Phage-GnRH Constructs for Population Control of Feral Animals: Evaluation in Cats

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 15, 2018

Keywords: contraception, feral cats, filamentous phage, gonadotropin-releasing hormone,
overpopulation

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Abstract

      The overpopulation of cats is a problem in the United States and worldwide due to public health

concerns and their role as a predator of wildlife species. The focus of this research is the development of

anti-fertility vaccines composed of whole phage particles carrying peptides with contraceptive

properties for use in feral animals. The vaccines are designed to trigger antibody production against

gonadotropin releasing hormone (GnRH). The antibodies inactivate GnRH, causing reduced release of

gonadotropic hormones and gonadal atrophy. Phage-GnRH constructs with potential contraceptive

properties were generated via selection from a phage display library using cat and dog GnRH antibodies

as selection targets, allowing identification of phages displaying GnRH-like peptides. When tested in

mice, these constructs invoked the production of antibodies against GnRH and suppressed serum

testosterone. The goal of this study was to evaluate the potential of these vaccines in cats. Sexually

mature male cats were characterized as to their reproductive parameters and injected with a phage-

GnRH vaccine according to the following treatment groups: single phage-GnRH vaccine with adjuvant

(group 1, n=5), a phage-GnRH vaccine without adjuvant and a booster one month later (group 2, n=5), or

a phage-GnRH vaccine with adjuvant and two boosters three months apart (group 3, n=5). Anti-GnRH

antibodies and testosterone in serum, testicular volume by ultrasound, and quality and quantity of

sperm were evaluated monthly for 7-9 months following immunization. All cats developed anti-GnRH

antibodies of varying levels following immunization and all but two cats still had detectable antibody

levels at the conclusion on the study. Serum antibody levels increased after booster immunization in

groups 2 and 3 and the highest antibody levels were reached by the cats in group 3 that received three

total vaccinations. When the cats were evaluated as treatment groups as a whole, mean serum testosterone was not significantly reduced except at eight months after primary immunization for four cats in Group 3. The total testicular volume (TTV) decreased in four cats in Group 1 by a range of 24-42% and all five cats in Group 3 by a range of 15-36%, indicating potential gonadal atrophy in these treatment groups. All cats still produced sperm at the conclusion of the study, but the majority of the immunized cats had a decrease in the percentage of morphologically normal sperm cells compared to pre-immunization and this change was significant at multiple post-immunization time points for each group. On histological examination, treatment resulted in vacuolation of the corpus of the epididymis. One of the adjuvants used in this study caused unacceptable injection-site reactions. This study demonstrated the potential of phage-GnRH vaccines for immunocontraception of cats, but further refinement, including optimization of phage dose, adjuvant used, age of immunization, and immunization regimen, must be performed to see if sterility can be induced.

Acknowledgments

First and foremost, I would like to thank Dr. Tatiana Samoylova for accepting me into her laboratory, entrusting me with this project, and providing guidance along the way. I would like to thank my committee member, Dr. Douglas Martin, for sharing his advice and knowledge. I would like to express my utmost gratitude to my final committee member, Dr. Aime Johnson, not only for sharing her knowledge and providing support, but also for her assistance with all the animal-related portions of the project. Additionally, Jessica Cannon and Melissa Korbely for assisting with sample collections, and they and the rest of the cat colony staff who helped to take great care of the cats involved in this project. Thank you Ann Cochran and Alex Samoylov for passing on your knowledge and assisting with laboratory procedures to help this project run smoothly. I would also like to thank Cynthia Hutchinson, Dr. Russell Cattley, and Dr. Catherine Picut, for their help with testes histology. Thanks to Dr. James Wright for assistance with statistics.

I would also like to thank my family for their support, patience, and understanding.

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List of Abbreviations

ANOVA Analysis of variance

AV   Artificial vagina

BSA   Bovine serum albumin

CDC   Center for Disease Control

CSD   Cat scratch disease, cat scratch fever

ECH   Epididymal cribiform hyperplasia

EEJ   Electroejaculation

ELISA   Enzyme-linked immunosorbent assay

FCA   Freund's Complete Adjuvant

FDA   Food & Drug Administration

FEDIAF European Pet Food Industry Federation

FeLV   Feline leukemia virus

FIV   Feline immunodeficiency virus

FSH   Follicle-stimulating hormone

GDP   Guanosine diphosphate

GLM   General linear model

GnRH   Gonadotropin-releasing hormone

GnRHR Gonadotropin-releasing hormone receptor

GTP   Guanosine triphosphate

hCG   Human chorionic gonadotropin

HPG   Hypothalamic-pituitary gonadal

HSUS   Humane Society of the United States

H&E   Hematoxylin and eosin (stain)

FSH   Follicle-stimulating hormone

FSHR   Follicle-stimulating hormone receptor

hCG   Human chorionic gonadotropin

IACUC Institutional animal care and use committee

K91BK Kanamycin resistant E. coli K91BluKan

KLH   Keyhole limpet hemocyanin

LH   Luteinizing hormone

LHR   Luteinizing hormone receptor

MA   Megestrol acetate

MGA   Megestrol acetate

MPA   Medroxyprogesterone acetate

NAP   Nucleic acid preservation buffer

NWRC   National Wildlife Research Center

NZY   Medium – casein hydrolysate enzymatic from bovine milk

OD   Optical density

OHE   Ovariohysterectomy

OPD   *o*-phenylene diamine

PAS   Periodic Acid Schiff (stain)

PBS   Phosphate-buffered saline

PEG   Polyethylene glycol

PLC   Phospholipase C

PLGA   Poly(lactic-co-glycolic acid)

pZP   Porcine zona pellucida

TBS   Tris-buffered saline

TNR   Trap-neuter-release or trap-neuter-return

TSH   Thyroid-stimulating hormone

ZP   Zona pellucida

CHAPTER 1

LITERATURE REVIEW

1.1   Cat Overpopulation and Its Implications

1.1.1   Overpopulation of Feral Cats and Their Reproductive Capacity

The overpopulation of cats is a concerning problem worldwide. In 2011, the number of pet cats

in the United States was estimated to be around 80 million [1]. A 2008 national survey suggested a

population of around 8.5 million pet cats in Canada [1]. A FEDIAF (European Pet Food Industry

Federation) Facts & Figures report from 2010 reported an estimated population of 8 million pet cats in

the United Kingdom, nearly 11 million cats in France, 8 million cats in Germany, 18 million cats in Russia,

and 84.7 million cats in all of Europe [1]. Their 2016 report reflects growth with an estimated European

population of 102.7 million cats. Rivaling the number of pet cats are the numbers of unowned, free-

roaming cats. The sometimes elusive behavior of these animals, as well as the fact that they are not

consistently accounted for by humans, makes the numbers of free-roaming cats hard to estimate. In the

United States, the numbers are typically estimated to be somewhere between 50 and 90 million cats [1,

2]. Figure 1.1 shows some feral cats in a colony in Auburn, Alabama.

The free-roaming, or "feral", cat population is made up of different classes of cats, with different

names and different definitions. It consists of animals with varying degrees of interaction with and

socialization with humans, from owned cats who spend their time outside, to unsocialized cats fed by

humans, to cats who have no direct contact with humans at all and hunt for their own food. Other

terms used sometimes interchangeably, sometimes differentially based upon cat-human contact, are

"stray", "abandoned", "community" cat, and "street" or "alley" cat [3, 4]. A working definition of the

term feral cat set forth by Gosling *et al.* in 2013 is "a cat that is unapproachable in its free-roaming

environment and is capable of surviving with or without direct human intervention, and may

additionally show fearful or defensive behavior on human contact" [3]. The purpose of all these terms

are to differentiate these animals from pet cats who have an owner who assumes responsibility for

them.

It is suggested that the huge numbers of feral cats come from the reproduction of existing feral

cats, the reproduction of unsterilized owned but lost or abandoned cats, and owners allowing their

unsterilized cats to breed freely or reproduce once prior to sterilization [4]. Cats are prolific breeders.

They are seasonally polyestrous but have been found to reproduce outside of the typical spring and

summer breeding period when environmental conditions are favorable. Typical litter size is between 2-5

kittens per litter but can vary higher or lower, and a single female cat may produce 1-3 litters per year

[5]. Cats often reach sexual maturity as early as 5-6 months of age and females often produce their first

litter before reaching one year of age [4, 5]. Left unchecked, reproduction occurs rapidly and the

population of free-roaming cats grows.

1.1.2   Implications of Cat Overpopulation

1.1.2.1 Public Health, Spread of Zoonotic Diseases

Cats are able to transmit zoonotic infectious diseases and parasites. The most concerning is

rabies. The rabies virus infects the central nervous disease and is ultimately fatal. Transmission usually

occurs through the bite and saliva of an infected animal [6]. Without human caretakers, feral cats are

unlikely to be regularly vaccinated against rabies. Although rabies is now mostly circulated amongst

wildlife species in the United States, 303 cases of rabies in cats were reported in 2010 [7]. In other parts of the world without strict vaccination laws, unvaccinated dogs are the most common vector for rabies [4].

Cats are the definitive host of *Toxoplasma gondii*, the organism causing the disease toxoplasmosis. The parasite produces oocysts in an infected feline, which are then excreted in the feces and transmit infection when ingested orally. Oocysts are environmentally resistant [8]. They can survive for years in the soil and be spread in the environment by many organisms [9]. This disease is often asympotomatic in humans unless they are immunocompromised, but infection during pregnancy can lead to the disease being congenitally acquired. This disease can cause severe birth defects such as blindness and mental impairments. Toxoplasmosis acquired in immunosuppressed humans may result in severe encephalitis and lymphadenopathy [9]. *T. gondii* has also been shown to cause death amongst the endangered southern sea otter [10].

Several concerning zoonoses of cats are associated with the flea. Cats may serve as hosts for several different species of the common ectoparasite, but the most common appears to be the cat flea, *Ctenocephalides felis*. The cat flea is the primary arthropod vector for the bacterium *Bartonella henselae* [11]. *B. henselae* is the cause of the disease known as cat scratch fever or cat scratch disease (CSD). Fleas acquire the infection while feeding on the blood of an infected animal, and then shed it in their feces. Cats can transmit this disease to humans through contamination of an open wound, such as a direct bite or scratch [4, 11]. The cat flea is also the primary vector and reservoir for *Rickettsia felis*, the pathogen that causes cat-flea typhus in humans [11, 12]. Cats are also susceptible to the plague (caused by *Yersinia pestis*) and can transmit infection directly to humans or by transporting infected fleas. Fleas may become infected by feeding on an infected host, and then transmit the infection when they feed on another host. Plague is a life-threatening illness. Most commonly, it results from cutaneous exposure,

and without antibiotic treatment may spread to the lungs to develop into pneumonic plague. Humans can also become exposed through respiratory exposure to develop pneumonic plague. Without prompt treatment, pneumonic plague is usually fatal within 3-4 days [11].

Humans are not the only species that feral cats may transmit diseases and parasites to. Feral cats without veterinary care may come into contact with pet cats, particularly those allowed to roam outdoors. The viruses feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) are not transmissible to humans, but may be spread from feral cats to pet cats, and can have serious adverse health effects [4]. Fleas are also capable of transmitting animal specific pathogens between cats [11]. Cats are also an intermediate host of *Sarcocystis neurona*, a protozoan that causes the serious neurological disease, equine protozoal myeloencephalitis, in horses [4]. *Toxoplasmosis gondii* has also been shown to cause death amongst the endangered southern sea otter [10].

1.1.2.2 Impact on Wildlife

Free-ranging cats, including both feral cats and owned cats who roam outdoors, can have a substantial impact on wildlife populations. Cats are effective hunters who may prey upon many species of birds, mammals, reptiles, amphibians, and fish. Despite the thought that providing food for cats will lead to them not having to catch wildlife to eat, even well-fed cats will hunt and kill prey [13]. It is estimated that free-ranging domestic cats kill 1.3-4 billion birds and 6.3-22.3 billion mammals annually in the United States [14]. Many more wounded animals are turned over to wildlife rehabilitators. A wildlife rehabilitation facility in California, The Lindsay Museum of Walnut Creek, reported that 24% of birds, 12% of mammals, and 15% of the reptiles they received between January 1 and September 14, 2003, were admitted because of cat-related injuries [15]. While predation of native small mammals is a concern due to loss of biodiversity and decreased prey availability for native mesopredators, the hunting

of nonnative small animals such as pest rodents is generally considered a desired behavior [16].

Nonnative pest rodent species in the United States includes the Norway rat (*Rattus norvegicus*), black

rat (*Rattus rattus*), and the house mouse (*Mus musculus*), and they are undesirable due to the damange

they cause to crops and buildings and their ability to carry harmful diseases [16]. Many people,

particularly in rural and agricultural settings, keep free-roaming cats for the purpose of hunting rats and

mice. A survey conducted in Michigan reported that free-ranging cats killed from 0.7-1.4 birds per week,

representing 23 species and two species of conservation concern [15]. It has been suggested that cats

have been involved in the extinction of more bird species than any cause besides habitat destruction

[17] and indeed domestic cats have been implicated in the extinction of at least 33 species of birds [16].

Many bird species are restricted to certain ranges or habitats, and birds in these populations are

particularly vulnerable to predation. The effect of predation of bird species on islands can severely effect

bird populations in these fragmented habitats that are especially susceptible to ecological disturbances

[16]. In Florida, domestic cats have been recognized as predators of several federal listed species,

including the Key Largo cotton mouse, rice rat, Key Largo woodrat, Lower Keys marsh rabbit,

Choctawhatchee beach mouse, Perdido Key beach mouse, Anastasia Island beach mouse, Southeastern

beach mouse, green sea turtle, roseate tern, least tern and the Florida scrub-jay [18]. Study conducted

by researchers from Auburn University has confirmed that domestic cats prey upon beach mice, which

are found only in the southeastern United States and are important in the dune ecosystem [18].

1.1.2.3   Cat Welfare

Another ethical concern with feral cat overpopulation is the welfare of the cats themselves.

Although cat welfare can vary by situation, there is concern that the cats live short lives punctuated by

injury, illness, and harsh conditions that often lead to their mortality. The Humane Society of the United

States (HSUS) states that free-roaming cats are often hit by cars or have death caused by disease, poison, starvation, or attacks by other animals [18]. Also according to HSUS, free-roaming cats have an average lifespan of three years, compared to 12-18 years for indoor cats [19]. In one study following the survival of kittens born in a monitored cat colony, 75% of 169 kittens died or disappeared before they reached six months of age and median survival time was only 113 days, Reported causes of deaths included motor vehicle accidents, dog attacks, and other types of trauma [5].

1.2   Current Methods of Feral Cat Population Control

1.2.1   Relocation: Shelters and Sanctuaries

One method of attempting to control the free-roaming cat population is the temporary housing of cats in shelters or permanent relocation to sanctuaries. The cats may be trapped and housed in an animal shelter, where it typically stays for an obligatory "stray hold", depending on state and local laws and ordinances. After that period of time is up, the animals may be put up for adoption. Unfortunately, feral cats who have had little or no contact with humans may be unsocialized and unsuitable for adoption. Kittens under four months are the best candidates for adoption, and young cats may still be able to be socialized, but many adults are not able to adjust to living with humans. Depending on the shelter's policies, cats that are not adopted or can't be adopted are typically euthanized. Animal shelters across the Unites States receive several million unwanted cats each year and it is estimated that approximately 75% of these cats are euthanized [13]. In rare cases, they may be transferred to a cat sanctuary.

Sanctuaries serve as permanent homes for cats who cannot be pets. They are, however, few and far between, and have limited space and funds. They are typically run as nonprofit organizations and run

on donations. They can only house as many cats as they can provide resources and space for, and they cannot alone tackle the problem of cat overpopulation.

An important concern with any management strategy that removes the cats from the environment is that other cats will simply move in and fill that niche. With a nearly unlimited population of cats, where there are resources, another cat will be able to move in and take the place of the cat that was removed.

1.2.2   Trap-Neuter-Release Program

Trap-neuter-release, also known as trap-neuter-return (TNR), is a popular method of population control. TNR involves the capture and surgical gonadectomy of free-roaming cats. It also usually involves some form of permanent identification indicating that the animal has been sterilized, such as ear tip removal, and vaccination for at least rabies. Sometimes additional vaccinations or anti-parasite medications are administered. The goal of TNR programs is to ultimately decrease or at least stabilize the population of free-roaming cats humanely using sterilization. Importantly, the cat holds its place in the colony, but cannot contribute to population growth. This prevents other, potentially intact cats from taking that cat's place.

Studies have shown that well-implemented TNR programs can be successful. A study evaluating TNR conducted in Florida found it be more efficient and cost-effective than extermination, and resulted in fewer admissions to the local animal shelter [20]. Implementation of a TNR program on a university campus also in Florida over an 11-year period resulted in a significant reduction in cat numbers and no new kittens were observed five years after the program began [21]. One study took place on a feral cat colony at the Zoological Park in the city of Rio de Janeiro. Cats were captured biannually and

hysterectomy was performed on all captured female cats over six months of age. This program resulted in restriction of growth and reduced the number of kittens born into the colony [22]

Unfortunately, not every TNR program is successful. It seems that when there is significant immigration into a managed colony, the efforts of some TNR programs are not sufficient to decrease the population size. A study of managed colonies in Rome, Italy, where there is a no-kill policy for feral cats, reported that the spay/neuter campaigns brought about a general decrease in numbers of feral cats, but the high percentage of immigration offset the success of the TNR program [23]. A study in Miami-Dade County, Florida, reported that the number of original colony members decreased over time but that immigration by illegal dumping of unwanted cats and attraction of stray cats negated population reduction [24]. These studies highlight that the cat overpopulation issue cannot be effectively managed without the cooperation and education of the public and pet owners.

The practice of TNR is controversial and hotly debated. For one, some consider the return of the cats to the colony to be abandonment [15]. Some municipalities actually have laws preventing abandonment of animals that make the implementation of a TNR program illegal. There is also the concern that even neutered (and sometimes vaccinated) cats may be detrimental to public health. It is also unpopular among wildlife advocates who are concerned about the prey items of the cats.

TNR programs have limitations. The process of trapping cats, transporting them to a site where sterilization can be performed, the sterilization procedure itself, and the transport back to the original site of capture are resource-intensive in cost and labor. A licensed veterinarian, facility for aseptic surgery, and drugs to induce/maintain/reverse anesthesia must all be available [2]. A TNR program in Florida, Operation CatNip, is estimated to cost US $17 per cat but relies extensively on volunteers [4]. In addition to the logistical challenges of implementing TNR, some cats will likely evade capture and continue to reproduce [25].

The practice of TNR is considered by many to be a safe and humane compromise between dispatching feral cats and letting them continue to reproduce and overpopulate. There is evidence that under the right conditions, TNR can effectively control feral cat populations, and is a humane alternative to methods such as extermination. Funding, availability of motivated volunteers, and education of the public are vital to the success of a TNR program.

1.2.3   Surgical Sterilization

The surgical removal of the reproductive organs is the most commonly used sterilization method in companion animals. When surgical sterilization is performed, the animal's gonads are completely removed. In females, the procedure is commonly called a "spay" and is most commonly the removal of the ovaries and the uterus, or an ovariohysterectomy (OHE). Females may also undergo an ovariectomy, where the ovaries alone are removed, or a hysterectomy or ovary-sparing-spay, where only the uterus is removed. The typical male castration procedure, or the "neuter," is the removal of the testicles. Males may also undergo a vasectomy, which is a procedure that sterilizes them while leaving the testicles intact. The ducts that transport sperm, the vas deferens, are clamped, cut, or ligated so that the sperm produced cannot be ejaculated. The removal of the gonads leads to complete, permanent sterilization of the animal. Furthermore, it also stops the production of sex steroid hormones which can cause undesirable sex-related behaviors such as marking. The ovary-sparing-spay or vasectomy methods of surgical sterilization leave sex hormones intact. Surgical gonadectomy is the "gold standard" of animal sterilization, and currently non-surgical methods have drawbacks that make them less preferable for various reasons.

In cats, spaying and castration are typically recommended early in life. Female cats that undergo ovariohysterectomy experience decreased risk for mammary neoplasia, with female cats left intact

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reported to have seven times the risk of the development of mammary tumors as spayed queens [26]. The reported drawbacks of the procedure include obesity, surgical complications, and possible increased incidence of diabetes mellitus [26, 27]. The primary benefits of castration for male cats are those associated with reducing reproductive behaviors, such marking, aggression, and wandering. The reported drawbacks are the same as for female cats [26, 27]. In dogs, more drawbacks to surgical sterilization have been reported: increased incidence of some cancers, including transitional cell carcinoma, osteosarcoma, hemangiosarcoma, lymphosarcoma, and mast cell tumors; increased incidence of joint/orthopedic problems, including cranial cruciate ligament injury and hip dysplasia; obesity, which may itself be associated with some of the other reported detriments; and increased incidence of urinary incontinence in female dogs [26, 27]. In female dogs, the benefits to gonadectomy include decreased incidence of mammary neoplasia, and it eliminates the risk of ovarian tumors, ovarian cysts, and pyometra (uterine infection, a serious and life-threatening condition). In male dogs, the primary benefits to surgical castration are eliminated incidence of testicular neoplasia and testosterone-dependent disease including benign prostatic hypertrophy [26, 27]. While it is reported that castration may decrease the nuisance behaviors associated with reproduction, training of the dogs is also effective for this purpose and may be required to address these behaviors even in the case of castration.

1.2.4   Non-Surgical Sterilization

There are several characteristics that might make up the ideal method of non-surgical fertility control (Figure 1.2). First and foremost, the product must be safe for the animals and safe for humans who will administer the product to handle. A product must also be long-lasting or permanent after a single treatment as re-capture of a feral cat is difficult and not ideal. Ideally, the product would be effective in both males and females to have the greatest impact on controlling the population. The

product would need to be more cost-effective than surgical sterilization to be able to compete. Table 1.1 briefly describes current non-surgical methods for feline contraception.

Progestin contraceptives are options for short term contraception for dogs and cats. While these produces have some effect in males, they are typically used to suppress estrus in female animals. Different variations include injections that may last several months or oral medications that need to be regularly dosed. Synthetic progestins used for this purpose include megestrol acetate (MA or MGA), medroxyprogesterone acetate (MPA), and proligestone. Effectiveness and duration varies by brand and formulation. These products can be accessed in the U.S. but are not typically recommended due to adverse side effects, including increased prevalence of mammary neoplasia and uterine disease leading to life-threatening pyometra. Risk of side effects increases with longer use of the synthetic sex hormones [27].

The product Zeuterin™ (marketed as EsterilSol™ in some other countries), is composed of zinc gluconate and arginine and sterilizes males by intratesticular injection. It is the only non-surgical sterilant with U.S. Food & Drug Administration (FDA) approval. However, it is only approved for use in male dogs. It also has regulatory approval for use in Mexico, Colombia, Panama, and Turkey, and is approved in Colombia for male cats. It was launched in 2014, but is no longer being produced or distributed in any country due to financial challenges of the manufacturer [27]. Calcium chloride is also being researched as an intratesticular injectable sterilant for male companion animals. It currently is not approved by any regulatory agency and is only created through compounding ingredients. The use of this product is still considered experimental at this point [27].

The product Suprelorin® (deslorelin acetate) is a gonadotropin-releasing hormone (GnRH) agonist. GnRH is described in detail in Section 1.3. It has regulatory approval in the European Union, Australia, and New Zealand for male dogs. It is a subcutaneous implant designed to provide temporary,

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reversible contraception. It releases a slow and continuous dose of a GnRH agonist that suppresses the reproductive endocrine axis. It is available as a 4.7mg implant with a minimum duration of six months or a 9.4mg implant with a minimum duration of 12 months. Although it is not marketed for cats, studies have indicated that it is also effective in male and female cats, but with variable onset and duration of efficacy [27].

One method of immunocontraception has been the creation of vaccines targeting the zona pellucida (ZP). This is the extracellular matrix that surrounds the egg in mammals and serves as a protective coating as well as plays an important role in the binding of spermatozoa to the oocyte [28]. Although there is species specificity, the glycoproteins making up the zona pellucida have some homology across species, indicating potential for adequate cross-reactivity across species to be useful for immunocontraception [28, 29]. Native porcine ZP (pZP) isolated from pig ovaries has been used successfully for contraception in several species, including horses, deer, and rabbits [2]. A weakness of this approach for contraception of feral cats (besides only being effective in females) is that it does not suppress estrous cyclicity. In a study testing a commercial vaccine based on a porcine zona pellucida glycoprotein, SpayVac, female cats were not rendered infertile and immunohistochemistry revealed that serum antibodies produced by the vaccinated cats reacted only to pig oocytes and not cat oocytes [30]. In another study, vaccines were constructed using native soluble-isolated ZP from cows, cats, ferrets, dogs, and mink. Although all cats produced antibodies, the antibodies had low reactivity for feline zona pellucida and immunohistochemistry did not detect any reactivity against feline ovaries. During a breeding trial, all cats became pregnant [31]. No study has clearly demonstrated efficacy of a ZP-based immunocontraceptive vaccine in cats as of yet.

The other popular target for immunocontraception is GnRH. Commercial GnRH-vaccines have been developed and shown to have efficacy in various species besides felines, which has been reviewed elsewhere [28, 32]. One of these, GonaCon, has been previously tested in cats. GonaCon was developed

by the National Wildlife Research Center. It is composed of synthetic GnRH coupled to keyhole limpet

hemocyanin (KLH) combined with AdjuVac adjuvant, a mineral-oil based adjuvant containing

Mycobacterium avium, and made into an emulsion. A newer formulation, GonaCon-B, uses blue protein

(*Concholepas cocholepas*), but it is the older formulation using KLH that has been studied in cats. In the

first study, male cats were singularly immunized with GonaCon and six of nine vaccinated cats were

identified as responders based upon high antibody titers, undetectable serum testosterone by 3 months

post-injection, reduction in scrotal volume, absence of testosterone-dependent penile spines, and lack

of motile sperm upon semen analysis [33]. All but one cat was fertile again by three years post-

immunization [2]. Another study tested the vaccine in female cats and reported long-term

immunocontraception in 11 of the 15 cats singularly immunized, with four cats still infertile five years

after vaccination [34]. Most recently, GonaCon was tested again in female cats living in colony

conditions [35]. Only 30% of the treated cats were infertile for a minimum of one year and 60% of the

treated cats became pregnant within 4 months of introduction to males, though average litter size was

significantly lower in treated cats when compared to control cats [35].

1.3   Gonadotropin-Releasing Hormone as a Target for Contraception in Feral Cats

1.3.1   Gonadotropin-Releasing Hormone Structure

GnRH is a ten-amino acid-long peptide (a decapeptide). It is considered the master reproductive

hormone since it controls the release of the other major reproductive hormones. There are over 20

isoforms of GnRH, with two that are found in most mammals. A third form of GnRH is present in some

vertebrate species. For the purposes of this paper, GnRH refers to GnRH-1. It is worth noting that the

structure of Gnrh-II is ubiquitious in all vertebrates, suggesting it is the earliest evolved isoform [36].

Except for the guinea pig, the GnRH peptide amino acid sequence is identical across all mammalian species. The amino acid sequence of the GnRH peptide is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2. Throughout the isoforms, the first four and last three amino acids (the amino terminus and the carboxyl terminus) are the most conserved, which suggests they are most important for giving the peptide its characteristic structure and allowing receptor binding [36].

GnRH takes on a folded conformation when it binds to its pituitary receptor by bending around the glycine in the sixth position. Substitution of this glycine with d-amino acids can actually stabilize the folded conformation and increase receptor binding affinity. The arginine in the eighth position is required for receptor binding and substitution with another amino acid leads to low affinity with the mammalian GnRH receptor [37, 38].

1.3.2   Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is the central endocrine axis that controls mammalian reproduction. It consists of three functional regions of the body secreting endocrine signals that communicate between them: the hypothalamus, the pituitary gland, and the gonads. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), the pituitary gland secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the endocrine products of the gonads are the sex steroids, primarily androgens and estrogens. The functionality of the HPG axis undergoes changes during development as well as throughout female cyclicity. The sex steroids produced by the gonads have a negative feedback effect on the hypothalamus and the pituitary. As the sex steroids (i.e. testosterone, estradiol, progesterone) are produced, the axis is regulated at the level of the hypothalamus and the pituitary to inhibit gonadotropin stimulation of the gonads. The HPG axis is shown in Figure 1.3 [39].

Endocrine control of reproduction begins in the brain. Specialized neurons in the hypothalamus secrete GnRH. Normal reproductive function is dependent on pulsatile release of GnRH, rather than continuous. Continuous GnRH stimulation causes desensitization of the receptor and loss of normal reproductive function, which is the mechanism for some methods of contraception [1]. GnRH is processed from a precursor polypeptide and stored in granules until those granules are transported down axons to the base of the median eminence [36].

GnRH travels from the hypothalamus to the anterior pituitary, or the adenohypophysis. The pituitary gland plays an intermediary role in regulation of reproduction. It plays the middle role in signal exchange between the hypothalamus and the gonads. It is connected to the hypothalamus by the median eminence through the hypophyseal or infundibular stalk. Neural signals and neuroendocrine secretions pass through the hypothalamic-hypophyseal portal system for hypothalamic control of the pituitary gland [40]. GnRH travels through the hypophyseal portal system to specialized cells in the anterior pituitary known as gonadotrope cells. The gonadotrope cells express the GnRH receptor on their surface. These cells are responsible for the secretion of the next major reproductive hormones, the gonadotropins. The gonadotropins are luteinizing hormone and follicle-stimulating hormone, which travel through the mammalian bloodstream and have effects on the gonads. Luteinizing hormone and follicle-stimulating hormone are collectively referred to as the gonadotropins. They are dimeric glycoprotein hormones consisting of an α and a β subunit. The α subunit (αGSU) is shared amongst the two gonadotropins as well as with the other glycoprotein hormones thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). The β subunit for each hormone is distinct and confers the essential unique functions [41].

The GnRH receptor is a G protein-coupled receptor. It has seven hydrophobic alpha helix transmembrane domains, three hydrophilic extracellular loop domains, four hydrophilic intracellular loop domains, an extracellular NH_2 terminus and an intracellular COOH terminus [42]. The extracellular

domains function in ligand binding. The intracellular domains are important for G-protein activation. The transmembrane regions act as signal transmitters and are important for receptor activation[43]. When GnRH binds to GnRHR, GnRHR undergoes a conformation change in its alpha helix bundles. This triggers switching of GDP for GTP and activates the associated PLC protein. This triggers a signal cascade that induces the release of calcium stores that activates calcium channels to cause the influx of more calcium into the gonadotrope. This leads to depolarization. With a pulse influx of calcium, a burst of LH and FSH is generated [44].

The ovaries are the female gonads. The ovaries are responsible for producing the oocyte and produce the female sex steroid hormones, primarily the estrogens. Luteinizing hormone drives the production of progesterone from the granulosa cells of the preovulatory follicle during the late stages of follicular growth in the female. In addition to stimulating follicular development, FSH is involved in estradiol secretion from granulosa cells. The testes are the male gonads, and they function to produce sperm and androgens, primarily testosterone. Pulses of luteinizing hormone drive the production and release of testosterone from Leydig cells. Sperm production is dependent upon FSH and testosterone and the Sertoli cells support the development of germ cells into spermatozoa [45].

1.4 Filamentous Bacteriophage as a Platform for Vaccine Development

1.4.1 Phage Structure and Phage Vectors

Bacteriophages are viruses that infect only bacteria, making them nonpathogenic for humans and other animals. Filamentous phages (genus *Inovirus*) of the Ff class are long, thin viruses composed of a single stranded circular DNA encapsulated in a protein coat. These bacteriophages are commonly investigated for use as a display vector for antigenic displays [46].

Ff class bacteriophages are rod-shaped and approximately 1μm long and 7nm thick. The genome encodes for eleven genes; five of these genes encode phage coat proteins. Proteins III, VI, VII, and IX (encoded for by the corresponding gene numbers) are considered minor coat proteins and are present in low copy numbers (five of each). Proteins III and VI are on one end of the phage particle and proteins VII and IX are on the other end. Protein III is important in mediating infection of bacteria and formation of a stable phage particle. The other minor coat proteins have roles in assembly and stability of the phage particle. Gene 8 encodes protein VIII, the major coat protein. This protein forms the capsular coat surrounding the phage DNA. The protein pVIII is present in around 2700 copies in wild type phage, and varies depending on the size of the DNA, reaching around 4000 copies in some recombinant phages [47]. The protein is made up of 50 amino acids [46]. Figure 1.4A shows the structure of filamentous bacteriophage.

The most common coat proteins engineered for the display of fusion peptides are genes 3 and 8 due to the exposure of pIII and pVIII on the surface of the phage. The number of copies of recombinant fusion proteins depends on the type of phage vector. Phage vectors of the types 33 or 88 contain two genes: a recombinant protein and the wild-type protein. This causes the phage to display a combination of both fusion proteins and wild-type proteins, which leads to more flexibility in the size and composition of the gene insert. The number of fusion proteins per virion that can be incorporated using a type 88 vector is dependent upon fusion protein size and composition, but displays of 150-300 fusion proteins per phage virion are common [48, 49]. Phage vector types 3 and 8 contain recombinant gene only, with no wild-type protein expressed. Thus, all copies of the phage coat protein are modified with fusion peptides. This creates constraints when using phage vector type 8, since there are high numbers of pVIII copies that are very close to each other and interact with each other. Inserts can only contain up to six amino acids or they interfere with assembly and stability [50]. Some inserts with certain amino acid compositions may still not be able to assemble or be unstable. If stability can be achieved, phage

17

constructs using this type of vector are immunologically advantageous. Each phage particle displays approximately 4000 copies of the fusion peptide displayed in a dense, highly organized pattern [47]. This pattern allows for a strong and persistent immune response due to the ability of B-cell receptors to bind and experience cross-linking activation [46, 51]. Figure 1.4B shows filamentous phage modified with phage vector type 8.

1.4.2   Approaches to Engineering Phage-Based Vaccines

Phages for vaccine use can be generated by cloning an oligonucleotide encoding the desired peptide sequence in a phage vector. This method can only be used when the fusion peptide sequence, and the nucleotide sequence encoding it, are known. This method uses standard cloning techniques and does not require a selection target, but the phage clones generated can be genetically unstable and/or have low propagation rates [46].

Antigenic phages can also be selected from a phage display library containing many (billions) pre-engineered phages displaying random peptides, as in Petrenko *et al.* 1996 [47]. This method requires the use of selection targets, such as antigen-specific antibodies. Phage display libraries are available commercially or may be constructed in-house. Phages in phage libraries are genetically stable and propagate well. For selection of phage from phage display libraries, the specific selection target must be incubated with the library, then the phages that don't bind to the target must be removed, and then the phages bound to the target must be recovered. This selection process is repeated three to five times. The disadvantages of this method for generation of phage-peptide fusions are that the selection target has to be available and well characterized, and an appropriate phage display library must be available, whether acquired commercially or constructed in the laboratory [46]. Another method is the chemical conjugation of synthetic peptides to phage surface proteins. The advantages of this approach

are that there is flexibility with the size and composition of fusion proteins that can be conjugated successfully, and that the preparations are relatively chemically stable if stored properly. The disadvantages are that the number of fusion peptide copies that conjugate to the phage particle varies between batches, and that the conjugation sites are not specific and thus not well defined molecularly [46].

1.4.3   Phage for Immunocontraception

There are many factors that make filamentous phage an advantageous platform for vaccine development. As described above, methods of engineering phage-peptide fusions are well established. Phage can be produced in large quantities relatively easily and without the use of special equipment, making the cost of phage production low. Phage is also stable to varying environmental conditions, such as temperature and pH. This makes phage well-suited for shipping, storage, and delivery in field conditions, and also able to be delivered orally. Phage is very immunogenic and stimulates humoral and cytotoxic immune responses, and has been shown to be safe in multiple mammalian species. Phage also retains its immunogenicity even when inactivated [52].

Phage-based vaccines for immunocontraception have been investigated for various reproductive targets such as LH and its receptor, FSH and its receptor, sperm-specific antigens, and zona pellucida (ZP) proteins, as is reviewed in Samoylova *et al.* [46]. For example, recombinant filamentous phages displaying three overlapping N-terminal decapeptides of FSHR have been tested in multiple mammalian species and found to induce neutralizing antibodies blocking FSH interaction with FSH receptor [53, 54]. They induced a reversible inhibition of ovulation rate in ewes and impaired fertility in female mice [54]. Immunization of three-week-old mice delayed sexual maturity and reduced litter size [55]. In male goats, immunization resulted in low, prepubertal circulating levels of testosterone that

persisted for several months [55]. Immunization of adult male bonnet monkeys with phage-FSHR vaccines given monthly caused reversible infertility and azoospermia without affecting serum testosterone levels [56].

Samoylov *et al.* generated phage-GnRH constructs for immunocontraception from a phage display library using multiple types of GnRH antibodies as selection targets, characterized these constructs, and then tested them in mice [52]. In this study, GnRH antibodies used were obtained from two cats and one dog immunized with Canine Gonadotropin Releasing Factor Immunotherapeutic as well as commercial rabbit antibodies. GnRH antibodies immobilized on magnetic beads were used as targets for selection of antibody-binding phages. Five phage constructs, chosen based upon sequence similarity to GnRH peptide sequence as well as their frequencies in the selection experiments and animal species used, were administered into mice as a single injection of consistent dose of $5x10^{11}$ vir/mouse with no adjuvant. All phage constructs stimulated the production of specific GnRH antibodies with varying titer and duration. Phage construct displaying EHPSYGLA peptide (amino acid residues that are the same as the GnRH peptide are shaded) was then tested in mice at a higher dose of $2x10^{12}$ vir/mouse as well as in combination with Thermogel, a PGLA-PEG-PLGA co-polymer designed to be liquid at 4°C but solid at 37°C. As such, it solidifies after *in vivo* injection and provides a slow release as the polymer degrades. These experiments resulted in an increase in GnRH antibodies compared to the lower dose and led to significant suppression of testosterone in immunized mice, indicating that the antibodies produced in response to the vaccine were neutralizing [52]. This phage construct was the one used to immunize cats in the present study.

Table 1.1: Non-Surgical Options for Feline Contraception

Product	Species	Drawbacks
Zeuterin™/Esterilsol™ Zinc gluconate intratesticular injection	Regulatory approval for dogs; some data on cats	Not currently manufactured in U.S., data on cats showed some non-responders and partial responders
Suprelorin® (deslorelin acetate) GnRH agonist implant	For dogs, some data on cats showing efficacy	Wide variation in onset and duration of efficacy
Progestin contraceptives (multiple options available)	Some use in cats	Only effective for short time, must be given continuously, side effects include increased prevalence of mammary neoplasia and uterine disease leading to pyometra

Figure 1.1: Feral cats around us. Feral cats are a problem worldwide and right here at home. Multiple feral cat colonies exist around the city of Auburn and Auburn University's campus. A small feral cat colony exists behind the QV Gas station and adjacent business at the corner of Webster Road and Wire Road, just half a mile down Wire Road from JT Vaughan Large Animal Teaching Hospital.

Properties of the ideal contraceptive agent for use in feral and/or shelter cats:

Safe: for the environment, animals, and anyone handling the product

Low cost/affordable

Relatively easy to manufacture

Effective in both males and females of any age

Permanent or long-lasting

Should not require surgery/general anesthesia

Completely effective in a single treatment

Ablates sex steroids and their effects

Figure 1.2: Properties of ideal contraceptive vaccines for feral cats.
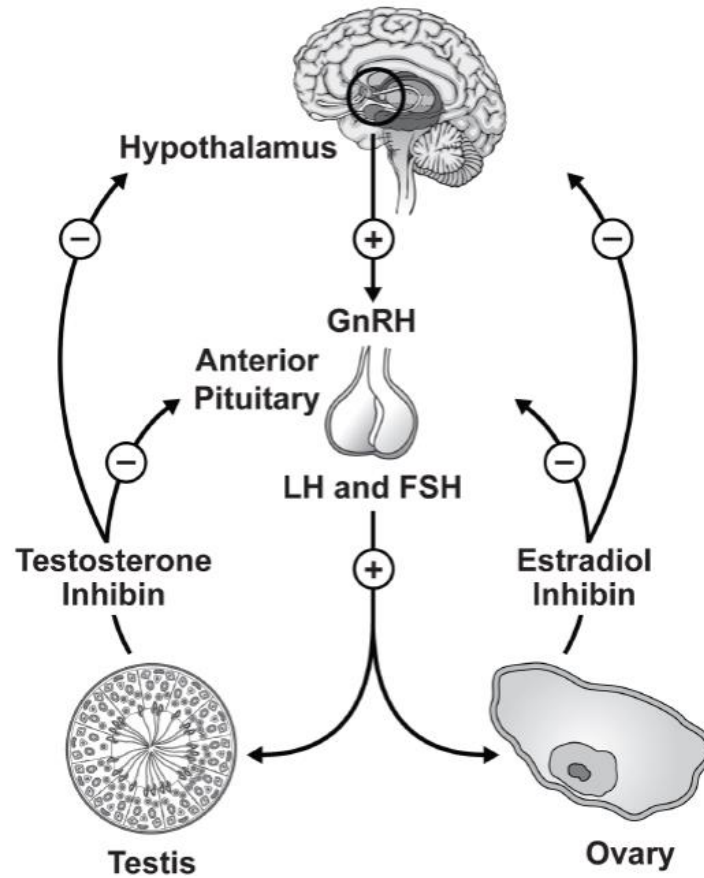
Figure 1.3: Hypothalamic-pituitary-gonadal axis. The Hypothalamic-Pituitary-Gonadal (HPG) axis, involving the hypothalamus, pituitary gland, and gonads (testes or ovaries), supports endocrine regulation of reproduction in male and female mammals. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which acts on cells in the anterior pituitary gland to support production of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). In turn, LH and FSH act on target cells in the gonads to support endocrine functions (ex. production of steroids and inhibin) and gametogenesis (spermatogenesis in males; oogenesis in females). Hormonal products of the gonads feedback on components of the HPG axis to support and regulate reproductive function. Inhibition of HPG functionality impedes reproduction. Image taken from Whirledge and Cidlowski; Minerva Endocrinol 35:109(2010) [39].
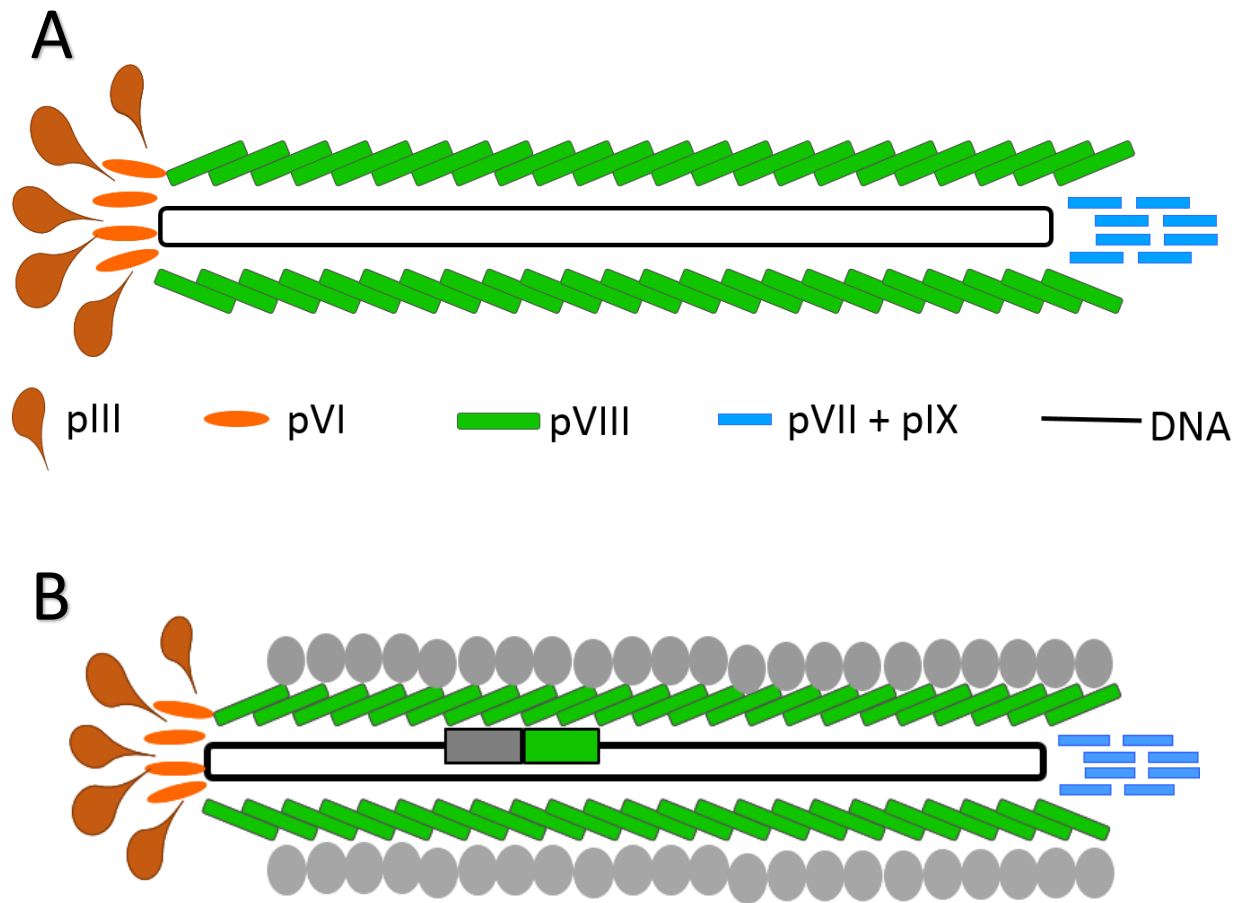
Figure 1.4: Filamentous bacteriophage. (A) Schematic structure of a filamentous phage. Coat proteins encapsulate a long, circular, single strand of DNA. Phage coat proteins pIII, pVI, pVI, and pIX are present in low copy numbers on the ends of the phage particle, while the major coat protein pVIII, present in ~2700 copies in wild-type phage, makes up the majority of the surface of the phage. (B) Phage display. Phages can be modified to display fusion peptides. Using phage vector type 8, phage can be generated to display fusion peptides in all copies of pVIII. Recombinant phages of this type were selected from a phage display library using anti-GnRH antibodies as selection targets in a previous study [52]. One of these phages was used for immunization of cats in the present study. Figure adapted from Samoylova *et al.* [46].

CHAPTER 2

MATERIALS AND METHODS

2.1 **Animals**

The cats used in this study were obtained from and maintained at a breeding colony at the

Scott-Ritchey Research Center, Auburn University College of Veterinary Medicine. Fifteen intact male

domestic cats made up three experimental groups (each n=5). Cats were housed in small groups of

males only in indoor runs. This study and all animal procedures were approved by Auburn University's

Institutional Animal Care and Use Committee (IACUC).

The ages of the cats used in this study are shown in Table 2.1. Four of the cats in Group 1 were

9-10 months old at the time of immunization; the fifth was 18 months old. The five cats in Group 2 were

12-13 months old at the time of immunization, and the five cats in Group 3 were 11-12 months old at

the time of their primary immunization. The groups were not run concurrently, but rather sequentially

as age-matched groups of cats reached sexual maturity. All cats were identified by either numbered ear

tag or microchip.Prior to vaccination, cats were assessed for general health by a complete physical

examination, complete blood count, and serum chemistry. A second complete blood count and serum

chemistry was performed for each cat at the conclusion of each experiment to ensure that the overall

health of the animals had not changed. Cats were neutered surgically at the completion of the study. All

cats deemed adoptable based upon health and sociability were adopted out to private homes at the

conclusion of the experiment.

2.2   Study Design

Blood was collected weekly for at least three weeks before primary immunization and then every month postimmunization for the rest of the experimental period. Reproductive parameters were also assessed prior to immunization; sperm was collected and evaluated and total testicular volume was measured at least once. Following immunization, sperm collection and analysis and testicular measurements were performed monthly. Groups 1 and 2 were castrated seven months after immunization; Group 3 was castrated nine months after immunization. The experiment was concluded following surgical castration. Figure 2.1 shows details of the study design. Table 2.2 summarizes the immunization information for each experimental group. Phage dose, route of administration, and adjuvant differed between experimental groups. The adjuvants used in this study are discussed in detail in section 2.4.

Group 1 received one-time immunization. A total phage dose of 8×10^{12} vir was split into two equal injections. The injections were given subcutaneously proximal to the left and right shoulders. One of the injections contained phage mixed with AdjuVac adjuvant and the other contained phage in PBS. This was done so that the immune system was exposed immediately to the phage in PBS, but also exposed to phage in a slow-release manner.

Group 2 received a prime immunization followed by a booster immunization one month later. The primary immunization was made up of a total phage dose of 4×10^{13} vir split into two equal injections. The injections were given subcutaneously proximal to the left and right shoulders. These injections contained phage in PBS only with no adjuvant. The booster immunization was a total phage dose of 2×10^{13} vir split into two equal injections. These injections were given subcutaneously proximal to the left and right shoulders above the site of the previous injections.

Group 3 received a prime immunization and two booster immunizations. The primary immunization was exactly like that of Group 1 except for the route and location of administration: a total phage dose of 8×10^{12} vir was split into two equal injections. The injections were given intramuscularly in the left and right rear legs. One of the injections contained phage mixed with AdjuVac adjuvant and the other contained only phage in PBS. They received their first booster vaccination three months later: it consisted of a phage dose of 4×10^{12} vir as one injection mixed with AdjuVac adjuvant. It was given intramuscularly in the rear leg that received phage in PBS only with the primary immunization. They received their second booster vaccination an additional three months later (six months after initial immunization). This booster was a total phage dose of 4×10^{12} vir combined with Thermogel adjuvant given intramuscularly in the left front leg proximal to the shoulder.

2.3   Phage Methods

The phage clone used in this study was obtained from an f8-8 landscape phage display library through selection using anti-GnRH antibodies purified from a cat previously immunized with Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health, Exton, PA, USA) that was shown to produce neutralizing anti-GnRH antibodies. All copies of phage major coat protein VIII in the library are modified with random 8-mer fusion peptides [52]. The selection of the phage clone used in this study was described in a previous study [52]. This phage clone was previously shown to induce the production of antibodies against GnRH and suppress serum testosterone in mice [52]. This phage clone displays the peptide sequence EHPSYGLA, where the shaded amino acid residues are identical to those in the native GnRH sequence.

2.3.1   Phage Propagation

To prepare injections, phage was propagated and purified as in Brigatti *et al.* [57]. Phage was propagated from purified phage stock using starved cells. The *Escherichia coli* strain K91BlueKan was used to prepare starved cells according to the following procedure. A tube with 2 mL of NZY broth containing 100μg/mL Kanamycin was inoculated with K91BK from one colony on a plate. This was shaken overnight in an incubated shaker at 200rpm and 37˚C (Excella E24 Incubator Shaker, New Brunswick Scientific). In the morning, 300 μL from the overnight culture was added to a 300mL sidearm flask with 20mL NZY. This was incubated at 37˚C with shaking at 200rpm until the optical density at 600nm reached 0.45. Optical density (OD) was measured at 600nm using the GENESYS™ 20 Visible Spectrophotometer (Thermo Scientific™). Once an OD of 0.45 was reached, shaking was reduced to 50rpm and the flask was incubated for an additional 5-8 minutes to allow F pili to regenerate and the final OD was approximately 0.48. The culture was then transferred to 50mL tubes (Oak Ridge) and centrifuged at 550xg for 10 minutes at 4˚C using a Sorvall Lynx 6000 Centrifuge (Thermo Scientific™). After centrifugation, the supernatant was gently pipetted off and the cells were resuspended in 20mL of 80mM NaCL. They were then transferred to a 125 mL culture flask and put in the incubator shaker at 50rpm for 45 minutes at 37˚C. The contents were then again transferred to 50mL tubes and centrifuged at 550xg for 10 minutes at 4˚C. The supernatant was gently removed and the cells were resuspended in 1mL of NAP buffer and then stored on ice until ready for use.

Phage was propagated in 1 L volumes as needed throughout the project. In an Eppendorf tube, 25μL of purified phage stock was added to 1 mL of starved cells at room temperature and incubated for fifteen minutes. This was then added to a flask containing 0.2 μg/mL tetracycline in 1L NZY and shaken in the incubator shaker at 200 RPM and 37˚C for 45 minutes to allow tetracycline resistance to develop. Tetracycline stock solution (20mg/mL) was added for a concentration of 20 μg/mL and the flask was allowed to shake overnight at 200 RPM and 37˚C. The next day, the solution containing phage was

centrifuged for 10 minutes at 3000 xG and 4°C. The supernatant was removed, placed into a fresh tube, and centrifuged again at 12000 xG and 4°C for ten minutes. The supernatant was pipetted into a new tube, to which 16.7% PEG/3.3M NaCl was added. The tube was mixed by inverting 100 times. This was placed on ice for at least four hours or overnight at 4°C. Precipitated phage was collected by centrifuging for 15 minutes at 31,000 xG. The pellet was then dissolved in TBS and the phage transferred to a microcentrifuge tube. It was centrifuged for two minutes at 4°C and 16,100 xG using a microcentrifuge (Eppendorf Centrifuge 5415 R). The supernatant was then pipetted into a new tube and 16.7% PEG/3.3M NaCL was added. The tube was inverted 100 times to mix and then left on ice for at least two hours. It was then centrifuged again using the microcentrifuge for 10 minutes at 16,100 xG and 4°C. The supernatant was removed and the pellet was dissolved in TBS and stored at 4°C until further use.

2.3.2   Phage Purification

Endotoxin removal from phage preparations was done using Triton X-114 phase separation. 1 mL of TBS was used to dilute 100 μL of phage. Triton X-114 was added at 1% and the solution was vigorously vortexed. The solution was then placed in an ice bath for five minutes. The tube was then centrifuged at max speed in a microcentrifuge for seven seconds. It was then placed in an incubator at 37°C for one hour. The tube was centrifuged again for 30 seconds. Then the aqueous phase containing phage was carefully removed and placed into a fresh tube. Then 1/6 volume of PEG/NaCl was added to the tube for phage precipitation. The tube was incubated on ice for at least one hour and then centrifuged for ten minutes at 16,100 xG and 4°C to pellet phage. The supernatant was then discarded and phage was resuspended in PBS. At this point, the concentration of phage could be determined. Phage was stored at 4°C until use.

2.3.3   Phage Quantitation

Phage was quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™). The wavelength used for measuring was 269 nm. Phage concentration was calculated by multiplying the absorbance at wavelength 269 nm by 6.5×10^{12} to find the number of virions per milliliter (vir/mL).

2.4   Adjuvants

AdjuVac was used as an adjuvant for Group 1 and the prime immunization and first booster for Group 3. AdjuVac is the adjuvant used in the GnRH immunocontraceptive vaccine GonaCon, developed by the National Wildlife Research Center (NWRC) for white-tailed deer population control (Figure 2.2A). AdjuVac is oil-based and contains a small amount of *Mycobacterium avium* that acts as an immunostimulant [58, 59]. Combination of phage in PBS with AdjuVac forms a stable water-in-oil emulsion that provides a depot effect for the vaccine and protects the antigen (phage) from rapid destruction by macrophages. It provides for a prolonged immune response because of the slow release rate of the antigen at the injection site [58]. AdjuVac was provided by Douglas Eckery of the USDA APHIS National Wildlife Research Center.

Thermogel was used as the adjuvant for the second booster immunization for Group 3. It is a commercially available product denoted AK097 (PolySciTech®, West Lafayette, IN, USA) (Figure 2.2B). Thermogel is a PLGA-PEG-PLGA co-polymer [(Poly(lactide-co-glycolide)-b-Poly(ethylene glycol)-b-Poly(lactide-co-glycolide)] that is designed to be liquid at 4˚C and solid at 37˚C. It solidifies after *in vivo* administration and provides a slow release effect as the polymer degrades. This product was used in combination with phage in mice and led to enhanced antibody response [52].

2.5   Preparation of Injections and Immunization of Cats

2.5.1   Preparation of Injections

Prior to injection, phage preparations were tested for sterility by plating a small amount onto a plate containing media without any antibiotics, incubating the plate for 48 hours and checking the plate for bacterial growth.

Phage concentrations were calculated based on measurement of NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Injections containing phage alone were prepared in PBS. Injections containing AdjuVac (USDA, Fort Collins, CO, USA) were prepared by mixing phage in PBS with AdjuVac at a 1:1 ratio. Phage in PBS and AdjuVac were drawn up into separate luer lock tip glass syringes and all bubbles were removed. The syringes were connected by a luer lock connector and the solution was moved back and forth between the two syringes until thoroughly mixed (Figure 2.3A). It was then separated into separate injections and stored on ice until immunization. To prepare injections containing Thermogel (AK97; Polyscitech, West Lafayette, IN, USA), the polymer was first solubilized in PBS at 1:5 w/v ratio and then mixed with phage in PBS. Injections were separated out and stored on ice until immunization. All injections were delivered in a volume of 0.5 ml using 1 ml syringes with 23 G needles.

2.5.2   Immunization of Cats

The day before or morning of immunization, the temperature, respiration, and pulse were taken for each cat and recorded. Food was withheld on the morning of immunization. The cats were sedated with a dose of dexmedetomidine appropriate for their weight given intramuscularly (0.5mg/mL, Zoetis). At this time, blood was collected to use as time-point zero. The area for injection was shaved and

surgically scrubbed with chlorhexidine and rinsed with alcohol to minimize the potential for skin

bacterial contamination. Groups 1 and 2 received subcutaneous injections (Figure 2.3B); Group 3

received intramuscular injections (Figure 2.3C). The injection site was marked with a tissue pen so it

could be monitored for reaction. After immunization, the sedation was reversed using equal volume of

atipamezole (5 mg/mL, Zoetis). The cats were monitored closely for at least 30 minutes following

recovery to ensure there were no immediate adverse reactions to the vaccine. Access to food was

allowed once the cats were adequately reversed and completely awake. Temperature, respiration, and

pulse were taken for 3-5 days after immunization and the cats were monitored closely throughout the

study for any adverse effects.

2.6   Collection and Evaluation of Cat Blood Samples

If the cat was fractious for blood collection, the cat was sedated with a solution of

dexmedetomidine appropriate for its weight given intramuscularly (0.5mg/mL, Zoetis). The cats were

placed in a restriction bag. A two-three mL blood sample was collected from the jugular vein. If sedated,

the cat's sedation was reversed with atipamezole (5 mg/mL, Zoetis) after blood collection and the cat

was monitored until completely awake.

After collection, the blood was allowed to clot for 20 minutes and then centrifuged at 850 xG

(Sorvall RT 6000D, H1000B rotor). Sera was separated from the blood cells and aliquoted into multiple

tubes. It was then stored at -20˚C until assayed for antibodies or testosterone.

2.6.1   Detection of Anti-GnRH Antibodies in Sera

GnRH antibodies were detected by ELISA using a synthetic GnRH peptide (New England Peptide, MA, USA) biotinylated at C terminus (NH_2 -EHWSYGLRPG-Lys-biotin-$CONH_2$) to serve as the detector molecule, as is described in Samoylov *et al.* [52].

Streptavidin (Amresco) was diluted in TBS at 25 µg/mL and added to 96-well plates (80 µL/well) for overnight incubation at 4°C. The following day, streptavidin was removed and the wells were washed three times TBS, 150 µL/well. GnRH peptide was added to streptavidin-coated wells at a concentration of 250ng/well in volume of 80µL/well. This was allowed to incubate overnight at 4°C or 37°C for one hour. Next, the peptide was removed and 1% BSA in TBS was added to the wells at a volume of 150 µL/well for one hour at 37°C to block non-specific binding. Then the blocking solution was removed and the wells were washed three times with a solution of TBS containing 1% BSA and 0.05% Tween-20 using a volume of 150 µL/well. Sera collected from cats was diluted to an appropriate concentration and added to wells for two hours at 37°C. Then the wells were washed three times with a solution of TBS and 0.05% Tween-20 (volume of 150 µL/well). Anti-cat IgG secondary antibody (peroxidase-conjugated goat anti-cat IgG (H + L); Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:2000 in TBS containing 1% BSA and 0.05% Tween-20 and added at a volume of 80 µL/well. This was incubated for one hour at room temperature. The wells were then washed five times with 150 µL/well of TBS/0.05% Tween-20. They were then reacted with *o*-phenylene diamine (OPD) substrate (Acros Organics via VWR, Atlanta, GA) solution for eight minutes. The reaction was stopped with 8N H_2SO_4 (80µL/well) and the plate immediately read at 490nm using Synergy HT reader (BioTek, Winooski, VT, USA).

Cat serum was diluted 1:100 and then tested at two-fold dilutions to endpoint titers. Cats served as self-controls; negative control pre-immunization sera for each cat was also tested at each two-fold

dilution. The endpoint titer was determined to be the highest dilution at which the value was at least two times that of the negative control.

2.6.2   Detection of Testosterone in Sera

Testosterone was measured using a mouse/rat testosterone ELISA assay kit (ALPCO, Salem, NH, USA) using the procedure recommended by the manufacturer. The kit includes six calibrators for creation of a standard curve at concentrations of 0 ng/mL, 0.1 ng/mL, 0.4 ng/mL, 1.5 ng/mL, 6.0 ng/mL, and 25.0 ng/mL. The kit includes a microtiterplate (12 x 8 strips with 96 wells coated with anti-testosterone antibody), incubation buffer, enzyme conjuguate (testosterone conjugated to horseradish peroxidase), substrate solution (contains tetramethylbenzidine and hydrogen peroxide in a buffered matrix), stop solution (2 N hydrochloric acid solution), and wash solution. The kit was stored at 4°C and all reagents were allowed to come to room temperature before use. Two controls of known testosterone concentrations in rat serum (Rat Control Set, ALPCO, Salem, NH, USA) were run with each assay and cat serum obtained from neutered cats originating in the same colony was utilized as a negative control.

Each calibrator, control, and sample (undiluted cat serum) was dispensed into appropriate wells at a volume of 10 µL. Next, 100 µL of incubation buffer was added to each well, followed by 50 µL of enzyme conjugate. This was incubated at room temperature for one hour while shaking on a microplate mixer. The contents of the wells were discarded and the wells were washed four times with 300 µL of diluted wash solution. Then 200 µL of substrate solution was added to each well and the plate was incubated for 30 minutes in the dark (covered in aluminum foil). The reaction was stopped using 50 µL of stop solution. The plate was read immediately at 450 nm using the Synergy HT reader (BioTek, Winooski, VT, USA).

2.7   Collection and Evaluation of Semen Samples

The cats were anesthetized with a solution of equal parts dexmedetomidine (0.5 mg/mL, Zoetis) and ketamine (100 mg/mL, Zoetis) appropriate for their weight given intramuscularly. Blood was collected first, followed by testicle measurements, and then the cats underwent collection of sperm via electroejaculation. The cats' anesthesia was supplemented with isoflourane gas delivered in oxygen if needed. Anesthesia was reversed after the procedure using atipamezole (5 mg/mL, Zoetis). Sperm was evaluated after each individual cat.

The media used for semen extender was prepared using Ham's F-10 (Thermo Fisher Scientific, Waltham, MA, USA) with 25 mM Hepes, 1mM pyruvate and glutamine, and 5% fetal bovine serum [60].

2.7.1   Electroejaculation

The electroejaculation procedure was as described in Johnson, 2018 [60]. The cats were subjected to three sets of stimulus cycles using an electro-ejaculation machine (P-T Electronics, Boring, OR, USA). A rectal probe one cm in diameter and 12-13 cm long with three longitudinal, stainless-steel electrodes was used to deliver the electric stimuli (Figure 2.4). The probe was lubricated and introduced rectally with the electrodes oriented ventrally. The probe was inserted approximately five-seven cm into the rectum and positioning was adjusted if indicated to be necessary by the response of the cat. At the beginning of each set, a sterile plastic 1.5 mL tube (Eppendorf) was placed over the extruded penis to collect a semen sample (Figure 2.4). The tube was directed ventrally to allow the ejaculate sample to flow by gravity into the tip of the tube and prevent the ejaculate from becoming lost. Set one consisted of 10 repeats of setting two volts, 10 repeats of setting three volts, and 10 repeats of setting four volts. Set two consisted of 10 repeats of setting three volts, 10 repeats of setting four volts, and 10 repeats of setting five volts. Set three consisted of 10 repeats of setting four volts, 10 repeats of setting five volts,

and an additional 10 repeats of setting five volts. Between repeats of ten within each set, a prostate

massage was done for approximately 15 seconds by exerting slight downward pressure while moving

the probe one-two cm cranial and caudally. There was a rest period of three-five minutes between sets.

After each individual set, the volume of semen collected was measured in microliters. An appropriate

amount of semen extender (minimum 1:1) was immediately added to each sample in order to have

adequate volumes to evaluate and to prevent loss of the sample through evaporation of seminal

plasma. Tubes containing sample were kept warm using a slide warmer (SireMaster, Ice Corporation,

Manhattan, KS, USA) until semen collection was complete. In between sets, the penis was manually

extruded and examined for penile spines.

2.7.2   Semen Quantity and Quality

Concentration of sperm in the sample was evaluated using a nucleocounter (SP-100

Nucleocounter, ChemoMetec, Allerod, Denmark) and total sperm number was calculated using the

concentration and volume. CASA software motility analysis was used to evaluate the motility

characteristics of each sample. Progressive and total motility were recorded. Sperm morphology was

evaluated by combining a small amount of the sample with eosin-nigrin stain on a slide, allowing it to

dry, and evaluating 100 cells for defects. All defects were counted, even if one cell had multiple defects.

The defects evaluated were classified as abnormal heads, abnormal midpieces, tailless, proximal

droplets, distal droplets, bent tails, or coiled tails. Cells with no defects were considered morphologically

normal.

2.8   Measurement of Testes Size by Ultrasound

Testicle length (L), width (W), and height (H) were measured ultrasonographically using a 7.5 MHz sector probe (CA123 Transducer and Acuson P300™ Ultrasound System, Siemens, Mountain View, CA, USA) (Figure 2.5). A single theriogenologist performed all measurements throughout the study. At least two measurements were taken for each parameter and then averaged. Testicular volume for each testicle was calculated by multiplying L x W x H x 0.5233, and the total testicular volume was recorded by adding the volume of both testicles together. This formula is derived from the formula for the volume of an ellipsoid, L x W x H x $\pi/6$ [61].

2.9   Collection of Testicles and Histology of Tissues

At the conclusion of the study, the cats were neutered and the testicles were submitted for histopathology. A routine closed neuter was performed by the study veterinarian. The tunica vaginalis tissue was removed from each testicle and each testicle was weighed. The poles of each testicle were perforated using a 21 gauge needle to allow penetration of fixative into the tissue. The testicles were placed into Modified Davidson's fixative for 24 hours then transferred to 70% ethanol. Tissues were transferred to the laboratory of Dr. Russell Cattley (Auburn University College of Veterinary Medicine) for processing. The tissues were processed as is standard for paraffin-embedded tissues and sections were prepared at 3 μm thickness. Hematoxylin and Eosin (H&E) staining and Periodic Acid Schiff (PAS) staining were performed. Pathological analysis was done by Dr. Russell Cattley and by Dr. Catherine Picut (Charles River Laboratories, LLC).

At the time of surgical castration, punch biopsies of injection sites were taken from any cats with palpable injection site reactions at the conclusion of the study. These samples were submitted for histopathology. They were placed into formalin, also fixed in paraffin, and H&E staining was done.

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## 2.10   Statistical Analysis

Data were analyzed using the general linear model (GLM) for repeated measures analysis of variance (ANOVA). For each group of cats each time point post-immunization was compared to pre-immunization month 0 using multiple contrasts. $P \leq 0.05$ shown on graphs by asterisks.

Table 2.1:   Cat Ages at the Time of Primary Immunization with Phage Vaccines

Age of cats					
Group 1	months	**Group 2**	months	**Group 3**	months
cat 1.1	9	**cat 2.1**	13	**cat 3.1**	11
cat 1.2	9	**cat 2.2**	13	**cat 3.2**	11
cat 1.3	9	**cat 2.3**	12	**cat 3.3**	11
cat 1.4	9	**cat 2.4**	13	**cat 3.4**	11
cat 1.5	18	**cat 2.5**	12	**cat 3.5**	12

Table 2.2: Cat Groups: Experimental Design of Immunization

| Group/Immunization	Phage dose	Injection site	Adjuvant
Group 1 (n = 5)			
Single Immunization	8×10^{12}	SC shoulder; left and right	AdjuVac (left only)
Group 2 (n = 5)			
Prime Immunization	4×10^{13}	SC shoulder; left and right	none
Booster Immunization	2×10^{13}	SC shoulder; left and right	none
Group 3 (n = 5)			
Prime Immunization	8×10^{12}	IM rear leg; left and right	AdjuVac (left only)
Booster Immunization	4×10^{12}	IM rear leg; right	AdjuVac
Booster Immunization	4×10^{12}	IM front leg; left	Thermogel
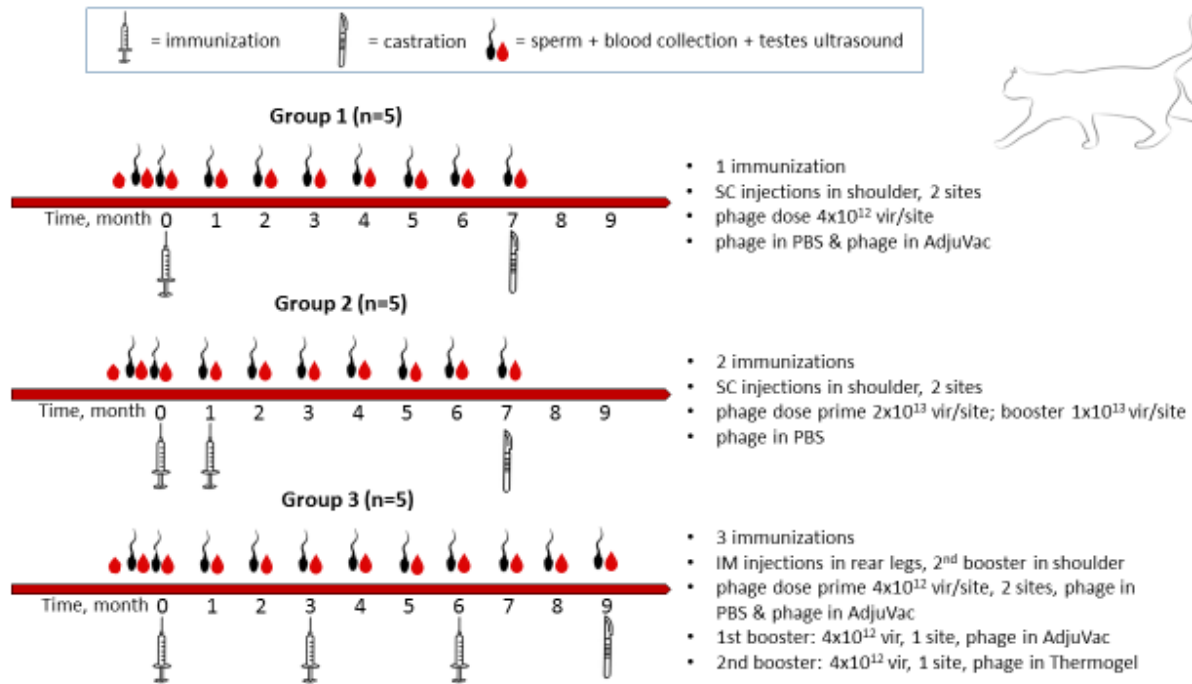
Figure 2.1: Study design. Blood was collected from each cat weekly for at least three weeks prior to immunization and then monthly for the duration of the experiment. Reproductive parameters were also assessed prior to immunization; sperm was collected and total testicular volume was measured at least once. Following immunization, sperm collection and testicular measurements were performed every month. Groups 1 and 2 were castrated seven months after immunization; Group 3 was castrated nine months after immunization. The experiment was concluded following surgical castration. Group 1 received one-time immunization. A total phage dose of 8×10^{12} vir was split into two equal injections containing either phage with AdjuVac or phage with PBS given subcutaneously. Group 2 received a primary immunization followed by a booster immunization one month later. The primary immunization was made up of a total phage dose of 2×10^{13} vir split into two equal injections with no adjuvant that were given subcutaneously. The booster immunization was a total phage dose of 1×10^{13} vir split into two equal injections that were given subcutaneously. Group 3 received a primary immunization and two booster immunizations. The primary immunization was a total phage dose of 8×10^{12} vir was split into two equal injections containing phage combined with AdjuVac or PBS given intramuscularly. The first booster vaccination was given three months later: it consisted of a phage dose of 4×10^{12} vir as one injection mixed with AdjuVac adjuvant and was given intramuscularly. They received their second booster vaccination an additional three months later (six months after initial immunization); this booster was a total phage dose of 4×10^{12} vir combined with Thermogel adjuvant given intramuscularly as one injection.
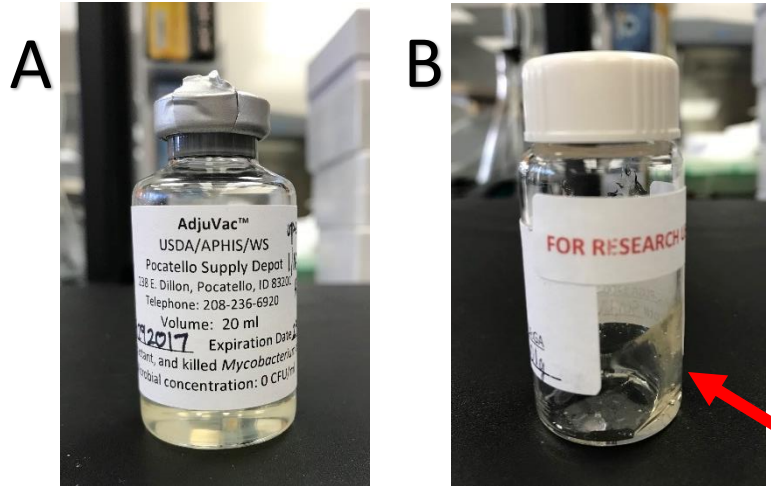
Figure 2.2: Adjuvants. (A) AdjuVac. This adjuvant was developed for use with the GnRH immunocontraceptive vaccine known as GonaCon. It is an oil-based adjuvant containing a small amount of *Mycobacterium avium*. (B) Thermogel. This is a PLGA-PEG-PLGA co-polymer that is liquid at 4˚C and solid at 37˚C, so it is designed to solidify after *in vivo* administration and provide a slow release effect as the polymer degrades. The red arrow shows solid Thermogel against the side of the vial as received from the manufacturer.
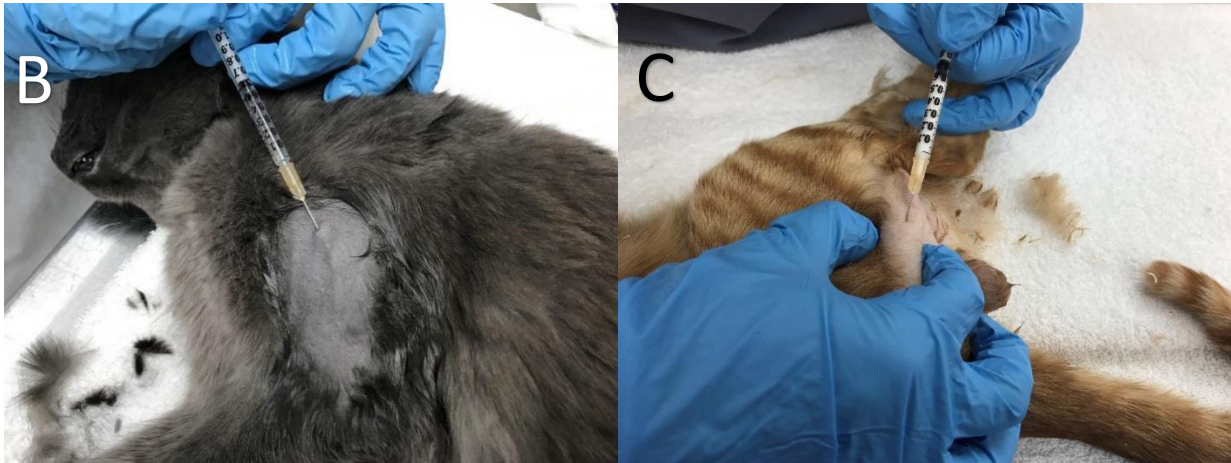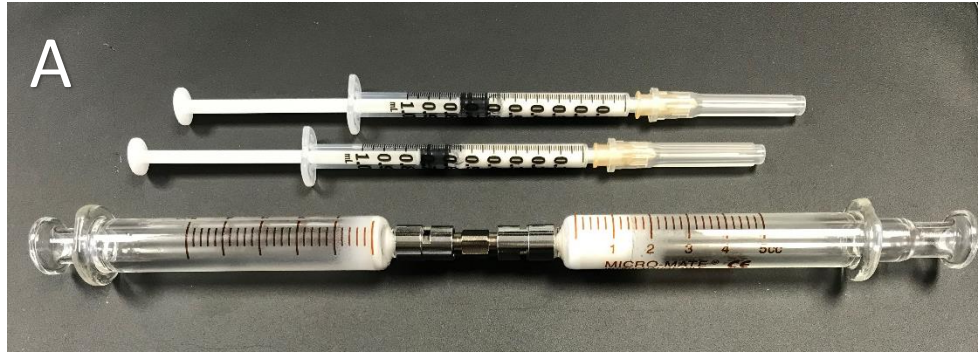
Figure 2.3: Preparation of injections and immunization of cats. (A) Preparation of injections containing AdjuVac. A 1:1 ratio of phage to adjuvant was used. Phage in PBS and AdjuVac adjuvant were drawn up into separate luer lock tip glass syringes and all bubbles were removed. The syringes were connected by a luer lock connector and the solution was moved back and forth between the two syringes until thoroughly mixed. It was then separated into separate injections (0.5mL each). (B) Immunization of cat subcutaneously at shoulder. (C) Immunization of cat intramuscularly in the rear leg. The cats were sedated prior to immunization to ensure consistent and proper placement of injections. The injection sites were shaved and scrubbed as if for surgery using chlorhexidine and alcohol. Injections were given using a one mL syringe and sterile 23 gauge needle.

Figure 2.4: Collection of cat semen by electroejaculation. Positioning of the probe and a collection tube is shown. The cat is under general anesthesia for the procedure. Electroejaculation is performed using a rectal probe one cm in diameter and 12-13 cm long. The probe is lubricated and inserted into the rectum approximately five-seven cm with the electrodes oriented ventrally. The penis is manually extended and a sterile vial is placed over the penis with the vial directed ventrally. The electroejaculation machine is used to deliver a series of electrical stimuli for three sets of stimulus cycles of gradually increasing voltage.
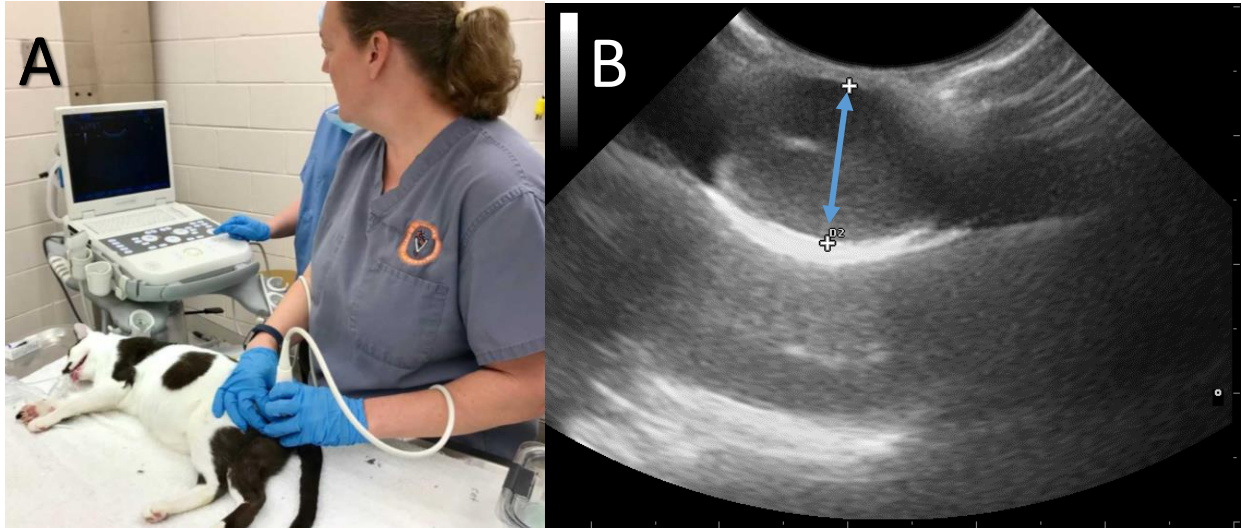
Figure 2.5: Measurement of testes size by scrotal ultrasound. (A) Representative photo of procedure. The ultrasound probe is placed on the external surface of the scrotum. At least two measurements are taken of the length, width, and height of each testicle. The measurements are averaged. Testicular volume is calculated by multiplying the length x width x height x 0.5233 and adding the results for both testicles together for the total testicular volume. (B) Representative ultrasound image of a cat testicle, where the distance between the two crosses (indicated by the blue line) is being measured.

CHAPTER 3

RESULTS AND DISCUSSION

The phage-GnRH construct used to immunize cats in this study was first generated and tested in mice as in Samoylov *et al.* [52]. It displays peptides with the sequence EHPSYGLA, where the shaded amino acid residues are identical to those in the native GnRH sequence. This phage construct was obtained from a phage display library using GnRH antibodies from a cat immunocastrated with Canine Gonadotropin Releasing Factor Immunotherapeutic. In initial experiments using a phage dose of $5x10^{11}$ vir/mouse, this phage construct had the most long-lasting response compared to the other phage constructs evaluated. It was then tested in mice at a higher dose of $2x10^{12}$ vir/mouse as well as in combination with Thermogel as an adjuvant. These experiments resulted in a multifold increase in GnRH antibodies compared to the lower dose and led to significant suppression of testosterone in immunized mice, indicating that the antibodies produced in response to the vaccine were neutralizing [52]. The goal of the present study was to evaluate this phage construct in one of the target species, the cat. Cats were immunized with the phage as described earlier in the study design and a number of parameters, including serum GnRH antibodies, serum testosterone, testicular volume and histology, and sperm quantity and quality, were evaluated.

3.1 Anti-GnRH Antibodies

All cats immunized in the study developed antibodies against GnRH over the course of the study. Antibody response, including the highest level of antibodies obtained and duration of time between immunization and antibody development, varied substantially amongst the individual animals. For group 1, the antibodies generally peaked around months 3-5 (Figure 3.1). For Group 2, antibodies peaked around 1.5-2 months after primary immunization, and then decreased (Figure 3.2). They plateaued around 3-4 months after immunization and stayed at approximately the same level until the end of the study. For Group 3, the highest levels of antibodies were generally reached between 6-9 months after primary immunization (Figure 3.3). Cat 3.5 reached the highest level of antibodies of all cats in the study, with a titer of 1:6,553,600, which was attained at month six and persisted through the end of the study. Out of Group 1, Cat 1.3 developed the highest level of antibodies with an endpoint titer of 1:819,200. Cats 1.4 and 1.5 developed the lowest levels of antibodies with maximum titers of 1:6,400. Cat 2.1 developed the highest level of antibodies for the cats in Group 2, which was 1:409,600. Cat 2.5 developed the lowest level of antibodies out of the cats in Group 2 with maximum titer of 1:25,600. Cat 3.4 developed the lowest level of antibodies for the cats in Group 3 with a maximum titer of 1:102,400.

Cat 1.3 was the first to develop antibodies with detectable levels as early as two weeks after immunization. Most cats in Groups 1 and 3 had detectable levels of antibodies one month after immunization. Only two cats in Group 2 had detectable antibodies one month after immunization, but all five had them at 1.5 months after primary immunization (two weeks after booster vaccination). Cats 1.1 and 1.5 were slow to respond and developed detectable antibodies at four months after immunization and cat 3.4 was somewhat slow to respond and developed detectable antibodies two months after immunization. Two cats had undetectable levels of antibodies at the end of the study: cat 1.4 and cat 2.5. These cats may be considered short term responders. Antibodies were undetectable five months after immunization for cat 1.4 and four months after primary immunization for cat 2.5.

3.2   Serum Testosterone

Baseline values in serum testosterone in Group 1 cats ranged from 0.76 to 3.13 ng/ml. Throughout the post-immunization study period, there was no significant change in testosterone concentrations in these cats compared to baseline values (Figure 3.4). Baseline values in serum testosterone in Group 2 cats ranged significantly: from 0.54-2.12 ng/ml in cats 2.2-2.5 to 9.08 ng/ml in cat 2.1. Also, cat 2.1 had much higher serum testosterone levels than any of the other cats in Group 2 post-immunization reaching 50 ng/ml at months six and seven. Therefore, testosterone for Group 2 cats was analyzed for four cats excluding cat 2.1 as well as for all five cats (Figure 3.5). Using either approach, there was no statistically significant difference in serum testosterone for Group 2 cats throughout the study period. Similar to Group 2 cats, baseline values in serum testosterone in Group 3 cats ranged significantly: from 2.43-7.55 ng/ml in cats 3.2-3.5 to 27.95 ng/ml in cat 3.1. Also, cat 3.1 had much higher serum testosterone levels than any of the other cats in Group 3 post-immunization reaching 50 ng/ml at month four and five. Therefore, testosterone for Group 3 cats was evaluated for four cats excluding cat 3.1 as well as for all five cats (Figure 3.6). When analyzed as a group of four cats, the mean testosterone was significantly reduced at eight months after initial immunization ($P < 0.05$). Interestingly, for both groups (2 and 3), the shape of the curve for four cats was very similar to the curve for five cats (Figures 3.5 and 3.6), indicating that the number of cats in analysis did not influence trends in testosterone changes notably, but did influence statistical significance.

3.3   Testicular Volume and Histology

The mean total testicular volume (TTV) for each group are shown in Figure 3.7. Total testicular volume was decreased from the beginning to the end of the study in four cats in Group 1 (excluding cat 1.2). The maximum decrease of 45.81% was observed in cat 1.5 and the smallest decrease of 24.53%

was observed in cat 1.4. Cat 1.2 had an increase of 19.79%. Thus, when evaluated as a group, the differences between pre- and post-immunization TTV values were not statistically significant although the curve is downward. There was no significant changes of mean TTV values for Group 2 cats except at two separate post-immunization time points (months 3 and 6) when the testicular volume was significantly increased. The mean TTV declined for all five cats in Group 3, but TTV fluctuated throughout the study. Differences in TTV were more pronounced for all Group 3 cats at seven months post-immunization than at nine months post-immunization. For example, Cat 3.1 had a decrease in TTV of 29.04% at month seven, but only 2.97% at month nine. The maximum reduction in TTV from pre-immunization to the end of the study was observed in cat 3.3. The observed reduction in TTV for the group was statistically significant at multiple time points: months 2, 3, 4, 5, 6, and 8 (Figure 3.7).

Histological examination by a pathologist was performed on the testes and epididymides collected from Group 3 cats, which had a significant reduction in TTV. Tissues were compared to two untreated, age-matched cats from the same colony, raised under the same conditions. All cats, treated and untreated, had sperm present in the epididymal tubules and active spermatogenesis in the testes. Upon subgross examination of H&E stained sections of the testes, the testes of the five experimental cats were uniformly slightly smaller in diameter than the testes of the two control cats. Upon microscopic examination of the testes, cats 3.1 and 3.2 had a mild, focal to focal-locally extensive, degeneration of tubules (Figure 3.8). This change was characterized by a small cluster of tubules lined by a mild paucity of germ cells and dilatation of tubule lumens. This change could be spontaneous and unrelated to the treatment.

The epididymides of all Group 3 cats had minimal to mild vacuolation in the corpus of epididymis that was not present in the untreated cats (Figure 3.9). Vacuolation was characterized by macrovesicles with proteinaceous material within the epithelial lining. Occasionally these vacuoles had a

rim of epithelial cells, compatible with the pseudoglandular appearance of cribiform change. Many vacuoles had no epithelial lining and were interpreted to be intracytoplasmic vacuoles.

No biologically significant differences could be detected in the size of the testicular and epididymal tubules, thickness of spermatogenic epithelium in the testes, or density of sperm in the epididymial tubules.

3.4   Sperm Parameters

All cats were still producing sperm at the end of the study period, and the total sperm number (TSN) for each cat varied throughout the study (Figure 3.10). Quality of sperm was evaluated by examining morphology and motility. The majority of the immunized cats (86.67%) showed a decrease in the percentage of morphologically normal sperm cells produced at the end of the study compared to pre-immunization. For group 1, the percentage of normal sperm cells was significantly decreased compared to pre-immunization at months 5, 6, and 7 (Figure 3.11 A). The percentage of normal sperm cells in group 2 was significantly lower from month 2 until the end of the study (Figure 3.11 B). Group 3 did not have a significant decrease, but had the lowest mean percentage of normal sperm cells to start with and the values at the end of the study were lower than those obtained prior to immunization (Figure 3.11 C). The pre-immunization average normal morphology for group 3 was 20.2% compared to 31.2% and 39.2% for groups 1 and 2, respectively. Pre-immunization percentage of normal morphology ranged from 9% in cats 3.1 and 3.2 to 51% in cat 2.1. Common morphological defects observed included coiled or bent tails, abnormal midpieces, and abnormal heads (Figure 3.12). There were no significant differences observed in progressive motility for any of the groups (Figure 3.13). Pre-immunization progressive motility ranged from 20-65%, with the lowest group average occurring in group 3.

3.5 Reactions to Immunization

Body temperature remained normal following immunization and no cats experienced any immediate adverse reaction. No systemic reaction was observed in any of the immunized cats. Four of the cats in Group 2 (who did not receive any adjuvant with immunization) experienced short-lived inflammation at the site of injection that resolved within two-eight days. These palpable tissue reactions ranged from 0.25-2 cm in size. Interestingly, the cats in groups 1 and 3 did not have any inflammation at the sites of injections containing phage in PBS without adjuvant.

All cats in Group 1 developed variable-sized, firm nodules at the site of immunization with phage-GnRH combined with AdjuVac (Figure 3.14 A). These first appeared approximately two weeks after immunization. The nodules were firm, nonpainful, and located in the subcutaneous space under the skin. Generally, the nodules increased in size initially, then slowly decreased in size over the later months of the study. The nodules were measured monthly throughout the seven-month period post-immunization, and those results are shown in Table 3.1. After the official conclusion of the study, only two cats had palpable nodules nine months after immunization (the last point at which the cats were assessed). Nodules were biopsied at the time of neuter and confirmed to be inflammatory masses with no evidence of neoplasia (Figure 3.14 B).

All cats in Group 3 also developed inflammatory masses at the sites of immunization with phage-GnRH combined with AdjuVac (Figure 3.14 D). Four of the five cats developed palpable nodules in response to the initial immunization and all five cats developed nodules in response to the first booster immunization. Size of these nodules ranged from less than 1 cm to 7 cm at the widest point. Generally, these nodules increased in size for the first month or two and then decreased in size throughout the study and completely disappeared in three of the cats before the conclusion of the study. The nodules were measured monthly throughout the post-immunization period, and those results are shown in

Table 3.1. The nodules were firm, nonpainful, and did not interfere with movement. One cat (cat 3.1)

developed a sterile abscess at the second injection site six months after the booster immunization

(Figure 3.14 C). This particular mass was partially excised at the time of neuter and completely healed

approximately one month later. The cats in Group 3 did not have any reactions to phage + Thermogel

(or phage alone). All nodules remaining at the time of neuter were biopsied and confirmed to be

inflammatory nodules with no evidence of neoplasia.

3.6   Discussion

In this study, immunization with a phage-GnRH vaccine induced the production of anti-GnRH

antibodies in all 15 intact male cats that were immunized. The expected result of sufficient neutralizing

anti-GnRH antibodies in serum is reduction in gonadal hormones, cessation or reduction of reproductive

function, and gonadal atrophy. Serum testosterone in immunized cats varied substantially, and when

analyzed as a whole experimental group, mean testosterone was only statistically significantly reduced

at eight months after immunization in Group 3 experimental cats. Total testicular volume decreased in

80% of the cats in Group 1 and 100% of the cats in Group 3, indicating some degree of gonadal atrophy.

Although all cats still produced sperm at the end of the study, the majority of immunized cats showed a

decrease in the percentage of their sperm cells that exhibited normal morphology.

While all cats in all three experimental groups developed anti-GnRH antibodies, their level and

duration varied significantly within each group as well as between groups. Out of the 15 total

experimental cats, 13 cats had detectable GnRH antibodies in serum at the conclusion of the study

(seven months post-immunization for Groups 1 and 2 and nine months post-immunization for Group 3).

AdjuVac stimulated significantly higher GnRH antibody responses and supported their high-level

presence in cat sera for extended time periods as compared to cats immunized without the adjuvant.

Cats immunized with higher phage doses produced higher antibody responses. Booster immunization, with or without adjuvant, improved antibody response. Cats in Group 3, who received two booster vaccinations three months apart injected intramuscularly, achieved the overall highest level of antibodies and all had detectable antibodies at the conclusion of the study. The antibodies were detectable in 80% of Group 3 cats one month after immunization and in all Group 3 cats two months after immunization. Group 1, who received the same initial vaccine dose and components injected intramuscularly, had one cat produce an antibody response as soon as two weeks after immunization, but it was not until four months after immunization that 100% of cats in Group 1 had developed detectable levels of antibodies.

In this study, cats were injected with moderate phage doses, as this was the first instance of phage being injected into cats. Increasing the phage dose in future formulations may lead to a more potent immune response. In mice, injection with a higher phage dose led to a significant increase in serum antibodies [52]. The highest reported phage dose injected into animals was a dose of $2x10^{14}$ vir/animal in rhesus macaques, and no adverse side effects were reported [62]. Sheep and goats were injected with $1x10^{14}$ vir/animal with no adverse effects [63]. There were no serious adverse effects in response to phage alone in this study, even with booster immunization. Thus, increasing phage dose should be explored as a way to improve the vaccine efficacy.

Statistically significant decrease in testosterone was only present at one time point in Group 3 cats when analyzed excluding the outlier, cat 3.1. However, testosterone was only analyzed at one time point for each month from each cat, and compared to the average of three pre-immunization time points one week apart. Testosterone secretion by Leydig cells, in response to a pulsatile episode of elevated LH, is also pulsatile [45]. Thus, serum testosterone concentrations can vary significantly over the course of even a single day, making it difficult to assess potentially subtle changes or trends in

testosterone. Testosterone was monitored in the case of substantial decreases that reached statistical significance.

A decrease in total testicular volume (TTV) was seen in Groups 1 and 3 and found to be statistically significant at multiple time points for Group 3. It is likely that the reduction in TTV in Group 1 was not significant due to the increase in TTV in cat 1.2. It is unknown why this increase occurred in one cat. It is possible that testicle size increased because the immunized cats were young and likely still growing. Although all measurements were carefully performed by a single researcher, we cannot rule out an error in the initial measurement on this cat. To prevent the possibility of an error in the initial measurement in subsequent cats, pre-immunization testicle size was measured on two separate occasions at least one week apart for all following groups of cats.

At this time, histological examination of the testes has not revealed a clear reason for the reduction in TTV seen in Group 1 and 3 cats. No biologically significant differences could be detected in the size of the testicular and epididymal tubules, thickness of spermatogenic epithelium in the testes, or density of sperm in the epididymial tubules. The only effect seen in the tissues from Group 3 cats that appears to be experimentally-induced (based upon occurrence in all Group 3 cats, and lack thereof in the control tissues) is mild vacuolation in the corpus of the epididymis. In some cases, these vacuoles appeared consistent with cribiform change, and in other instances appeared to be intracytoplasmic vacuoles. Intracytoplasmic vacuolation has been published as an effect of toxic compounds targeting the epididymis that induce phospholipidosis [64]. Testosterone deficiency may result in cribiform change associated with epithelial cell loss, as well as single cell necrosis of the epididymal epithelium and/or decreased tubular diameter [64]. Vacuolation is associated with epididymal cribiform hyperplasia (ECH), which has been associated with testicular atrophy [65].

Electroejaculation was the method of collecting sperm utilized in this study because training

male cats to ejaculate naturally into an artificial vagina (AV) may require months of conditioning and

training, and training may not be successful in all cats. EEJ consistently provides sperm for evaluation,

but it is known that the total sperm number obtained may vary with each collection attempt [60]. The

variable quantities of sperm obtained in this study are likely due to the method of collecting sperm. For

this reason, statistical analysis was not done on TSN, and focus was on quality of sperm produced by the

cats. Thirteen of the fifteen immunized cats (all except 3.1 and 3.2) showed a decrease in the

percentage of morphologically normal sperm that they produced. Cats 3.1 and 3.2 only had 9% normal

morphology prior to immunization, and normal morphology at the conclusion of the study for the two

cats was 9% and 12%, respectively, so there was not a substantial increase. Decreases in percentage of

normal morphology were significant for Groups 1 and 2. Teratospermia, defined as less than 40%

morphologically normal spermatozoa, is common in small populations of cats where inbreeding occurs,

such as the research colony where these cats were obtained [66]. In some species, morphology and

motility are often closely correlated and ejaculates with a low number of morphologically normal

spermatozoa also have poor motility. This is not observed in the cat, and teratospermic cats appear to

compensate for the lower number of morphologically normal sperm by increasing spermatogenesis and

frequency of copulatory activity [66, 67]. Morphology and motility did not appear to be correlated in this

study, as no significant decrease in progressive motility of spermatozoa was observed.

This is the first study testing a phage-based vaccine in cats, but there are several other studies

evaluating GnRH-based immunocontraceptive vaccines in cats, which were described in Section 1.2.4.

The immunization of 9-12 month old male cats with GonaCon led to 6/9 vaccinated cats responding to

the vaccine with undetectable serum testosterone, reduction in testicular volume, regression of penile

spines, and lack of production of motile sperm [33]. Although our study also resulted in high anti-GnRH

antibody titers, the results on reproductive parameters in our study were not as clear, as serum

testosterone was not consistently significantly decreased, penile spines were still present, and motile sperm was produced by all vaccinated cats.

Although the vaccine formulation tested in this study demonstrated potential for use for contraception of cats, the severity of the injection site reactions where phage-GnRH constructs were injected in conjunction with the AdjuVac adjuvant points to the need for use of a different adjuvant. The AdjuVac adjuvant has been used in cats in other studies. Levy *et al.* 2004 immunized 12 male cats with a GnRH/KLH/AdjuVac vaccine, followed them for six months after immunization, and did not report any site reactions to the vaccine [33]. Levy *et al.* 2011 immunized 15 female cats with the same vaccine and reported granulomatous injection sites in five of the cats [34]. The largest of these was reported to be 4 cm x 5 cm, and the size of the masses increased and decreased throughout the duration of the study [34]. Vansandt *et al.* 2017 utilized a slightly different vaccine formulation that also contained AdjuVac in six female cats and reported that four of them developed lesions at the site of immunization [68]. They immunized three cats once with the vaccine, and two developed masses at 18 days post-injection and 249 days post-injection, respectively. They also injected three additional cats twice (booster 60 days after primary immunization). Of those cats, two developed masses only at the site of the second injection, both within a month of booster immunization [68]. Fischer *et al.* 2018 immunized 20 female cats in a simulated colony environment with GonaCon and reported injection site reactions in 9 of those cats [35]. Those cats were reported to develop soft tissue masses or swelling near the site of the injection. Six of the masses developed between 1 and 2 months after immunization and resolved within 2-3 months after immunization. Reactions for three of the cats were first detected during or after 7 months post-immunization and persisted through the termination of the study [35].

Injection site reaction severity in our study varied significantly between cats. It is likely that the severity of the reaction is dependent on unknown factors particular to each individual cat. Higher antibody titers were not necessarily always associated with a more severe or prolonged injection site

reaction. Two cats, cats 1.2 and 3.4, had a mass become completely nonpalpable and then reappear later. The reappearance of the mass did coincide with increase in antibody titer. Cat 1.4 had among the lowest antibody levels achieved in the study but also had one of the most persistent, large reaction site masses. In a less formal estimation of mass size following the official conclusion of the experiment, the two cats in Group 3 that still had significant masses were littermates. In general, the masses were smaller and shorter-lived in the cats that received intramuscular injections, but the potential for interfering with quality of life was worse in that location and potential removal more complicated. The development of a draining lesion from one cat injected at this location was also concerning. Importantly, the injection of phage-GnRH alone did not cause severe reactions, indicating the safety of the use of phage alone in cats and caution when using adjuvants.
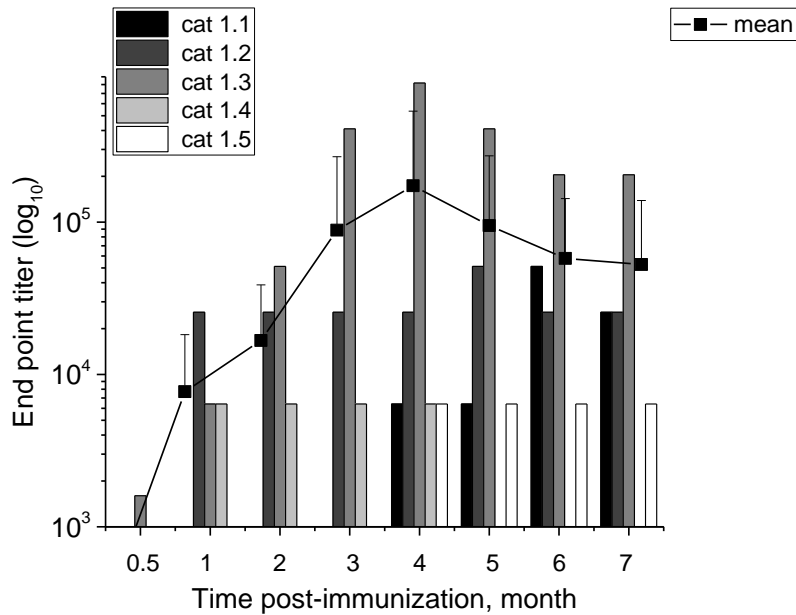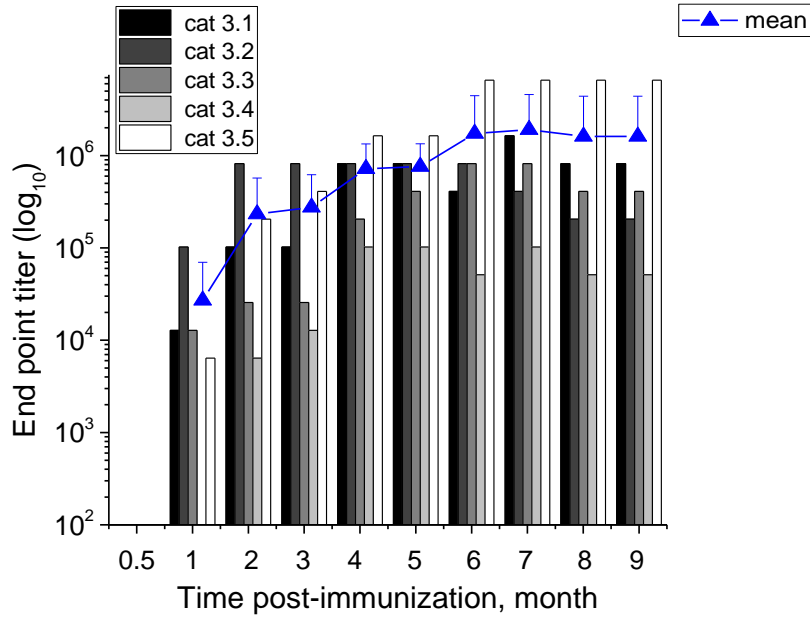
Figure 3.1: GnRH Antibody responses in sera collected from Group 1 cats (by ELISA). Cats were inoculated with GnRH-like (EHPSYGLA) phage at one time point (week 0) via subcutaneous administration in shoulder. Phage dose was $4x10^{12}$ vir/site (two injection sites). The data is presented as endpoint titers, which were determined at two weeks after immunization and monthly thereafter. Cat serum was diluted 1:100 and then tested at two-fold dilutions to endpoint titers. The measurements were performed in duplicate. Different shaded bars represent individual cats and the horizontal line with squares represents the group mean.
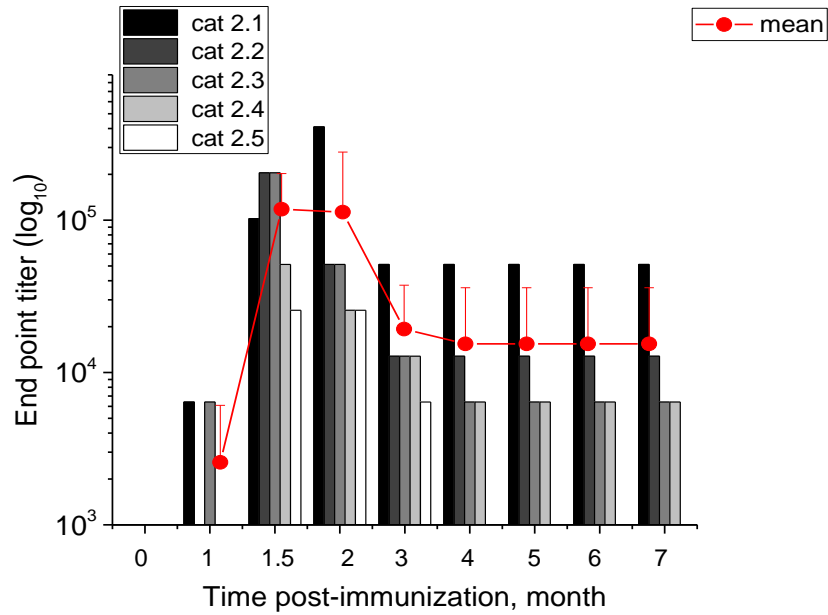
Figure 3.2: GnRH antibody responses in sera collected from Group 2 cats (by ELISA). Cats were inoculated with GnRH-like (EHPSYGLA) phage at two time points (week 0 and 4) via subcutaneous administration in shoulder. Phage dose for prime immunization was $2x10^{13}$ vir/site (two injection sites). Phage dose for booster immunization was $1x10^{13}$ vir/site (two injection sites). The data is presented as endpoint titers. Cat serum was diluted 1:100 and then tested at two-fold dilutions to endpoint titers. The measurements were performed in duplicate. Different shaded bars represent individual cats and the horizontal line with circles represents the group mean.

Figure 3.3: GnRH antibody responses in sera collected from Group 3 cats (by ELISA). Cats were inoculated with GnRH-like (EHPSYGLA) phage at three time points (week 0, 12, and 24) via intramuscular administration. Phage dose for prime immunization was 4×10^{12} vir/site (two injection sites). Phage dose for the first booster immunization was 4×10^{12} vir/site (one injection site). Phage dose for the second booster immunization was 4×10^{12} vir/site (one injection site). The data is presented as endpoint titers, which were determined at two weeks after immunization and monthly thereafter. Cat serum was diluted 1:100 and then tested at two-fold dilutions to endpoint titers. The measurements were performed in duplicate. Different shaded bars represent individual cats and horizontal line with triangles represents the group mean.
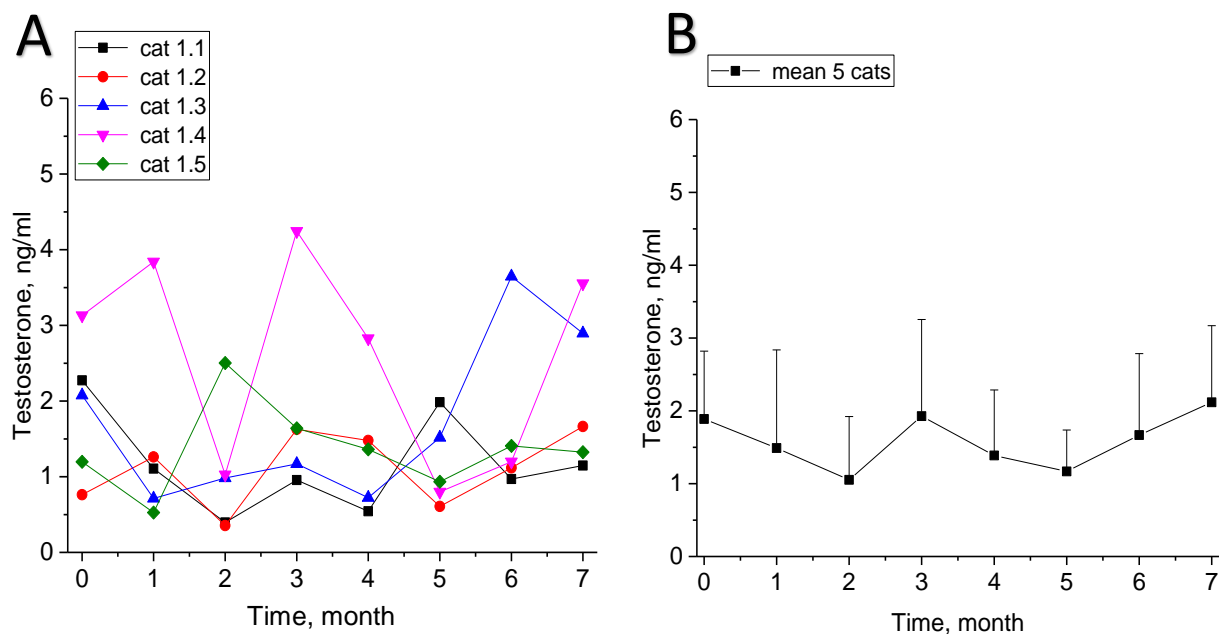
Figure 3.4: Testosterone in sera collected from Group 1 cats. Sera were collected before immunization and monthly for the duration of the study period after immunization. Serum samples were evaluated using ELISA and performed in duplicate. (A) The individual responses of each cat in the group, where each line represents an individual cat's testosterone levels each month throughout the study. (B) The mean serum testosterone for the five cats in Group 1 monthly throughout the study. Data are presented as group means ± SD.
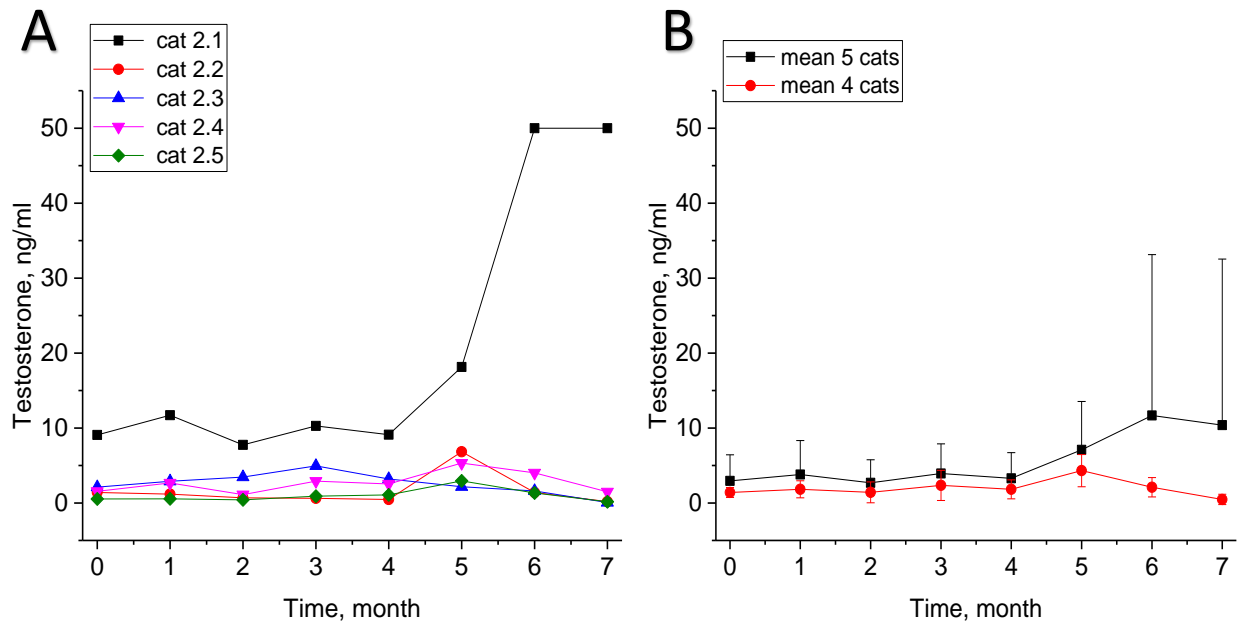
Figure 3.5: Testosterone in sera collected from Group 2 cats. Sera were collected before immunization and monthly for the duration of the study period after immunization. Serum samples were evaluated using ELISA and performed in duplicate. (A) The individual responses of each cat in the group, where each line represents an individual cat's testosterone levels each month throughout the study. (B) The group mean for all five cats (black line) and the group mean for four cats excluding cat 2.1 (red line). Data are presented as group means ± SD.
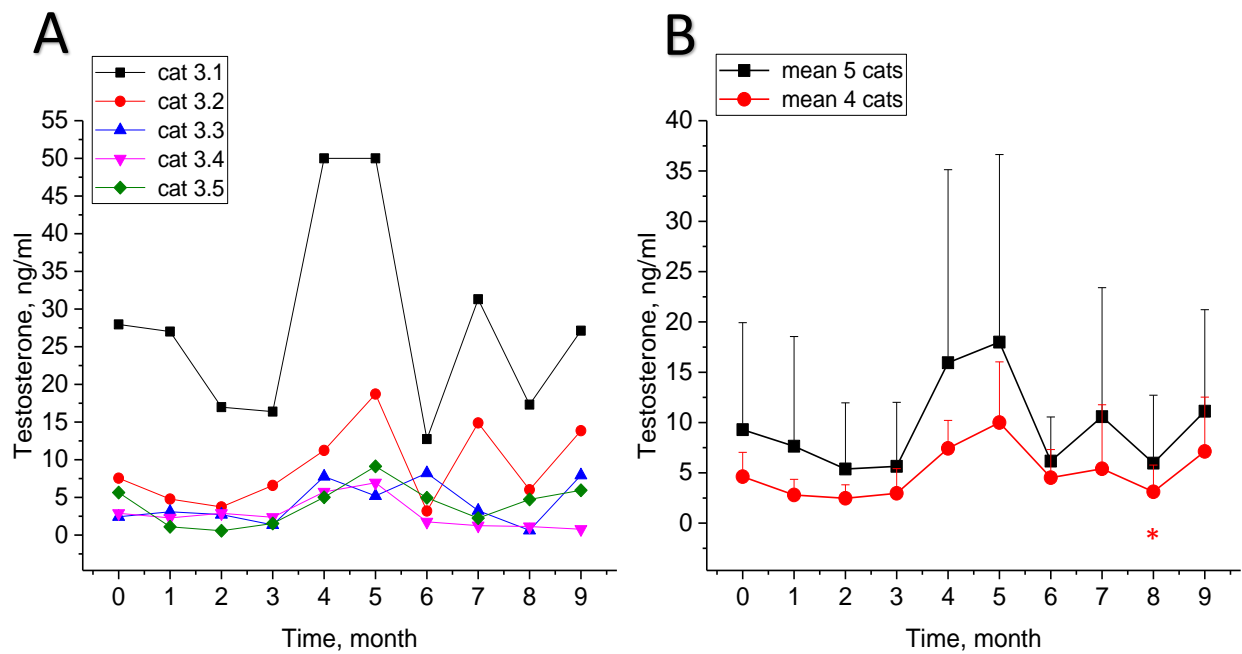
Figure 3.6: Testosterone in sera collected from Group 3 cats. Sera were collected before immunization and monthly for the duration of the study period after immunization. Serum samples were evaluated using ELISA and performed in duplicate. (A) The individual responses of each cat in the group, where each line represents an individual cat's testosterone levels each month throughout the study. (B) The group mean for all five cats (black line) and the group mean for four cats excluding cat 3.1 (red line). Data are presented as group means ± SD. Asterisk denotes P < 0.05.
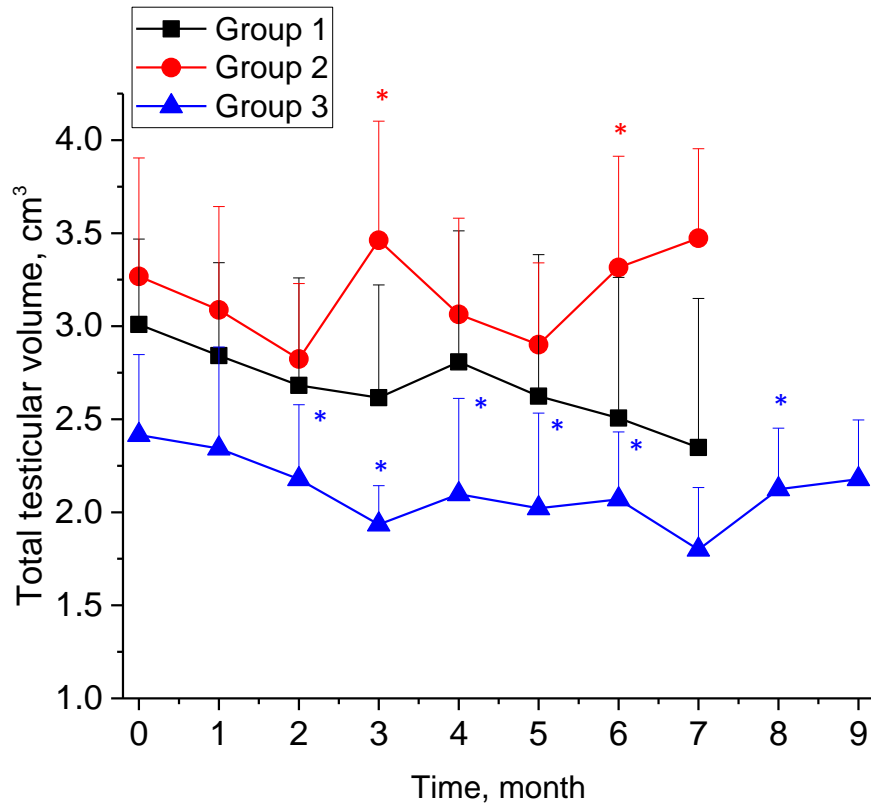
Figure 3.7: Total testicular volume in cats immunized with phage-GnRH. Measurement of testicular size was performed on each cat monthly. Testicular size was measured via scrotal ultrasound. The procedure was performed by placing an ultrasound probe on the external surface of the scrotum and performing duplicate measurements of the length, width, and height of each testicle. Testicular volume was calculated by multiplying the length x width x height x 0.5233, and the total testicular volume for each cat was found by adding the volume of both testicles. Data are presented as group means ± SD where each line represents the mean for each treatment group. $P < 0.05$ is noted with an asterisk.
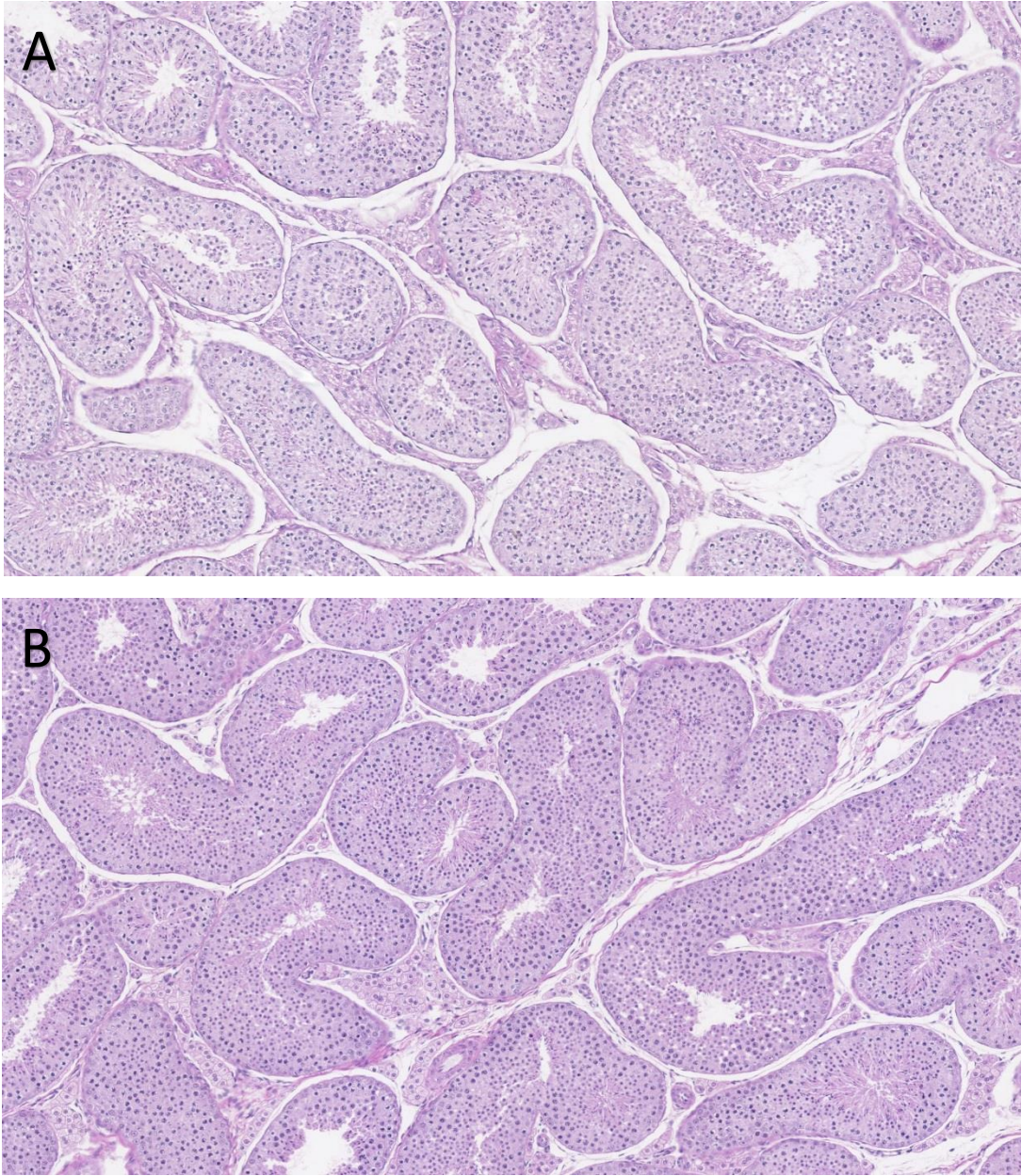
Figure 3.8: Histological examination of the testes. Selected scanned slides are shown at 10x from (A) treated cat (cat 3.1), and (B) an untreated, age-matched cat from the same colony. Cat 3.1 had some mild degeneration of tubules and some sloughed cells were seen. There was no significant difference in tubule diameter between the treated and untreated cats, and dilatation of the tubules is present in both.
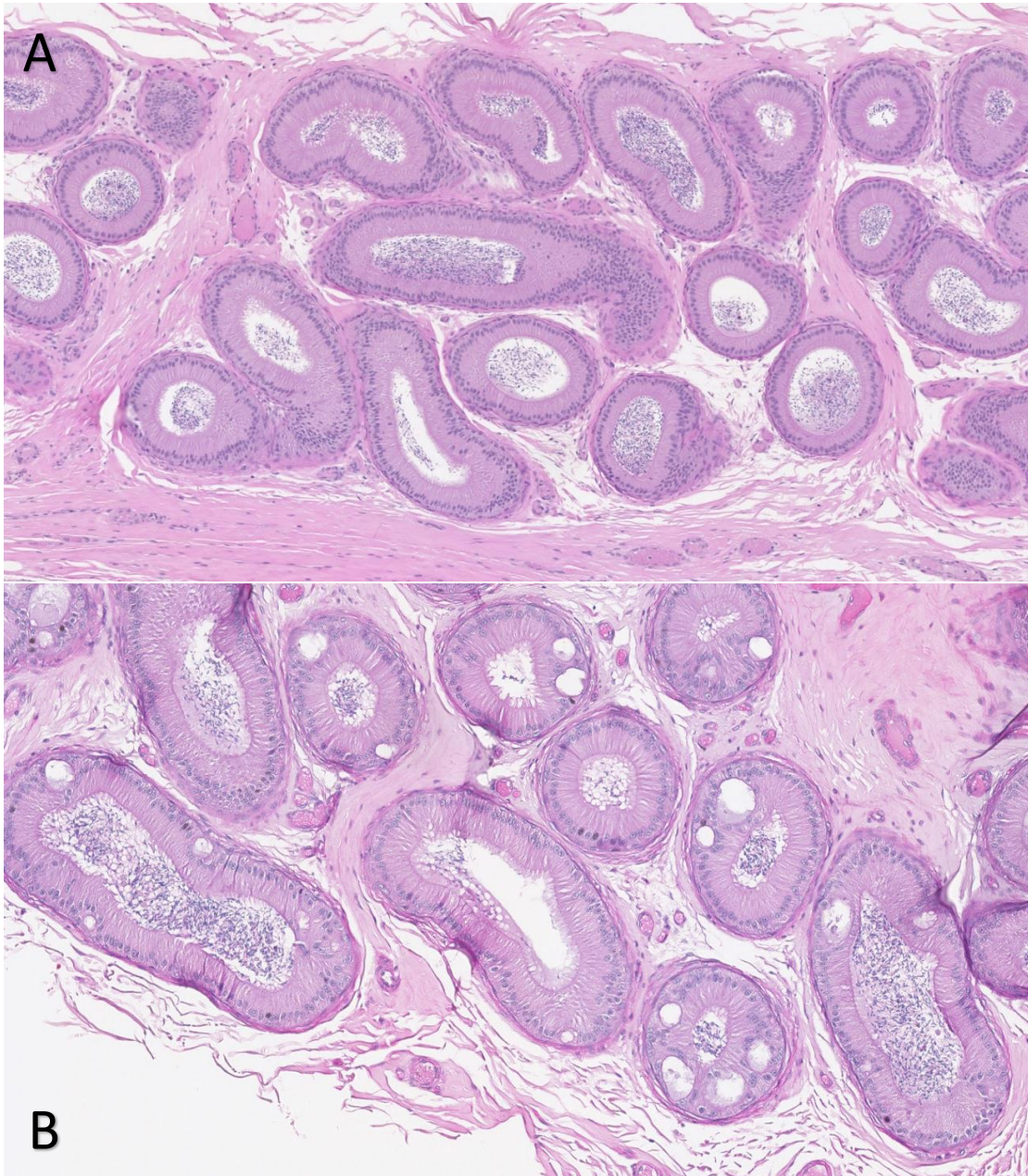
Figure 3.9: Histological examination of epididymis. Representative scanned slides from untreated (A) and treated (B) cats are shown at 10x magnification. Note the vacuolation of the corpus epithelium present in the treated cat (cat 3.2). The untreated cat for comparison was an age-matched control from the same colony.
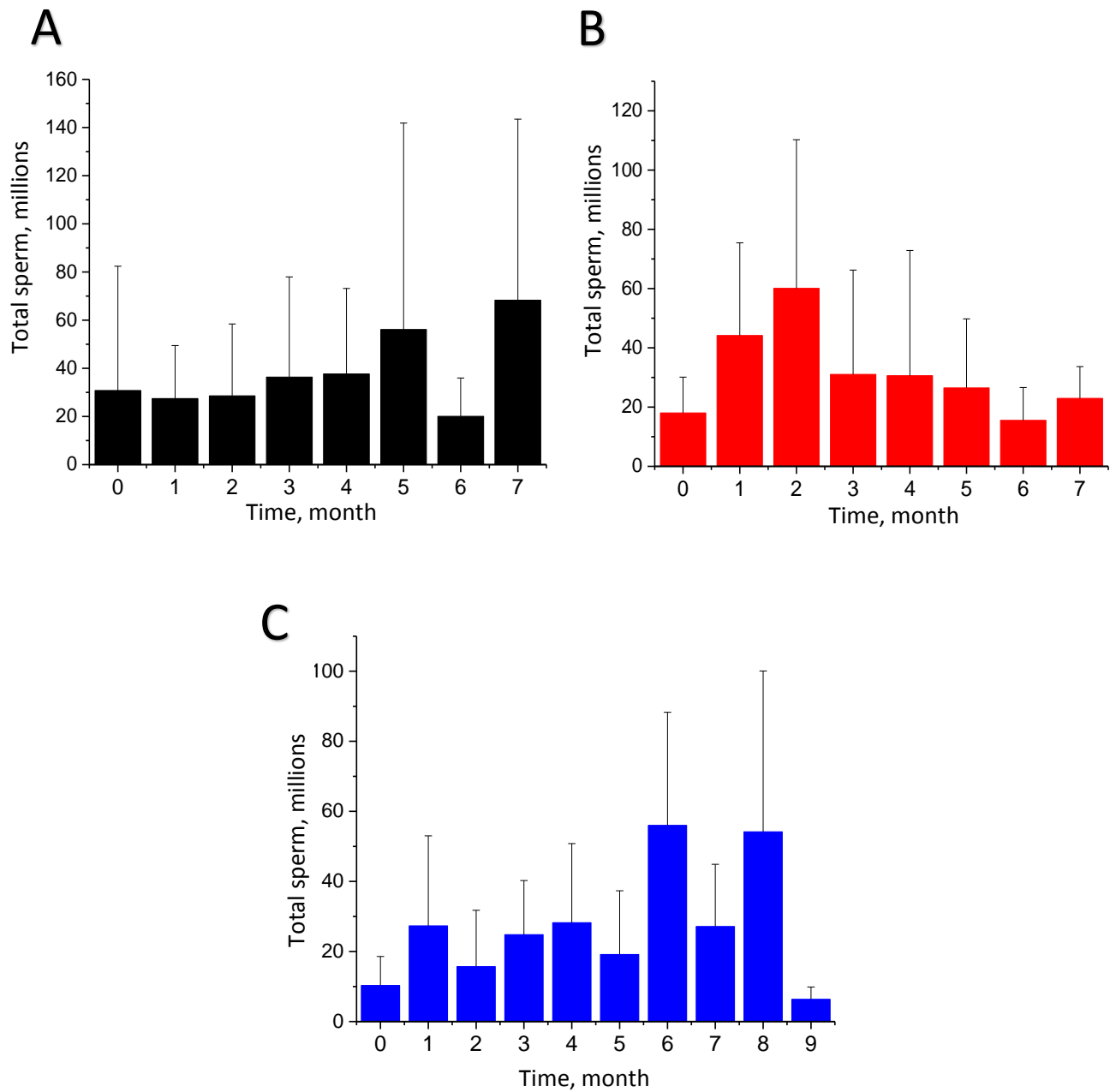
Figure 3.10: Total sperm number collected from cats immunized with phage-GnRH. Semen collection was accomplished by electroejaculation under general anesthesia. Total volume of the collection was measured using a micropipette and the concentration was measured using a nucleocounter. The volume and concentration were then used to calculate the total sperm number (TSN) for each monthly collection. The mean number of sperm cells in millions for each group ± SD is shown. (A) Group 1. (B) Group 2. (C) Group 3.
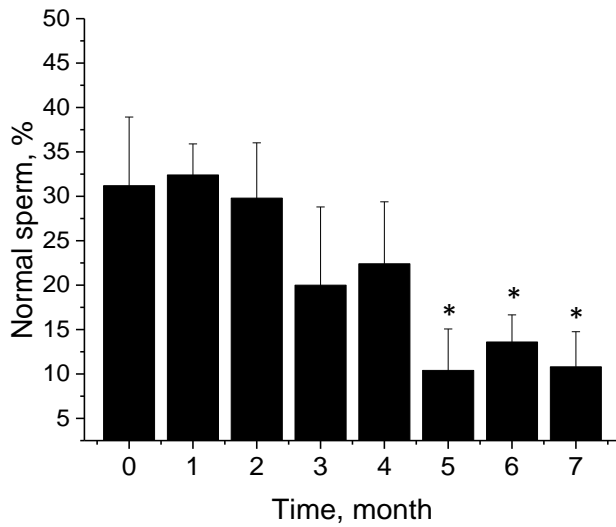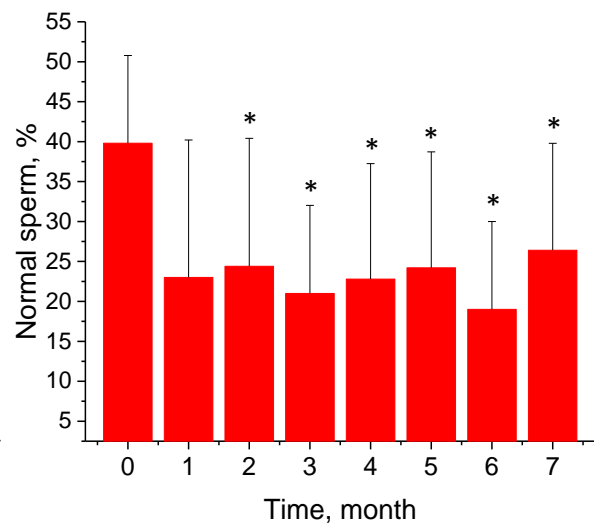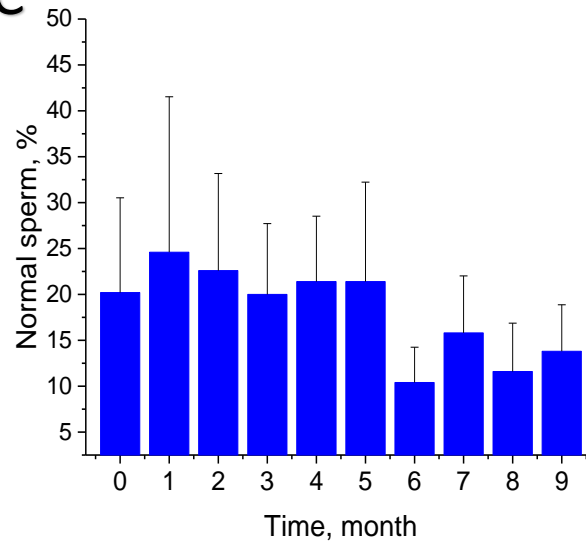
Figure 3.11: Relative quantity of morphologically normal sperm cells in cats immunized with phage-GnRH. Semen collection was accomplished by electroejaculation under general anesthesia. The morphological analysis was performed using eosin-nigrin stain and evaluating 100 cells for defects. The mean relative number (%) of normal cells for each group ± SD is shown. Asterisk denotes P < 0.05. (A) Group 1. (B) Group 2. (C) Group 3.

Figure 3.12. Representative sperm abnormalities in semen samples. Common morphological defects observed included coiled or bent tails, abnormal midpieces, and abnormal heads.
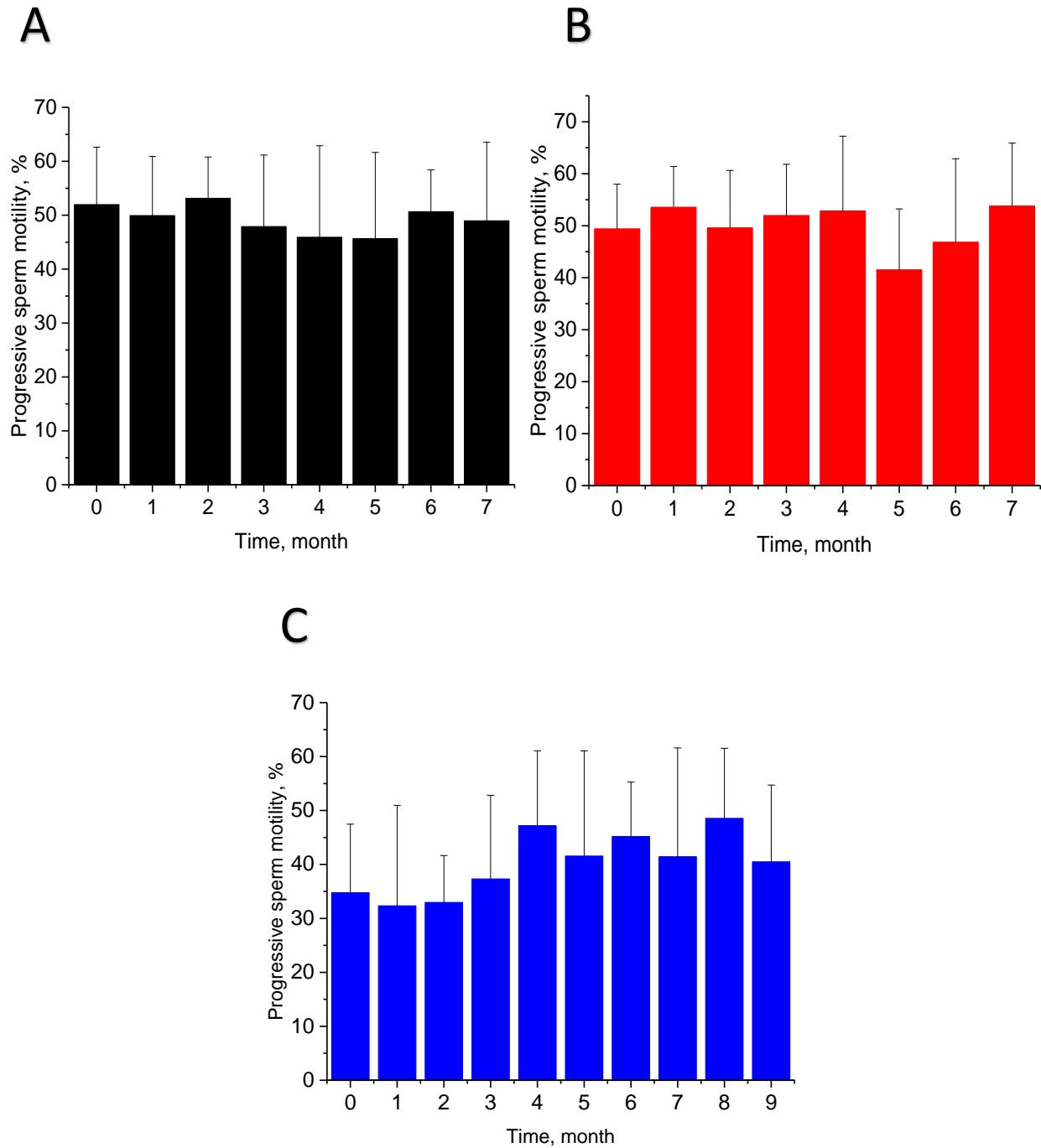
Figure 3.13: The progressive sperm motility of cats immunized with phage-GnRH. Sperm collection was accomplished by electroejaculation under general anesthesia. Computer-assisted-sperm-analysis (CASA) software was used to evaluate the motility of each sample. The mean relative number (%) of progressively motile cells for each group ± SD is shown. (A) Group 1. (B) Group 2. (C) Group 3.
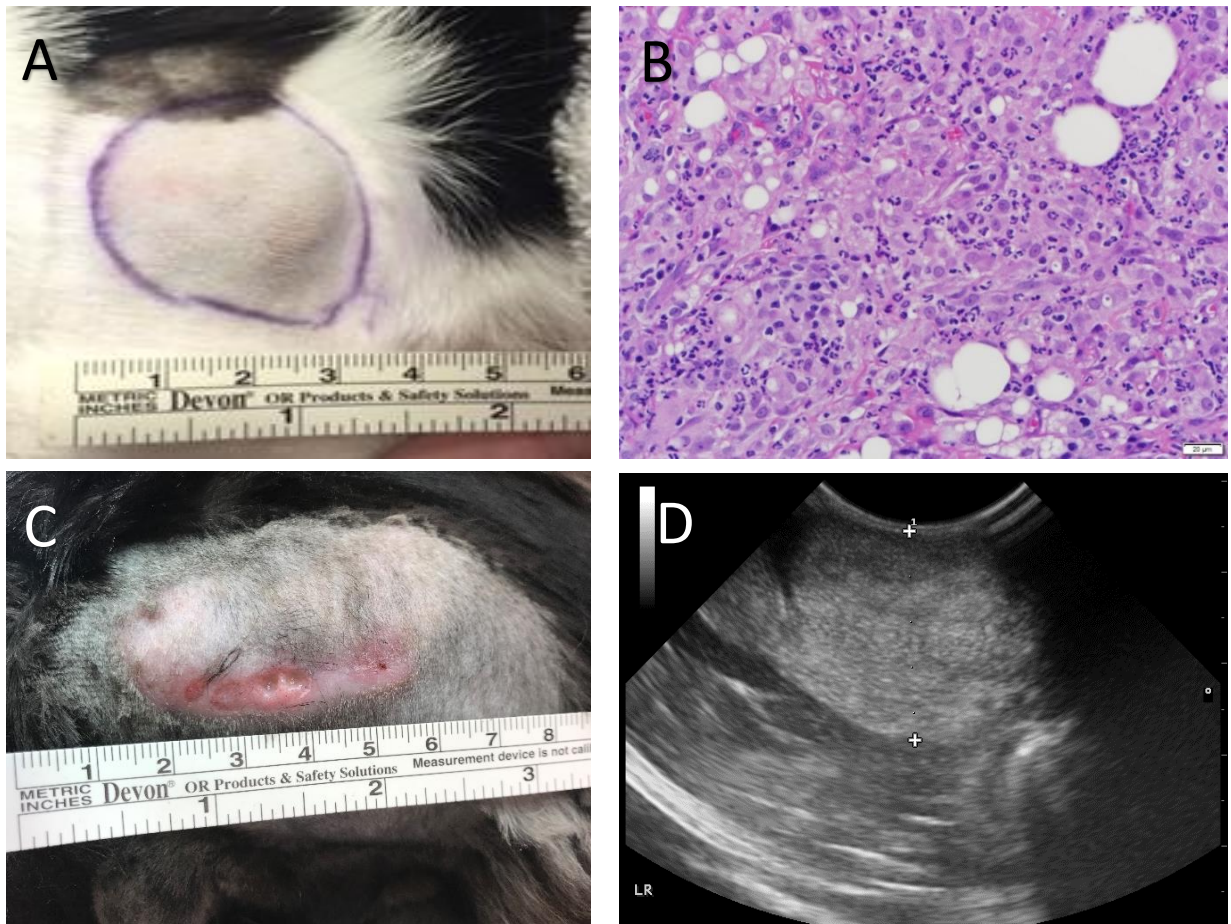
Figure 3.14: Injection site reaction in cats immunized with phage-GnRH combined with AdjuVac. (A) Physical appearance of injection site in cat 1.3, representative of the masses that developed in Group 1 cats (subcutaneous immunization). (B) Representative histological image of soft tissue masses in Group 1 (from cat 1.3) biopsied from the injection site. (C) Physical appearance of injection site in cat 3.1 (Group 3, intramuscular injection) after development of a draining lesion. The reaction appeared at the site of the first booster. This was the worst reaction of all cats in Group 3; no other masses developed into draining lesions. (D) Ultrasound image of the injection site mass from primary immunization in cat 3.1. The mass is characterized by hyperechogenicity (area between two crosses).

Table 3.1: Injection site reactions at the site(s) of immunization using AdjuVac adjuvant for cats in groups 1 and 3. Individual cats are listed on the left side of the table. Size of masses was estimated for each month of the experiment. Group 1 cats received only a single injection containing AdjuVac on their left side. Group 3 cats received AdjuVac on the left side at the time of primary immunization and received a booster vaccination containing AdjuVac adjuvant three months after primary immunization.

Cats		*Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
** Group 1										
1.1		+ + +	+ + +	+ + +	+ + + +	±	±	±		
1.2		+ + + +	±	-	-	+ + + + +	+ + + + +	+ +		
1.3		+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
1.4		+ + +	+ + + + +	+ + + +	+ + + + +	+ + + + +	+ + + + +	+ + + +		
1.5		+ + +	+ + + + +	+ + + +	±	±	±	±		
*** Group 3										
3.1	left	+ +	+ + + + +	+ + + + +	+ + +	+ + +	+ + +	+ + + +	+ + + +	+ + +
	right				+ + +	+ + + +	+ + + +	+ + +	+ + + +	+ + + + DL
3.2	left	-	-	-	-	-	-	-	-	-
	right				+ +	+ +	+	±	-	-
3.3	left	+	+ +	-	-	-	-	-	-	-
	right				+	-	-	-	-	-
3.4	left	+	-	-	±	-	-	-	-	-
	right				+	-	-	+	+ +	+ + +
3.5	left	+ + + + +	+ + +	+ +	+ +	±	-	-	-	-
	right				+ + +	+ + +	+	-	-	-

* Months are numbered relative to primary immunization with AdjuVac.
** Group 1 received one injection that contained AdjuVac adjuvant. Duration of experiment was 7 months.
*** Group 3 received two injections that contained AdjuVac: primary (left leg) and booster (right leg, at month 3).
 Longest dimension of mass: + = ≤ 1 cm, + + = 1.1-2.9 cm, + + + = 3-4.4 cm, + + + + = 4.5-5.9 cm, + + + + + = ≥ 6 cm; ± = thickening present but not defined mass; - = no mass palpable; DL = draining lesion.

CONCLUSIONS

The goal of this study was to evaluate a phage-based vaccine against GnRH (previously developed and tested in mice) for its potential to suppress reproductive characteristics in cats, one of the target species for animal fertility control. The vaccine and vaccination regimen parameters that were tested included phage dose, number of immunizations, use of adjuvants, and delivery routes. Based on the obtained data, the following major conclusions have been outlined. Independently from the vaccination regimens, all cats immunized with phage-GnRH vaccine developed antibodies against GnRH. Out of the 15 total experimental cats, 13 cats had detectable GnRH antibodies in serum at the conclusion of the study (seven months post-immunization for Groups 1 and 2 and nine months post-immunization for Group 3). The antibodies were detectable in some cats as soon as two weeks after immunization and in the majority of cats one month after immunization. Although the vaccine stimulated production of GnRH antibodies in all immunized cats, their level and duration varied significantly within each group as well as between groups. The immunization regimen specific for each cat group appeared to be very important. Cats immunized with higher phage doses produced higher antibody responses. Booster immunizations increased antibody responses significantly. The cats in Group 3, who received two booster vaccinations three months apart, achieved the overall highest level of antibodies and all had detectable antibodies at the conclusion of the study. The antibody response occurred the fastest when cats were injected subcutaneously with phage adjuvanted with AdjuVac. AdjuVac stimulated significantly higher GnRH antibody responses and supported their high-level presence in cat sera for extended time periods as compared to cats immunized without the adjuvant.

Total testicular volume (TTV) decreased in the treatment groups that received phage adjuvanted with

AdjuVac. Although all cats still produced sperm at the end of the study, the percentage of

morphologically normal sperm decreased in all cat groups. The changes observed were temporary and

the permanent sterilization of cats was not achieved under the study conditions. To further refine

immunization regimens, it is recommended that certain parameters are optimized. (1) Phage dose.

Phage doses of at least $5x10^{13}$ vir/cat are recommended since lower doses did not achieve sterilization

in our study. (2) Number and frequency of booster immunizations. Although a single injection

contraceptive vaccine would be ideal for feral cats, there are still potential applications for a vaccine

that requires a booster to achieve permanent sterilization in pet or captive wild animals. Multiple or

continuous injections will likely be required to achieve long-lasting or permanent infertility. (3) Phage

delivery routes. The first three months post-immunization for Groups 1 and 3 provide a direct

comparison of subcutaneous injection (Group 1) vs. intramuscular injection (Group 3) as that was the

only difference between the two groups. Overall, antibody response for the first three months was

higher and more consistent for group 3, indicating that intramuscular may be the better route for

immunization. (4) Type of adjuvant. The addition of adjuvant to the vaccine definitively prolonged and

increased antibody response. The AdjuVac adjuvant used in this study in combination with phage-GnRH

produced more severe reactions in cats than were indicated by the existing body of literature. The

severity of the reactions contraindicates the use of the AdjuVac adjuvant in combination with phage-

GnRH for future studies in cats. The Thermogel adjuvant used in this study seemed to be well tolerated,

but was only tested as a booster and not as a single immunization. Immunization with phage-GnRH

alone did not cause severe reactions and is concluded to be safe for cats by itself. Testing of novel

adjuvants that are more potent and well tolerated by target animals is recommended. (5) Age of study

animals. It is recommended to study the vaccine in cats of different age groups since the vaccine may be

more effective in younger animals that are not yet sexually mature. To summarize, the phage-based

vaccine against GnRH evaluated in this study demonstrated a potential for fertility impairment in cats. Future research is required to optimize the vaccine regimens and to identify animal age groups that are most responsive to the vaccine. If permanent contraception (highly desirable in feral cats) cannot be achieved, the vaccine has a potential to be used in zoo animals where the vast majority requires temporary and reversible contraception. Since GnRH peptide is conserved in all mammals, contraceptive vaccines against GnRH might be effective in multiple animal species. There is also potential for this vaccine to be used in some groups of owned domestic animals, such as pedigree or show animals, where reversible contraception may be desirable and booster immunization is feasible.

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