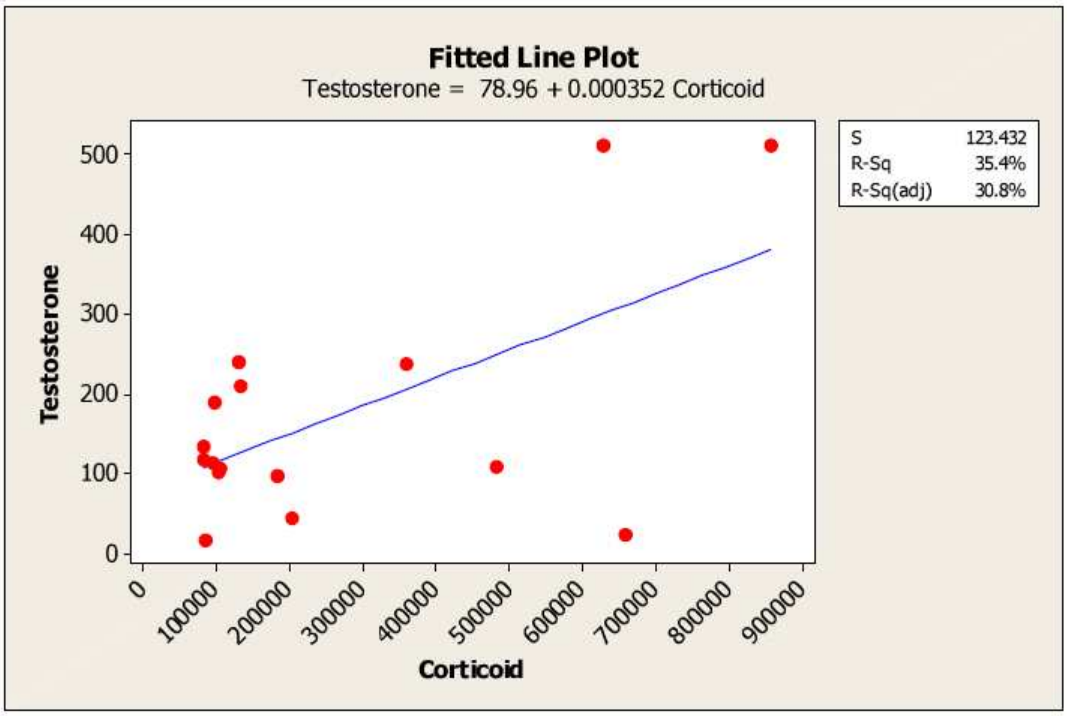

Figure 3. Positive correlation (0.60; $P < 0.05$) between fecal corticoid and androgen metabolites in male jaguar # 2 during ACTH challenge.