DISSERTATION

REPRODUCTIVE, BEHAVIORAL, AND FIRST GENERATIONAL EFFECTS OF GONADOTROPIN RELEASING HORMONE VACCINATION IN FEMALE ROCKY MOUNTAIN ELK (*CERVUS ELAPHUS NELSONI*)

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2011

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ABSTRACT

REPRODUCTIVE, BEHAVIORAL, AND FIRST GENERATIONAL EFFECTS OF GONADOTROPIN RELEASING HORMONE VACCINATION IN FEMALE ROCKY MOUNTAIN ELK (*CERVUS ELAPHUS NELSONI*)

Free-ranging wildlife species in North America are owned by no one but are managed in public trust by State and Federal government agencies. Wildlife resources and their habitat are conserved for a wide variety of reasons; from preservation of biodiversity, to utilization for hunting purposes, to satisfying human desires for connection to the natural world. The principles of the North American Model of Wildlife Management were developed and adopted around the turn of the twentieth century when the take of many wildlife species required regulation or even total protection to prevent irreparable changes in species frequency and distribution. At that time the overarching societal sentiment was wildlife conservation for sustainable future use. With increased urbanization, decreased reliance on wildlife species to sustain human populations, and increasingly anthropomorphic relationships with animals, American society has moved from a primarily utilitarian focus to a progressively more mutualistic view of wildlife. At the same time several wildlife species, including Rocky Mountain elk, have adapted to human influenced environments and, without the regulatory effects of a full suite of predators, have become locally overabundant. Changing views of *why* wildlife should be conserved have precipitated questions of *how* wildlife should be managed.

Stakeholders have encouraged the wildlife community to develop non-lethal management techniques particularly in areas where traditional tools such as hunting, trapping, and habitat modification are not feasible or are undesirable due to human dominance of the landscape. Fertility control is one potential method of regulating the size of wildlife populations without lethal removal. In the past four decades significant resources have been devoted to investigation and development of products intended to inhibit reproduction in wild ungulates. While considerable progress has been made in achieving suppression of individual animal fecundity, fertility control is not routinely used to regulate free-ranging wildlife populations. Barriers to using reproductive inhibitors in free-ranging wildlife are biological, ecological, regulatory, and sociological in scope.

While public discussion and discourse is needed to resolve questions of when and why fertility control may be an appropriate technique for limiting populations, managers and regulators need sound biological science on which to base these decisions. One reproductive inhibitor which has demonstrated promise as a multi-year ungulate wildlife contraceptive after a single application is the gonadotropin releasing hormone (GnRH) vaccine GonaCon. However, questions remain regarding its use in pregnant animals, long-term efficacy, possible effects on socio-sexual behaviors, and potential pathological side-effects.

Using captive pregnant Rocky Mountain elk as a model cervid species, we investigated the effects of intramuscular GnRH vaccination on maintenance of pregnancy, subsequent pregnancy rates, reproductive behaviors, and inflammatory lesions at the site of injection as well as systemic indicators of inflammation. We found a

single vaccination during mid-gestation did not disrupt the current pregnancy and decreased subsequent pregnancy rates for three years. Reproduction generally resumed by year four. GnRH vaccination was associated with persistence of male and female pre-copulatory reproductive behaviors throughout the breeding season, while sham-vaccination was not. Vaccination of either type was associated with robust immune and inflammatory responses at least four years post vaccination. Calves nursing from GnRH-vaccinated females developed strong passive GnRH immunity through colostral antibody consumption.

Calf maternal colostral GnRH antibodies waned over time and by six months of age were no longer detectable in serum. Exposure to high GnRH antibody titers during the neonatal period did not affect reproductive development or maturation. Male calves had normal secondary sexual characteristics including antler, scrotal, and neck growth parameters and produced satisfactory semen samples during their first breeding season (~ 1.5 yr. of age). Male and female calves responded to GnRH agonist stimulation with a typical increase in concentration of serum luteinizing hormone prior to the second reproductive season. All female calves became pregnant during their second breeding season (~ 2.5 yr. of age). There were no differences in histologic structure of the hypothalamic-pituitary-gonadal axis between antibody exposure groups at the time of necropsy (~ 3 yr. of age). There was no delay in puberty or long-term alteration of reproductive function in calves exposed to GnRH antibodies during the neonatal period.

These findings extend the physiologic and applied understanding of GnRH vaccination in elk. While abscessation at the site of injection was the only clear pathologic side-effect of GnRH vaccination in our study, questions regarding the

ecological consequences of immunocontraception remain. Potential effects on; herd social structure, synchrony of reproductive seasons and habitat quality, and heritability of immune response, remain important knowledge gaps which need to be addressed prior to consideration for widespread application as a wildlife reproductive inhibitor. Public dialog between stakeholders including wildlife managers, scientists, hunters, residents affected by human-wildlife conflicts, and those interested in the welfare of free-ranging species will help define under what circumstances wildlife fertility control is, or is not, a useful and desirable tool for wildlife management.

This dissertation is dedicated to Vedauwoo; the only beast who could live with me through graduate school not once but twice.

I would like to acknowledge and thank my family, friends, and the small army of collaborators who made this project possible. I cannot imagine a better group of people to work in the company of, learn from, and play with in the future.

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Chapter 1: Introduction

The Evolution of Wildlife Management and Fertility Control

History of Wildlife Management

The science of wildlife management is a relatively new but influential discipline in the history of natural resource utilization. Until the mid nineteenth century plentiful natural resources including clean water, vast forests, abundant minerals, and copious wildlife were the rule rather than the exception on the North American continent [1]. Early colonial Americans found this bounty irresistible after the relative paucity and classist distribution of resources in Europe [2]. During the sixteenth and seventeenth centuries Americans desiring access to natural resources needed only to move further west in order to find more. The prevailing societal approach to natural resources was to "conquer" and "tame" additional land for civilization and human use. National success and progress were measured in acres of land recovered from wilderness and put to productive use by humans. There was little consideration of the finite nature of resources as their bounty appeared limitless [3]. The paradigm for natural resource use was primarily one of personal gain and exploitation for commercialization. Conservation and preservation of natural resources appeared unnecessary and unduly restrictive therefore few laws or regulations existed to temper overuse of the natural world [1, 3, 4].

Similar to other natural resources, wildlife it seemed did not need regulation. However, late in the nineteenth century, with the extinction of the passenger pigeon (*Ectopistes migratorius*) [5], near extinction of the plains bison (*Bison bison*) [1], and rapid decline in other large game species such as white-tailed deer (*Odocoileus virgineanus*) [6] and elk (*Cervus elaphus)* [7] as well as fur-bearing species, particularly

beaver (*Castor canadensis*) [8], due to commercialization and demand by a growing population in the Eastern States and Europe, American society began to recognize that without regulation the abundance, and in some circumstances the existence, of certain wildlife species would be permanently altered. During the Industrial Revolution (late eighteenth through the nineteenth centuries), the United States (US) urban workforce grew substantially and was often fed with wild game. At the same time markets for wild game hides, feathers, and other parts were burgeoning in the US and Europe [4, 7, 9]. This led to unsustainable market hunting of wildlife to feed and adorn a significant proportion of the American and European public. Concurrently, as life became increasingly mechanized and civilized, an urban class of society emerged who had the time and money to engage in sport hunting. Sport hunting differed from market hunting in that participants endorsed the principles of fair-chase, self-restraint, and pitting the skills of an individual against nature rather than using any means necessary to kill as many wild animals as the market would bear [4, 9]. Conflicts between market and sport hunters, dwindling wild game populations, and the well publicized wasteful bison slaughter led to advocacy for elimination of wildlife markets, allocation of wildlife by law rather than privilege, and restraint on the take of wildlife except for legitimate purposes [9]. Thus, sustainable harvest became the first principle of wildlife management.

The 1842 US Supreme Court ruling in Martin v. Waddell was also paramount in the development of contemporary wildlife management practices. This case set the precedent that wild natural resources are owned by no one and are to be held in trust by government for the benefit of the citizenry now and in perpetuity. This principle, now

known as the Public Trust Doctrine, is the basis for state and federal government authority for wildlife management [10].

Early champions of wildlife conservation were George Bird Grinnell and Theodore Roosevelt. Both were avid hunters and they recognized that if long-term gain and human enjoyment were to come from co-existing with and hunting wild species, the human take of these species had to be regulated. Gifford Pinchot, the first chief of the US Forest Service, supported the idea of sustainable-use rather than outright preservation of natural resources. While Grinnell, Roosevelt, and Pinchot did not always agree with early preservationists including John Muir, George Wright, Margaret Murie and others, on the ideas of sustainable-use versus preservation they all recognized that wildlife habitat was crucial to healthy and productive wildlife populations. These leaders and other founders of the field of wildlife management fought convincing political battles to establish large areas of publicly owned land to maintain wildlife habitat and they helped shape the basis for the contemporary North American Model of Wildlife Conservation [4, 11].

North American Model of Wildlife Conservation

In contrast to the traditional European system of wildlife management, which embodies the principle that most land, and therefore wildlife habitat, is privately owned often limiting wildlife harvest to only the wealthy, the North American Model is based on the following seven principles [4, 9, 12, 13].

1. *Wildlife is held in public trust by the state and federal governments for all citizens.* The decision in Martin v. Waddell, which ruled all citizens, not only landowners, could dredge for oysters in navigable waters off the coast of New Jersey, established the “Public Trust Doctrine”. The 1896

court ruling, Geer v. Connecticut, regarding illegal transport of game birds across state borders, solidified the concept of government management of wildlife in public trust.

2. *Wildlife will not be sold in commerce.* During the mid- to late-nineteenth century the commercial market demand for wildlife products was diverse and included meat, hides, and feathers. Because wildlife was owned by no one and available for all there was no method to regulate the take. By making the sale or traffic of wildlife products illegal the desire and demand for wildlife take was reduced.
3. *Take of wildlife will be allocated by law.* Once the market demand for wildlife was removed the question remained of how to decide who should have access to wildlife resources and how to set limits on wildlife take. The logical choice in a democratic nation was by law. This eliminated the allocation by privilege and allowed for input into wildlife management practices by all citizens.
4. *Hunting is democratized.* Because wildlife is owned by all citizens of the US all members of the public should have equal opportunity to hunt and fish. By giving people the right to use wildlife sustainably, the model also encourages those who value these resources to support their conservation. This empowers individual citizens and encourages protection of not only wildlife but also the habitat they depend upon.
5. *Wildlife should only be killed for a legitimate purpose.* Once the laws regulating who, what, where, when, and how many of a species could be

hunted, there still remained the question of "why" wildlife may be harvested. Consistent with early sport hunting traditions and the principles of conservation wild animals could only be killed for food, fur, self-defense, and property protection. While these categories are now broadly interpreted, the intent was to prevent frivolous take of wildlife.

6. *Wildlife is an international resource.* Early hunters and anglers recognized that human determined boundaries are not recognized by wildlife and policies of one nation can influence wildlife interests in another. Therefore, the only reasonable way to manage wildlife is in cooperation with other jurisdictions which share wildlife resources and their habitats.
7. *Science is the preferred tool for discharge of wildlife policy.* Curiosity and the desire to understand the natural world were important qualities in hunters and anglers at the turn of the twentieth-century. Theodore Roosevelt was a particularly avid hunter, naturalist and advocate of making decisions based on science rather than anecdotal evidence or special interest group whim. Therefore, it is not surprising that early wildlife management principles were based in scientific findings and accurate descriptions of species natural history.

This hunter/angler harvest driven model of wildlife conservation was developed for the state of the nation at the turn of the twentieth century. Although there are many challenges to these tenets in modern day wildlife management [10, 14], laws and policies supporting these concepts continue to be the profession's guiding principles today. Keys to success of this model are the sustained regulated use of wildlife species and

maintenance of sufficient habitat to support viable wildlife populations. This model has been self-supporting throughout the twentieth century with the majority of state funds dedicated to wildlife management activities derived from sales of hunting and fishing licenses as well as taxes derived from the Pittman-Robertson (1937), Dinglell-Johnson (1950), and Wallup-Breaux (1984) Acts [15]. These congressional legislative principles authorized taxation of hunting and fishing equipment to create revenue for use in fish and wildlife management. It is clear that maintaining wild game populations for hunting and angling purposes was the primary driver of the field of wildlife conservation at its inception. While there was no specific mention of non-game species in the North American Model, many of these species have benefited from the Model because of temporal or spatial association in habitat with game species [16]. However, with development of more refined understanding of ecosystem function, the importance of wide species diversity and preserving complete ecosystems rather than managing only for game species has become clear [17].

Changing Management Paradigms

The Endangered Species Act of 1973 was instrumental in recognizing and protecting the value of all species. This began a paradigm shift which emphasized preservation of rich species diversity rather than protection of only individual species which are valued for their direct benefits to humans. At the same time the American populace has become more urbanized and less likely to participate in hunting and angling opportunities. Wildlife related activities such as hunting, fishing, and even wildlife viewing have plummeted in the last quarter century [18]. As this happens the reasons for wildlife conservation inevitably begin to change. There is a growing desire to incorporate

wildlife into one's social environment or simply know it exists rather than to utilize it as a renewable harvestable resource [19]. Modernization and urbanization has resulted in an increasingly mutualistic view of wildlife. Mutualism is the view that wild animals are capable of relationships of trust with humans and is defined by a desire for companionship with wildlife [20]. This view has likely grown from the reality that we are no longer reliant on wildlife as a source of food or material and that people are more removed from wildlife in their daily lives. As a whole, Americans tend to learn of wildlife not from their own interactions but from removed sources such as the media, stories, and other's experiences. This filtered information is often portrayed in an anthropomorphic manner. It has been proposed that wildlife is perceived less as a natural resource or threat and more as a part of the social environment, having the potential for companionship [19, 20]. Although attitudes vary widely and have many driving forces, at a societal level this phenomenon has led to declining utilitarian and rising protectionist attitudes [21].

At the same time that attitudes are changing, many of the species nearly hunted or trapped to extinction have now recovered. Highly adaptive species, such as the coyote (*Canis latrans*), Canada goose (*Branta canadensis*), white-tailed deer, and elk, have learned to thrive in the face of anthropogenic change. This has led to locally burgeoning populations which are often associated with increased human-wildlife conflicts. When wildlife is no longer seen as rare or "special" and negative interactions override the desire to connect with wildlife they become pests [22]. As a result, there is a dichotomy in which some segments of society desire both protection for wildlife and the relief from agonistic interactions [21]. The value driven objectives of wildlife management and

conservation are changing from more traditional utilitarian goals to increasingly mutalistic aims.

A natural response to changing drivers of conservation has been the shift in methods of conservation. Mid to late in the twentieth century locally abundant populations of wildlife led to increased human-wildlife conflicts [23]. At the same time society's knowledge of animal welfare and acceptable methods of managing human and wildlife interactions grew. While hunting is still a large and influential part of American culture, access to land with large concentrations of game has become more limited as urban and suburban development expand into rural areas [10]. Government agencies, with wildlife preservation mandates but without hunting as their primary wildlife management tool, also experience locally overabundant wildlife populations [24]. Finally, animal and wildlife advocacy groups have formed and began speaking out against lethal removal of wildlife species where human-wildlife conflicts are present [25-27]. All of these factors, and arguably many others, have influenced current wildlife management techniques.

Changing Management Techniques

Where once sustainable harvest was the driving force and primary tool in the wildlife manager's toolbox, today wildlife professionals need a range of tools to deal with both game and non-game wildlife populations, particularly in areas where lethal take is either illegal or inaccessible. As stewards of wildlife resources held in public trust, their tools must be in accordance with changing cultural values. While many of these tools will focus on methods of preventing or mitigating wildlife impacts on the human environment (e.g. exclusion techniques, minimizing spatial and temporal overlap of humans or their

property and wildlife, encouraging tolerance of wildlife in human environments) others will focus on direct manipulation of wildlife population size or density. The four basic drivers of population size are reproductive rate, survival rate, emigration, and immigration [28]. Traditional wildlife management techniques for limiting population size (e.g., hunting, culling, translocation) focus on survival rate and mitigating immigration or encouraging emigration effects. During the 1960s scientists began to ask if it was possible to influence reproductive rates of wild species. From this the field of wildlife fertility control developed [29].

While the field of wildlife fertility control spans a variety of avian and mammalian taxa [30], most research has concentrated on the orders Artiodactyla (e.g., families Cervidae, Suidae), Perissodactyla (e.g, family Equidae), and Carnivora (e.g., family Felidae) [31]. Two cervid species native to North America, the white-tailed deer and elk, have become adept at living in close proximity to humans [7, 32]. This adaptive quality combined with shrinking habitat due to human encroachment and a scarcity of remaining natural predators has led to areas of locally overabundant cervid populations [16]. “Overabundant” is a value driven term implying there are too many animals. Caughley described four general situations where wildlife become locally overabundant 1) when animals threaten human life, livelihood, or property, 2) when animals depress densities of favored species of flora or fauna, 3) when animals reach densities that promote disease transmission or decrease fitness in the population, and 4) when their numbers or densities cause ecosystem dysfunction [33]. Both white-tailed deer and elk have met these criteria in a variety of urban, suburban, rural, and wild habitats precipitating human-wildlife conflicts. The combination of changing societal attitudes

which emphasize connection with wildlife contrasting with mounting antagonistic human-wildlife conflicts may have driven the emergence of fertility control methods in cervids and other wildlife.

The following chapter will summarize the historical and current state of the science of fertility control methods applied to cervids. This will be followed by two research papers which expand our understanding of one potential method for curtailing wildlife reproduction. Finally, I will describe the most critical technical and sociological barriers to using fertility control in free-ranging wildlife as a viable method of population management.

Chapter 2: Literature Review

Fertility Control Alternatives in Female Cervids

Requirements for a Fertility Control Agent

Cervids have a polygamous breeding structure [34, 35] and females are polyestrus with estrous cycles continuing into the late winter and early spring if pregnancy is not achieved [36, 37]. While mature males dominate breeding activity, subordinate males also contribute to reproduction [34, 35, 38]. It is generally accepted that decreasing female rather than male fecundity is necessary to reduce the rate of population growth or initiate population decline [28, 39, 40]. Relatively few methods for manipulating female reproductive function have been investigated as potential fertility control techniques in cervids. To date three basic means of inhibiting reproduction that have received the most scrutiny are steroid hormone administration (e.g., estrogens and progestins), non-steriodal hormone delivery, and immunocontraception [30, 41, 42]. Physical disruption of the reproductive tract has also been explored [43-45].

It has been suggested that the ideal fertility control agent would be 1) highly effective, 2) free from toxicity and harmful side-effects for the target animal, 3) reversible to preserve the reproductive capacity of the individual and genetic integrity of the population, 4) inexpensive, 5) have little if any impact on social interactions and behavior, 6) be effective with a single administration preferably through remote delivery, and 7) be incapable of passing through the food-chain to predators, scavengers, or humans [42]. The remainder of this review will evaluate to what degree investigated methods of fertility control in female cervids meet the above criteria. Given that the majority of the costs incurred with fertility control in free-ranging wildlife are in handling

the animal [46] and actual cost of the drug or method is either inconsequential or unknown due to the experimental nature of the agent, costs will not be explicitly described.

Steroid Hormones

Early investigation into limiting the reproductive rate of wild species applied knowledge derived from contraception methods used in humans and domestic animals [29, 47]. The first inquiry into chemosterilization of elk [45] and deer [48-55] used synthetic estrogen (i.e., diethylstilbesterol [DES]) and/or progestins (i.e., altrenogest, melangesterol acetate [MGA], levonorgestrel) which directly affected feedback at the level of the reproductive tract, pituitary and hypothalamus thus preventing ovulation, implantation, or maintenance of pregnancy. Oral, subcutaneous, and intramuscular administration of these compounds have been applied [48, 51], prior to conception [48, 49] and during gestation [45, 52]. Levongesterol was not effective in suppressing pregnancy in deer [52, 53], whereas oral or parenteral MGA [50, 54, 55] and parenteral DES or altrenogest were highly effective [48, 51, 55]. A more contemporary application of the synthetic progestin norgestomet through remotely delivered biodegradable implants (i.e., biobullet), was highly successful in preventing pregnancy for one year [56, 57].

While good or even excellent success in preventing or terminating pregnancy has been achieved with many of these regimens and they are used routinely in captive wildlife medicine [29], steroidal contraceptive and contragestive measures have not been pursued as management alternatives in free-ranging wildlife for a variety of reasons. The first are logistical constraints. Oral administration of MGA requires daily administration

to maintain adequate peripheral progestin concentrations to suppress ovarian function [49, 54, 58]. This creates the challenge of maintaining daily intake in free-ranging species and preventing consumption by non-target species. To date these challenges are insurmountable in free-ranging cervids. While not as application intensive, parenterally administered steroids generally require yearly retreatment prior to the breeding season [56, 57] although some slow release implants may be effective for two years [50, 55]. These methods may be feasible if sufficient numbers of animals can be treated on regular basis [59, 60].

The second and likely more significant set of reasons for avoiding steroid hormone contraception in wildlife are related to the health and welfare of the treated animal and the potential or perceived potential for non-target animal effects through the food chain. Few studies have investigated the long-term effects of steroid based contraceptives in cervids [31]. In other mammalian species, primarily carnivores and primates, high dose exogenous progestins are associated with a variety of pathologies including cancer, inflammatory endometrial and ovarian disease, and diabetes [61]. Diethylstilbesterol is a potent teratogen and mild carcinogen in humans but apparently not in cattle [62-64]. While adverse effects, other than pyometra, have not been reported in cervids, the potential for pathologic changes exist and the stigma associated with their use likely influence wildlife manager's decisions. Finally, there is little information on the potential behavioral effects of estrogen/progestin use in cervids, but given that estrogens and progestins can be used to regulate estrous behavior in other ruminants [65, 66] it is likely these hormones would similarly effect cervid sociosexual behaviors.

Whereas the health effects of exogenous estrogens or progestins in target cervid species are not well defined, potential effects in non-target species, including humans, through the food chain are even more elusive [57, 67]. Two potential exposure risks exist: 1) through consumption of an implant that has residual bioactive hormone or 2) by consuming the tissues of a treated animal. It is routine practice in North America to use combinations of slow release estradiol, progesterone, and androgen implants in cattle and sheep production [62]. While the influence of low level chronic exposure to these exogenous hormones through the food chain is not well understood, it has been suggested that they may have effects on the developing male human fetus [67, 68]. There is a growing body of evidence that environmental exposure to a variety of endocrine disrupting compounds, which could include growth promotants, have negative long-term effects on human male fertility [69]. The US Food and Drug Administration (FDA) regulates the requirements for use of these pharmaceuticals in the US and the International Joint Food and Agriculture Organization/ World Health Organization Expert Committee on Food Additives (JECFA) have published acceptable daily intake for all hormones currently used as growth promotants [67]. Despite these safeguards the European Union does not allow the use of growth promotants which has roused trade disputes and confusion amongst the public [63, 67, 70]. The true risk of altering population level or even individual fertility in either non-target wildlife predators/scavengers or humans through consumption of cervids treated with exogenous hormones is remote given the exceptionally low exposure potential; however, lingering questions and the perception that steroid hormones present an environmental hazard has

likely influenced the wildlife fertility control community to avoid steroidal methods of altering fertility [42, 57, 71].

Non-Steriodal Hormones

Methods of non-steriodal manipulation of the hypothalamic-pituitary-gonadal (HPG) axis of deer and elk, for the purpose of decreasing reproduction, include the use of GnRH agonists to down-regulate GnRH receptors in the pituitary or administration of prostaglandins to induce abortion. Continuous exposure to GnRH agonists causes desensitization and internalization of the homologous receptor and elimination of gonadtotrope response to endogenous GnRH signaling [72, 73]. More than 2000 GnRH analogues (agoinsts and antagonists) have been synthesized since the structure of GnRH was reported [74]. Strong receptor binding affinity, high receptor activation, and slow degradation rate contribute to the enhanced anti-fertility effects observed with 'superagonists' [75]. Continuous high dose administration of the GnRH agonists leuprolide acetate or histrelin acetate suppress reproductive function in female deer [76, 77] and elk [71, 78, 79]. In fact, a 10 or 32.5 mg leuprolide dose delivered in a slow release implant was 100% effective in suppressing reproduction for 1 year in both mule deer and elk respectively. Although there were no differences in time spent foraging, resting, or moving between agonist treated and control animals [79] male precopulatory behaviors displayed toward treated females were persistent throughout the breeding season whereas those displayed toward control females declined with time. However, precopulatory behaviors were not observable during the post-breeding estrous transitional season [71, 76]. At this time GnRH agonists must be administered parenterally in wildlife because their oral bioavailability is extremely low [80]. This characteristic prevents non-

target species exposure and effectively eliminates food-chain reservations [71]. A drawback to using GnRH agonists is that they must be reapplied yearly prior to the breeding season to be effective. This poses logistical challenges to treating sufficient numbers of animals at a time when they are typically highly dispersed throughout their habitat [81].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), is a paracrine and autocrine cellular messenger derived from arachidonic acid which is instrumental in regulating function of the corpus luteum (CL) [82, 83]. It is used extensively to manipulate reproduction in domestic livestock and has been investigated as a reproductive inhibitor in deer [84-86]. Prostaglandin $F_{2\alpha}$ is produced by the uterus as well as the CL, and initiates luteal cell apoptosis and luteolysis [83]. White-tailed deer are dependent on luteal progesterone during at least the first 156/200 days of gestation [87] and elk are likely similarly reliant on the CL [88, 89]. Treatment with prostaglandin $F_{2\alpha}$ has been used to terminate pregnancies in elk although they are moderately refractory to its luteolytic effects [89, 90]. Deer also appear to require extended exposure to $PGF_{2\alpha}$ to realize the abortifacient effects [86]. Terminating pregnancy early in the reproductive season may lead to re-breeding whereas inducing abortion late in the breeding season may lead to increased complications due to pyometra or dystocia [91, 92]. Only a small window exists for effective use of $PGF_{2\alpha}$ as a fertility control agent in extensively managed free-ranging cervid populations. Two benefits of $PGF_{2\alpha}$ are that it is FDA approved for use in food producing animals and that it is rapidly metabolized in the lungs to biologically inactive break-down products [93], thus effectively eliminating food-chain concerns [84]. Behavioral effects have not specifically

been investigated; however, they would likely depend on the timing of pregnancy termination in relation to seasonal reproductive patterns.

Immunocontraception

By far, the most extensively studied cervid reproductive inhibitors are the immunocontraceptives [30, 41, 94, 95]. The host is immunized with one of several potential antigens required for reproductive function. Once sufficiently stimulated the host's immune system produces antibody (B lymphocyte) and cell-mediated (T lymphocyte) responses that prevent ovulation, conception, or implantation. Vaccines have been created to target a variety of antigens and several have been tested in female cervids. They include; 1) porcine zona pellucida (PZP or pZP) [36, 94, 96-106], 2) gonadotropin releasing hormone (GnRH) [107-111], 3) sperm proteins [112], and 4) chorionic gonadotropins [113]. Neither sperm protein nor chorionic gonadotropin vaccines have proven effective in cervids despite their efficacy in other species [114, 115]. Alternatively, PZP and GnRH vaccines have proven moderately or even highly effective (~ 50 – 100%) at preventing pregnancy, with newer vaccine formulations inducing prolonged (> 1 yr) sub-fertility after a single vaccination in cervid species [106, 109, 110, 116, 117].

Immunocontraceptive vaccine targets are self-antigens and therefore must be combined with strong adjuvants and/or coupled with immunogens novel to the mammalian immune system to produce adequate immune response [112]. Due to their broad immune-stimulatory effects, Freund's complete and incomplete adjuvants (FCA/FIA) were the primary adjuvant system of choice in early studies, however, due to regulatory and animal welfare concerns alternative adjuvants are desirable [30, 118]. At

this time, FCA/FIA continue to be used in some contraceptive vaccine formulations. Regardless, effective adjuvants are often associated with granulomatous inflammation at the site of the injection and may incite inflammation in the local lymph nodes draining the injection site [105, 110, 119-121]. These inflammatory lesions do not appear to be overtly painful or limiting to daily activity, however, they do raise animal welfare concerns. Antibodies stimulated by contraceptive vaccines are not likely to pose a problem for scavenger, predator, or human food chains because they degrade in the gastrointestinal tract. Similarly, there is no evidence that ingestion of the vaccine depot, which may remain in muscle tissue of vaccinated animals, is a human or non-target animal health concern, however, little research has been conducted to validate this hypothesis [122].

The majority of immunocontraception work in wildlife species has been performed using PZP vaccines. Both antibody and cell-mediated immunity directed towards specific outer surface proteins of domestic pig oocytes have been implicated in the mechanism of action of PZP vaccines [123]. Antibodies prevent fertilization, presumably by occluding sperm attachment sites on the zona, although ovarian oophoritis likely also contributes to the long-term contraceptive effect [123]. The PZP antigen vaccines have been tested in more than 70 captive wildlife species [94] and there have been extensive field trials in free-ranging white-tailed deer [46, 101, 106, 124-126] as well as limited studies in free-ranging elk [102, 127]. Porcine zona pellucida vaccines are the only fertility control agents that have been applied in long-term management level studies, and have proven successful at achieving modest population decreases in small closed populations of long-lived wildlife species including deer [126, 128]. While

efficacy, durability, and population level effects of PZP vaccination are impressive, immunization is consistently associated with continued estrous cycling beyond the typical cervid breeding seasons [98, 103, 116, 127]. This can prolong breeding behaviors and if contraception fails late in the season may lead to late season pregnancies which have been observed [129]. It has been argued that late season breeding is not damaging to the individual or the population, because decreased energy demands in non-pregnant females and seasonal limitations of male mating activity will offset potentially increased energy demands of an extended breeding season [94, 98, 129]. While this may be true, there is also the potential for decreased fawn survival with late season births and changes to the basic ecology of cervid reproduction which is typically well synchronized with habitat nutrient availability [34].

Gonadotropin releasing hormone (GnRH) vaccines have also received significant scrutiny as wildlife reproductive inhibitors [30, 41]. GnRH is a small neuropeptide which initiates the endocrine cascade that eventually results in reproduction. It is naturally secreted in a pulsatile pattern from neurons in the hypothalamus, released into the hypothalamic-pituitary portal vasculature, and signals gonadotroph cells in the anterior pituitary to synthesize and release luteinizing hormone (LH) and to a lesser extent follicle stimulating hormone (FSH) [130, 131]. GnRH along with ovarian and pituitary derived endocrine, paracrine, and autocrine signaling is responsible for gonadotrope function [132, 133] and ultimately gametogenesis [134]. GnRH is not generally immunogenic but can be made so by conjugation to a large, highly immunogenic carrier protein [135]. When combined with a potent adjuvant, this vaccine stimulates a persistent immune response resulting in prolonged antibody production against GnRH, the carrier protein,

and the adjuvant [108]. Although there are competing theories of action [136], the prevailing hypothesis suggests that antibodies to GnRH likely induce transient infertility by binding to endogenous GnRH in the hypothalamic-pituitary portal vessels, thus preventing attachment to receptors on gonadotropes, and suppression of pulsatile luteinizing hormone (LH) secretion. GnRH-antibody titers are associated with suppression of the reproductive system and infertility in a variety of species [107, 137-140].

Single administration of a GnRH vaccine has proven effective in both deer and elk at limiting individual animal fertility [108-110], but has not yet been evaluated for population management. Because GnRH vaccination suppresses the reproductive axis it is likely that estrus and associated breeding behaviors will be eliminated in immunized cervids. Preliminary findings in white-tailed deer lend support to this hypothesis [107]; however, the question has not been posed in elk nor extensively studied in deer. Finally, GnRH vaccination during pregnancy has not been investigated in cervids. It has been suggested that GnRH vaccination may act as a contragestive by inducing luteal insufficiency and decreased progesterone secrection [107]. Although CL function and progesterone production is regulated by LH signaling during the estrous cycle [141], it is not likely that elimination of GnRH signaling and pulsatile LH release during pregnancy will significantly alter CL function [142, 143]. However, the possibility of luteolytic effects in cervids has not been investigated. Finally, the potential effects of maternal passive transfer of GnRH antibodies to offspring of vaccinated cervids are unknown. While GnRH vaccination is a promising wildlife fertility control agent, significant questions remain unanswered.

Although immunocontraception is generally reversible over variable time periods, permanent sterility in a proportion of the population is a potential consequence that has not been fully investigated in the many versions of PZP and GnRH vaccines [96, 117, 119, 144-146]. Ovarian lesions including oophoritis and follicle depletion are often associated with PZP vaccination [121-123]. Inflammatory lesions of the median eminence have been observed after active vaccination against GnRH and were associated with degree of HPG axis suppression [136]. These changes have been attributed to T-cell interactions with self-antigens and have the potential to persist indefinitely [123, 136]. Despite these autoimmune reactions, daily life activities and function do not appear to be compromised [121, 122]. It has also been suggested that seasonal return to estrous cyclicity may self-inoculate and increase humoral immunity [121]. These mechanisms may contribute to long-term or even permanent infertility in a proportion of a population treated with either PZP or GnRH vaccines. This phenomenon may be advantageous when trying to most efficiently control reproduction in a population; however, it may be unacceptable when the genetic contribution from individual animals is desired [112, 147].

Other Potential Reproductive Inhibitors

Other reproductive inhibitors investigated in female cervids include gonadotrope toxins, intrauterine devices, and surgical sterilization [43, 44, 148, 149]. Gonadotrope toxins have been created by conjugating GnRH agonists to potent cytotoxic agents such as pokeweed antiviral protein or doxorubicin [150, 151]. While suppression of gonadotropin secretion has been demonstrated in ovariectomized sheep and deer, and testis volume decreased in treated domestic dogs, the ability to decrease female fertility has not yet been tested [148, 152, 153].

Physical disruption of the reproductive tract through oviduct ligation [43] or intrauterine device application [44] has also been explored. Both of these methods have been successful in limiting deer reproduction, however, they are logistically difficult and not likely to be adopted on a population level scale. Neither method removes HPG axis endocrine signaling; therefore, estrous cycling is likely to continue until seasonal anestrus ensues. All three of these methods would require further development in captive cervids prior to use in free-ranging animals.

Conclusions

While there is no panacea for controlling female cervid fertility, one of the most promising agents, for use in free-ranging wildlife, is the GnRH vaccine. However, questions remain regarding its long-term efficacy and effects on reproductive behavior, current pregnancy and physiological side-effects, particularly in elk.

Chapter 3: Effects of GnRH Immunization on Reproductive Function and Behavior in Captive Female Rocky Mountain Elk (*Cervus elaphus nelsoni*)

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Abstract

Fertility control is a potential method for managing overabundant wildlife populations; however, current technology is limited by duration of treatment efficacy and unacceptable side-effects. The objective of this study was to determine the efficacy of a single immunization with gonadotropin-releasing hormone (GnRH) vaccine to suppress reproductive function in pregnant female elk and to evaluate potential behavioral and pathological side-effects of treatment. Eighteen captive adult female elk were randomly allocated to one of two experimental groups. Ten females were administered a conjugated and adjuvanted GnRH vaccine intramuscularly and eight elk received a sham vaccine without conjugated GnRH. We compared success of existing pregnancy, neonatal survival, subsequent fertility, reproductive behavior rates, and side-effects of treatment between January 2006 and January 2010. GnRH vaccination did not affect existing pregnancy or calf survival during the year that it was applied; however, it reduced the proportion of pregnant females for three years. Male precopulatory behavior rates exhibited toward GnRH vaccinated females tended to be greater than those directed at

[1] Manuscript has been provisionally accepted (4/11) by the journal Biology of Reproduction
[2] Work was supported by the Morris Animal Foundation grant # D05ZO-303 to JGP
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sham-vaccinated females during the second half of the breeding season, when GnRH vaccinates continued to be proceptive. Strong immune and inflammatory responses, including robust GnRH-antibody concentrations in GnRH vaccinates, and sterile pyogranulomatous injection site abscesses in both groups, were consistent with vaccination. In conclusion, this GnRH vaccine resulted in prolonged, though reversible impairment of fertility, and is associated with extended reproductive behaviors and partial suppression of hypothalamic-pituitary-gonadal axis function in captive female elk.

Introduction

Regulating the abundance of ungulate populations has become a significant issue for natural resource managers in many areas of North America [24, 33, 154-159]. This is particularly true for protected environments, such as national parks and conservation areas, where unregulated populations, if left unchecked, can have adverse effects on natural and human-dominated systems [157, 160]. Hunting, culling, and trapping have traditionally been used to regulate animal numbers but there are a growing number of circumstances where these methods pose significant liability [161, 162] and, as a result, resource managers are seeking alternative approaches to population control [47, 163].

Fertility control offers a potential non-lethal method for controlling the growth of overabundant ungulate populations and considerable research has been directed toward the development of different contraceptive technologies [30]. Models have been developed to characterize effects of fertility control on population dynamics of wild ungulates [59, 60, 164, 165]. Yet, current technologies for altering wildlife fertility suffer from a variety of technical, physiological, and regulatory challenges [30]. As a result, only modest successes have been achieved in developing a safe, practical, and feasible

method of controlling reproduction in free-ranging wild ungulate populations [126, 128].

A potential alternative to current fertility control products, which may overcome some of these challenges, involves active immunization against gonadotropin-releasing hormone (GnRH), a small, 10 amino acid, neuropeptide produced in the hypothalamus, which signals via receptors on gonadotroph cells in the anterior pituitary. GnRH along with ovarian and pituitary derived endocrine, paracrine, and autocrine signaling is responsible for gonadotrope function [132, 133] and ultimately gametogenesis [134]. GnRH is not generally immunogenic but can be made so by conjugation to a large, highly immunogenic carrier protein [135]. When combined with a potent adjuvant, this vaccine stimulates a persistent immune response resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant [108]. Although there are competing theories of action [136], the prevailing hypothesis suggests that antibodies to GnRH induce transient infertility by binding to endogenous GnRH in the hypothalamic-pituitary portal vessels, thus preventing attachment to receptors on gonadotropes, and suppression of pulsatile luteinizing hormone (LH) secretion. GnRH-antibody titers are correlated with suppression of the reproductive system and infertility in a variety of species [107, 137-140].

Numerous GnRH vaccines have been developed and are successful in interrupting the hormonal cascade that controls ovulation. These vaccines are used in physiologic research with a variety of domestic ungulates including horses (*Equus caballus*) [166], cattle (*Bos taurus*) [167], swine (*Sus scrofa*) [168], and sheep (*Ovis aries*) [169, 170] and in reproductive management of domestic livestock [171-173]. However, the use of GnRH vaccines as contraceptive agents in wildlife management has been limited by effective

duration, multiple treatments to stimulate adequate antibody response, and deleterious side-effects associated with the use of the controversial Freund's complete adjuvant (FCA) [107, 119]. Recently, an alternative adjuvant (AdjuVac), containing mineral oil and mycobacteria derived from a Johne's disease vaccine (Mycopar, Fort Dodge Animal Health, Iowa), has been developed and may be as effective as FCA but associated with fewer lesions [118].

Based on results from studies in captive and free-ranging wild or feral ungulates, a single application of this GnRH vaccine (GonaCon-B) may prove to be a safe and effective multi-year immunocontraceptive agent for free-ranging wildlife. A single application, formulated with the same or a similar GnRH-protein conjugate, has been shown to provide multiple years of decreased fecundity in several species including feral horses (*Equus caballus*) [104, 174], bison (*Bison bison)* [175], elk [109], feral pigs (*Sus scrofa*) [176] and white-tailed deer (*Odocoileus virginianus)* [108, 110]. However, few studies have rigorously investigated the secondary effects of this vaccine on social behaviors or ecological consequences in any wildlife species [31].

Elk are polyestrus short day ovulators with the peak of breeding season in North America between mid-September and mid-October [177, 178]. Waves of follicle development occur during the ovulatory [179], anovulatory [180], and transitional [37] seasons. Ovulation occurs approximately every 20-21 days [179, 181] and the ovulatory season can persist as late as April although is, on average, 142 days ending in mid-February [37]. Pregnancy rates for mature females (2-12 yr) can approach 1 calf/female/year in populations which are not forage limited [182, 183]. Approximately 50% of yearling females become pregnant at 16-18 months of age if an appropriate body

mass is achieved (~220kg, 10% body fat) [177, 183, 184, 185]. Reproductive senescence is not well described but fertility may decline between 13 and 17 years of age [182]; however, effects of body condition are likely more important to fertility than age [181, 185]. Elk are monotocous and twinning is exceptionally rare [177, 184]. Calving occurs from late May to early July after a gestation of 247-262 days and coincides with spring nutrient flush in most northern latitudes [34, 177].

Application of GnRH vaccine would be most practical during the winter period (Dec – Feb) when elk are concentrated in large, primarily single sex herds [81] and temperatures and snow cover are conducive to chemical or physical capture [186]. At this time, most adult female elk are in mid- to late-gestation and any contraceptive treatment must be demonstrated to be safe for the developing fetus and health of the neonate [42, 187, 188].

In light of these issues we examined the contraceptive efficacy and potential side-effects of GnRH vaccination by testing the following four hypotheses using captive female elk. A single immunization with GnRH vaccine during mid-gestation in elk will: 1) not affect existing pregnancy, 2) suppress future fertility with contraceptive effects waning as GnRH-antibody concentrations decrease, 3) suppress reproductive behaviors, and 4) be associated with localized inflammatory reactions but not other pathological side-effects.

Materials and Methods

Animals and Vaccine

This study was reviewed and approved by the Colorado State University (#05-187A-01) and the Colorado Division of Wildlife (#07-2005) Institutional Animal Care

and Use Committees. Animals were housed at the Colorado Division of Wildlife, Foothills Wildlife Research Facility in Fort Collins, Colorado, USA (40°35'46" N, 105°9'29"W). Eighteen female elk (1.5 – 12 years of age at study onset; 220 – 275 kg) and two reproductively proven mature male elk (5 and 7 years of age; 400-450 kg) were used for this experiment. The majority of experimental elk were long-term residents of the facility, most having been born in captivity. To meet sample size requirements, two free-ranging three year-old females from a local wild population were captured and brought into the captive herd in the spring of 2005. Female elk were trained for repeated handling in isolation pens, alleyways, and a handling chute, and for blood sampling and ultrasound imaging procedures. Female elk were maintained throughout the experiment in two fenced paddocks (5.0 ha) with minimal native forage and fed a diet of ad libitum alfalfa-grass hay mix, trace mineral blocks, and water, as well as limited pelleted grain supplement. Male elk were similarly maintained in a paddock, physically removed but within sight of females. During breeding seasons (late September – late November), males were maintained with females. All biological samples were collected and hands-on measurements made while female elk were lightly sedated using xylazine hydrochloride (30-250 mg/animal i.m.; TranquiVed, Vedco, Inc. St. Joseph, MO) in a non-squeeze chute. Tranquilizer effects were reversed after each sampling session with either yohimbine hydrochloride (30 mg/animal i.v., Wildlife Pharmaceuticals, Fort Collins, CO) or tolazoline hydrochloride (600 mg/animal i.m., Tolazine; Akorn, Inc., Decatur, IL).

Experimental vaccines were prepared as previously described [108]. Briefly, the GnRH vaccine consisted of multiple copies of synthetic GnRH peptide linked to hemocyanin protein (Blue Carrier) from the Chilean mollusk (*Concholepas concholepas*;

CCH), and combined with a water-in-oil adjuvant containing killed *Mycobacterium avium* ssp. *avium*. The sham vaccine was similarly prepared but without conjugated GnRH.

Experimental Protocol

Data were collected between January 2006 and January 2010. At the start of the experiment, all 18 females were 80-100 days pregnant, as determined by serum pregnancy specific protein B (PSPB) assay [189], rectal palpation [190, 191], and/or transrectal ultrasound [192]. One sham vaccinated female (20 mo. of age) aborted her pregnancy by late February 2006.

Approximately half of the females had been previously exposed to a *Brucella abortus* Strain 19 vaccine. To avoid potential confounding anemnestic response effects, which may arise from antigenic stimulation with similar intracellular pathogens (*B. abortus* and *M. avium*) [193] or age related fertility affects [182], we blocked sample units (female elk) with respect to brucella vaccination history and age (1, 2-10, ≥11 years of age) and then randomly assigned animals to either the GnRH vaccine (n = 10) or sham vaccine (n = 8) group. GnRH-vaccinated females were administered 1.5 mg GnRH-Blue Carrier protein conjugate with adjuvant (1.5 ml) into the left biceps femoris muscle using a hand-held three ml syringe and 3.8 cm, 18 gauge needle. Sham-vaccinated females received a similar volume of carrier protein and adjuvant but without conjugated GnRH. Injection site location was similar in all animals with placement consistently at the leading edge of the cream colored rump patch and at the same height as the tuber ischii. To facilitate pre-treatment ultrasound examination described below, hair was removed

from the injection site and surrounding skin using electric clippers with a no. 40 blade and the area was cleaned using isopropyl alcohol.

Females were divided into two pens (each pen with 5 GnRH vaccinates and 4 sham vaccinates) and placed in separate paddocks. In late September of each year (2006-2009) one male was placed in each pen for 62-65 days. Females remained in the same pen throughout the study while males were rotated between pens each year to minimize effects due to potential random differences in male fertility and individual male/female interactions.

Pregnancy and Ovarian Measurements

We investigated the effects of GnRH immunization on the existing corpora lutea (CL), pregnancy, and calving (2006) by comparing monthly (January – May 2006) serum progesterone concentrations, calving success, and calf survival and growth. Calves were weighed 12-24 hours after birth, and then at two to four week intervals for the first three months of life. Calves remained with their dams until weaning on September 1, 2006. Subsequent pregnancy rates were determined each January over a four year period (2007-2010) using methods described above. Once pregnancy was confirmed in subsequent years, abortion was induced using two doses of prostaglandin $F_{2\alpha}$ six hours apart (25mg i.m.; Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) [89, 90]. Abortions were conducted for management purposes.

We measured ovarian follicular and luteal structures in GnRH and sham-vaccinated females at single time points the first year post-vaccination (2006-2007) during the early ovulatory (mid September), late ovulatory (early January), transitional (late February), and anovulatory (late April) seasons using transrectal ultrasound imaging

(5 MHz linear array transducer; Televet 1000, Classic Universal Ultrasound, Tequestra, FL) [37, 179, 180]. January imaging occurred coincident with pregnancy diagnosis; at all other time points females were not pregnant. Images were saved on a laptop computer for later evaluation. We measured the total number of antral follicles, follicular diameter (mm) and the presence or absence of ovarian structures consistent with luteal tissue. We then grouped follicles into small (< 4 mm), medium (4 to < 7 mm), and large (≥ 7 mm) classes, and calculated total follicular volume combining data from both ovaries for each individual.

Analysis of Hormone Concentration and Antibody Titer

Blood samples (10-30 ml) were collected via jugular venipuncture using a 20-gauge blood collection needle, tube holder, and 10 ml blood tubes without anticoagulant (BD Vacutainer SST; Becton, Dickinson, and Co., Franklin Lakes, NJ). Blood was allowed to clot at room temperature, centrifuged for 10 min at 1500 x *g*, and serum was decanted to polypropylene tubes and stored at -80° C until assays were performed. Monthly (January – May 2006) serum concentrations of progesterone were measured by radioimmunoassay (RIA) [194]; a technique previously validated in elk [71]. All samples were run in duplicate in a single batch. The range of the standard curve was 0.39 ng/ml (80% ligand labeled progesterone) to 15.0 ng/ml (20% ligand labeled progesterone). Intra-assay coefficients of variation were 14.1% for the low reference sample and 7.1% for the high reference sample.

Serum GnRH antibodies were measured prior to vaccination, monthly until calving, and prior to introducing males into female pens each year (September 2006-2009). The concentration of unbound GnRH antibodies was estimated by measuring ^{125}I-

GnRH binding capacity in peripheral serum [135, 170]. Serum samples were diluted between 1:2 and 1:100,000 using 0.05 M ethylenediaminetetraacetic acid (EDTA) in 0.01 M PBS with 0.1% gelatin (EDTA-PBS gel). Two hundred µl of diluted test sera, 100 µl EDTA-PBS gel, and 100 µl ^{125}I-GnRH (specific activity ~ 1850 Ci/mmol; ~ 60,000 dpm) were added to 5 ml glass tubes. Solutions were vortexed and incubated for 24 hours at 4° C. One ml of 20% polyethylene glycol solution (6000 mw; diluted with PBS) was added and tubes were vortexed. Tubes were incubated for 20 minutes at 4° C, then centrifuged for 30 minutes at 980 x *g*. Supernatants were decanted and tubes placed in a gamma counter (efficiency ~ 80%; Micromedic Systems, Horsham, PA) to record radioactivity (cpm). Total radioactivity was measured in 100 µl ^{125}I-GnRH. Non-specific binding was measured by adding 100 µl ^{125}I-GnRH to 300 µl of EDTA-PBS gel and handling similarly to test samples. High antibody titer positive control sera from rabbits [135] and elk previously vaccinated against GnRH (data not shown) as well as negative control elk sera were included with each batch. All samples were analyzed in duplicate. To provide a similar comparison between animals, we selected the 1:1000 dilution to estimate antibody concentration (pmol/ml) and re-analyzed all samples in a single batch. The intra-assay coefficient of variation was 3.5%. GnRH-antibody response is presented as pmol of ^{125}I-GnRH bound per ml of serum.

Physiological Side-Effects

Mean serum chemistry and hematology parameters, *M. avium* antibody status, and prevalence of injection site reactions were compared between groups. Blood was collected as described above but with the addition of a 10 ml blood tube with EDTA anticoagulant for hematology. Samples were taken prior to vaccination for each assay and

then at 30, 90, and 340 days post-vaccination for hematology, chemistry, and *M. avium* assays respectively. Whole blood and serum were submitted to the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO) for analysis. Hematology parameters measured included: total nucleated cells, segmented neutrophils, lymphocytes, monocytes, eosinophils, platelets (all x 10^3/µl), plasma protein (g/dl), red blood cells (x10^6/ µl), hemoglobin (g/dl), packed cell volume (%), mean corpuscular volume (fl), mean corpuscular hemoglobin concentration (g/dl), and fibrinogen (mg/dl). Chemistry profile parameters included: glucose, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, total bilirubin (all mg/dl); enzymes including creatinine kinase, aspartate aminotransferase, gamma-glutamyltransferase, sorbitol dehydrogenase (each IU/l); electrolytes including sodium, potassium, chloride, and bicarbonate (each mEq/l). *M. avium* antibody concentrations were measured using an enzyme-linked immunosorbant assay (HerdChek ELISA; IDEXX Laboratories, Westbrook, ME) and reported as optical density of the sample.

We used palpation and ultrasonography to evaluate injection sites for evidence of localized inflammation prior to vaccination, at monthly intervals until calving (February – May 2006), and prior to each breeding season (September 2006 – 2009). Injection sites and surrounding skin were shaved as described above. Qualitative changes in skin temperature, erythema, and swelling were assessed by comparing the injection site with adjacent skin. We used ultrasonography to evaluate muscle tissue for subcutaneous and intramuscular signs of inflammation and injury including lesions such as hematoma, abscess, scar tissue, cellulitis, and myositis which are associated with characteristic changes in muscle echogenicity and architecture [195-197]. Longitudinal and transverse

ultrasound images of skin, fat, fascia, and muscle at the site of injection were collected using a 5 MHz linear array transducer to a depth of 80 mm with a focal zone at 40 mm and stored on a computer for future analysis. Ultrasound images were described to indicate qualitative degree of change between pre-vaccination and post-vaccination sampling dates in both muscle echogenicity (e.g., hyperechogenic, hypoechogenic) and architecture (e.g., fiber length, fiber pattern). All ultrasound evaluations were performed and read by a single technician. When images were consistent with a well-defined abscess accessible by percutaneous needle aspiration or if a soft, fluid-filled swelling was palpated, it was aspirated and aerobic, including mycobacterial, and anaerobic cultures were performed. Two abscesses were lanced, drained, and flushed because the welfare of the animal was a concern.

Each animal was observed for the presence or absence of lameness (e.g., limping, gait alterations, reluctance to stand or bear weight on a limb) each time they were moved from pens to the handling chute. Additionally, caretakers observed each female daily during feeding and noted if swelling or discharge was evident at the site of injection and if there were overt signs of lameness.

During the course of the experiment, three GnRH-vaccinated females died due to handling difficulties (n = 1, February 2006) or chronic wasting disease (n = 2, August 2007). Chronic wasting disease is endemic within the facility and was diagnosed in study animals using immunohistochemistry of rectal mucosa associated lymphoid tissue, retropharyngeal lymph nodes and brainstem at the level of the obex [198, 199]. Complete necropsies were performed by a veterinary pathologist and injection sites along with

standard tissues (e.g., representative tissues of each organ system) were removed and preserved in 10% neutral buffered formalin for histopathological evaluation.

Reproductive Behaviors

We compared rates of reproductive behavior interactions (behavior events/hour) of male and female elk from 25 September - 12 November, 2006 as previously described [71]. Individually identifiable numeric/color coded collars were placed on each female in both treatment groups. Behaviors were observed during the morning (0400-0600), evening (1600-2000) and at night (2200-2400) from a tower which provided good visibility of both pens. An infrared night vision monocular was used to observe behaviors during dark hours and a spot light was used to confirm collar identification. Four technicians similarly trained in behavior identification and blinded to treatment group performed observations. Based on previously reported elk reproductive behaviors, we identified and recorded 13 different interactions associated with harem tending, proceptivitity, receptivity, and mating [71, 81, 178, 200] (Table 1). Only male/female interactions were recorded. All behaviors were attributed to the individual female displaying or receiving the behavior. If the male displayed behavior directed at more than one female (e.g. herd tending) it was recorded as a separate behavior for each female. Due to small sample size, we grouped these specific behaviors into four general behavior categories: general breeding, male precopulatory, female precopulatory, and copulatory (Table 1). A total of 112 two-hour sampling periods were recorded in 47 days during 38 morning (74.1 hr, 35%), 43 evening (81.4 hr, 38%), and 31 night sampling periods (58.5 hr, 27%). Length of behavioral interactions was typically short compared to sampling interval, so each interaction was recorded as an event. Behavior rates were calculated as

events per female per hour and estimated mean weekly behavior rates were compared between treatment groups.

Statistical Analysis

Pregnancy status is reported as proportion pregnant (number of pregnant females in each treatment group / number of females exposed to bulls from late September to late November each year). Proportions of pregnant elk were compared with one-tailed Fisher's exact tests. The difference in proportions was used to estimate treatment effect. We used normal approximations to binomial distributions to compute confidence limits for the differences between proportions.

To evaluate the probability of pregnancy for a given GnRH antibody concentration, we used simple logistic regression with pregnancy status, breeding season, and treatment group as classification variables and GnRH-antibody concentration as the continuous variable (PROC PROBIT, SAS 9.1, SAS Institute Inc., Cary, NC). The probability of pregnancy after treatment with the GnRH vaccine was calculated for a theoretical sample population with an intrinsic pregnancy rate of 1.0 calf/female/year and this study's sham-vaccinated control population, which had an intrinsic pregnancy rate of 0.96 over the four experimental years. Using a cut-off value of $\geq$ 20 pmol GnRH antibody/ ml of serum to indicate infertility, we estimated the type 1 (false positive) and type 2 (false negative) error rates by calculating the specificity (proportion of correctly classified non-pregnant females) and sensitivity (proportion of correctly classified pregnant females) of using antibody concentration as a diagnostic indicator of pregnancy status. Specificity was calculated as the number of true non-pregnant females indicated by the antibody concentration divided by the total number of non-pregnant females.

Sensitivity was calculated as number of true pregnant females indicated by the antibody concentration divided by the total number of pregnant females. Pregnancy data were combined over four years for specificity and sensitivity calculations.

We tested the hypothesis that mean behavior rates between treatment groups were different using a mixed linear ANOVA model to account for both random (individual animal) and fixed (treatment, date, male, and time of day) effects in a repeated measures structure (PROC MIXED, SAS 9.1). For each of the four behavior categories, a global model was constructed which included treatment group, time of day, and male along with all of their first order interactions. Date was included as an additive trend factor. Next, variance structures, heterogeneous versus homogeneous both within and between individual females were added to the model and Akaike's Information Criterion adjusted for small sample size (AICc) was used to select the best fit model. Similarly, three covariance structures, none, compound symmetry, and spatial power were analyzed and AICc was used for model selection. Finally, after fitting variance and covariance structures, six reduced models were run to find the most parsimonious model to estimate treatment effects. Using the best model, mean behavior rates (± SEM) were estimated using least squares analysis, and hypothesis tests were based on type III generalized estimating equations which account for sample size imbalance.

Similar methods were used to compare mean (± SEM) concentrations of progesterone, ovarian follicle number, size and volume. Fixed effects in the analyses were treatment status and date, and individual females were evaluated as a random effect. Differences in calf weight during the first three months of life were analyzed in the same way with dam treatment status, sire, sex, and age as fixed effects. Differences in

proportions of small, medium, and large size follicles were analyzed using a Chi-square test with treatment status and date as classification variables (PROC CATMOD, SAS 9.1). A paired Student's T-test (PROC TTEST, SAS 9.1) was used to evaluate differences in mean optical density readings from *M. avium* ELISA. Descriptive statistics were used to explain the similarity of hematology profiles, presence or absence of luteal tissue during early ovulatory and transitional periods, and occurrence of lesions at the site of injection.

Results

Pregnancy and Calving

GnRH vaccination did not affect calving success in female elk treated at approximately 80-100 days of pregnancy. Serum progesterone concentrations during the second half of gestation did not differ between treatment groups ($P = 0.849$) (Fig. 3.1). Progesterone concentrations varied by month ($P < 0.001$) but there were no treatment by month interactions ($P = 0.619$). All females, except the single 20 mo old sham-vaccinate which had mid-gestation pregnancy loss, delivered full term calves. One calf born to a GnRH-vaccinated dam died during parturition due to dystocia. All calves born alive (n = 15) survived the neonatal period and were weaned at approximately three months of age prior to the 2006 breeding season. Dam vaccination exposure did not affect calf weight at any time point prior to weaning ($P = 0.448$) (data not shown).

GnRH Vaccine Efficacy and Duration

Pregnancy proportions in GnRH-vaccinated females were lower ($P \leq 0.05$) than in sham-vaccinated females during the first three years of the experiment (Table 3.2). Treatment effect decreased between year one and year four ($P = 0.009$) from a high of

0.90 in 2007 to a low of 0.12 in 2010 (Table 3.2). Antibody concentrations in GnRH-vaccinated elk were detectable 1 month post-inoculation, peaked between four and eight months after vaccination, and waned during the course of the study (Fig. 3.2). GnRH antibodies were not detectable in sham-vaccinated females (data not shown). There was a strong inverse relationship between GnRH-antibody concentration and the probability of becoming pregnant ($P < 0.001$) (Fig. 3.3). At 30 pmol/ml of free GnRH-antibody binding capacity in the peripheral serum, the logistic model predicted a pregnancy rate of 0.10 in a population whose intrinsic pregnancy rate approached 1.0 (Fig. 3.3). Only one GnRH-vaccinated female with lower than 20 pmol/ml concentrations of antibody (approximately 2-17 pmol/ml) did not become pregnant during the first three breeding seasons. Conversely, one female with consistently high serum antibody concentrations (> 20 pmol/ml) did become pregnant during the third and fourth breeding seasons. Using a GnRH-antibody concentration of 20 pmol/ml during September as the cut-off point, the assay had a sensitivity of 0.85 and specificity of 0.87 to predict whether a female elk would be infertile during the current breeding season.

Reproductive Behaviors

General breeding and male precopulatory behaviors were the most prevalent interactions recorded. Copulatory behaviors were observed too infrequently for meaningful analysis. While differences in mean male precopulatory behavior rates only approached significance ($P = 0.073$), those directed towards GnRH-vaccinated females (0.45 ± 0.06 behaviors/hr) were 30% greater than those directed towards sham-vaccinated females (0.33 ± 0.06 behaviors/hr). This response became apparent after the initial two weeks of observation (Fig. 3.4). Individual males were a significant covariate in male

precopulatory behavior ($P = 0.002$) as were date and time of day ($P < 0.001$) and there was an interaction between bull and time of day ($P < 0.001$). Female precopulatory behaviors were not different between treatment groups ($P = 0.720$); however, precopulatory behavior of GnRH-vaccinated females persisted throughout the sampling period, whereas this behavior rate dropped to nearly zero in sham-vaccinated females after the first three weeks of observation (Fig. 3.4). Date tended to be an important covariate in female precopulatory behavior ($P = 0.055$). There were no differences in mean general reproductive behavior rates between treatment groups ($P = 0.794$). Time of day and date were significant ($P < 0.001$) covariates for general reproductive behaviors.

Physiological Side-Effects

Most biochemistry and hematology parameters were within clinically normal ranges for all elk [177]. Two females, one from each treatment group, demonstrated leukocytosis (22-26 x 10^3 nucleated cells/µl) one month post-injection which was attributable to neutrophilia (9-12 x 10^3 /µl) and lymphocytosis (9-11 x 10^3 /µl). The GnRH-vaccinated female demonstrated mild hyperfibrinogenemia (300 mg/dl) and thrombocytopenia (57 x 10^3 /µl). An additional four GnRH-vaccinated females had mild hyperfibrinogenemia (300-400 mg/dl). Although all globulin levels were within the clinically normal range (1.9-4.3 mg/dl), serum globulins increased between 0.1 and 0.6 mg/dl at four months post-inoculation in every female in both groups.

All animals were seronegative for Johne's disease prior to vaccination. One year post-injection *M. avium avium* ssp. *paratuberculosis* (MAP) antibody concentrations, indicated by an increase in optical density, tended to be greater than zero ($P = 0.059$) in both treatment groups. With one exception, all females showed an increase in MAP

antibody concentrations and 3/17 (18%) females had sufficiently robust responses to be classified as seroconverted and Johne's disease positive.

Mean number of follicles were greater in GnRH-vaccinated females (5.6 ± 0.44) than sham-vaccinated females (2.46 ± 0.48) ($P = 0.005$). Mean size of all imaged follicles in GnRH vaccinates (2.35 ± 0.29 mm; range 0.3 – 12.6 mm) was smaller than those of sham vaccinates (4.73 ± 0.84 mm; range 1.2 – 16.9 mm) ($P = 0.014$). There was no difference in total follicular volume between groups ($P = 0.240$). Date was not a significant covariate ($P \geq 0.05$) in any of the analyses and there were no date by treatment interactions. GnRH-vaccinated females had more small follicles (< 4 mm) but fewer medium (4 to < 7 mm) and large (≥ 7 mm) follicles ($P < 0.001$) than sham-vaccinates. Corpora lutea were observed in 6/22 (27%) images collected from sham-vaccinated females during transitional season periods. In contrast, corpora lutea were never observed in GnRH-vaccinated females during the same time periods. Luteal tissue was not observed in any study animal during the anestrus.

Between 15 and 52 months post-injection, 6/17 (35%) females (n = 4 GnRH-vaccinated and n = 2 sham-vaccinated) developed clinically apparent abscesses (e.g., large soft swelling with purulent material) at the site of injection. No aerobic, including mycobacterial, or anaerobic bacterial growth was detected in cultures (n = 4). Two of the four GnRH-vaccinated animals described above died of causes unrelated to vaccination (i.e., chronic wasting disease) 19 months post-vaccination. Both possessed large (~500 ml), purulent, multiloculate, encapsulated abscesses, at the site of injection. Histopathology revealed pyogranulomatous inflammation and multiple acid-fast bacilli

within the capsule and surrounding muscle tissue. The acid-fast bacilli were consistent with mycobacteria. We did not observe lameness at any time point in study animals.

Muscle architecture and/or echogenicity was altered in 10/17 (~60%) females (n = 5 from each treatment group). The earliest changes in echogenicity were observed two months post-injection; however, the most severe changes, including images consistent with abscessation were seen five to 30 months after vaccination. Regardless of treatment group, there was a wide variation in severity of change, from muscle fiber disruption and diffuse fiber hyperechogenicity to large hypoechogenic multiloculated areas within the muscle or between fascial planes. All of the animals with clinical abscesses had evidence of muscle fiber disruption in one or more ultrasound images prior to observing external manifestations of the abscess.

Discussion

A single vaccination against GnRH during mid-gestation did not disrupt pregnancy but did decrease subsequent pregnancy rates in female elk for three years post-treatment. The efficacy of the GnRH vaccine decreased each year following treatment. These findings supported our predictions that this GnRH vaccine delivered to pregnant female elk suppressed fertility with decreasing effectiveness over time. These findings were also consistent, in part, with those reported for non-pregnant female elk treated with GnRH vaccine using keyhole limpet hemocyanin (KLH) as the carrier molecule rather than CCH [109]. However, in contrast to our measurements, Killian et al. (2009) showed a weak inverse relationship between contraceptive efficacy of the vaccine and antibody levels; increasing effectiveness with decreasing antibody titers. Their result is difficult to explain biologically in light of our observations and others [104, 110] that support a

strong positive association between infertility and antibody levels. Their finding may be an artifact of small sample size. Despite the association between antibody titer and contraception, we did not identify a GnRH-antibody concentration cut-off that predicted contraceptive effects with both high sensitivity and specificity. The most useful diagnostic assays have wide separation between negative and positive cut-off values [201, 202]. Because there was overlap in pregnancy status at given antibody concentrations, GnRH-antibody concentration, as a diagnostic test, may be a good indicator of herd or population level infertility but is a less reliable predictor of individual animal fertility.

The wide variation in antibody concentrations observed in our study were not surprising given that individual humoral responses to a foreign antigen are known to be influenced by many physiological factors including nutrition, previous exposure to the same or similar antigens, age, persistence of the antigen, current immune stimulation by other immunogens and genetics [201]. We attempted to control for the first four potentially confounding factors, however, the latter two factors were not measured or controlled and may have influenced individual responses. Of more interest was the persistence of GnRH-antibody concentrations over the course of four years in GnRH-vaccinated animals. Most commercially available vaccines require an initial series of two or three vaccinations with annual re-vaccination to maintain significant serum antibody concentrations [201]. Previous wildlife immunocontraceptive vaccines have required similar vaccination strategies [36, 94, 97, 111, 124]. Only recently have single dose applications been effective at inducing long-term antibody production and corresponding infertility [104, 105, 110, 116, 203]. It has been suggested that a combination of depot

effect produced by a non-biodegradable oil in water based emulsion along with an optimized concentration of immunostimulatory killed mycobacteria is responsible for the prolonged antibody effect in GnRH-vaccinated deer [105, 108]. Our finding of extensive localized inflammation at the site of injection nearly four and a half years post-vaccination supported this hypothesis of a depot effect, particularly given the retained and apparently dead mycobacteria within the sterile lesions. Additionally, increased *M. avium* antibody concentrations that were often sufficiently elevated to indicate disease status seroconversion, supported the assertation that a generalized and robust humoral immune response to the vaccine was critical to its efficacy.

Both GnRH-vaccinated and sham-vaccinated groups had follicles ranging in size from less than two mm to more than 12 mm in diameter during the transitional and anestrous seasons. Additionally, during transitional seasons, ultrasound evidence of luteal tissue was only observed in sham-vaccinated females. Although we did not intensively measure follicular development and document ovulation, these data suggested that 12 – 18 months post GnRH-vaccination, at least some females had large pre-ovulatory sized follicles, which likely did not ovulate. The range of follicular size was consistent with earlier data from untreated elk in the transitional and anestrous periods [37, 180]. While a full range of follicular sizes was observed in our study, mean follicular size was smaller but antral follicles were more numerous in GnRH-vaccinated as compared to sham-vaccinated females. Our observations are similar, although less extreme, than those of Seekallu et al. (2010) [170]. These authors found complete cessation of follicular waves and development of large sized follicles in domestic sheep after a series of two vaccinations against GnRH. Our measurements were made 12 – 18 months post

immunization after a single vaccination, whereas, they made measurements 26-66 days post-booster vaccination. Their more intensive vaccination and monitoring schedule may explain the more complete hypthamic-pituitary gonadal axis suppression observed.

Our data indicated fewer follicles developed to the pre-ovulatory stage which was likely due to incomplete gonadotropin support. Attenuation of follicle development into large antral stages likely resulted in less negative feedback from dominant follicles secreting inhibin and estradiol. An increase in small follicle recruitment due to less negative feedback from dominant follicles and early regression due to incomplete gonadotropin support could account for the increase in numbers of small follicles observed in GnRH-vaccinated elk. Although follicles were on average smaller in GnRH-vaccinated females, antral follicle development remained active and suggested that gonadotropins, particularly follicle stimulating hormone (FSH), continued to stimulate early follicular development. While luteinizing hormone (LH) secretion is closely regulated by GnRH [131], FSH synthesis is only partially regulated by GnRH and is primarily constitutively secreted [204, 205]. It has been suggested that a component of basal LH secretion is similarly free from GnRH regulation [206]. In the current study, continued antral follicle development was consistent with continued FSH stimulation and possibly basal LH signaling, albeit at a suppressed level. Absence of observed luteal tissue in GnRH-vaccinated females was consisten with a probable lack of sufficient estradiol to initiate a LH surge and ovulation. Experiments to intensively measure individual animal follicular dynamics and concurrent gonadotropin concentrations are required to test these speculative hypotheses.

Contrary to our prediction, reproductive interactions during the breeding season were not eliminated in vaccinated females. Estrous behavior in domestic ruminants is influenced not only by the presence or absence of progesterone and estradiol but also the concentration and timing of these steroid hormones [207]. Continuous, high concentrations of progesterone (e.g. during pregnancy) usually inhibit expression of estrous behavior regardless of estradiol concentrations [66]. In contrast, sexual receptivity can be triggered by estradiol alone in most species, although often in the pharmacological range, as long as progesterone is not inhibitory [65, 66]. In this study, despite the apparent absence of ovulation, both female attractiveness, measured by male precopulatory behaviors, and female proceptivity, measured by female precopulatory behaviors, were maintained throughout the first breeding season post immunization in GnRH-vaccinated and consequently non-pregnant animals.

Interestingly, domestic male cattle will spend equal time with young nulliparous females in estrus or in the luteal phase, finding them equally attractive [208]. Male elk may similarly investigate non-pregnant females regardless of estrous status. A combination of low progesterone due to lack of ovulation and the presence of limited estradiol, due to continued follicular development, may account for continued expression of precopulatory behaviors in female elk. These mechanisms could account for pregnancy status being a stronger indicator of reproductive behavior than vaccination status. Because we observed too few copulatory behaviors to evaluate differences between treatment groups, it is unclear if vaccinated females were receptive to copulation or were only displaying proceptive behaviors. Because GnRH-vaccinated animals did not likely experience progesterone priming prior to estradiol exposure, which is important for the

display of full estrous behaviors in domestic sheep [65, 209], we speculate that copulation failed to occur in non-pregnant GnRH-vaccinated animals. Regardless, it is apparent that in the relatively small captive environment of this experiment, with intensive male/female interactions, GnRH vaccination was associated with persistent breeding behaviors. While these observations may be an artifact of captivity, the consequences in free-ranging populations are unclear but could be significant and deserve further investigation into potential ecological consequences prior to use in a management application.

In accordance with our hypothesis regarding physiological side-effects, GnRH-vaccination did not significantly affect blood chemistry or hematology parameters but did result in considerable injection site reactions. Two females showed evidence of a systemic inflammatory response and four additional animals had mildly elevated fibrinogen, an indicator of inflammation. Every animal had increased serum globulins. These findings indicated a robust immune response to both the GnRH conjugate and sham vaccines. Abscesses were confirmed in 35% of females which was consistent with others using a similar formulation of this vaccine in white-tailed deer [110, 119]. However, ultrasound changes indicative of myositis, trauma, and abscessation [195-197] suggested a higher incidence of injection site inflammatory lesions. It was unclear if the pyogranulomatous inflammation at the site of injection would have resolved with time. Evaluation of the long-term physiological consequences of injection site reactions in animals treated with this vaccine will be essential for managers making choices based on animal-welfare concerns.

In addition to antibodies produced in response to GnRH, antibodies were also produced to other elements of the vaccine. Mycobacteria and their cellular components are particularly immunostimulatory [210]. Antibodies produced in response to *M. avium* ssp. *avium* appear to cross-react well with commercial assays for Johne's disease, a gastrointestinal disease of domestic cattle and occasionally wild ruminants such as elk [211]. Vaccination with GonaCon-B and/or the adjuvant components increased mycobacterial antibodies in nearly every animal and induced clinical seroconversion in 18% of animals. Managers concerned with regulatory issues or Johne's disease management should be aware of this finding prior to using this vaccine in free-ranging ungulates.

In conclusion, a single vaccination during mid-gestation with the described GnRH vaccine decreased pregnancy rates for three years post-treatment without compromising the existing pregnancy. This result extended potential use of contraception by providing multiple years of decreased fertility when applied to either pregnant or non-pregnant female elk. Hematology and serum chemistry parameters were normal and vaccinated females appeared healthy. Furthermore, the vaccine appeared to be safe for the developing fetus and neonates born to vaccinated females. By contrast, the vaccine prolonged reproductive behaviors during the breeding season, a finding that has potential ecological effects that require further study. The most apparent pathological side-effect of the vaccine was related to the development of sterile abscesses at the site of injection. Although lameness was never observed, most GnRH and sham-vaccinated females showed some level of tissue inflammation or abscess. Finally, we demonstrated that GnRH vaccination inhibited but did not eliminate follicular development despite the

absence of pregnancy, possibly due to continued FSH secretion, indicating continued partial function of the hypothalamic-pituitary-gonadal axis. Our findings extend both the fundamental and practical understanding of GnRH vaccination in pregnant elk, which may assist wildlife managers in their pursuit of science-based population management.

Acknowledgements

The authors thank the Colorado Division of Wildlife for providing the resident elk and handling facilities used in this investigation. We offer thanks to USDA/APHIS, National Wildlife Research Center (L. Miller, J. Rhyan, K. Fagerstone) for the generous donation of the GnRH vaccine. We are particularly grateful to T. Davis, A. Mitchell, M. Fisher, J. Spaak, and I. LeVan for technical assistance in animal handling, sample collections, and husbandry. We are thankful for the many hours D. Germann, K. Kearl, and M. Paulek spent recording elk behaviors, for histopathological examination provided by T. Spraker, and for the endless logistical support provided by C. Broomfield and T. Powers. A special thanks to M. Allen and C. Magee who trained multiple people in the art of RIA, and to A. Graham and A. Latimer for laboratory assistance. Thank you to G. Sargeant, who provided statistical advice and improved our figures. This manuscript was significantly improved by the review and comments of M. Phillips, and T. Shenk of the Colorado Division of Wildlife and three generous anonymous reviewers. A special thanks to M. Wild for supporting our pursuits of excellent research and professional development.

Tables

Table 3.1. Thirteen individual reproductive behaviors and associated behavior categories observed and recorded 8 – 10 months after immunization in GnRH-vaccinated and sham-vaccinated elk (*Cervus elaphus nelsoni*).

Behavior Category	Reproductive Behaviors
General Breeding	Male behavior related to establishing, maintaining and defending a group or harem of female elk (i.e., herding, guarding)
Male Precopulatory	Male courtship behavior directed toward an individual female to induce or detect estrus (i.e., Flehmen for urine testing, chivy, sniff and/or lick body, rub body, pre-copulatory mount)
Female Precopulatory	Female courtship behavior directed toward dominant male to arouse copulatory behavior (i.e., sniff and/or lick body, rub body, circle, mount, lordosis)
Copulatory	Male behavior directed toward a receptive female (i.e., intromission)

Table 3.2. Mean yearly pregnancy proportions (no. pregnant/ no. exposed to fertile bull) and estimates of treatment effect size (difference in proportions) with 95% confidence intervals for GnRH- and sham-vaccinated female elk (2006-2010).

Years post-treatment	**Proportion Pregnant**		**Treatment Effect**
	GnRH-vaccintes [n]	*Sham-vaccinates [n]*	*Difference (95% CI)*
0 (2006) pre-treatment	1.0 [10]^{ax}	1.0 [8]^{ax}	0.0
1 (2007)	0.10 [10]^{by}	1.0 [7]^{ax}	0.90 (0.71 – 1.0)
2 (2008)	0.25 [8]^{byz}	1.0 [7]^{ax}	0.75 (0.50 – 1.0)
3 (2009)	0.50 [8]^{byz}	1.0 [7]^{ax}	0.50 (0.15 – 0.85)
4 (2010)	0.75 [8]^{az}	0.86 [7]^{ax}	0.12 (0.0 – 0.29)

Proportions with different superscripts are significant ($P \leq 0.05$) letters a and b are between treatment groups within a given year, letters x, y and z are between years.

Figures

Figure 3.1. Mean monthly serum progesterone concentrations ± SEM in GnRH- (filled circles, n = 10) and sham- (open circles, n = 7) vaccinated female elk (*Cervus elaphus nelsoni*) between the time of GnRH immunization (January 2006) and parturition (May-June 2006). Means with different letters indicate differences ($P < 0.05$) between months.

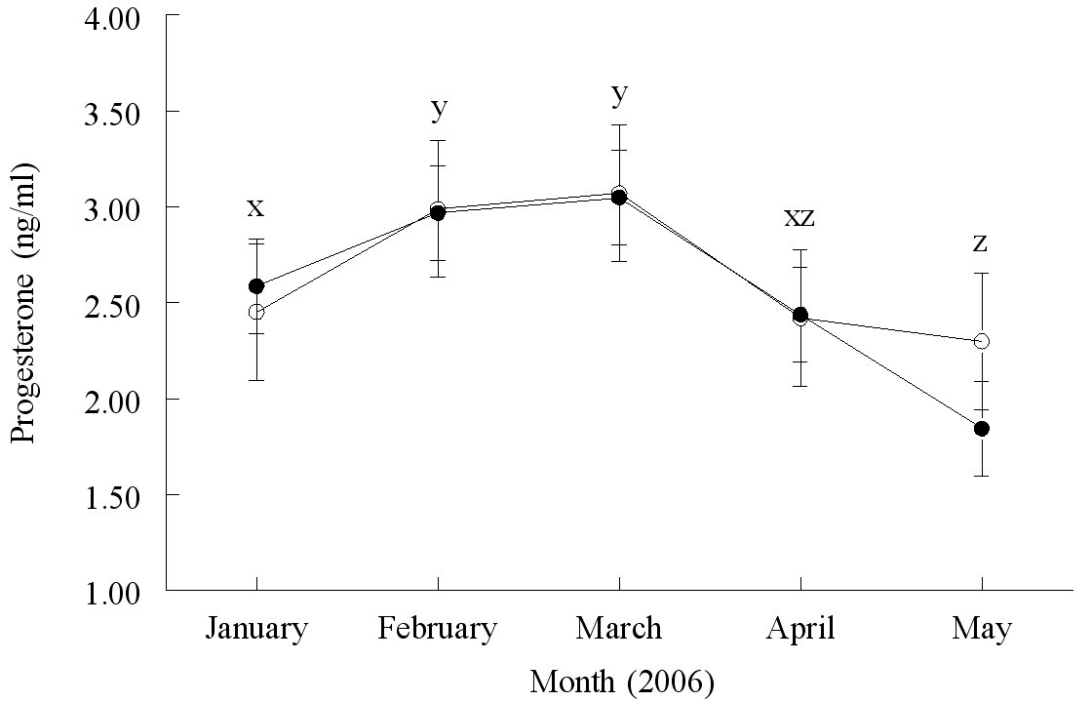


Figure 3.2. Persistence of GnRH antibodies measured via ^{125}I-GnRH binding capacity in peripheral blood of GnRH-vaccinated female elk. Top panel shows antibody concentrations in females which did not become pregnant post-vaccination (n = 4/10) throughout the four year study. Two animals were lost from the study after year two. Lower panel shows antibody concentrations from females which either did not experience infertility (n = 1/10) or returned to fertility during the study (n = 5/10). Solid lines indicate non-pregnant females. Dotted lines indicate pregnant females. Two females became pregnant after antibodies were measured in September 2009. Arrows indicate date when males were placed in female pastures (September 2006 - 2009).

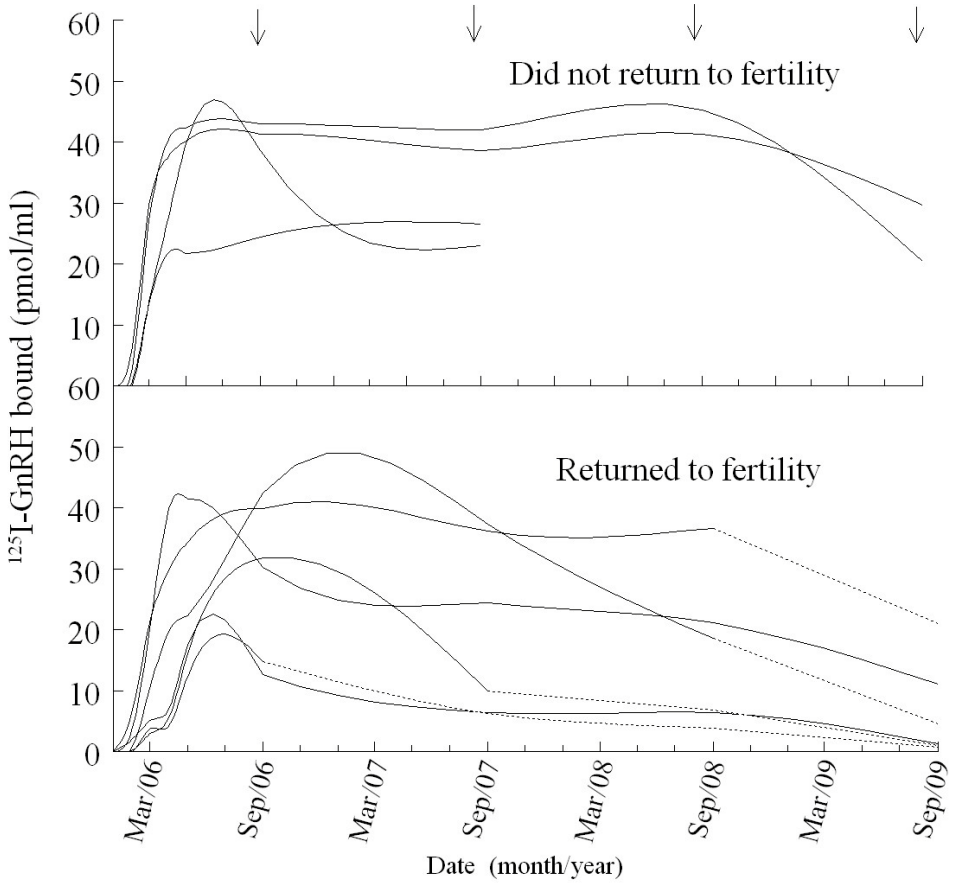


Figure 3.3. Predicted relationship between ^{125}I-GnRH binding capacity (antibody concentration) and the probability of infertility in GnRH-vaccinated female elk, modeled with (dashed line, probability of pregnancy if untreated = 0.96) and without (solid line, probability of pregnancy if untreated = 1.0) intrinsic infertility.

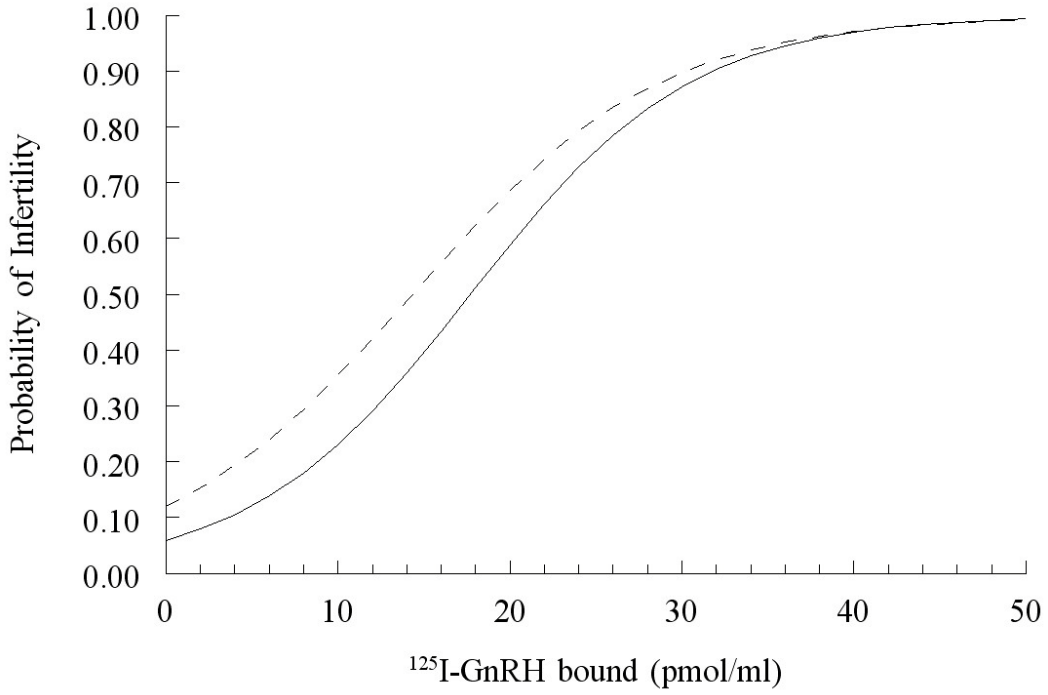
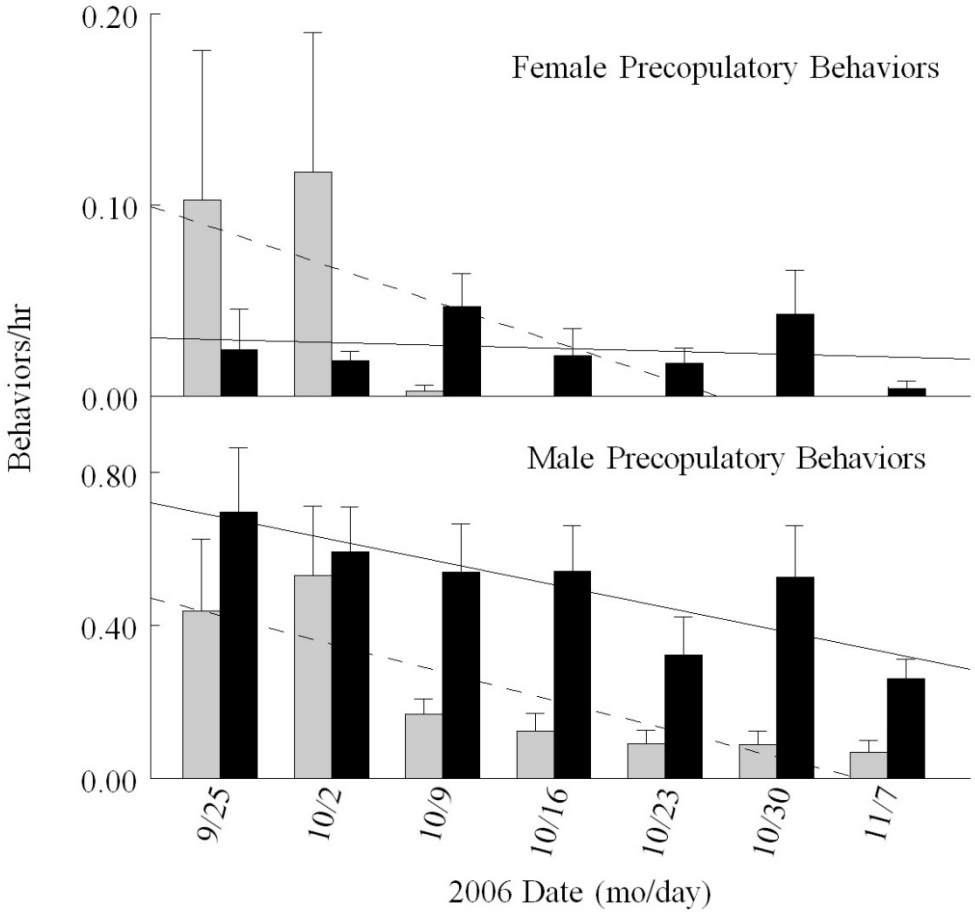


Figure 3.4. Mean ± SEM weekly female precopulatory behavior rates demonstrated by GnRH-vaccinated (black bars and solid lines, n = 10) and sham-vaccinated (grey bars and dashed lines, n = 7) female elk (top panel) and mean weekly male precopulatory behavior rates received by female elk (n = 10 GnRH vaccinated; n = 7 sham-vaccinated) (bottom panel) during the 2006 breeding season (25 September – 12 November). Regression lines illustrate persistence of precopulatory behaviors demonstrated and received by GnRH-vaccinated females.



Chapter 4: Maternal Passive Transfer of GnRH Antibodies Does Not Change Reproductive Development in Elk (*Cervus elaphus nelsoni*) Calves

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Abstract

Gonadotropin-releasing hormone (GnRH) is intermittently released from the hypothalamus in consistent patterns from before birth until final maturation of the hypothalamic-pituitary-gonadal axis at puberty. Disruption of this signaling can alter reproductive development. In this study elk calves (*Cervus elaphus nelsoni*) were exposed to high concentrations of GnRH-antibodies through maternal passive transfer immediately after birth. We investigated the long-term effects of antibody exposure on reproductive axis maturation and function. We found male and female calves had similar growth rates, endocrine profiles, and gametogenesis between antibody exposed and unexposed groups. Pituitary stimulation with GnRH analog prior to the second reproductive season induced secretion of luteinizing hormone in all elk and increased concentrations of serum testosterone in males. All females became pregnant during their second reproductive season. There were no differences in hypothalamic GnRH content, pituitary gonadotropin content or gonadal structure between antibody exposed or unexposed groups at nearly three years of age. We conclude that suppressing GnRH

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[9] This work was supported by the Morris Animal Foundation grant # D07ZO-054 to TMN

signaling through immunoneutralization during the neonatal period does not alter long-term reproductive function in elk.

Introduction

Episodic secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus and signaling at gonadotroph cells of the anterior pituitary is required for normal steriodogenesis and gametogenesis in reproductively mature mammals [132]. Increases in pulse frequency mark the shift from reproductive quiescence to activity during puberty and transition from anestrus to breeding condition in seasonal breeders [21]. The hypothalamic-pituitary-gonadal (HPG) axis is functionally active in sheep by 70 days of gestation [213] but is not fully mature until the pre-pubertal period [214]. There is a biphasic increase in gonadotropins during pre-pubertal maturation in both female cattle and sheep [215]. Heifer calves experience rapid ovarian development [216] and corresponding increases in gonadotropins [217] between 2 and 14 weeks of age which subsequently remain constant until final peri-pubertal maturation at 50 to 60 weeks of age [218]. Similarly, cross bred ewe lambs experience an increase in antral follicle number and serum concentrations of follicle stimulating hormone (FSH) between 4 and 16 weeks of age respectively. Both subsequently decline until final pre-pubertal maturation (~ 32 wks) [219, 220]. Additionally, luteinizing hormone (LH) and FSH secretion as well as folliculogenesis are elevated in ewe lambs between 5 and 10 weeks of age but not in older pre-pubertal lambs [221].

Similar to heifers, pre-pubertal bulls have a critical period of HPG axis maturation between 6 and 10 weeks of age, with significant increases in GnRH and estradiol receptors in the anterior pituitary gland and ensuing increases in LH secretion during this

period [222]. Ram lambs have elevated concentrations of LH and concomitant testosterone as early as 3 days after birth which are robust by 28 days of age [223]. Attainment of reproductive maturity can be hastened in bull [224-226] or heifer [224] calves by administration of GnRH during early post-natal life. Alternatively, puberty can be delayed in pre-pubertal bulls [227] or heifers [228] by inhibiting GnRH secretion during this early period of development. GnRH signaling during the post-natal/ pre-pubertal time period is important for final maturation of the HPG axis in domestic ruminants.

Vaccination against GnRH is one method of functionally removing the hormone and its effects *in vivo*. Several research groups have investigated the effects of removing GnRH on reproductive system function by using active and passive GnRH vaccination in mature [139, 229] and juvenile [230, 231] ruminants. In adults, reproductive function generally returns as antibody titers wane [232, 233]. In contrast, immunization of neonatal or pre-pubertal animals can have a prolonged effect on reproductive function, despite progressive reduction in GnRH antibodies [146, 169, 234-236]. In fact, Brown and colleagues (1994) [169, 235] found that active vaccination against GnRH during the neonatal period caused permanent suppression of reproductive function in a subset of mature domestic sheep of both sexes in spite of a lack of measurable GnRH-antibodies after the pre-pubertal period. Few studies have investigated the long-term (> 1 year) reproductive effects of GnRH vaccination during the neonatal or pre-pubertal period.

GnRH vaccination has been proposed as a method of wildlife contraception [30]. In a previous study, we actively vaccinated mid-gestation female elk to evaluate the safety, efficacy, and duration of a recently registered GnRH vaccine (GonaCon) intended

for use in wild ungulates as a contraceptive (Powers et al., in review). Calves born to these females experienced neonatal passive transfer of GnRH antibodies.

The purpose of this study was to investigate the long-term reproductive effects of maternal passive transfer of GnRH antibodies to male and female elk calves immediately after birth. We tested the hypothesis that passive transfer of maternal GnRH antibodies during the neonatal period will delay the onset of puberty in both male and female elk calves and permanently suppress function of the HPG axis leading to decreased fertility.

Materials and Methods

Animals and Experimental Approach

This study was reviewed and approved by Colorado State University (#07-146A-01) and the Colorado Division of Wildlife (#09-2007) Institutional Animal Care and Use Committees. All animals were housed at the Colorado Division of Wildlife, Foothills Wildlife Research Facility in Fort Collins, Colorado, USA. We previously reported the effects of active vaccination against GnRH in mature female elk during mid-gestation (Powers et al. in review). The current study used calves born during our previous study. Fifteen elk calves (n = 8 male; n = 7 female) born to females immunized with GnRH vaccine or a sham vaccine (carrier and adjuvant but no GnRH) during mid-gestation (~100 d of 255 ± 7 d gestation period [177]) were divided into two groups based on the presence of serum GnRH antibodies measured 24 hours after birth. Dam vaccination status was not necessarily indicative of calf GnRH antibody exposure status due to nursing from multiple females early in the neonatal period. Calves with robust neonatal titers (n = 10; 6 males/4 females) were designated as the exposed group and those with low or undetectable titers (n = 5; 2 males/3 females) the unexposed group. Calves were

dam raised in mixed exposure status groups until 3-4 months of age when they were weaned (September 1, 2006). One male calf exposed to GnRH antibodies was less than 30 days of age at weaning and was euthanized due to welfare concerns. At weaning calves were separated into same sex groups, maintained in fenced paddocks (5.0 ha) and fed a diet of *ad libitum* alfalfa-grass hay mix, limited supplement, trace mineral blocks, and water. Prior to experiments, calves were trained to repeated handling, blood sampling, isolation pens, alleyways, and a handling chute. All biological samples, with the exception of semen collection which required complete immobilization (see fertility section below), were collected and hands-on measurements made while elk were lightly sedated using xylazine hydrochloride (30-150 mg/animal i.m.; TranquiVed, Vedco, Inc. St. Joseph, MO) in a non-squeeze chute. Tranquilizer effects were reversed after each sampling session with either yohimbine hydrochloride (30 mg/animal i.v., Wildlife Pharmaceuticals, Fort Collins, CO) or tolazoline hydrochloride (600 mg/animal i.m., Tolazine; Akorn, Inc., Decatur, IL). During the experiment one additional male calf exposed to GnRH antibodies died as a result of a venomous snake bite in May 2007. The study was conducted from birth (May-August 2006) to 3 years of age (March/April 2009) (Fig. 4.1).

Antibodies and Growth Rates

To measure maternal antibody transfer to calves, we opportunistically collected blood from calves prior to first nursing (n = 2; one from each exposure group) and from every calf 24 hours after birth, then at approximately two week intervals for the first two months of life and monthly until six months of age. Calves were weighed (kg) at the same time points. Thereafter, similar measurements were made sporadically but no less

than every six months. Final antibody concentration measurements were made prior to the 2008 reproductive season (mid September – mid November). Blood samples (10 to 30 ml) were collected via jugular venipuncture using a 20-gauge blood collection needle, tube holder, and 10 ml blood tubes without anticoagulant (BD Vacutainer SST; Becton, Dickinson, and Co., Franklin Lakes, NJ). Blood was allowed to clot at room temperature, centrifuged for 10 min at 1500 x *g*, and serum was decanted to polypropylene tubes and stored at -80° C until assays were performed. Peripheral GnRH antibody concentrations (pmol of ^{125}I-GnRH bound/ml at 1:1000 dilution) were measured using a modified radioimmunoassay technique as described previously (Powers et al. in review).

Puberty and Fertility

Males: We measured and compared mean monthly serum concentrations of testosterone (ng/ml) between exposure groups from 14 to 21 mo. of age [237]. Blood was collected as described above at single time points at the beginning of each month between August 2007 and March 2008. In addition, we measured secondary sexual characteristics including antler length (cm), scrotal circumference (cm), and neck girth (cm) at monthly intervals from 9 to 21 mo. of age (March 2007 – March 2008) as well as prior to the 2008 reproductive season (August 2008). Finally, we measured antler complexity (number of branch points), and weight (g) prior to the first and second potential breeding seasons (August 2007, 2008) when hardened antlers were removed for management purposes. Antlers were consistently removed 1 cm above the ridge of bone where the antler and pedicle meet using a Giglis wire saw.

To estimate fertility and confirm pubertal maturation we collected semen samples at monthly intervals from August 2007 to March 2008 (14 – 21 mo.). Semen samples

were collected via electro-ejaculation [238] using a 60 mm diameter rectal probe (Pulsator III, Lane Manufacturing, Inc., Denver, CO) while elk were chemically immobilized (18-23 mg butorphanol tartrate, 15-19 mg azaparone tartrate, and 6-8 mg medetomidine hydrochloride i.m.; Wildlife Pharmaceuticals, Fort Collins, CO). An endotracheal tube (20 mm internal diameter) was placed during the procedure to prevent laryngeal collapse and hypoxemia. No supplemental oxygen was administered. Sedative effects were reversed once procedures were completed (10 mg atipamazole hydrochloride i.m.; Phizer Animal Health, Exton, PA; and 500 mg tolazoline hydrochloride i.m.). Total and progressive sperm motility (%) as well as components of velocity (straight line [VSL], curvilinear [VCL], and average path [VAP]; μm/s), were evaluated immediately using computer assisted sperm analysis (IVOS, Hamilton Thorne Biosciences, Beverly, MA) [239, 240]. If necessary, samples were diluted with semen extender (E-Z Mixin – "BF", Animal Reproduction Systems, Chino, CA) prior to motility analysis. Undiluted semen was smeared, stained with eosin-nigrosin (Hancock Stain, Animal Reproduction Systems, Chino, CA), and slides were stored at room temperature for future evaluation. Sperm morphology (% normal) was evaluated by a single technician according to standards used for bovine semen [241].

Females: We estimated age at the onset of puberty in females by measuring peripheral serum concentrations of progesterone (ng/ml) every 10 days (estrous cycle length = 21-22 days, luteal phase = 13-17 days; [179, 180, 243, 244]) between August 2007 – April 2008. Serum concentrations of progesterone ≥ 1.0 ng/ml were considered indicative of a functional corpus luteum (CL) and signified females were post-pubertal [88, 243]. Female fertility was evaluated by assessing pregnancy after exposure to proven

herd sire males for 63 days during the second breeding season (September – November 2008) at 2.5 years of age. Pregnancy status was determined using pregnancy specific protein B assay [189], transrectal palpation [190, 191] and transrectal ultrasound [192] 45 days after bulls were removed from paddocks. For management purposes, once pregnancy was confirmed, abortion was induced using 2 doses of prostaglandin $F_{2\alpha}$ 6 hours apart (25mg i.m.; Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) [89, 90].

Hypothalamo-Pituitary-Gonadal Axis

Pituitary responsiveness to GnRH was measured prior to the second breeding season (August 2008). All elk were fitted with non-surgical indwelling jugular catheters (14 ga 14cm Abbocath, Abbott Laboratories, Abbott Park, IL) and administered the GnRH analog d-Ala^{6}-GnRH-Pro^{9}-ethylamide (1µg/ 50kg body weight i.v.; Sigma Chemical Co., St. Louis, MO). Blood samples were collected prior to treatment then at hourly intervals for 8 hours [71, 244]. Blood was handled as described above and serum was analyzed for LH (ng/ml) using RIA [245]. All blood samples collected from males were also analyzed for testosterone (ng/ml) [237]. Progesterone was measured in pre-treatment serum samples collected from females [194]. Gonadotrope response to GnRH analog challenge was assessed in two ways: 1) mean maximum concentration of LH over all time points; and 2) total amount of LH secreted (ng ml^{-1} min^{-1}) estimated by calculating the area under the curve for LH response [246]. To avoid confounding female pregnancy estimates, stimulation with GnRH analog was repeated in males but not females during the breeding season (early November 2008).

In addition to challenge with GnRH, we evaluated potential changes to the HPG axis by collecting hypothalamus/median eminence, pituitary, and gonads on 25 March

(males) and 14 April 2009 (females). We assessed morphologic and morphometric differences in gonads between exposure groups. Gonad mass (g) was determined for each animal. Transverse sections of testes were cut, weighed, and frozen until sperm per gram of testicular tissue was measured [247]. Remaining testes tissue and ovarian tissue was preserved in Bouin's fixative (Polysciences, Inc., Warrington, PA). Pituitary and hypothalamus were hemi-sectioned through the mid-sagittal plane. One half of each brain tissue was preserved in 4% paraformaldehyde and the other half wrapped in aluminum foil and frozen at -80° C. Frozen sections of hypothalamus were assayed for total content of GnRH (ng) [248]. Frozen pituitary sections were processed as described by Hart and colleagues [248] and analyzed for content of LH (mg/g tissue) and FSH (mg/g tissue) [249]. Fixed tissues were trimmed, placed in cassettes, maintained in 10% neutral buffered formalin, and sectioned using standard histology techniques at the Colorado State University Veterinary Diagnostic Laboratory. Hematoxylin and eosin stains were used for histological examination. A veterinary pathologist qualitatively examined sections of gonad, pituitary, and hypothalamus at 2 to 40 times magnification for pathological changes and evidence of differences in morphology.

Hormone Analysis

Concentrations of progesterone [194], testosterone [237], LH [245], FSH [249], and GnRH [248] were measured using RIA. Samples were run in duplicate in single batches for each hormone at each time point. Intra-assay coefficients of variation for the upper and lower reference standards (20% and 80% ligand labeled hormone bound) were 4-15% for progesterone, 3-17% for testosterone, 3-11% for LH, 3% and 6% for FSH, and 5% and 10% for GnRH. Inter-assay coefficients of variation were 2% for progesterone,

and less than 20% for testosterone and LH. Single assays were run for FSH and GnRH. Mean limits of detection for each hormone (1 SD of the assay) are as follows: progesterone 5 pg, testosterone 2.5 pg, LH 30 pg, FSH 0.2 ng, and GnRH 0.5 pg.

Statistical Analysis

Dependant response variables including body weight, concentrations of progesterone, testosterone, and LH measured at multiple time points, sperm parameters measured after electroejaculation and secondary sexual characteristics were analyzed using one-way ANOVA models for a non-randomized design with a repeated measures structure (SAS 9.2, Proc MIXED, SAS Institute, Cary, NC). The independent variables exposure status, sire, and date/time, were included as fixed effects. Sex was also treated as a fixed effect if a response variable was measured in both sexes. Individual animal was included as a random effect. We first modeled the variance–covariance structure for each dependent variable using the restricted maximum-likelihood method, with the most global model of fixed effects (response variable = exposure status + sire + date or time as categorical variables +/- sex). We modeled the following variance–covariance structures appropriate for unequal time intervals; variance components (VC), compound symmetric (CS), and spatial power [SP(POW)]. We selected the most appropriate variance–covariance structure using AICc (Akaike's Information Criterion with correction for small sample sizes) and then used the top-ranked structure for subsequent modeling of fixed effects. The best structure for all models had constant variance within an exposure group; some models allowed heterogeneous variance between exposure groups whereas others were homogeneous for all study animals. The best covariance structure for all variables except body weight was VC, which implies no covariance between the repeated

measurements. The best covariance structure for body weight was SP(POW). Because date and time were the only significant fixed effects in the model describing total LH released after GnRH analog stimulation, a student's T test was used to compare differences between sexes and between months for males. Single time point concentrations of LH, FSH, and GnRH at necropsy as well as gonad mass, and sperm per gram of testis were analyzed using generalized linear ANOVA models (Proc GLM), with exposure status, sire, and sex (if appropriate) as classification variables. Arcsine transformation was performed for all data expressed as percent. Means and standard errors were estimated using least squares analysis and tests for differences between antibody exposed and unexposed groups were based on Type III generalized estimating equations. Descriptive statistics were used to explain concentrations of GnRH antibody.

Results

GnRH Antibody Concentrations and Growth

Antibodies to GnRH were undetectable prior to nursing in blood samples taken from two calves, one with a GnRH-vaccinated dam and the other with a sham-vaccinated dam. Neonatal antibody concentrations in exposed calves were often higher (35.3 ± 5.0 pmol/ml; range 3.2 to 48.9 pmol/ml) than those previously measured in their dams at similar time points (May 2006) (28.8 ± 3.8 pmol/ml; range 12.2 to 42.3 pmol/ml). Maternal GnRH-antibodies waned over time in exposed calves and were undetectable by six months of age (Fig. 4.2).

There was no effect of neonatal exposure to GnRH antibodies on body weight ($P = 0.968$) between birth and 3 years of age (Fig. 4.3). Although males were heavier than females at birth ($P = 0.024$) and at 3 years of age ($P < 0.001$), neither sex ($P = 0.905$) nor

sire ($P = 0.913$) were significant variables in the model. Age was the most important variable describing body weight ($P < 0.001$) and there was an age by sex interaction ($P = 0.001$) with males gaining weight faster than females. Males gained approximately 0.83 kg/day during the first 100 days and then gained more slowly at 0.2 kg/day between 200 and 800 days of age. Females gained an average of 0.75 kg/day during the first 100 days but decreased their growth rate to 0.1 kg/day between 200 and 800 days of age.

Puberty and Fertility

Males: There was no effect of exposure on mean monthly concentrations of testosterone ($P = 0.659$) between 15 and 21 months of age. Likewise, sire did not affect concentrations of testosterone ($P = 0.275$), however, levels did vary by month ($P = 0.001$) (Fig. 4.4). With the exception of one calf exposed to GnRH antibodies which had a maximum concentration of 1.7 ng/ml during the month of November, all males had at least one testosterone measurement ≥ 2 ng/ml.

Similar to endocrine results, in male calves neither neonatal antibody exposure nor sire had an effect on semen parameters or most secondary sexual characteristics including antler mass, neck girth, and scrotal circumference (Table 4.1, Fig. 4.5). Date was an important variable describing differences in antler length ($P = 0.002$), antler mass ($P = 0.007$), neck girth ($P = 0.0003$), and scrotal circumference ($P < 0.0001$) but not semen parameters ($P > 0.05$). Only antler length was affected by exposure status ($P = 0.037$) with a single male exposed to GnRH antibodies contributing the majority of the variance. This animal had an unusually short antler, which may have been physically damaged, during his second reproductive season but had a similar number of branch points (5) as other males (5 or 6 per antler). Regardless of exposure group, all males had

at least one semen sample which met criteria for adequate progressive motility (≥ 30%) and morphology (≥ 70%) of sperm in yearling male domestic cattle [242] and was consistent with acceptable sperm from red deer (> 40% motility, > 40% normal morphology) [250]. Scrotal circumferences were consistent with those reported for two year old elk [238].

Females: Monthly serum concentrations of progesterone in females were not affected by presence of neonatal antibody titer ($P = 0.727$) or sire ($P = 0.805$), however, concentrations varied by month ($P = 0.002$). All females had evidence of CL formation and regression based on progesterone profiles, suggesting puberty had been reached by November 2007 (Fig. 4.6). All had cyclic changes in serum concentrations of progesterone with multiple samples > 1 ng/ml until March 2008 (Fig. 4.7). Females were exposed to proven herd sire bulls between September and December 2007 at 28 – 32 months of age. All females (7/7) were pregnant when examined in January 2008. Transrectal palpation, ultrasound examination, and PSPB results were concordant.

Hypothalimic-Pituitary-Gonadal Axis Function and Structure

In August 2008, prior to their second potential reproductive season, both males and females responded to GnRH analog stimulation with an acute LH release that peaked between two and five hours and returned to near baseline levels by eight hours. While mean maximum concentration of LH was not statistically different between sexes ($P = 0.069$) females (16.1 ± 2.3 ng/ml) had nearly twice the maximum release of LH as males (9.4 ± 2.4 ng/ml). Exposure to GnRH antibodies during the neonatal period did not affect total secretion of LH (ng ml^{-1} min^{-1}) in either males ($P = 0.668$) or females ($P = 0.333$) (Figs. 4.8, 4.9). Neither mean maximum concentrations of LH or testosterone were

different between months ($P > 0.05$) for males; however, total LH and testosterone released in August was greater than in November ($P < 0.001$). Concentrations of testosterone and LH were positively correlated ($r = 0.36$). A single male with unusually high concentrations of LH (14-22 ng/ml) accounted for most of the variability (data not shown). Concentrations of progesterone in all females were below the limit of detection at the time of GnRH stimulation in August 2008 indicating lack of luteal tissue.

Endocrine profiles in serum and tissue at the time of necropsy were similar between exposure groups and sexes. There was no effect of exposure to GnRH antibodies on pituitary concentrations of LH ($P = 0.525$) or FSH ($P = 0.349$) in males or females (Table 4.2). Content of GnRH in hemi-hypothalmi was not different between exposure groups ($P = 0.979$) or sexes ($P = 0.980$). Serum concentrations of testosterone in males and concentrations of progesterone in females were nearly undetectable and did not differ between exposure groups ($P > 0.05$). Gonad mass (g) did not vary by exposure status or sire ($P > 0.05$) (Table 4.2).

There were no observed differences in gross or histological structure of the hypothalamus, pituitary, testes, or ovaries between antibody exposed and unexposed elk (Fig. 4.10). There was no evidence of overt inflammation or change in structure in the median eminence of any study animal. While gonadotropes were not specifically identified, adenohypophesial structure was similar between exposure groups and was within normal limits for ruminant pituitaries. All ovaries and testes showed evidence of gametogenesis. Ovaries had primoridal through Graffian follicles and testes had seminiferous tubules which contained spermatocytes. The only sign of inflammation was

infiltration of atretic follicle with eosinophils in 4/7 (n = 2 from each exposure group; ~60%) females.

Discussion

Passive transfer of maternal GnRH antibodies to elk calves shortly after birth did not affect long-term reproductive development. We found no differences in growth, time of pubertal onset, or structure and function of the HPG axis between exposure groups in males or females. Our findings suggested that the presence of high concentrations of passively transferred GnRH antibodies during the first 60 days of life did not permanently alter the reproductive function of elk. This is in contrast with findings in domestic sheep actively vaccinated against GnRH at two weeks of age [145, 169, 235] and male rats passively immunized five days after birth [234, 236, 251]. Brown and Clarke found that neonatal vaccination against GnRH caused permanent HPG axis suppression during adulthood, in a subset of vaccinated males and females, despite a lack of GnRH antibodies. This was caused by decreased GnRH secretion rather than a decrease in GnRH content of the median eminence [146]. Bercu and Vogel found permanent suppression of rat testicular development after neonatal passive vaccination despite normal post-pubertal gonadotropin levels during adulthood [234, 236]. They suggested that there is a critical window of testicular development in rodents at this time that is permanently altered without the timely support of gonadotropins. In our study we investigated both the potential for passive neonatal vaccination to permanently decrease GnRH secretion and the potential for transient removal of GnRH signaling to alter the structure or function of the pituitary or gonad even if long-term GnRH secretion was

unimpaired during adulthood. We found neither of these altered reproductive function in elk from this study.

Gonadotropin releasing hormone is secreted from hypothalamic neurons originating within the blood brain barrier but terminating near blood vessels of the hypothalamic-pituitary portal system outside of the blood brain barrier. After active GnRH vaccination in male pigs at 10 and 18 weeks and subsequent necropsy at 26 weeks of age, Molenaar and colleagues found lesions including fibrosis and scar tissue formation within the median eminence which were positively correlated with GnRH antibody titer and testicular atrophy [136]. They suggested that T cell-mediated autoimmune reactions directed at GnRH neurons or retrograde transport of GnRH antibodies might result in destruction of the neurons or their processes. They demonstrated inflammatory lesions of the median eminence consistent with the speculation that anti-GnRH IgG in addition to interleukin cytokines may be responsible for permanent changes to function of GnRH neurons. Their study was different from ours in two important ways. First, the lesions they described were present in animals which had concurrently high concentrations of GnRH antibodies which may have indicated the inflammatory processes were actively happening at the time of death but that lesions could have been transient. Second, pigs were actively vaccinated with Freund's complete and incomplete adjuvants; potent stimulants of both humoral and cell-mediated components of the immune response [210]. In contrast, elk in our study did not have detectable antibody concentrations by six months of age and most response variables were measured between nine and 30 months of age. More importantly, elk in the current study were passively immunized with maternal antibodies rather than stimulating their

own immune system to initiate a humoral and cell-mediated response. While concentrations of GnRH antibodies were certainly higher in our study 24 hours after birth because theirs were not actively vaccinated until 10 weeks of age, due to reporting inconsistencies it was not possible to compare concentrations of antibody between the two studies at later time points. Regardless, our elk were not exposed to a full complement of humoral and cell-mediated components of the immune response whereas pigs exposed to Freund's complete adjuvant along with GnRH conjugated to keyhole limpet hemocyanin (KLH) likely had robust stimulation of the entire immune system. While we did not examine the histological structure of the median eminence to the same degree that Molenaar et al. did, there was no apparent evidence of inflammation or fibrosis in hypothalmi and there was no clinical evidence of HPG axis dysfunction in our elk. Our findings suggested that elevated antibody titer alone was not sufficient to induce a permanent decrease in GnRH secretion.

In altricial species such as the rat, HPG axis maturation is incomplete at birth [252]. In precocious species such as sheep, and likely elk, the fetal HPG axis is functional by mid-gestation [213]; however, hypothalmic signaling is required for development of gonadotroph cells during the last month of gestation [253] and puberty is reliant on an adequate gonadotrope population [253]. It is unknown exactly when the permanent structure or function of the HPG axis is complete in elk, but our findings suggest that elimination or at least a decrease in GnRH signaling during the first 60-180 days after birth does not delay the process. Because we did not measure gonadotropin secretion during the neonatal period we do not know if there were adequate concentrations of GnRH antibodies to completely remove GnRH signaling. Our previous study in adult

female elk with similar or lower concentrations of GnRH antibodies showed suppression of fertility but not complete inhibition of final stages of follicular development indicating a partially intact HPG axis. Alternatively, ewes with lower concentrations of GnRH antibodies but more aggressive active vaccination schedule had complete cessation of follicular wave development [169]. It is likely that antibody concentrations were sufficient to influence GnRH signaling in the current study. This finding added strength to our speculation that antibodies alone do not account for the long-term suppression of the HPG axis observed in previous studies.

Our investigation of endocrine changes, direct and indirect measures of fertility, and development of secondary sexual characteristics through puberty in male elk provided results which are similar to those seen in untreated red deer (*Cervus elaphus*). Red deer experience the highest mean levels of testosterone in late summer and early fall when antlers are hard and velvet has been shed [255-257]. Conversely, in the spring, at the time of antler casting, testosterone is nearly undetectable and testes are at their smallest [256, 257]. Elk experience a similar pattern [238]. Likewise, we measured the highest mean concentrations of testosterone in late summer when antlers were hard and concentrations fell as winter advanced. Scrotal circumference mirrored testosterone levels with the smallest measurements in late spring and largest in late summer; however, as expected, scrotal circumference did not regress to pre-pubertal dimensions. Male elk pituitary response to stimulation with GnRH analog prior to and during the second reproductive season was similar to that seen in red deer stags [255, 256]. Maximum concentrations of both LH and testosterone, induced by GnRH stimulation, were higher in August than in November. Interestingly, the single outlier male with an unusually large

maximum concentration of LH had a relatively modest corresponding maximum concentration of testosterone. This pattern of large release of LH with little secretion of testosterone is characteristic of the non-reproductive season in red deer [255]. It was our observation that this individual was socially the least dominant male and he did not produce an acceptable semen sample until December 2007. This male was apparently the last to mature within his cohort.

In elk populations which are not nutrient limited, males are generally reproductively competent at 14-16 mo. of age [177, 238] with similar fertility rates as those seen in older more mature bulls when competition is removed [38, 258]. While we did not directly evaluate fertility we found no differences in semen quality or secondary sexual characteristics associated with fertility between exposure groups. Sperm motility, velocity, and morphology parameters met or exceeded standards for domestic bulls [241] and were similar to findings in red deer [250, 259]. Semen evaluation and sperm analysis have remained elusive as a reliable indicator of fertility in domestic animals [260, 261]; however, these assays may be more useful in wild species where strong artificial selection pressures for increased fertility are not in affect [262]. Swimming velocity and sperm morphology account for differences in fertility rates of Iberian red deer (*Cervus elaphus hispanicus*) [240]. Additionally, antler size and complexity are associated with testes size and sperm velocity [259]. Finally, despite seasonal testicular atrophy, all males in our study had maturing spermatocytes within the seminiferous tubules during the early spring at approximately 33 months of age. Our data suggested that all males were reproductively mature and likely fertile by the end of their first reproductive season at 15 to 21 months of age.

Puberty and ultimately fertility in female elk is dependent upon body condition. Approximately 50% of yearling females will become pregnant at 16 to 18 months of age if an appropriate body mass (~220 kg, 10% body fat) is achieved [177, 182, 184, 185]. The first estrous cycle of the reproductive season often has a short interovulatory interval [37] followed by regular ovulations every 20 to 24 days [179, 181]. In our study every female displayed regular increases in serum concentrations of progesterone at approximately 20 day intervals. Most females had evidence of functional CL by mid October and all were cycling by early November. This is later than for mature female elk in North America [37, 263] but is consistent with what others have observed in two year old females [184]. Three of seven females continued to have elevated concentrations of progesterone in mid to late March when sampling was terminated. This is in accordance with previous research which demonstrated estrous cycling is possible in late March, though in mature females typically ends in late February [37]. Pituitary stimulation with GnRH analog prior to the breeding season induced similar LH release to that seen in non-pregnant mature female elk during the reproductive season [244]. In our confirmatory test of fertility all females became pregnant. These results substantiate that there was no effect of exposure on maturation of the HPG axis or fertility in female elk calves.

In conclusion we found no effect of exposure to high concentrations of maternally transferred GnRH antibodies on the long-term structure or function of the hypothalmo-pituitary-gonadal axis of elk. The elk HPG axis is likely structurally mature at birth and transient disruption in GnRH signaling through antibody neutralization was not sufficient to permanently change function.

Acknowledgements

We would like to thank the Colorado Division of Wildlife, Foothills Wildlife Facility Staff for excellent animal care and handling (I. Levan, J. Spaak, M. Fisher, T. Davis, A. Mitchell). We thank M. Allen, A. Latimer, A. Graham, and A. Lothridge for laboratory assistance with RIA procedures. We thank P. Moffett for assistance with semen collection and evaluation. M. Conner provided most helpful comments and advice on statistical analyses.

Tables

Table 4.1. Measures of semen quality and secondary sexual characteristics in male elk between 15 and 22 months of age. Asterisk denotes a difference between exposure groups.

Response Variable	Exposed				Unexposed			
	Mean Est.	±	SEM	Range	Mean Est.	±	SEM	Range
Normal morphology (%)	59.0	±	1.6	15-86	67.5	±	2.4	35-91
Total motility (%)	60.9	±	1.5	7-98	83.0	±	1.8	48-98
Progressive motility (%)	39.1	±	1.3	1-79	64.7	±	1.5	32-81
Sperm velocities VAP	74.5	±	10.0	31-116	97.6	±	11.1	76-116
(µm/s) VSL	62.6	±	10.0	19-100	83.1	±	11.1	67-104
VCL	119.2	±	121.2	52-174	153.8	±	13.5	115-178
Antler length (cm)	148.8	±	3.1*	123-187	166.3	±	3.8*	129-197
Antler mass (cm)	2805.5	±	244.4	1040-4420	2622.3	±	299.3	1330-4500
Neck girth (cm)	72.7	±	0.5	62-80	74.8	±	0.7	68-80

Table 4.2: Hormone levels and gonadal measurements in GnRH antibody exposed and unexposed male (m) and female (f) elk calves at the time of necropsy (March/April 2009) at approximately 3 years of age. GnRH and gonadotropins were measured in both sexes, testosterone in males, and progesterone in females.

Response variable	Exposed	Unexposed
	Estimate ± SEM	Estimate ± SEM
FSH (µg/g pituitary)	(m) 1368 ± 268	(m) 1660 ± 328
	(f) 2262 ± 268	(f) 1200 ± 568
LH (µg/g pituitary)	(m) 70 ± 67	(m) 105 ± 82
	(f) 215 ± 67	(f) 309 ± 142
Hypothalmic GnRH (ng)	(m) 7.1 ± 2.2	(m) 5.3 ± 2.7
	(f) 5.3 ± 2.2	(f) 6.9 ± 4.7
Serum testosterone (ng/ml)	0.41 ± 0.73	0.00 ± 0.00
Serum progesterone (ng/ml)	0.18 ± .07	0.07 ± 0.08
Testes mass (g)	48.5 ± 5.5	42.6 ± 4.5
Ovary mass (g)	3.1 ± 0.5	2.2 ± 0.2
# Sperm x10^6 (/g of testis)	79.9 ± 7.0	77.1 ± 0.6

Figures

Figure 4.1. Timeline for measurements of hypothalamic-pituitary-gonadal axis function in elk calves exposed or unexposed to GnRH antibodies.

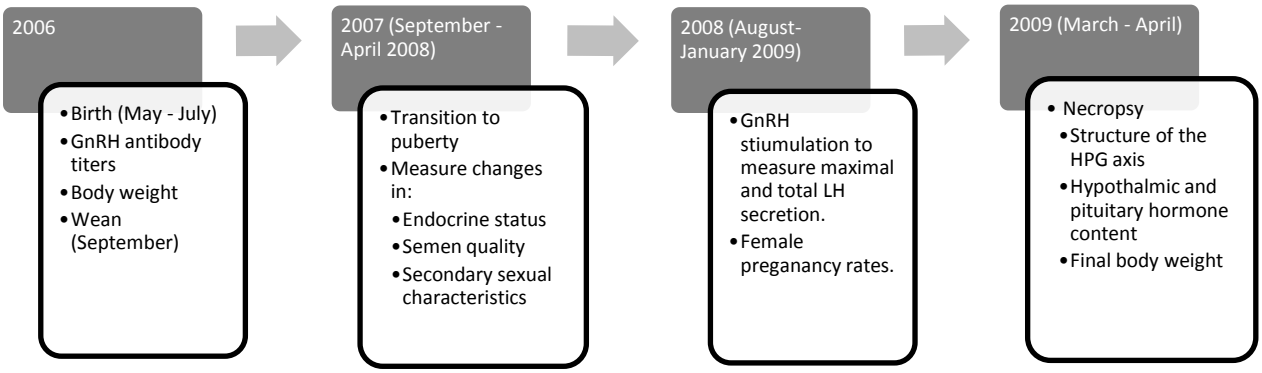


Figure 4.2. Persistence of maternal antibody from birth to 1 year in elk calves (n = 6-8 males, 2 died Aug ‘06, May ‘07; n = 4 females) exposed to colostral transfer of GnRH antibodies during the first 24 hours of life. Unxposed titers not shown.

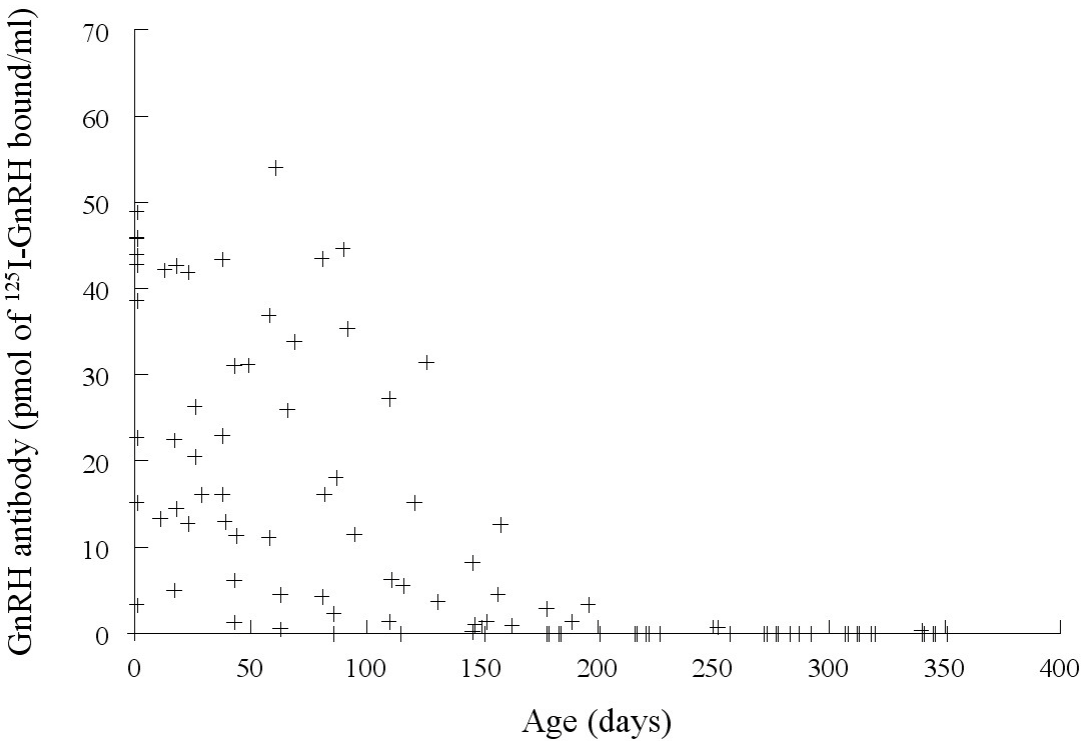


Figure 4.3. Growth curves from birth to three years of age for male (n = 6-8; 2 died Aug '06, May '07) and female calves (n = 7) exposed (●) or unexposed (○) to GnRH antibodies during the neonatal period.

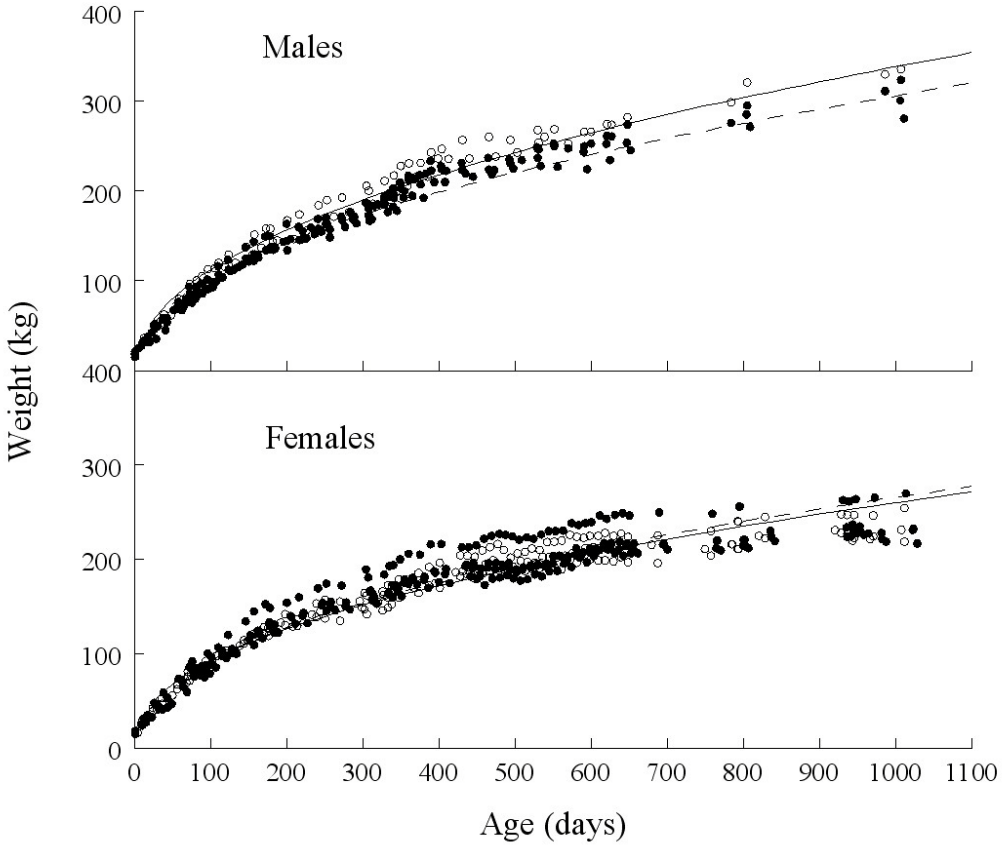


Figure 4.4. Mean monthly concentrations of testosterone ± SEM, between 14 and 21 months, in male elk exposed (n = 4; ●) and unexposed (n = 2; ○) to neonatal GnRH antibodies. Letters denote differences between months ($P < 0.05$); there were no differences between exposure groups within months.

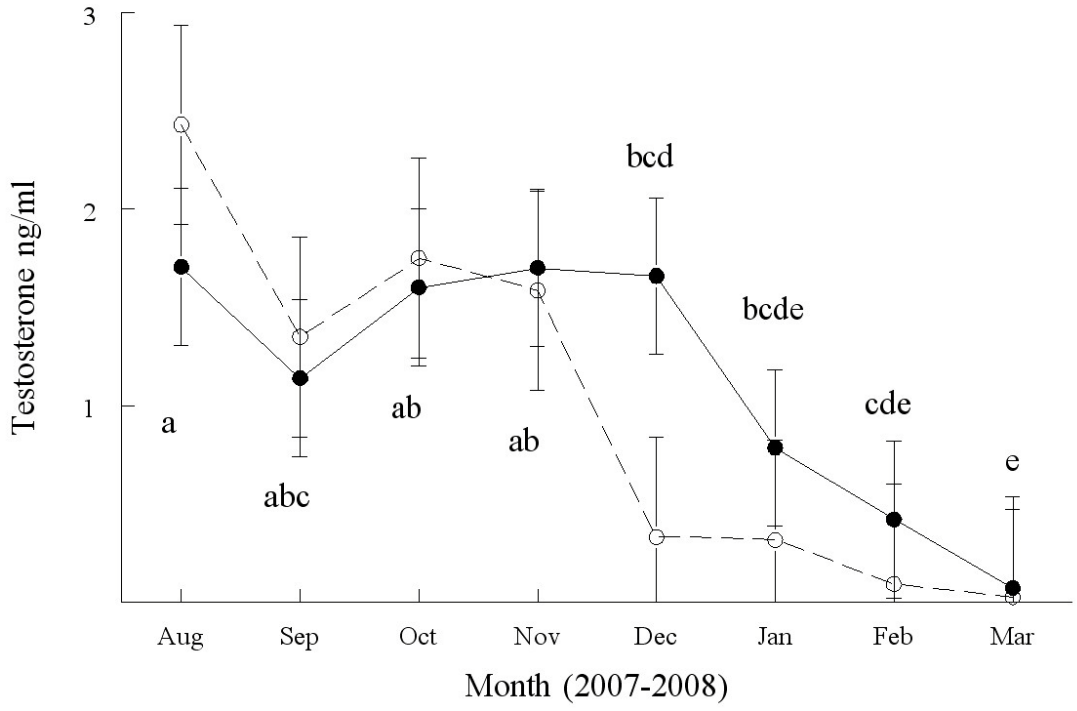


Figure 4.5. Mean scrotal circumference ± SEM, measured between 10 and 27 months of age, in male calves exposed (●) and unexposed (○) to GnRH antibody during the neonatal period. There were no differences between exposure groups.

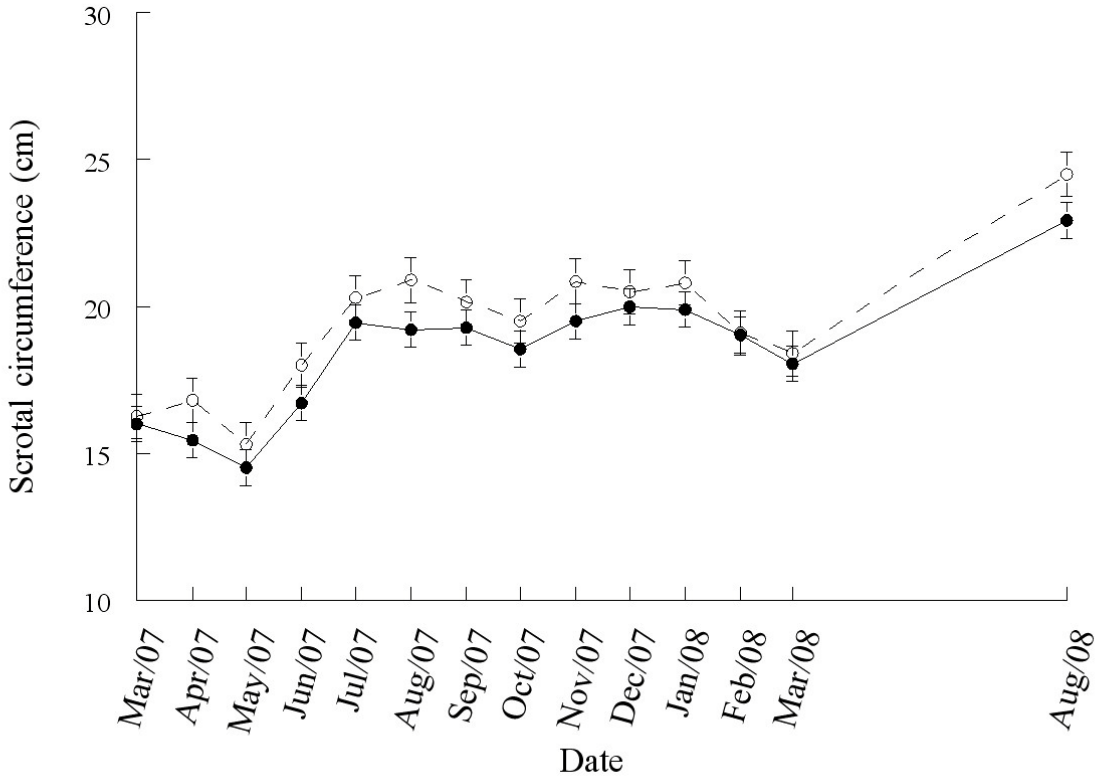


Figure 4.6. Mean monthly concentrations of progesterone ± SEM, from August 2007 to March 2008, in female calves exposed (n = 4; ●) and unexposed (n = 3; ○) to GnRH-antibody during the neonatal period. Letters denote differences between months ($P < 0.05$); there were no differences between exposure groups within months.

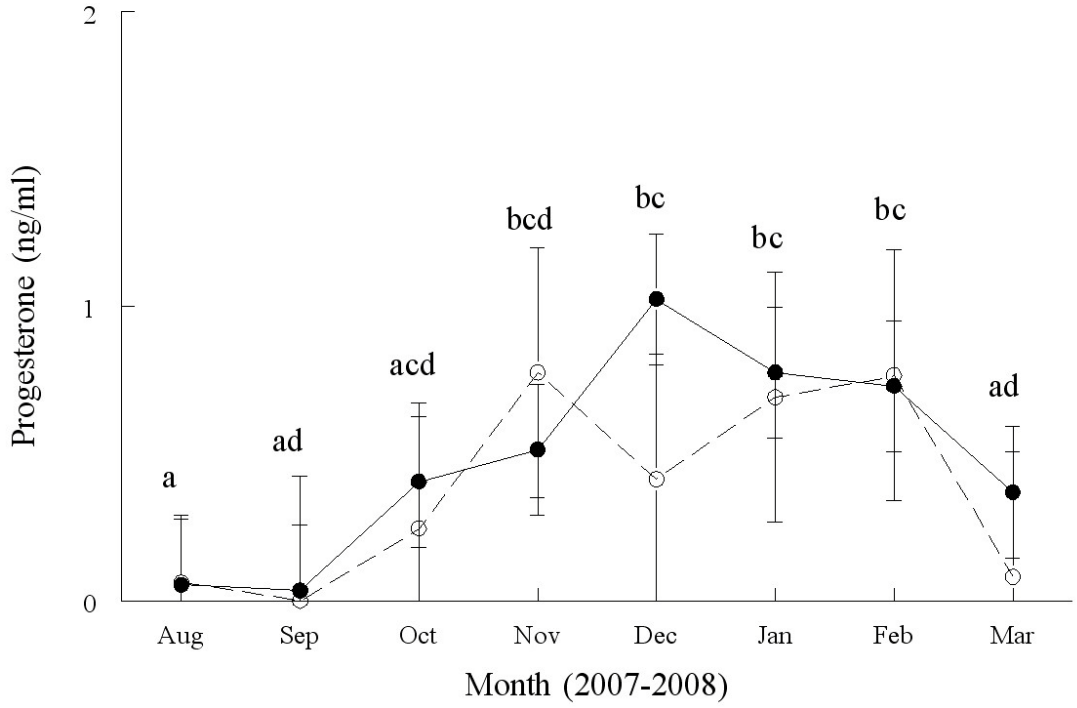


Figure 4.7. Representative progesterone profiles from female elk calves exposed (●) and unexposed (○) to GnRH antibody during the neonatal period. Sampling occurred at 10 day intervals between 15 and 22 months of age.

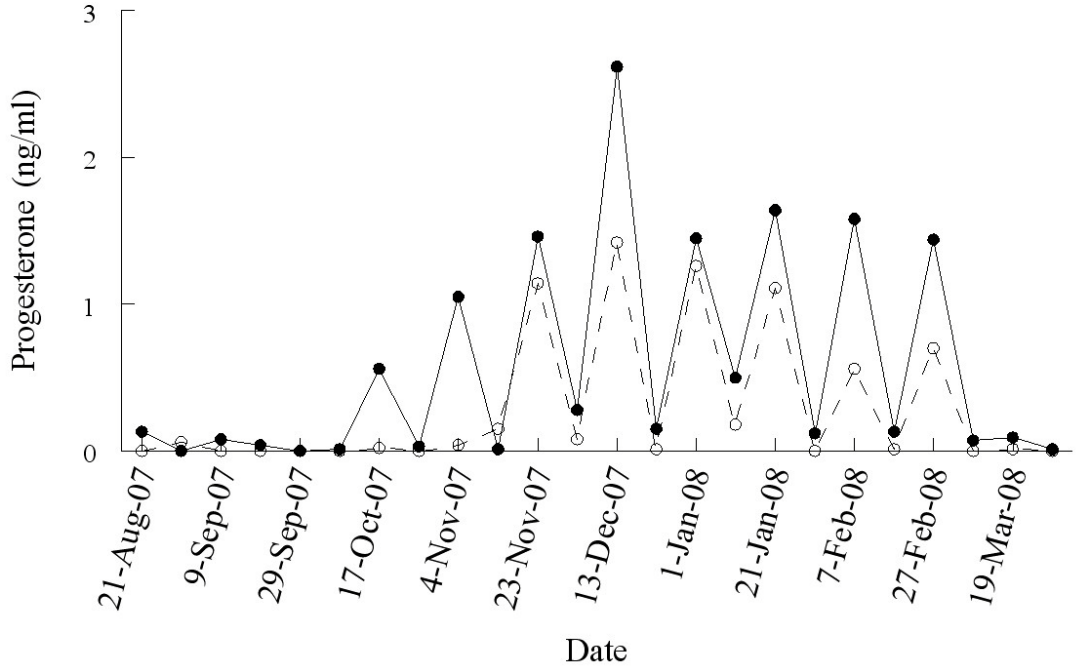


Figure 4.8. Mean concentrations of LH (○ ●) and testosterone (□ ■) ± SEM after GnRH agonist treatment in male calves (27 to 30 mo.) exposed (filled solid line, n = 4) and unexposed (open dashed line, n = 2) to GnRH antibodies during the neonatal period. Asterisk denotes significant difference between exposure groups.

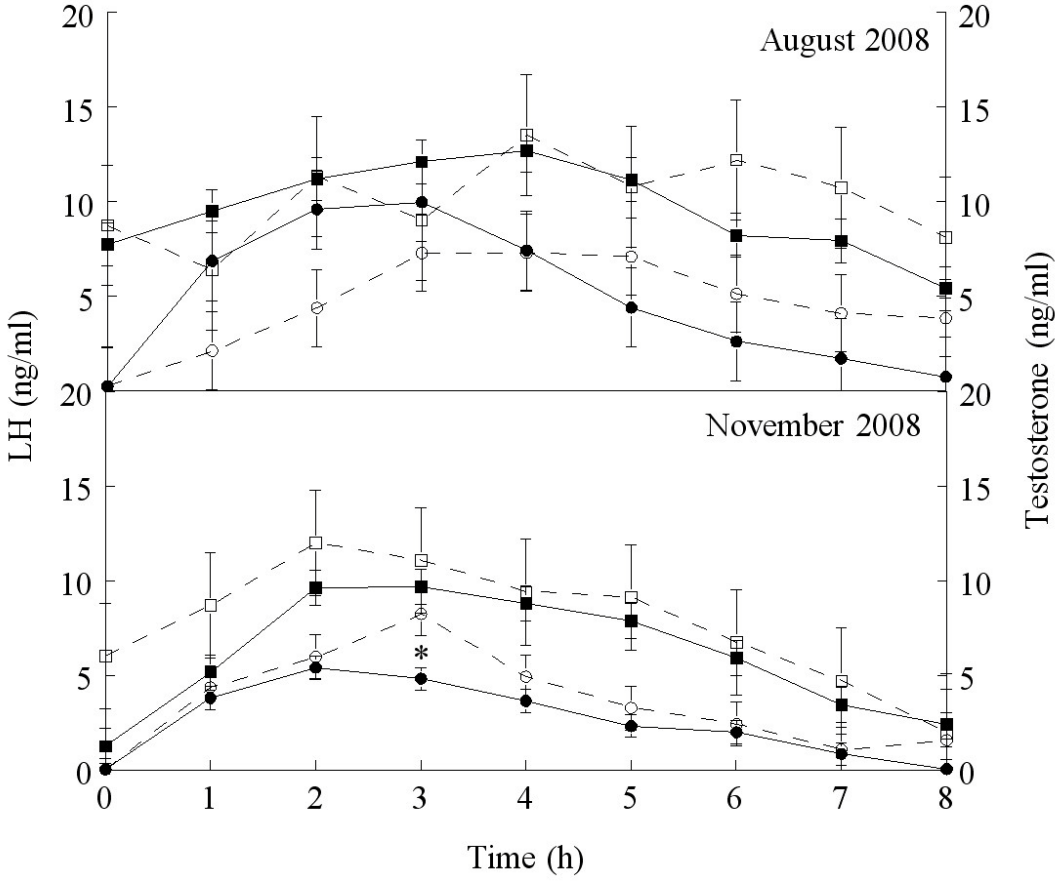


Figure 4.9. Mean LH concentrations ± SEM, after GnRH agonist treatment, in female elk calves (27 to 28 mo) exposed (●, n = 4) and unexposed (○, n = 3) to GnRH antibody during the neonatal period.

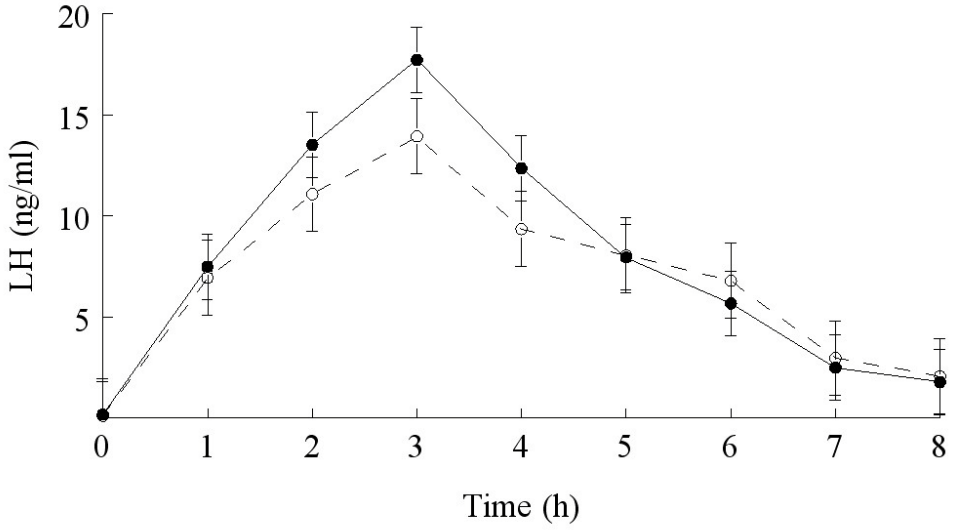
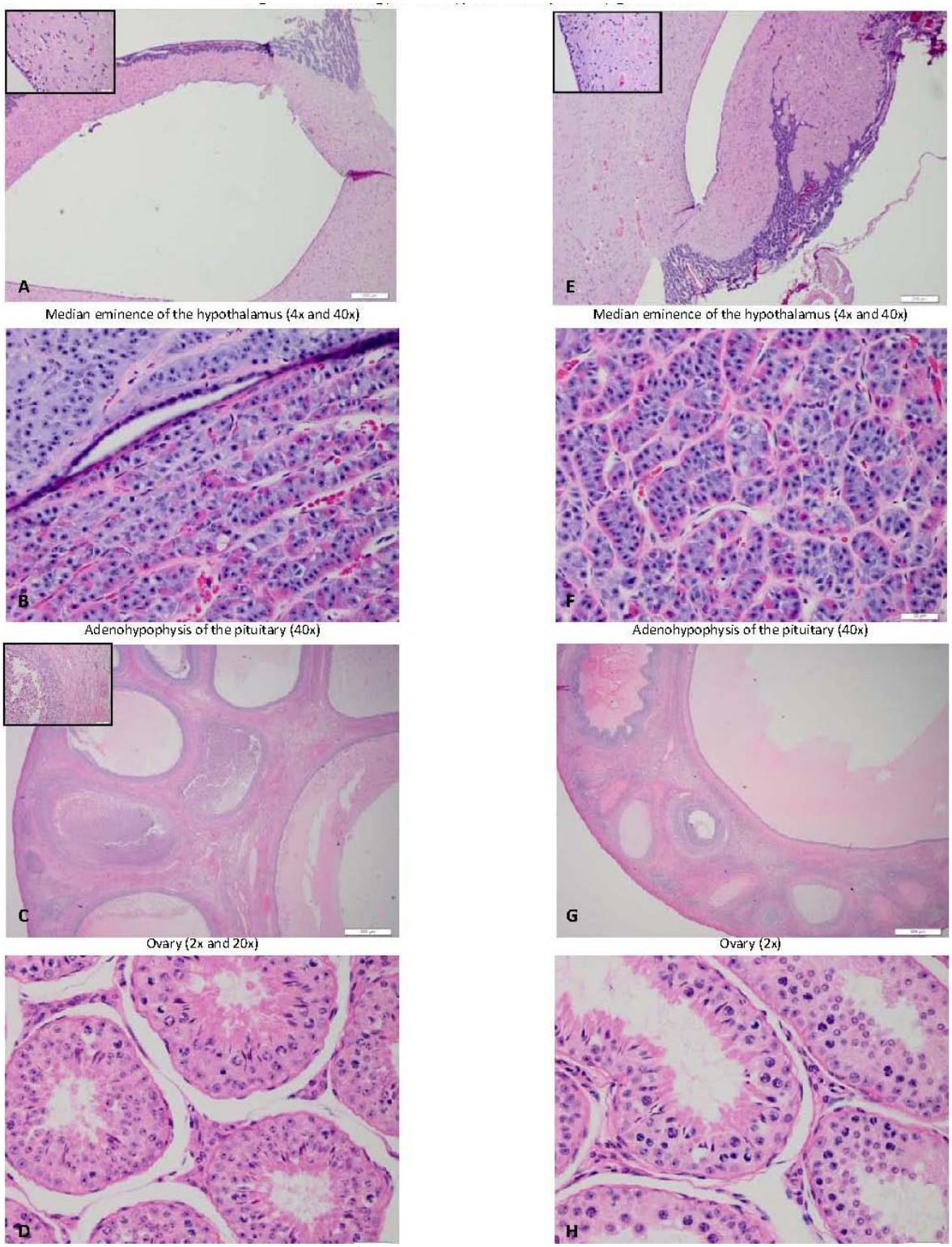


Figure 4.10. Histology of the hypothalamic pituitary gonadal axis. Images A-D are typical of antibody exposed and E-H are typical of antibody unexposed elk.



Chapter 5: Conclusion

Challenges and Future Directions in the Use of Wildlife Fertility Control

Obstacles to the Use of Wildlife Fertility Control

The study of wildlife fertility control has evolved substantially in the last two decades. Research has moved from primarily efficacy studies to investigation of collateral effects at both the individual animal and less frequently the population level [31]. Immunocontraception, with either porcine zona pellucida or gonadotropin releasing hormone antigens, has emerged as the most promising option for fertility control in ungulates, particularly feral horses and suburban white-tailed deer [30]. Both vaccines have been developed to the point of having multi-year efficacy with few individual animal pathological side-effects of welfare concern [110, 117, 203, 264]. Despite 30 years of concerted scientific efforts and modest management investigation, fertility control is rarely used as a primary means of controlling free-ranging wildlife populations. Barriers to its widespread use vary by wildlife jurisdiction and include biological, ecological, economic, political, and regulatory obstacles. While remaining biological and even ecological questions can, and more than likely will, be answered through scientific investigation, economic, political, and regulatory concerns will ultimately be resolved through public discourse and debate between agency, academic, political, and public stakeholders who value their common wildlife resources for a wide variety of reasons. Here I will highlight issues limiting the use of fertility control agents in general, and more specifically immunocontraceptives, as wildlife management tools. Some are technical in nature while most are sociological in scope. Stakeholders will eventually have to decide if, when and under what circumstances fertility control agents and their collateral effects

are acceptable. Additionally, there must be debate over how public and private resources will be used to implement any wildlife management technique including fertility control.

Biological Barriers

One consistent biological characteristic of PZP immunocontraception is repeated estrous cycling. Although there is conflicting research, likely due to study design limitations, breeding behaviors have been demonstrated outside of normal breeding seasons in both cervids and horses [98, 117, 127, 265]. Subsequent fawning and foaling seasons are also extended if vaccine failure occurs [97, 120, 265]. Behavioral consequences of GnRH vaccination are equivocal. Early studies in deer and horses indicated a decrease in estrous behaviors which were inversely associated with GnRH antibody titer [107, 173]. However, others have found erratic suppression of reproductive behaviors [266, 267] or delayed estrous cycling and fawning season [111]. We found prolongation of precopulatory behaviors directed towards and displayed by GnRH vaccinated female elk throughout the breeding season similar to that seen in female elk and deer treated with GnRH agonists [71, 76]. We did not investigate persistence of reproductive behaviors in the non-breeding season. The ecological implications of changes to sociosexual behaviors and/or birthing seasons are not fully understood with either vaccine; however, there is the potential to change social structures as well as alter the synchrony of forage and reproduction cycles [98, 265]. Additional population level studies will assist in quantifying the likelihood and intensity of these effects. Potentially more revealing will be sociological studies or management discussions which address the acceptability of these outcomes.

A second biological effect is initiation of inflammation at the site of vaccine injection and possibly systemically. Both PZP and GnRH vaccines often induce granulomatous injection site lesions in deer when vaccines are adjuvanted with killed mycobacteria and non-biodegradable mineral oil [110, 119, 121]. It has been suggested that deer vaccinated with Freund's complete adjuvant (FCA) may have increased incidence of pneumonia and granulomatous lesions in dispersed organ systems similar to those seen in laboratory animals [119, 210]. Local and systemic inflammatory effects have not been fully evaluated in horses due to lack of post-mortem examination. While inflammation and lesions may be decreased by using AdjuVac, as we did in our study, rather than FCA as an adjuvant these reactions are far from eliminated [118]. While animals continue to eat and ambulate apparently normally, we demonstrated that GnRH vaccination using the adjuvant AdjuVac can cause large pyogranulomatous abscesses. The Animal Welfare Act Regulations advise that procedures which can reasonably be expected to cause more than slight or momentary pain or distress in a human should be considered to cause pain in an animal (9 CFR §1.1). I offer that a 500 cm^3 abscess or any space occupying lesion of this size, in a large muscle mass would cause pain in a human. This is not meant to imply that the vaccine should not be used for population management only that the potential benefits of non-lethal population management must be weighed against the animal welfare concerns associated with vaccination.

Ecological Barriers

While the physiological effects of immunocontraception have received much theoretical and experimental attention, the potential genetic consequences have not begun to be addressed. In 1997 Nettles questioned the soundness of using a wild animal's

immune system to select for the ability to reproduce [268]. Since this time several authors have repeated this apprehension [188, 269, 270]. Artificial selection can theoretically occur in two ways. First, by purposeful or inadvertent non-random selection of animals targeted for contraception, mangers may prevent reproduction in wildlife in ways that change gene flow in a population. This would have genetic effects similar to those that may be applied through traditional means of wildlife management such as lethal removal with non-random removal of individuals from the gene pool. The second and potentially far more important mechanism is that inferred by the animal itself. Neither the GnRH nor PZP vaccines are 100% effective at preventing pregnancy and both impose their effects through stimulation of the immune system. Depending on the proportion of heritability as opposed to environmental influence on the immune response, it is possible to select for decreased immune function and likely decreased fitness in relatively few generations. For example, if the phenotype of failure to mount a significant contraceptive response to the vaccine has 80% heritability with 10% of the females not responding to vaccination, and continuing to become pregnant, approximately 20% of female progeny will likewise not respond [269]. Thus the trait has doubled in a single generation time when all females within the population are vaccinated. This has the potential to lead to immune incompetence and resistance to immunocontraception in the population. If immune responses responsible for contraception are mediated through the same or similar genetic pathways as those responsible for responding to pathogens and disease states it is possible and even likely to select for decreased population fitness.

Alternatively, if a large proportion of the variation in contraceptive response is attributable to the environment then even intense selection will have little effect on the

phenotype of future generations [271]. Difficulties in estimating heritability and finding reliable, accurate, and sensitive indicators of changes in phenotype of immune function, given multi-gene effects on the system, pose significant challenges to resolving this question. However, there is evidence from studies with mice, chickens, and pigs which strongly suggest that antibody production, delayed type hypersensitivity, and phagocytic activity are heritable traits [272-275]. In controlled environments, significant changes in both humoral and cell-mediated immune responses can be achieved in as few as three generations [274]. However, genetic variability in phenotype can change in response to environmental conditions [276]. This emphasizes the need for both laboratory and field studies of immunocontraceptives when investigating variance in immune response.

The proportion of the population targeted for immunocontraception will also influence selection pressure applied by non-response. From a practical standpoint managers are likely to treat as many females as possible when beginning a fertility control program, particularly in extensively managed species such as cervids or in populations that are above population objectives, which will maximize non-response selection pressure [28]. Targeting for contraception may be more discriminating in intensively managed feral horse herds, by selecting only a proportion of the population and ensuring each female contributes a foal to the population prior to contraception [265]. This strategy would result in decreased selection for non-response. Additionally, immigration and emigration will affect gene flow in the population and dilute the selection pressure. Treatment application intensity, non-response rate, change in fitness, and migration will all influence the strength of artificial selection. This is one aspect of immunocontraception that wildlife managers have little if any valid data to make an

informed decision. A cross-disciplinary approach involving immunologists, reproductive scientists, population or conservation geneticists, and wildlife biologists may begin to answer these questions.

Beyond their influence on selection pressure, immigration and emigration significantly affect the likelihood of achieving population management goals. Even modest interchange between local populations can have large effects on achieving population reductions using fertility control [165, 277]. Porter et al. (2004) modeled scenarios with demographic data derived from suburban white-tailed deer and found with as little as 8% dispersal 68% of the females would need to be infertile to hold a the population constant at one-half of ecological carrying capacity [277]. In fact, after modeling an open deer population, with data collected from a fertility control project in Cayuga Heights, a suburban community in New York state, Merrill et al. (2006) concluded that even continued efforts using permanent sterilization would not reduce population density because immigration would continue to ply the population with fertile females, emigration would dilute the treatment effect, and stochasticity in the system would limit treatment success [165]. Accurate estimates of population parameters, including birth, survival, immigration, and emigration rates as well as population age and sex demographic data is required for useful predictive modeling of fertility control success. An estimate of ecological carrying capacity is also needed to approximate likely changes in future changes to population size and demographics based on density dependence. These measures are often quite expensive and laborious to collect in terms of personnel and in some situations aircraft time. Holding populations constant at or near carrying capacity requires less effort than managing populations with large growth

potential as reproductive and juvenile survival rates are inversely proportional to population density, encounter rates are higher, and a smaller proportion of the population requires treatment [101]. However, it is likely that human-wildlife conflicts necessitating management actions will occur at densities far below ecological carrying capacity thus increasing the effort required for success [28].

Most fertility control models have assumed populations are closed, with no immigration or emigration, which limit their applicability when considering arbitrary population boundaries which animals cross freely [59, 60, 147]. If a population is truly closed with physical barriers such as fences, island status, or geographic isolation, the possibility of extirpation using fertility control must also be considered. Using intensive efforts with long-term fertility control agents or methods it is possible to drive populations, particularly those that are small, to extinction [59, 60]. Site specific modeling efforts with accurate population parameter data are a technical limitation to using fertility control given that prudent wildlife managers are hesitant to embark on a long-term and resource intensive management strategy without a reasonable forecast of their success. However, given sufficient resources this data can be collected and reasonably accurate predictive population models created.

Technical Barriers

The final technical hurdle is one of vaccine delivery. Traditional versions of the PZP vaccine have long been delivered successfully through remote dart delivery systems [117]. The more contemporary pelleted versions offering more than one year of contraception with a single administration have not yet been delivered remotely [264]. The GnRH vaccine GonaCon has been delivered remotely through a dart [109], but

Fagerstone et al. (2010) offered that hand delivery is more desirable because individual animals can be marked [30]. Additionally, the GnRH vaccine GonaCon is labeled for use only by hand-injection to prevent non-target and environmental exposure [278]. While not trivial from a labor and feasibility standpoint, delivery technicalities will likely become surmountable in the future once darting mechanisms and regulatory issues begin to match vaccine technologies.

Regulatory Barriers

Regulation of wildlife contraceptives has significantly hindered their use in free-ranging populations. Until 2006 the Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) regulated fertility control agents similarly to other pharmaceuticals under the Federal Food, Drug, and Cosmetic Act [30]. As product development progressed to the point of efficacy testing potential fertility control agents were studied using Investigational New Animal Drug (INAD) exemptions or if already approved for use in other species were used in an extra-label manner for experimental use. Once sufficient data was collected the intention was to complete a FDA New Animal Drug Application (NADA) or to reject the product development [279]. Due to stringent and expensive testing requirements for NADAs, small commercial markets, and the limited scope of free-ranging wildlife studies no products were approved for use as wildlife fertility control agents. In response the FDA and the Environmental Protection Agency (EPA) agreed that the EPA would regulate contraceptives in free-ranging wildlife and feral animals while FDA would continue to regulate these products in captive animals including domestic livestock, companion animals, and zoological wildlife species [30]. The EPA exerts their regulatory authority under the Federal

Insecticide, Fungicide, and Rodenticide Act of 1972. Therefore, contraceptive agents used in free-ranging animals are considered pesticides. An Experimental Use Permit (EUP) from the EPA is required before studying a product in free-ranging populations. The intended end result of fertility control agent EUPs is a product that is registered for use by wildlife professionals with a pesticide applicator's license and jurisdictional authority for wildlife management. Once nationally registered, state pesticide control offices must register a product prior to use within their boundaries [280]. Currently (May 2011), the GnRH vaccine GonaCon is the only product registered for use in free-ranging white-tailed deer. There are no products registered for use in other ungulates.

Sociological Barriers

In addition to the complicated regulation of fertility control products, there is often confusion and consternation between state and/or federal agencies which have exclusive or shared authority for wildlife management in a given area. While free-ranging wildlife is a public resource, more than one agency, often with markedly different missions, will have authority over the same biological population residing on adjacent lands. Wildlife species generally do not stop at jurisdictional borders. Agencies may have conservation, preservation, or sustainable use missions and corresponding policies for the wildlife resources they administer. They are also subject different political and special interest group pressures. These goals and pressures may be different than those that their wildlife management neighbors experience, which can quickly create conflicts in management objectives and preferred methods to reach these objectives. Dialog and discussion remain powerful tools to resolve or at least articulate these differences and to identify what biological and ecological effects of wildlife management techniques are

acceptable under given circumstances. Scientists can contribute to this dialog by offering access to unbiased information, giving estimates of certainty to the biological, ecological, and sociological ‘facts’ surrounding wildlife management techniques, and interpreting potential outcomes of these techniques.

While fertility control has been successful in managing free-ranging populations in a few isolated cases, techniques have not been widely adopted [117]. This is likely due to a combination of real and perceived biological and ecological side-effects, intensity of management and resources required for success, conflicting political and social pressures between management agencies, and a healthy dose of skepticism. When we are considering something as cherished and emotionally charged as a nation’s wildlife resources erring on the side of caution with respect to novel, invasive, or manipulative tools of wildlife management that have the potential for unintended consequences appears a wise and sensible path forward.

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