

EVALUATION OF A GONADOTROPIN RELEASING HORMONE VACCINE  
FOR THE HUMANE CONTROL OF FEMALE FERAL CATS

By

JOHN FRIARY

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Abstract of Thesis Presented to the Graduate School  
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John Friary

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Chair: Julie Levy  
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The unwanted cat population in the United States numbers in the tens of millions and current control measures have only had limited success in reducing it. Immunocontraception has the potential to humanely reduce this population. The purpose of this study was to investigate the effectiveness of a GnRH-based vaccine for immunocontraception of female cats. It was expected that the treated cats would produce antibodies against GnRH and there would be a positive correlation between high titer and contraception. Adult female cats were divided into a sham group (n = 5) and a treatment group (n = 15) that was immunized once with 200 µg of synthetic GnRH coupled to keyhole limpet hemocyanin and combined with a mycobacterial adjuvant. GnRH antibody titer and serum concentrations of progesterone and estradiol-17β were determined monthly. For the duration of the study the daily photoperiod was manipulated in an attempt to induce estrus. A male breeding cat was housed with the females during the long-day periods, and continuous videography was used to monitor

for signs of estrus and breeding. GnRH antibodies were detected in all treated cats by 150 days after immunization, but when the titer in four cats fell below 16,000, they became pregnant and were classified as nonresponders. The titers of the remaining 11 cats (responders) never decreased below 16,000. These cats displayed no signs of behavioral estrus and did not become pregnant by the end of the study 24 months after immunization. All five sham cats became pregnant within one month of the introduction of the male cat. From 60 days after immunization until the end of the study, progesterone concentrations in all responders remained at basal levels, and increased two months before parturition in all cats that became pregnant. The responder cats gained more weight than the nonresponders during the 14 months after immunization ( $P = 0.004$ ), which is the same response observed in surgically sterilized cats. A single dose of GnRH vaccine resulted in contraception in 73% of the cats for at least 24 months.

## IMPACT OF FERAL CATS

The domesticated cat is the most numerous companion animal in the United States with 37.7 million households owning 90.5 million cats [1]. It is estimated that there are also an equal number of unowned, unwanted cats [2]. Considerable debate exists on what impact these cats have on human health through zoonotic diseases, animal health by acting as reservoirs for diseases that affect pet cats and other species, and wildlife through predation and competition. In addition, there is not agreement on the quality of life of the cats. The debate extends to the actions that should be taken to ameliorate the impact of these unowned cats.

Accurately assessing the impact and designing solutions is made more difficult because cats are assigned to subpopulations according to different criteria. Cats are often described according to their ownership status (owned or unowned), lifestyle (indoor, indoor/outdoor, outdoor), or degree of socialization [3] (tame, feral). In addition, during its lifetime an individual cat may move from one subpopulation to another. For instance, a cat living indoors may be abandoned, forced to live outside, and over time become untrusting of humans; this tame, indoor, owned cat has become an unowned, feral cat. Many reports on the impacts of cats fail to clearly define which subpopulation of cats is being studied. For the purposes of this thesis, a feral cat will be defined as any unowned, free-roaming cat, regardless of its socialization status.

Concerns have been expressed that feral cats serve as a reservoir for diseases that may be transmitted to humans, pet cats and wildlife [4,5]. In general, feral cats do not appear to pose a greater risk to humans or other cats than pet cats [3,6]. Cats can transmit disease directly through a bite or other physical contact; by shedding the pathogenic organism into the environment; or by concentrating pathogens that can be transferred to other hosts via vectors such as fleas, ticks and mosquitoes. The Center for Disease Control and Prevention considers eradicating wildlife to reduce disease reservoirs ineffective, but does support trap-vaccinate-release programs for wildlife [7].

### **Human Health**

The American Association of Feline Practitioners lists 40 potential feline zoonotic agents, but transmission of the disease from cat to human has not been documented or occurs only rarely with most of the organisms [8]. Rabies is often cited as a risk from feral cats, but none of the 57 human cases of rabies in the United States from 1980 to 2004 was attributable to cats [9,10]. The risk of rabies transmission from cat to human is very low.

*Bartonella henselae* and *Bartonella clarridgeiae* can both be passed from cats to humans by biting or scratching. Infection by these bacteria is the most common direct zoonosis associated with cats, and it results in 25,000 cases of cat scratch disease in the United States each year [8]. Cat scratch disease is generally a mild illness, but in rare cases it can lead to serious disease. In Randolph County, North Carolina, 93 of 100 (93%) feral cats and 48 of 76 (63%) pet cats were seropositive for antibodies against *B. henselae* [11]. In northern Florida 186 of 553 (33.6%) feral cats were seropositive for

antibodies against *B henselae*, which is within the range of values found in several studies of pet cats [6].

Roundworms, hookworms and tapeworms can be shed by cats in their feces and can potentially cause infection in humans. However, the larvae of roundworms and hookworms must mature in the environment for at least 3 days and 3 weeks, respectively, before they can infect a new host [8]. Fleas containing tapeworm must be consumed for infection to occur [8]. The infection rate of roundworms in feral (21%) and pet (18%) cats in Randolph County, North Carolina, was not statistically different [11]. However, a study of 80 feral and 70 pet cats in California found infection rates of roundworms and tapeworms significantly higher in feral cats than pet cats. The roundworm infection rates were 54% and 4% for feral and pet cats, respectively, and tapeworm infection rates were 26% and 4% for feral and pet cats, respectively [3].

The protozoa *Toxoplasma gondii*, *Cryptosporidium parvum*, and some species of *Giardia* are shed in the feces of cats and can infect humans through the fecal-oral route. Infection with *Cryptosporidium* spp. and *Giardia* spp. in humans is common, but they are rarely directly linked to cats [8]. In Randolph County, North Carolina, the feral and pet cats did not have significantly different prevalences of infection with *Cryptosporidium* (7% and 6%) or *Giardia* (6% and 5%) [11].

Domestic cats and other felids are the only definitive hosts to shed *T. gondii* oocytes in their feces, thus contaminating the environment with infectious organisms. The seroprevalence of *T. gondii* in feral cats (63%) in Randolph County, North Carolina, was higher than for owned cats (34%) [11]. Feral cats from Northern Florida were found to have a seroprevalence of 12.1%, lower than seroprevalence rates found for pet cats in

the United States (30%) [6]. Consumption of tissue containing *T. gondii* cysts allows transmission between intermediate hosts, such as pigs and humans. The relative importance of the two modes of infection in humans, ingestion of oocytes shed by cats and consumption of infected tissue, is unknown.

The natural oropharyngeal flora of healthy cats includes *Capnocytophaga* and *Pasteurella*, which are frequently found in cat bite infections. Untreated infections with these organisms can cause death [12]. However, most feral cat bites are provoked [3]. Advising the public to avoid direct contact with feral cats and implementation of trap-vaccinate-return programs should reduce the zoonotic disease risk from feral cats.

### **Animal Health**

The feline zoonotic diseases are transmissible between cats, but cats can also transmit diseases that do not affect humans, including feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), and feline coronavirus (FCoV). Some feel that kitten mortality rates, which may be as high as 75%[13], and the general health of feral cats are compelling reasons to euthanize these cats[5]. They believe that feral cats suffer higher rates of injury and disease than owned cats[5]. However, feral cats brought to trap-neuter-return (TNR) clinics in northern Florida were lean, but not emaciated [14], had death rates due to complications of surgery comparable to pet cats, and had a euthanasia rate for humane reasons of only 0.4%[14,15].

In addition, several studies have found that disease prevalence rates are very similar between feral and owned cats[6,11,16,17]. In 2004, 5,259 feral cats and 12,779 pet cats were tested for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) in 145 animal shelters and 345 veterinary clinics in the United States, Puerto

Rico and Canada. The infection rates for the feral cats were 2.0% (FIV) and 1.6% (FeLV) compared to the infection rates for the pet cats that were 2.7% (FIV) and 2.6% (FeLV) [16]. Between 1995 and 2000, a total of 1,876 feral cats from two trap-neuter-return programs in Gainesville, Florida and Raleigh, North Carolina, had infection rates for FIV and FeLV of 3.5% and 4.3%, respectively [17]. In addition, 553 feral cats in northern Florida had similar or lower prevalence rates of *Mycoplasma haemofelis*, *Mycoplasma Haemominutum* and *Bartonella henselae* compared to previous reports in pet cats [6].

However, it is possible that the overall health of feral cats brought in to TNR clinics is different than the health of feral cats in general.

### **Predation on Wildlife**

It has been well-documented that cats can have adverse impacts on wildlife, particularly on islands. Prey species on island ecosystems are especially vulnerable to the introduction of non-native mammalian predators such as the cat [18]. Cats have been introduced to at least 65 island groups and are a major threat to many island bird species [18]. In 1949, cats were introduced to Marion Island, a 112-square mile sub-Antarctic island in the Indian Ocean. By 1975, it was estimated that they were killing 450,000 burrowing petrels annually and probably had driven the common diving petrel to local extinction [19]. On an island in New Zealand, the last remaining Stephens Island Wren (*Xenicus [Traversia] lyalli*) was killed by a cat [20].

There is evidence that cats contributed to the total extinction of the Little Barrier snipe (*Coenocorypha aucklandica barrierensis*) and the local extinction of the North Island Saddleback (*Philesturnus carunculatus rufusater*) on Little Barrier Island, New Zealand [21]. However, introduced rats also had a detrimental effect on the bird

populations, and the relative contributions from the cat and the rat could not be determined [21]. In addition, seven years after eradication of cats was completed on the island, the bird numbers were similar to their numbers before the cat eradication program began [21]. It is believed that in some instances cats may actually protect island species by keeping the rodent population in check [19,22].

Determining the impact of cat predation in mainland settings is even more difficult than on islands because in most instances there are additional predators present. Outside of Canberra, Australia, domestic cats were found to be opportunistic predators, catching prey in proportion to the prey density. The conclusion reached was that cats had little effect on the local ecosystem [23]. Wildlife managers in Australia have been working to remove rabbits, another exotic species. However, in many ecosystems rabbits are the primary food for cats, and there is a fear that a sudden removal of rabbits will cause the opportunistic cats to switch to native prey. Individual ecosystems must be evaluated to determine the impact that cat predation has on wildlife and if removal is appropriate or necessary.

Possible transmission of *T. gondii* from cats to California sea otters (*Enhydra lutris nereis*) is of current concern. Between 1997 and 2001, the seroprevalence of *T. gondii* was 42% for live otters and 62% for dead otters [24]. The source of the *T. gondii* is believed to be cat feces from sewage and surface runoff from coastal communities. However, whether the feces came from litter boxes of indoor cats, from owned cats allowed outdoors, or feral cats has not been investigated. There is also concern that native marsupials in Australia, which has non-native cats, may be more susceptible to *T. gondii* than ecosystems with native cats[25].

Although the true impact of feral cats on the health and welfare of the public, animals, and the environment is difficult to quantify, it is appropriate public policy to develop effective cat control programs. The best approach to cat control is another area of intense controversy.

## METHODS TO MANAGE FERAL CATS

The most common action taken regarding feral cats has been to do nothing [26]. Historically, lethal methods of control have been used in an attempt to eradicate particular groups of cats. In the United States, euthanasia is commonly performed at animal control shelters. Worldwide, however, trapping, poisoning, and hunting have been the most common methods for eradication of feral cats. The use of biological vectors has been utilized in at least three island eradications [20,27]. In some areas of the world, including the United States, there has been strong public pressure to devise more humane ways of dealing with feral cats, including trap and relocate, and TNR.

### **Lethal Methods**

Since 1934, cats have been eradicated from 48 islands around the world. All but five of these islands had cat populations of less than 100, and only 10 of the islands are greater than 10 square kilometers [28]. The largest eradication effort occurred on Marion Island using trapping, poisoning, hunting with guns and dogs, and biological control, and took 19 years to complete [20]. It took three years to eradicate 151 cats from Little Barrier Island using traps, poison, hunting with guns and dogs, and biological control [21].

The poison used in most eradication campaigns was sodium monofluoroacetate (1080), which has been used since the 1950s in New Zealand for pest control [29]. Sodium monofluoroacetate kills by disrupting the Krebs cycle, thus inhibiting energy

production by cells. Long-term exposure to sub-lethal doses can be harmful to people handling the poison. Non-target animals are also at risk; dogs are especially susceptible to 1080 [29]. For these reasons, the use of 1080 in the United States is restricted to the protection of livestock from coyotes [30]. Secondary poisoning by ingestion of rats poisoned with the anticoagulant, brodifacoum, contributed to cat eradication on four islands [28].

Feline panleukopenia virus (FPV) was the biological control agent used on Jarvis, Marion, and Little Barrier Islands [28]. FPV virus is spread through feces, urine, saliva, and vomit. Felids are the primary hosts of FPV, but raccoons and a few other mammals can also contract the disease. On Marion Island, 96 cats were trapped and inoculated with 1000 TCID<sub>50</sub> of FPV and released by helicopter to 93 different locations on the island. The total cat population declined at a rate of 26% per year for five years, but stabilized as the population developed immunity [20]. On Little Barrier Island, the use of FPV was abandoned because the virulence among that population of cats was determined to be too low to be effective [21]. The use of biological controls has many inherent risks, including accidental release as happened with the rabbit calicivirus in Australia in 1995 [31], and non-target susceptibility. As was demonstrated on Marion and Little Barrier Islands, the target species may become resistant over time.

Eradication or reduction of cats on the mainland is more difficult than on islands for several reasons. Continuous influx of cats into the area is likely, the risk of non-target species death increases with poisoning and hunting with dogs, hunting with guns is heavily restricted for human safety reasons, and biological control mechanisms would be nearly impossible to contain.

Euthanasia at animal shelters is the leading cause of death of cats in the United States, with an estimated 3 million cats per year [26,32]. Euthanasia has not been shown to effectively reduce feral cat populations. In 1996 in Ohio 72.2% of cats admitted to shelters were euthanized and in 2004, the euthanasia rate was 68.8% [33]. It is hypothesized that removing cats from a habitat simply allows other cats to fill the vacated niche [26,34].

### **Non-Lethal Methods**

More humane solutions include trapping cats, sterilizing them, and adopting them into homes, relocating them to a more acceptable outdoor location, placing them in sanctuaries, or returning them to the location of their capture.

#### **Adoption**

Seventy-five percent of the cats euthanized in animal shelters in the United States are classified as adoptable [3], but there are not sufficient homes to accept them. Increasing the pool of cats waiting to be adopted with less socialized cats is clearly not a viable solution to the overall problem.

#### **Relocation Programs**

Relocation of cats to a non-enclosed site is difficult because it is time-consuming to acclimate the cats to their new environment and the cats often have low survival rates at the new sites [26].

#### **Sanctuaries**

Another alternative is to remove cats to permanent sanctuaries where they can live out their lives in confinement. Although sanctuaries can be a small-scale solution, the overall number of feral cats is too large to be accommodated in sanctuaries. Three

sanctuaries that have reached capacity and only accept cats on a very limited basis are Best Friends Animal Sanctuary in Utah, the Chico Cat Coalition in California, and a program instituted by the National Humane Education Society. In addition to their sanctuaries, Best Friends Animal Sanctuary and National Humane Education Society operate TNR programs [3].

### **TNR Programs**

The goal of TNR programs is to sterilize feral cats and return them to the location where they were trapped. TNR programs are generally run as grass-roots operations that depend on donations and volunteers. However, some municipalities, such as Orange County, Florida, use TNR programs as a cost-effective alternative to trapping and euthanizing cats and have incorporated TNR into their animal control efforts. The average cost of sterilizing each cat was \$56 compared to the estimated \$139 per cat for impounding, sheltering and processing [26]. Other agencies that have incorporated TNR into their animal control programs include Tomkins County Society for the Prevention of Cruelty to Animals, New York; Maricopa County Animal Care and Control, AZ; New York City Center for Animal Care and Control; San Francisco Society for the Prevention of Cruelty to Animals; and the American Society for the Prevention of Cruelty to Animals. At a minimum, TNR programs sterilize the cats and return them to their colonies. Some programs provide food, shelter and veterinary care, vaccinate against rabies and other diseases, and test for diseases such as FIV and FeLV. Removal of the tip of an ear is recognized internationally as a sign that the cat has been sterilized. For control to be effective it is essential that colonies are monitored for new arrivals and for

the birth of kittens. A TNR program was started and stopped on a Florida university campus; once the program stopped, the feral cat population began increasing [2].

One long-term goal of many TNR programs is to reduce the colony size through adoption and attrition. Complete elimination of large colonies is uncommon, but there are many examples of colonies being greatly reduced in size. In 1991, a TNR program was instituted on the University of Central Florida campus and by 2002, the original 11 colonies containing a total of 155 cats had decreased to eight colonies comprised of 23 cats [2]. In addition to a decrease of 132 cats, other benefits of the program included prevention of the birth of an estimated 300 to 700 kittens, and medical care or euthanasia for sick and injured cats in the colonies. In a three-year period ending in 2002, 1,116 California Veterinary Medical Association veterinarians neutered 170,334 feral cats through the Feral Cat Altering Program [35]. Maddie's Fund awarded nearly \$9.5 million to the California Veterinary Medical Association for the program [35]. A TNR program on a Texas university campus resulted in a 30% decrease in the feral cat population in two years [26].

Some wildlife advocates believe TNR is inappropriate because the cats are not removed from the wild, some critics believe euthanasia is more humane than returning the cats to their colonies, and others believe TNR is ineffective. Indeed, critics of TNR often cite two county parks in south Florida, A.D. Barnes Park and Crandon Marina, as examples of TNR failure. The high visibility of the TNR programs in these parks encouraged continual cat abandonment at the sites, resulting in a net increase in the colony size despite a decrease in the original cat populations [36].

Drawbacks recognized by proponents of TNR include expenses for traps, surgical equipment, medical supplies, and veterinary fees; intensive labor for trapping, transportation, surgery, recovery and return to the colony; and requirement for the technical expertise of veterinarians.

Mathematical models have been used to estimate the percent of feral cats that would need to be neutered in order to cause an overall decline in a population. One population model predicted that the annual percent sterilization necessary for stabilization of population growth would be 14% of the 241,000 cats in San Diego County, CA and 19% for the 36,000 cats in Alachua County, FL [34]. Another study [37] predicted that there would have to be a 75% neutering rate annually in order to have a population decline. This study predicted that a trap and euthanize program would only have to trap 50% of the population to have a population decline. A limitation to the mathematical models is the difficulty of determining parameters that determine feral cat reproductive capacity, such as the carrying capacity of a particular habitat [34].

While surgical TNR can be effective for reducing targeted cat populations, it is extremely resource-intensive and difficult to implement on a regional or national scale.

### **Non-Surgical Contraception**

Non-surgical methods of contraception or sterilization have the potential for more efficient implementation, less risk to cats, lower cost, and lower technical requirement when compared to surgery. Surgical sterilization achieves sterility by removal of the gonads, thus preventing the production of gametes. Successful non-surgical contraception or sterilization methods disrupt some aspect of the hypothalamic-pituitary-gonadal axis or interfere with fertilization of the egg or maintenance of the pregnancy.

### **Feline reproductive endocrinology**

Cats will only breed when they are in estrus, which is characterized by a rapid increase in estradiol-17 $\beta$  concentrations. The estrogen is released by the follicles, which are stimulated to grow by the gonadotrope, follicle-stimulating hormone (FSH). FSH is synthesized and released at a basal level, but the rate of synthesis increases greatly with the release of gonadotropin releasing hormone (GnRH). Female cats are photoperiod sensitive and they begin estrous cycling with an increasing daylight-length, as occurs in January and February in the northern hemisphere. Cats will stop estrus with a decreasing daylight-length as occurs in September or October. Cats in the laboratory can be induced to enter estrus by increasing the daylight-length to 14 hours or greater. It is possible that the pineal gland responds to increasing daylight-length by decreasing the production of melatonin, which inhibits the release of FSH [38]. Conversely, decreasing the amount of light appears to increase the production of FSH and the cats will enter anestrus, meaning that estrous cycling stops.

During estrus, female cats are receptive to male cats and sufficient breeding triggers a cascade that leads to ovulation. As an induced ovulating species, cats release GnRH within minutes of genital somatosensory stimuli, as occurs during breeding [39]. GnRH causes the release of a second gonadotrope, luteinizing hormone (LH), within 5 minutes of the GnRH release [40]. LH concentration increase does not necessarily lead to ovulation, however. With a single copulation only about 50% of cats will ovulate, but repeated copulations probably trigger successive GnRH releases which leads to cumulative increments in LH levels [39].

GnRH is synthesized in hypothalamic neurons whose axons extend into the pituitary stalk, which is not within the blood-brain barrier. Upon appropriate stimulation, GnRH is released by exocytosis into the capillary plexus that emanates from the superior hypophyseal artery. GnRH is carried down the pituitary stalk in portal veins which give rise to a second capillary plexus that supplies the endocrine cells of the anterior pituitary. GnRH binds to gonadotrophs which synthesize and release the two gonadotropins, LH and FSH. However, the duration and the amplitude of the gonadotropin release depends on the pulsatile and periodic release of GnRH. For a proper functioning hypothalamic-pituitary-gonadal axis (HPGA), GnRH must be released with the proper pulsatility and periodicity; the characteristics of the GnRH release differ in male and female cats, with the age of the cat, and the stage of estrus with the female cat.

LH binds to Leydig cells in the male cat and thecal cells in the female cat and stimulates the synthesis and secretion of androgens. FSH binds to Sertoli cells in the male cat and granulosa cells in the female cat and stimulates estrogen synthesis. FSH also stimulates synthesis in the Sertoli and granulosa cells of various protein products, including activin and inhibin, two proteins involved in feedback mechanisms within the HPGA. In addition, FSH increases the number of LH receptors on the granulosa cells, which amplifies the sensitivity of granulosa cells to LH.

If the female cat does not breed during estrus, or if there is insufficient breeding to induce ovulation, then the estrogen levels decrease after three to sixteen days [41] and the cat enters interestrous, or a phase between successive estrous periods. The cat will continue to cycle between estrus and anestrus until ovulation occurs or the breeding season comes to an end with decreasing daylight-length.

If the cat ovulates and the egg or eggs are successfully fertilized then the cat begins a gestation period of approximately 65 days. If the cat ovulates, but the egg or eggs are not fertilized, then the cat enters pseudopregnancy which lasts approximately 45 days. In both pregnant and pseudopregnant cats, the follicles that have matured have released their eggs from the ovary. The portion of the follicles that remain behind are termed corpus lutea and within 48 hours of ovulation they begin secreting progesterone.

Prior to the release of progesterone by the corpus lutea, the basal level of progesterone is less than 2 ng/ml. Within 14 to 18 days after ovulation the progesterone concentrations are greater than 20 ng/ml. The lifespan of the corpus lutea is approximately 35 days, so the progesterone concentration in a pseudopregnant cat returns to baseline after 35 or 40 days. However, a pregnant cat maintains progesterone concentrations above baseline because the placenta secretes the hormone after 30 days of gestation [41].

GnRH, the gonadotropes and the sex hormones form a complex feedback web. GnRH induces the release of FSH and LH, which in turn induce the release of testosterone and estrogen. However, testosterone and estrogen provide negative feedback and inhibit the release of LH and GnRH. In addition, the gonads produce three other hormones that act on the gonadotrophs. Activin stimulates the release of FSH, inhibin and follistatin inhibit release of FSH [41].

Additional hormones that are produced outside the gonads and affect fertility include leptin, prolactin, growth hormone and insulin-like growth factor 1 (IGF-1). Leptin is required for fertility [42], while high concentrations of prolactin inhibit the

secretion of FSH and LH in both sexes. Growth hormone stimulates synthesis of insulin-like growth factor 1 which stimulates sex hormone synthesis [42].

The reproductive system in cats provides a number of mechanisms and potential targets to inhibit fertility, including the use of steroid and peptide hormones to disrupt the necessary hormone balance, destruction of reproduction-related cells, and immunocontraception to bind a hormone or hormone receptor in the HPGA.

### **Steroid hormones and GnRH agonists**

Steroids related to estrogen and progesterone have been used since the 1960's for contraception of some species. Diethylstilbestrol (DES) is a synthetic estrogen that can reduce fertility in animals, but it must be administered with precise timing, lasts a limited amount of time, accumulates in body tissues, and has potential adverse health effects in treated animals [43]. These characteristics preclude DES and other estrogen-related compounds from intense use in feral cat population control. Progestins, which are synthetic progesterones, induce contraception, but the mechanism of contraception is not well understood. The mechanism most likely involves one or more of the following: negative feedback on GnRH release, disruption of oocyte transport and fertilization, and altered receptivity of the endometrium [44]. However, limited duration of contraception and potential harm to target animals also preclude progestins from use in feral cats. Administration of GnRH agonists disrupt the pulsatile release of GnRH necessary for secretion of pituitary follicle-stimulating (FSH) and lutenizing hormones (LH) [45]. This method disrupts gonadal hormone production to produce contraception, but the short duration of effectiveness makes this method unsuitable for feral cats.

**Cell destruction**

Targeted destruction of specific cells or tissues can be used to disrupt the reproductive process. A GnRH analogue conjugated to a cytotoxin, pokeweed antiviral protein, resulted in lower serum testosterone concentrations in dogs [46]. It is believed that the GnRH analogue portion of the conjugate attaches to GnRH receptors on the gonadotroph cells in the pituitary gland. The molecule is taken into the cells by endocytosis and the cytotoxic portion of the conjugate destroys the cell's ability to synthesize proteins, which leads to cell death. It was hoped that the effect would be permanent, but a portion of the treated dogs demonstrated increasing FSH and LH serum concentrations by week 36. Ovarian follicles in rats have been destroyed by administration of 4-vinylcyclohexene diepoxide (VCD), a metabolite of an industrial chemical [47]. Female mammals are born with their entire lifetime supply of oocytes contained within primordial ovarian follicles. VCD targets and destroys the primordial follicles and the oocytes they contain leaving the animal devoid of oocyte stores and unable to ovulate successfully. This method provides permanent sterility and cessation of estrus cycling. However, VCD has only proven safe and effective in rodents and more research needs to be done in other species. In addition, the current treatment requires daily doses for 15 days, which is not practical for feral cats.

## IMMUNOCONTRACEPTION

The goal of immunocontraception is to induce an immune response against critical elements of the reproductive process that are not used for other functions. There are several targets that meet this criterion and have been investigated for their immunocontraceptive potential, including riboflavin carrier protein (RCP), sperm proteins, zona pellucida (ZP), lutenizing hormone receptor (LH-R), and GnRH.

An acceptable immunocontraceptive agent for feral cats would be effective in both sexes and only a single treatment should be used because retrapping of free-roaming cats for repeated treatments would be impractical. In addition, the vaccine should block the production of sex hormones so as to eliminate nuisance behaviors such as calling, spraying, wandering, and fighting. In addition to these minimum standards, an ideal agent would be permanent, have a quick onset of contraceptive effect, be effective in all ages, be safe to the cat and the environment, be inexpensive, and be easy to administer.

### **Immunocontraception Targets**

#### **Riboflavin Carrier Protein**

RCP is the prime mediator of riboflavin supply to the developing zygote in mammals and is necessary for maintenance of pregnancy. Three monthly treatments with an RCP vaccine followed by repeated treatments every four months elicited high antibody titers and interfered with pregnancy in Bonnet monkeys, but it was not determined if conception or implantation was affected [45]. The need for multiple

treatments and failure to block sex hormone production make this approach impractical for feral cats.

### **Sperm Proteins**

Provoking an immune response against sperm proteins has the potential to be effective in both sexes because antibodies to the proteins could interfere with sperm development and viability in the male or after insemination in the female [43]. Targeting sperm would not interfere with sex hormone production, therefore, immunocontraceptives against sperm proteins are not the best candidate for feral cats.

### **Zona Pellucida**

Zona pellucida (ZP) is a glycoprotein layer surrounding the mammalian egg and is involved in sperm-egg interaction [43]. The use of ZP antigens for immunocontraception has been successful in preventing pregnancy in many species, but not in cats [49,50]. When ZP from pigs, cows, ferrets, dogs and mink was used, the cats developed high antibody titers to the xenogenic proteins, but these antibodies failed to cross-react with the cats' native ZP. Immunization with antigens from feline zona pellucida evoked a poor immune response in the cats, possibly because feline ZP was recognized as a self protein.

### **Hypothalamic-Pituitary-Gonadal Axis**

Seven adult female cats immunized against LH-R and given four booster treatments had absence of behavioral estrus for 500 days, at which point the cats showed signs of recovering normal ovarian function [51]. While immunization against LH-R has the potential to block the production of sex hormones, multiple treatments were used and the goal of a single treatment was not reached. However, it is possible that a variation on

this method that extended the duration of immunity could lead to an effective single-treatment vaccine.

In a previous study, six male cats immunized three to four times with GnRH conjugated to tetanus toxoid produced GnRH antibody titers, but there was no resulting decrease in testosterone concentrations below the contraceptive level [52]. In another study 10 female and four male cats were immunized against GnRH at eight weeks, boosted four weeks and 100 weeks later, and housed with a proven male. GnRH antibodies were detected in all 10 female cats. They had basal progesterone concentrations, did not display estrous behavior and failed to become pregnant for the entire observation period of 20 months. Three of the four males had castrate levels of testosterone for the entire duration of the study. A rise in testosterone in the 4<sup>th</sup> male was associated with a decline in GnRH antibody titer [53].

### **Vaccine Delivery**

Vaccines can be delivered orally, by a biological vector, or by injection. Delivery of the vaccine through bait would be easier than the other methods, but dose is much more difficult to control, and consumption of the vaccine by non-target species would be a risk.

The advantages of a biological vector for immunocontraception include the low expense for vectors that are self-disseminating; possibly low environmental impact compared to poisons; and it is considered humane [54]. Disadvantages include the risk to non-target species and the inherent risk associated with releasing a potential pathogen into the environment. As designed, the vector is not pathogenic, but there is always a chance for reversion to virulence or unexpected effects on non-target species. There is

great concern that other species would be transfected by the vector, so this approach could not be used where other potentially vulnerable species are present. This limitation applies to almost any mainland area. Biological vectors may play a role in immunocontraception of cats on islands where the risk of the vector reaching the mainland is low.

Injection requires the targeting of individual animals, which increases the effort required for control programs. Injectable vaccines are often delivered with a dart rifle for large animals [55], but it requires a skilled marksman and may not be practical or safe for cats due to their small size.

TNR programs typically use live traps to capture cats for transport to veterinary clinics the cats to veterinarians for neutering. The same method of capture would allow delivery of the vaccine by intramuscular or subcutaneous injection in the field without need for transporting the cats to a central facility for surgical sterilization. An implant, which would allow timed-release of a vaccine, could also be delivered in this way.

## GNRH VACCINE

Blocking GnRH from binding to the gonadotrophs disrupts the hormone cascade, leading to cessation of both fertility and sexual behavior. GnRH has the potential to satisfy many of the requirements of an ideal antigen for an immunocontraceptive vaccine.

Since GnRH is a decapeptide, it is a hapten which is a very weak immunogen. The National Wildlife Research Center (NWRC) developed a GnRH vaccine that uses keyhole limpet hemocyanin (KLH) as a protein carrier and AdjuVac™ as an adjuvant. The GnRH peptides are attached to the KLH in such a way as to mimic molecular patterns found on many microorganisms; these patterns activate the innate immune system [56]. AdjuVac™ was developed as an alternative to Freund's complete adjuvant (FCA). FCA is a very effective adjuvant, but it can result in harm to the immunized animal including necrosis and development of granulomas. AdjuVac™ contains inactivated *Mycobacterium avium* in oil.

In a previous pilot study in male cats, GnRH/KLH-AdjuVac™ resulted in testosterone concentrations below the contraceptive level following a single treatment in six of nine male cats for the duration of the 6-month observation period [57]. A single injection also prevented pregnancy in female feral pigs for 36 weeks [58] and was effective in female deer [55].

The hypothesis was that a single-dose GnRH vaccine, (GnRH/KLH-AdjuVac™), would produce long-term immunocontraception in female cats. The specific objectives

were to determine the proportion of cats that respond, the duration of the response, and the safety of the vaccine over a 24 month period.

## MATERIALS AND METHODS

### **Cats**

Twenty-four 8- to 14-month-old specific-pathogen-free female domestic shorthair cats were acquired from a commercial vendor (Liberty Research, Waverly, NY, USA). All 24 cats were group-housed in the Animal Care Services facilities at the University of Florida College of Veterinary Medicine, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Cat housing consisted of one large room with raised resting benches and was climate controlled to maintain ambient temperatures between 21 and 23°C with controlled lighting. Food and water were provided ad libitum. The experimental design was approved by the UF Institutional Animal Care and Use Committee. All cats and their offspring underwent surgical sterilization and were adopted to private homes at the conclusion of the study.

### **Vaccine Construction**

A GnRH vaccine was constructed using a synthetic GnRH peptide with the sequence [pEHWSYGLRPGGGC-SH] produced by the Fmoc/tBU protection method (Global Peptide Services, Fort Collins, CO, USA). Immunogenicity was enhanced by coupling GnRH peptides to a protein carrier, keyhole limpet hemocyanin (KLH; Pierce Endogen, Rockford, IL, USA), in a 1:3 GnRH:KLH mass ratio. The underlined amino acids represent the native GnRH molecule and “pE” signifies pyro-glutamate. Two glycines were added at the C terminus as a spacer and a cysteine was added to ensure consistent

alignment of the peptide to the maleimide-activated KLH. The aqueous-based GnRH-KLH conjugate (200 µg) was combined in a 1:1 ratio by volume with a novel adjuvant, AdjuVac<sup>®</sup>. AdjuVac<sup>®</sup> was produced by diluting a USDA-licensed Johne's disease vaccine containing inactivated *Mycobacterium avium* in mineral oil (Mycopar<sup>®</sup>; Fort Dodge Animal Health, Fort Dodge, IA, USA)

### **Treatment**

Upon arrival, the 24 cats were housed for 30 days in a photoperiod regime (8 hours light:16 hours dark) that is inhibitory of estrus. Fifty days prior to treatment (Day -50), the photoperiod regime was reversed to 16 hours light:8 hours dark, which stimulates estrous cycling within 15 days in 85% of cats [59]. Serum hormones (progesterone and estradiol-17β) were measured on Day -60, then every other day from Day -50 to Day -30. The magnitude, duration and rate of change of estradiol-17β concentrations in each cat from Day -50 to Day -30 were used to confirm normal estrous cycling in cats selected for this study.

Confirmation of normal hormonal responses to the lighting change, including evidence of estrous cycling and docile temperament, were used to select 20 of the 24 cats to continue in the study. The 20 cats were randomized into a sham group (n = 5) and a treatment group (n = 15) based on maximum estradiol-17β concentrations. The sham group received placebo vaccines containing all components except GnRH-KLH. The 15 treated cats received vaccines containing 200 µg GnRH-KLH. Brief anesthesia was induced by administration of isoflurane (IsoFlo<sup>®</sup>; Abbott Laboratories, North Chicago, IL, USA) by face mask. The hair of the right cranial thigh was clipped, and the injection site was cleaned with 70% isopropyl alcohol. The vaccine (0.5 mL) was injected into the

quadriceps muscle group. The right pinna was tattooed with a treatment code, specifically, C1 through C5 for the sham cats and T1 through T15 for the treatment cats.

Potential adverse reactions to treatment were evaluated by daily physical examination, including inspection of the injection site and measurement of body temperature for one week following treatment.

### **Blood Collection**

Blood (4 mL) was collected by jugular venipuncture monthly into serum separator tubes for determination of GnRH antibody titer and concentration of estradiol-17 $\beta$  and progesterone. In addition, blood for estradiol-17 $\beta$  and progesterone concentration determination was collected every other day for 20 days following each of four estrus-inducing photoperiod changes. Serum was separated by centrifugation and stored at  $-20^{\circ}\text{C}$  until analysis.

### **Detection of GnRH Antibodies**

Serum was tested for GnRH antibodies using an enzyme-linked immunoabsorbant assay (ELISA). Bovine serum albumen (BSA) was coupled to GnRH in a 1:1 GnRH:BSA mass ratio and 200 ng of GnRH-BSA buffered with 50  $\mu\text{l}$  bicarbonate was used to coat the wells in each 96-well microtiter plate and incubated overnight at  $4^{\circ}\text{C}$ . GnRH-BSA was used so that only anti-GnRH antibodies would be detected, not antibodies against the KLH component of the vaccine. The wells were washed twice with 200  $\mu\text{l}$  of phosphate buffered saline (PBS) and 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), blocked with salmon serum (SeaBlock, East Coast Bio, Inc., North Berwick, ME, USA) overnight at  $4^{\circ}\text{C}$ . The wells were aspirated and 100  $\mu\text{l}$  of cat serum diluted 1:1,000 in PBS was added in the top row of the plate, with each column

containing a sample from a different cat. Two negative controls were run on each plate; one negative control was buffer without cat serum and the other was pre-vaccination cat serum. The last column was used as a positive control with serum from a cat with a known high antibody titer.

All remaining wells were filled with 50  $\mu$ l PBS and serial dilutions were made from 1:1,000 to 1:128,000 by taking 50  $\mu$ l from each well in row 1 and mixing it into the corresponding well in row two. This process was repeated for the remaining six rows. The plates were then incubated for two hours at room temperature on a shaker. The wells were washed twice with 200  $\mu$ l PBS/0.05% Tween 20.

Antibody to GnRH was detected by adding 50  $\mu$ l goat anti-cat IgG (Sigma Chemical Co.) diluted 1:10,000 in PBS/1% Sea Block to each well. The plate was incubated at room temperature on a shaker for 2 hours. The wells were washed twice with 200  $\mu$ l PBS/0.05% Tween 20. Fifty microliters of rabbit anti-goat coupled to horseradish peroxidase conjugate (Sigma Chemical Co.) diluted 1:3,000 in PBS/1% Sea Block was added to each well and incubated for two hours at room temperature on a shaker. The wells were washed three times with 200  $\mu$ l PBS/0.05% Tween 20. Fifty microliters of Tetramethylbenzidine/phosphate-citrate buffer (Sigma Chemical Co.) was added to each well and the plate was incubated for three to five minutes or until a blue color change developed. Fifty microliters of 2M sulfuric acid was added to each well to stop the reaction and the plates were read on a plate reader (Dynatech, Horsham, PA) at 450 nm. The endpoint dilution was considered positive based on the positive control with the titer equal to the reciprocal of the endpoint dilution.

### **Determination of Serum Estradiol-17 $\beta$ and Progesterone Concentrations**

In cats, behavioral estrus is preceded by a surge in estradiol-17 $\beta$  released by developing follicles. After ovulation progesterone concentrations rise significantly within four days [60]. Serum was tested monthly for estradiol-17 $\beta$  and progesterone concentration. In addition, blood was collected every other day for 20 days following each of four estrus-inducing photoperiod changes. Serum samples were analyzed for total estradiol-17 $\beta$  and progesterone by radio-immunoassay (Coat-A-Count<sup>®</sup>; Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions. The manufacturer reports an estradiol-17 $\beta$  sensitivity of 8 pg/mL with within-run coefficient of variation (CV) of 4–7% and between-run CV of 4–8%, depending on the estradiol-17 $\beta$  concentration. The manufacturer reports a progesterone sensitivity of 0.02 ng/mL with within-run CV of 2–9% and between-run CV of 4–10%, depending on the progesterone concentration.

### **Breeding Trial**

#### **Estrus Induction**

The effects of treatment on normal hormone responses and fertility were evaluated beginning four months after treatment and continuing for the duration of the 26-month study. The photoperiod length was alternated between the estrus-inhibiting regime (16 hours dark:8 hours light) and the estrus-inducing regime (8 hours dark:16 hours light). From Day 0 to Day 120, Day 330 to Day 360 and Day 510 to Day 540, the cats were exposed to the estrus-inhibiting regime (26% of post-treatment time). For the remainder of the study the cats were exposed to estrus-inducing light regime (74% of post-treatment time) (Figure 1). The switch from the short-day photoperiod to the long-day photoperiod

was intended to induce estrus in any of the cats that were capable of an estrous response. A breeding male was housed continuously with the females during all of the long-day photoperiods after Day 120. Three breeding males were alternated to assure that inter-cat incompatibility was not an issue. Time-lapse videography was used to monitor breeding activity during periods of long-day photoperiod. The tapes were reviewed daily and the number of attempted and successful breedings was recorded. Distinctive shaving of the cats' fur was used to allow differentiation of similar-looking cats.

### **Assignment of Cats to Study Group**

During the pre-treatment long-day photoperiod (Day -50 to Day -30), 19 of 24 cats (79%) had a pattern of estradiol-17 $\beta$  concentrations consistent with estrus based on the peak concentrations and the rate of change of the concentrations. These 19 cats and one cat that did not display estrus were divided among the sham and treatment groups in such a way that the two groups did not have significantly different maximum estradiol-17 $\beta$  concentrations ( $P = 0.4$ ).

### **Fertility and Fecundity**

Fertility was defined by the proportion of cats becoming pregnant. Fecundity was defined as the number of live births. Pregnancy was scored as a treatment failure, and the cats were removed from the study after parturition. The treated cats were divided into responders, which failed to become pregnant during the 19 months following treatment, and nonresponders, which became pregnant during the study.

### **Body Phenotype**

It has been well-documented that cats gain weight after surgical neutering, but it is unknown if immunocontraception has the same effect. To evaluate for this effect, body

weight was recorded for each cat on Day 0 and Day 420, and percent weight gain was calculated. Radiographic measurement of the falciform fat pad was performed 14 months (Day 420) after treatment for further evaluation of phenotypic changes. For fat pad measurement, the cats were anesthetized with medetomidine 40 mcg/kg IM and radiographed in right lateral recumbancy. Using computed radiography (Kodak, Rochester, New York, USA), the depth (mm) of the falciform fat pad was measured by dropping a perpendicular line from the center of the body of the 12<sup>th</sup> thoracic vertebra to the ventral body wall and measuring the distance between the caudoventral angle of the liver and the ventral body wall. The area (mm<sup>2</sup>) of the fat pad was defined as the area outlined by the line used for the depth measurement, the ventral border of the liver, the diaphragm, and the ventral body wall (Figure 2).

### **Statistical Analysis**

Descriptive statistics (mean, error, range) were calculated for each group for hormone concentrations, GnRH antibody titers, fecundity and body weight. The fertility of the sham and treatment groups was compared by the two-sided Wilcoxon rank sum test. Fecundity of the sham and nonresponder groups was compared by one-way analysis of variance. Differences in GnRH antibody titer and hormone concentrations between the groups were tested by Kruskal-Wallis one-way analysis of variance on ranks (Kruskal-Wallis ANOVA). Differences between the three groups (responder, nonresponder and sham) with regard to body weight, percent change of body weight, falciform fat pad depth, and falciform fat pad area were analyzed using Kruskal-Wallis ANOVA. The Holm-Sidak method for pairwise comparison was used for all of the Kruskal-Wallis ANOVA tests that indicated a difference between at least two of the groups. Differences

were considered significant when  $P < 0.05$ . All tests were performed using SigmaStat<sup>®</sup> statistics software, version 3.0.1 (SPSS Inc., Chicago, IL, USA).

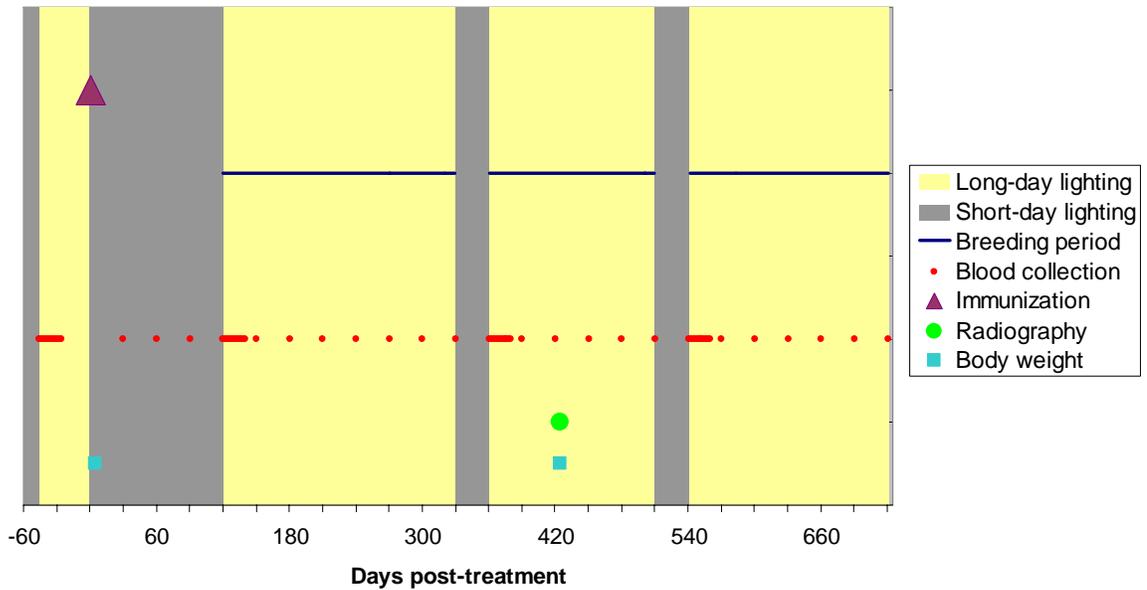


Figure 1. Timeline for 26-month study showing alternating periods of long-day (16 hr light:8 hr dark) and short-day (8 hr light:16 hr dark); periods during which the breeding male had access to the females (Breeding trial); blood collection times; the treatment (Day 0); falciform fat pad radiograph; and body weight times.

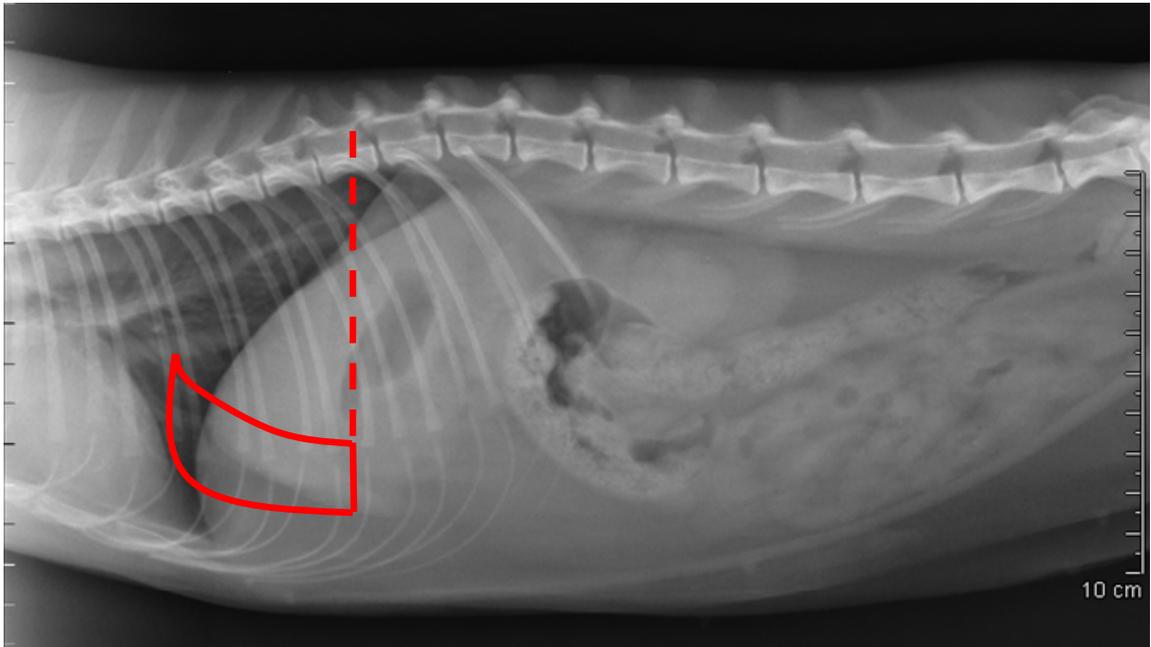


Figure 2. Lateral abdominal radiograph of a cat with falciform fat pad measurements marked. The vertical line extends from the center of the body of T12 to the ventral abdominal wall with the solid portion of the line measuring the fat pad depth. The three solid lines demarcate the area for the falciform fat pad area measurement.

## RESULTS

### **Reactions to Treatment**

Body temperature in all of the cats remained normal after treatment, and there was no inflammation or tenderness at the injection sites. One treatment cat, (T4), died suddenly 45 days after treatment; necropsy revealed no gross or histological abnormalities to explain the death. This cat was replaced with a two-year old cat of proven fertility from the research colony which was tattooed with the code “T16” and vaccinated with a vaccine containing GnRH. This replacement cat was exposed to the same lighting regimen and blood collection schedule as the other 19 cats.

Twenty-four months after immunization a 3 cm x 4 cm mass was discovered by palpation at the injection site. A biopsy was performed and the mass was found to be neither cancerous or infected. It was concluded that the mass was a granuloma that had formed in response to the adjuvant. One cat had multiple 1 cm masses near the injection site and three other cats had a single 1 cm mass near the injection site.

### **Breeding Trial**

#### **Detection of Estrus**

The breeding male cat was introduced on Day 120 at the beginning of the second long-day regime. All sham cats displayed behavioral signs of estrus and were receptive to breeding attempts by the male. Time-lapse videography revealed that the male bred each of the sham cats at least 15 times between Day 122 and Day 155. One cat was observed breeding in two intervals during gestation (days 7-11, 25-26 of gestation). Four treated

cats displayed estrous behavior and began breeding on Day 128 (T9), Day 418 (T8), Day 469 (T15), and Day 504 (T5). These four nonresponder cats were the treated cats with the lowest GnRH antibody titers.

### **Fertility and Fecundity**

Based on an average gestation period of 65 days, the date of parturition was used to estimate the date of successful breeding. All five sham cats became pregnant between five and 26 days after the male cat was introduced (Day 125 to Day 146) and four of them within six days of each cat's first observed breeding. Nonresponder cat T9 was bred in three different estrous intervals from Day 129 to Day 162 before becoming pregnant. In contrast, nonresponder cats T8, T15, and T5 became pregnant in their first breeding cycles (Day 418, Day 469, and Day 504, respectively) (Figure 3). The mean number of live kittens per litter was 3.8 (range 3 to 5) in the sham group, which was significantly higher ( $P = 0.03$ ) than the mean of 2.3 (range 1 to 3) live kittens in the nonresponder group. Based on maintenance of contraception in 11 of 15 treated cats over a 24-month period, the vaccine had a 73% success rate.

### **GnRH Antibody Titer**

The mean GnRH antibody titer for the responder group was significantly higher than the titer of the nonresponder group by Day 150 and remained significantly higher for most of the remainder of the study (Figure 4). All of the sham cats had negative titers throughout the study.

Three patterns of antibody response were observed among the treated cats: high titers, variable titers, and low titers. Five of the 11 responder cats (T1, T7, T10, T14, and T16) had GnRH antibody titers of 128,000 by Day 30 and maintained a titer of 64,000 or

above for the remaining 20 months of the study (Figure 5). The antibody titers of five other responders (T2, T6, T11, T12, and T13) peaked, then decreased and subsequently increased again. None of these variable titers decreased below 16,000, and after a trough level of three to six months, all the titers increased again to at least 64,000 by Day 540 (Figure 6). All four nonresponders became pregnant after their GnRH antibody titer decreased to 8,000 or below (Figure 7).

For the last seven months of the study, 10 of 11 responders had a GnRH titer of 64,000 or above. The responder cat with the lowest GnRH antibody titer, T3, remained at a titer of 16,000 for the last 13 months of the study. Two of the nonresponder cats had an initial high titer that was not sustained and two had titers at 32,000 or below throughout the study. These four cats became pregnant when titers decreased below 16,000, which suggests that a titer of 16,000 is contraceptive in female cats. Poor antibody responses in the nonresponder group delayed, but did not prevent pregnancy.

### **Hormone Concentrations**

#### **Progesterone**

In cats, ovulation accompanied by fertilization results in pregnancy, whereas ovulation without fertilization results in pseudopregnancy. In both cases, serum progesterone increases from a baseline of  $< 0.5$  ng/mL to a peak above 20 ng/mL between 16 and 26 days after ovulation [41]. In pregnancy, progesterone concentrations are higher than in pseudopregnancy and remain above baseline for the entire gestation period. In pseudopregnancy, progesterone concentrations return to baseline by 50 days.

The nine cats (five sham, four nonresponder) that became pregnant had substantial increases in progesterone concentrations for two months prior to parturition. This finding

was expected given the average gestation period of 65 days. Prior to the introduction of the male (Day 120), two of the sham cats, two of the responders, and two of the nonresponders had serum progesterone concentrations higher than 2.0 ng/mL, indicating ovulation. T9, the first nonresponder to become pregnant, had progesterone concentrations indicative of pseudopregnancy on Day -30 and Day 60. A second nonresponder, T5, had elevated progesterone concentration on Day 30. T1 and T2, both responders, had progesterone concentrations of 7 and 20 ng/mL respectively, on Day 30. It is likely that these cats experienced nonfertile ovulation and pseudopregnancy prior to the development of contraceptive titers of GnRH antibody. After Day 30, none of the responder cats had evidence of ovulation.

### **Estradiol-17 $\beta$**

It was expected that the sham group's estradiol-17 $\beta$  concentrations would be consistent with estrous cycling until they became pregnant. Four of the five control cats became pregnant during the first sustained estradiol-17 $\beta$  elevation (the first estrous cycle) following the introduction of the breeding male and one cat, C2, became pregnant during the second estrous cycle.

## **Body Condition**

### **Body Weight**

The mean weights of the sham, nonresponder, and responder cats on Day 0 were not significantly different from each other ( $P = 0.5$ ). Similar to cats undergoing surgical ovariectomy, responding cats had a higher percent gain in body weight than the nonresponder cats ( $P = 0.004$ ) and sham cats ( $P = 0.02$ ) (Table 1). The percent weight gain was not significantly different between the sham and nonresponder groups ( $P =$

0.08). The mean weight on Day 420 of the responder group was significantly greater than the nonresponder group ( $P = 0.002$ ), but not significantly greater than the sham group ( $P = 0.1$ ) (Table 1). The mean weights of the sham and nonresponder groups on Day 420 were not significantly different ( $P = 0.1$ ).

### **Falciform Fat Pad**

At 14 months post-treatment, the mean falciform fat pad depth for the responder group was greater than the mean for the nonresponder group ( $P = 0.046$ ) and sham group ( $P = 0.03$ ) (Table 2). The mean falciform fat pad areas of the three groups were not significantly different from each other ( $P = 0.1$ ) (Table 2).

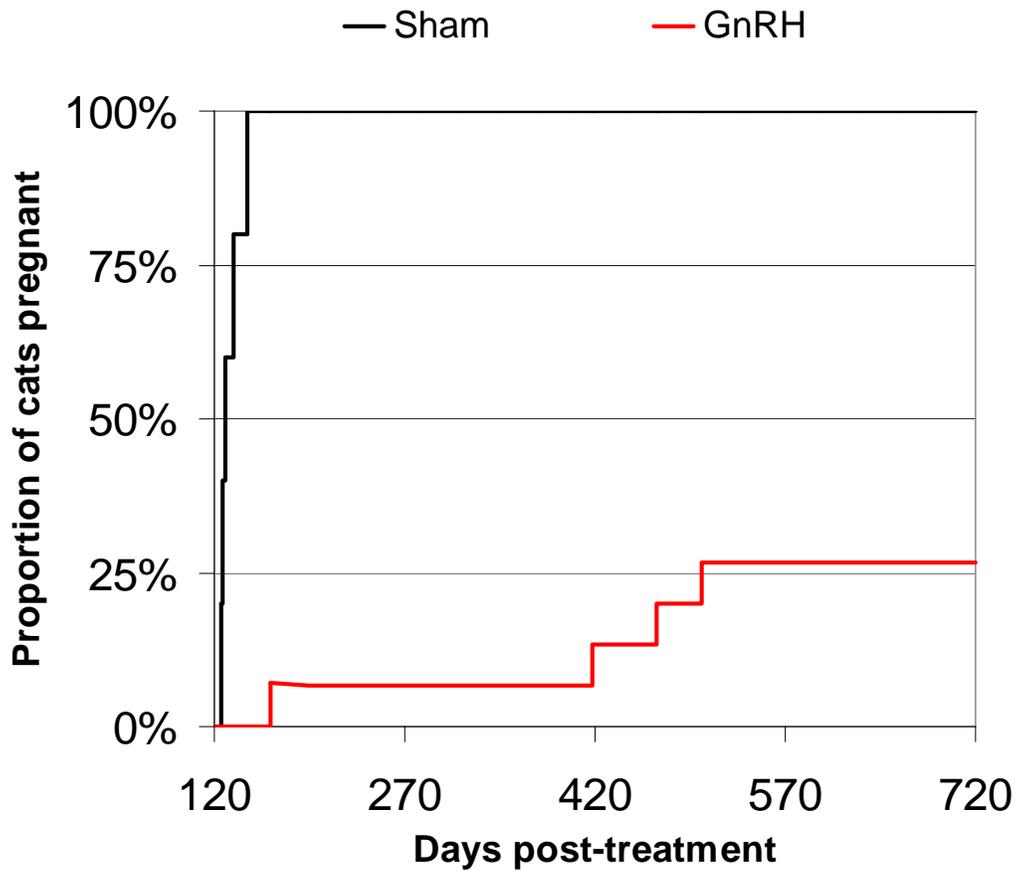


Figure 3. Breeding trial. Change to a long day-length cycle (Day 120) resulted in the rapid induction of behavioral estrus and breeding in all five sham cats, whereas only one of 15 immunized cats was observed to be breeding. All six cats that bred shortly after Day 120 became pregnant. During the same period three additional immunized cats displayed behavioral estrus and became pregnant.

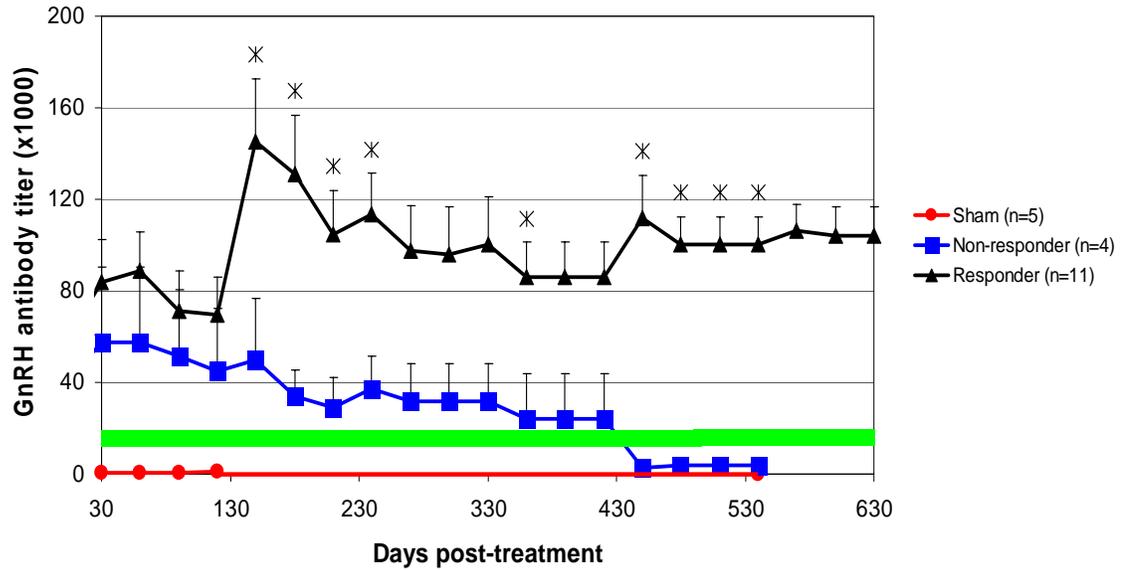


Figure 4. GnRH antibody titer for female cats (mean + SE). The sham group had baseline titer for the duration of the study. For nine of the 18 post-treatment time points the responder cats (cats that did not become pregnant during the study) had significantly higher GnRH antibody titers than the nonresponder cats. (\* $P < 0.05$ .) None of the responder cats had antibody titers that decreased below 16,000 and all four nonresponder cats (cats that became pregnant during the study) decreased below 16,000 and became pregnant within 100 days. The contraceptive titer for these cats (■) was 16,000.

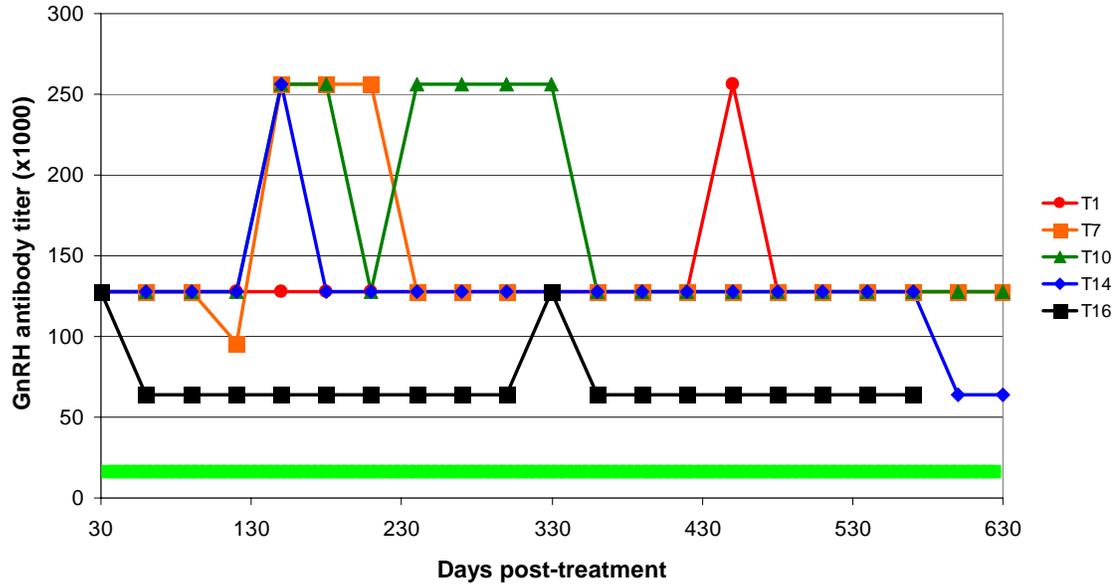


Figure 5. GnRH antibody titers for cats with high titers from Day 30 through Day 630. These five responders (cats that did not become pregnant during the study) (T1, T7, T10, T14, T16) had GnRH antibody titers of 128,000 by Day 30 and none decreased below a titer of 64,000 for the entire 24-month treatment period, which was above the contraceptive titer of 16,000 (—).

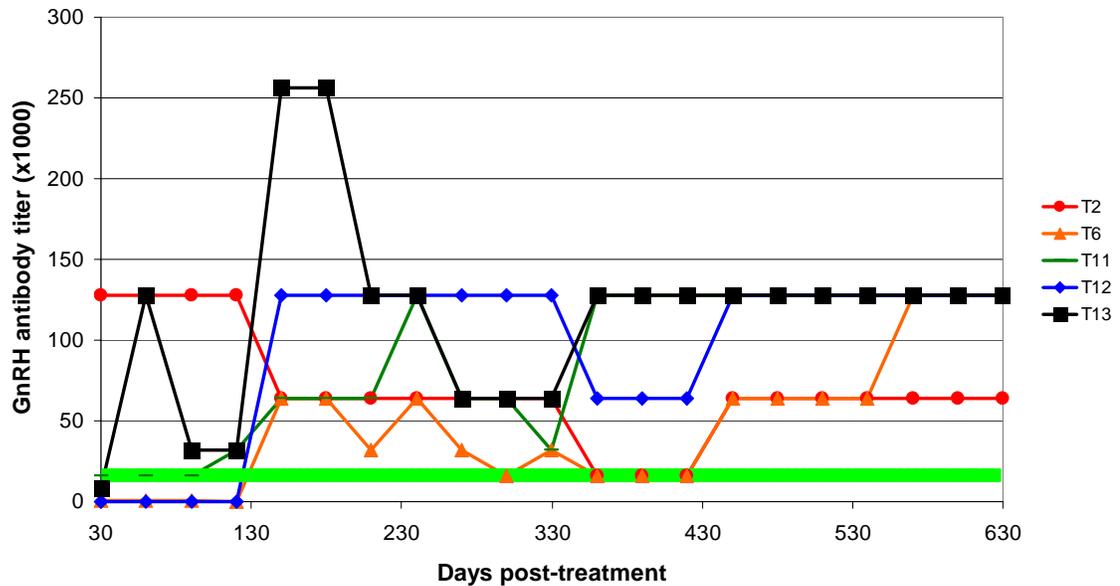


Figure 6. GnRH antibody titers for cats with variable titers. These five responders (cats that did not become pregnant during the study) (T2, T6, T11, T12, T13) had titers that peaked, decreased, and then increased again. After the second increase all five cats maintained this new titer through Day 630. The titers for these cats did not decrease below the contraceptive titer (—) of 16,000.

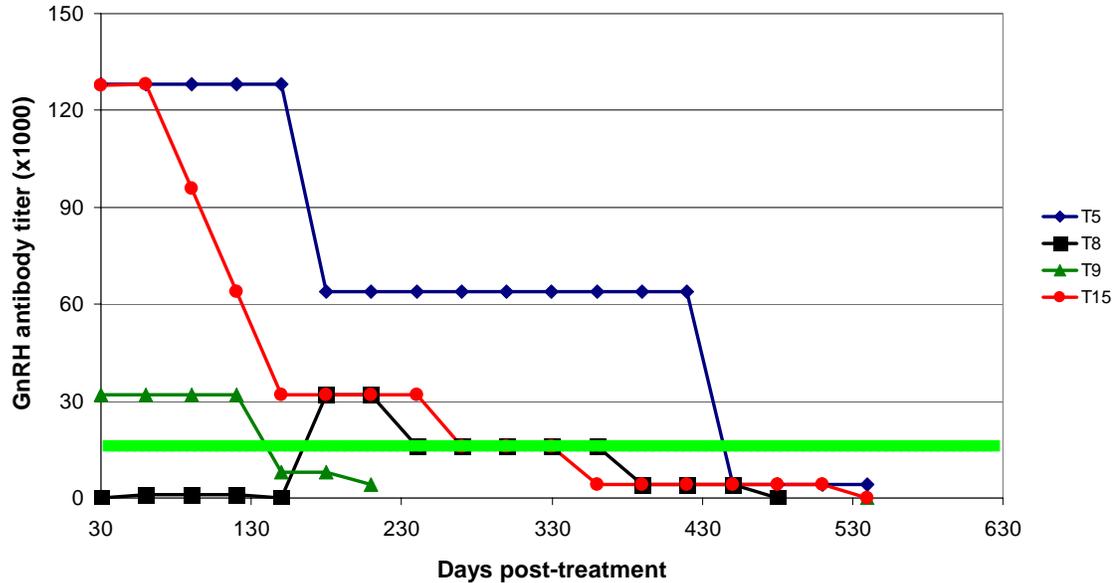


Figure 7. GnRH antibody titers of nonresponders (treated cats that became pregnant during the study). Cats became pregnant on Day 569 (T5), Day 483 (T8), Day 229 (T9), and Day 534 (T15). The titer of these cats decreased below the contraceptive titer (—) of 16,000.

Table 1. Body weight and percent weight gain in cats over a 14-month period following GnRH immunocontraception. The mean weights of the three groups were not significantly different from each other on Day 0. The percent weight gain of the responder group was significantly greater than the nonresponder group and sham group.

Group	Weight (kg), mean (range)		% Weight gain, mean (range)
	Day 0 (Treatment Day)	Day 420	Day 0 to Day 420
Sham	3.54 (3.30 – 4.18)	4.05 (3.48 – 4.66)	15 ( 1 – 41)
Nonresponder	3.21 (2.77 – 3.50)	3.31 (2.62 – 4.00)	3.5 (-14 – 31)
Responder	3.34 (2.64 – 4.20)	4.63 (3.70 – 5.98)	40 (12 – 73)

Table 2. Falciform fat pad depth and area in cats 14 months after GnRH immunization. Fat pad depth of the responder group was significantly greater than the nonresponder group and sham group. The falciform fat pad areas were not significantly different between the groups.

Group	Fat Pad Depth (mm) mean (range)	Fat Pad Area (mm <sup>2</sup> ) mean (range)
Sham	18.6 (13.2 – 23.8)	728 (367 – 1210)
Nonresponder	18.6 (15.2 – 22.4)	839 (543 – 1270)
Responder	27.8 (17.2 – 42.9)	1194 (489 – 2020)

## DISCUSSION

In this study, 15 sexually intact female cats were immunized once against GnRH. Twenty-four months after immunization it was discovered that five of the treated cats had granulomas near their injection site. There was no lameness evident and the masses did not appear to cause the cats any discomfort. It is possible that a lower dose of vaccine would still be effective, but not cause this type of reaction at the injection site.

No systemic or local adverse reactions were noted during the 24-month observation period. For the 24 months following treatment, 73% of the treated cats did not become pregnant or display signs of behavioral estrus. The four nonresponders had GnRH antibody titers at or below 8,000 when they became pregnant, while none of responders had a titer below 16,000 during the study. This suggests that the contraceptive titer in female cats is 16,000.

As expected, the responder cats reacted to the immunocontraceptive treatment in ways similar to cats that have undergone surgical sterilization. Behavioral estrus was suppressed and they gained more weight than the sham and nonresponder cats. However, surgically sterilized female cats have estradiol-17 $\beta$  plasma concentrations consistent with cats in anestrus, whereas the responder cats intermittently demonstrated estradiol-17 $\beta$  concentrations well above expected anestrus values and possibly indicative of estrus cycling. However, the females were never receptive nor attractive to the male, so if the cats were cycling, they were not cycling normally.

Many studies have demonstrated weight gain following surgical sterilization [61-63] and the percent weight gain in these studies (28% to 39%) was consistent with the 40% weight gain found in the responder cats in the present study.

The number of live kittens per litter born to the nonresponder cats was significantly less than the number for sham cats. Immunization against GnRH may not prevent pregnancy in all cats, but it might interfere with some aspect of pregnancy that results in lower fecundity. One possibility is that decreased LH, FSH, and estradiol-17 $\beta$  led to fewer follicles developing and fewer ovulated follicles. However, the sample size was very low and the difference found may have been due to normal variation.

In a study of nine male cats immunized with the same vaccine used in the current study (50 to 200  $\mu$ g), six cats had a GnRH antibody titer above 64,000, undetectable testosterone concentrations and testicular atrophy. The three cats with an antibody titer below 64,000 had measurable testosterone concentrations and normal semen quantity [57].

In a study that targeted a hypothalamic-pituitary-gonadal axis component downstream from GnRH, seven adult female cats were immunized with lutenizing hormone receptor (LH-R) encapsulated in an implant and given four booster injections. LH-R antibodies were detected, progesterone concentrations remained near basal levels, and no behavioral estrus was observed for approximately 500 days, at which point antibody titers began to decrease and the cats showed signs of recovering normal physiologic ovarian function. Estradiol-17 $\beta$  concentrations were not significantly different between the sham cats and treated cats, even though estrus was observed in the sham cats, but not in the treated cats

[51]. Determining hormonal estrous cycling based on estradiol-17 $\beta$  concentrations requires estrogen be measured very frequently over the time frame of interest. In the current study, there were four clusters of 11 every other day blood collection over a 26-month span. The four clusters of blood collections, each 20 days long, were likely to reveal an estrous cycle if one occurred during one of those intervals. However, it is possible that an estrous cycle occurred, but was missed, during the two- to seven-month intervals between these clustered blood collections. In addition, the basal levels and estrus profile of estradiol-17 $\beta$  concentrations are highly variable among cats and other components such as plasma protein content and steroid hormone binding protein may interfere with the measurement [64].

Increasing the frequency of estrogen measurement would aid in more precise determination of estrous cycling regardless of basal and maximum concentrations. However, continuous collection of blood samples would create welfare and health issues for cats and monitoring of fecal estrogen would necessitate individual housing. To increase the accuracy of the estradiol-17 $\beta$  assay, a portion of each sample can be used to prepare an estradiol-17 $\beta$ -free aliquot. The steroid is removed by passing the aliquot through a charcoal column. The estradiol-17 $\beta$  concentration in each sample is determined by subtracting the value in the aliquot (sample blank) from the estradiol-17 $\beta$  concentration value in the unfiltered portion [64]

Immunization against GnRH in other species has been used as an alternative to gonadectomy to prevent undesirable behavior or characteristics caused by sex hormones for more than 25 years [65]. It has been primarily used in juvenile male food animals and has resulted in decreased aggression, testosterone concentration, and testes size in bulls

[66-70], boars [58,71-73] and rams [74,75]. In female pigs [58,76], sheep [77] and horses [78] estrus was suppressed; concentrations of lutenizing hormone, progesterone, and inhibin A were decreased; and ovarian and uterine weights were reduced. Most of these animals received multiple treatments, and the observation periods were short since most of the animals were sent to slaughter.

GnRH immunization has been proposed as a humane solution to overpopulation of wildlife species and has been tested in deer, bison, rats, swine, rabbits, squirrels, coyotes and horses [79]. In white-tailed deer, three or four treatments of GnRH vaccine resulted in a fawning rate reduction up to 88% [55]. GnRH vaccination was 100% effective with three treatments in male and female Norway rats for up to 17 months [55]. A single treatment in male and female feral swine resulted in reduced testicular and ovarian sizes, reduced concentrations of testosterone and progesterone, and a 90% reduction in pregnancy for 36 weeks [58]. A single treatment in bison led to a decrease in progesterone concentrations and prevented pregnancies for one year [80].

There is a protein very similar to GnRH, named GnRH-II, whose function has yet to be elucidated. However, there is concern that a vaccine against GnRH could also bind to GnRH-II and disrupt a non-reproduction function [81]. The safety of the GnRH vaccine has been widely investigated, though, and no significant safety concerns have been found. A study in male rats and rabbits found no differences in hematological or biochemical findings between GnRH-immunized animals and surgically sterilized animals [82]. At necropsy the only abnormalities were detected in the reproductive organs. Studies in many species including horses [78], deer [83], and male cats [57], found no health concerns associated with GnRH immunocontraception.

Contraceptive GnRH antibody titer and effective dose may be different for male and female animals. The GnRH antibody titer needed to induce contraception in the current study in female cats (16,000) was lower than the antibody titer needed for male cats (64,000) treated with the same vaccine [57]. A similar sex difference has been noted in horses with a contraceptive titer in males of 1,000 and in females of 300 [78]. The effective dose of GnRH vaccine was found to be lower in male than female swine [58]. Sex differences may be related to the cyclic nature of hormone secretion in females that is largely absent in males [58]. Since males continuously produce GnRH, it is possible that the store of anti-GnRH antibodies is depleted more quickly at the sites of interaction or that GnRH must be suppressed to a greater degree to block reproductive functions in males than in females.

GnRH has been shown previously to be an effective immunocontraceptive target in many species, including cats, but in reports the contraceptive activity was not effective in all of the animals, required multiple treatments, and faded over time. In contrast, a practical contraceptive for feral cats must be effective in a large fraction of cats for a substantial duration following a single treatment.

In this report, a single treatment of GnRH conjugated with KLH and adjuvanted with *M. avium* and oil achieved long-term contraception in female cats, meeting the minimum requirements for contraception in feral cats. Targeting of GnRH had the additional benefit of curbing nuisance behavior associated with estrous cycling. Additional studies are needed to investigate the full duration of immunity, rate of efficacy, and safety in both sexes and all ages of cats.

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## BIOGRAPHICAL SKETCH

John Friary was born July 24, 1970, in Laconia, New Hampshire. He attended secondary school in St. Petersburg, Florida, studied for one year at the Georgia Institute of Technology in Atlanta and then moved to Gainesville, Florida. John graduated from the University of Florida with a B.S. in environmental engineering in 2004. John became interested in research to benefit feral cats after volunteering at Operation Catnip, a trap-neuter-return clinic in Gainesville, Florida.

He entered a graduate program at the College of Veterinary Medical at the University of Florida to investigate the potential for immunocontraception to reduce the population of feral cats under the advisement of Dr. Julie Levy. He received his M.S. in 2006.

John will continue research related to the humane control of feral cats.