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FIELD EVALUATION OF THE IMMUNOCONTRACEPTIVE GONACONTM IN REDUCING EASTERN GRAY SQUIRREL FECUNDITY IN URBAN AREAS

Murali Pai
Clemson University, mpai@clemson.edu

FIELD EVALUATION OF THE IMMUNOCONTRACEPTIVE GONACON™ IN REDUCING EASTERN GRAY SQUIRREL FECUNDITY IN URBAN AREAS

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Murali Pai
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Accepted by:
Greg K. Yarrow, Committee Chair
William C. Bridges
William W. Bowerman
David W. Tonkyn
Christopher J. Post

ABSTRACT

The purpose of this study was to evaluate the immunocontraceptive GonaCon™ in reducing fecundity in Eastern gray squirrel (EGS) (*Sciurus carolinensis*) in urban areas. Using a modified box trap design, 317 EGS were captured during four trapping sessions on a 5.66 ha site on Clemson University's main campus. EGS were handled using a restraint cone and sexed, weighed, ear-tagged and implanted with a microchip at the nape of the neck on all "original" captures and later identified in subsequent captures as "recaptures." Blood samples and morphometric data were obtained on EGS before the immunocontraceptive GonaCon™ was administered by injection during three trapping sessions to 33 EGS (17m, 16f) in trapping session 1 (TS1), 23 (14m, 9f) in trapping session 2 (TS2), and 11 (8m, 3f) in trapping session 3 (TS3) at a dosage rate of 0.4 ml containing 400 µg of GnRH-blue protein conjugate intramuscularly in the thigh. Control EGS were given a sham injection containing 0.4 ml saline- AdjuVac™ during the three trapping sessions: 22 EGS (16m, 6f) in TS1, 20 (12m, 8f) in TS2, and 8 (4m, 4f) in TS3. In the last trapping session (TS4) 35 EGS were necropsied to evaluate histological changes in testes and ovaries as potential metrics of GonaCon™ efficacy and to determine its potential side effects.

EGS density on the study area was estimated to be 9 ± (2.89) EGS/ha, based on the Lincoln-Peterson model. There were no significant differences in body weights of treated and control EGS by TS3 ($p = 0.40$), or testosterone (p

= 0.32) and progesterone (p = 0.68) levels. However, there were significant differences in antibody titers between treated and control EGS by TS3 in both males and females active antibodies seen in the treatment group ($\chi 2 = 5.656$, df = 1, p = 0.017). There were highly significant differences in scrotal size of treated and control males with a reduction in scrotal size being observed in treated males (t= 10.14, df = 8, p = 0.001). There were marked histological changes in treated EGS males and no observable histological changes in treated EGS females. Although there were no serious side effects to the vaccine; 6 EGS developed injection site abscesses. GonaCon™ may be a potential tool to manage EGS overabundance in urban areas, but additional research is needed.

DEDICATION

To my wife, Neena

my sons; Nakhul and Shravan

my mother; and in memory of my father

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I am grateful to Dr. Greg K. Yarrow, my major professor and committee Chair, for being the first to make this a reality. His vision, wit, and wisdom are surpassed only by his patience. I thank the members of my committee - Dr. William C. Bridges, Dr. William W. Bowerman, Dr. David W. Tonkyn and Dr. Christopher J. Post. They gave me much support and guidance during my graduate study.

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One other person uniquely shared the mid-life burst of ambition that brought me to Clemson University. My wife Neena continues to not only endure my long absences from home, but also raises my two sons in my absence. My journey of ten thousand miles from Mangalore to Clemson was a result of the initiative taken by family and friends in India. My brother Madhu not only nudged me in the right direction, but also gave generous financial support. Bhanu and

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CHAPTER ONE

IMMUNOCONTRACEPTION AS A TOOL TO MANAGE OVERABUNDANCE OF WILDLIFE POPULATIONS

One of the many spin-offs of a burgeoning human population is the shrinkage of wildlife habitats and the attendant overabundance of native species, or the invasion of introduced exotic species. This in turn shapes human tolerance of the presence and activities of free-ranging animals. The overabundances of native species with its negative ecological implications, and the invasion of exotic species with the consequent threat to native biota, are two faces of the same coin (Garrott et al. 1993). The Eastern gray squirrel (*Sciurus carolinensis*) is a prime example of both of these scenarios. This species not only thrives well in its native range in the United States, but it is also considered invasive in England, because it has displaced and almost taken over the range of the endemic red squirrel (*Sciurus vulgaris*). The crux of the invasion process occurs in six stages: introduction, establishment, naturalization, dispersal, population distribution, and invasive spread (Henderson et al. 2006). In essence, overabundance can be insidious, and such animals are often categorized as nuisance wildlife.

Inferences drawn from invasion biology (Davis 2009) can be applied to overabundance:

- Virtually all natural environments are prone to species invasion to some degree;

- The most reliable predictor of species invasion is resource availability, with both temporal and spatial variation in resources shown to be the primary mechanisms by which pools of resources are made available to incoming species;
- Enemy and facilitator-related processes can be important in accounting for invasion in some instances, but neither has proven to be as reliable a predictor of species invasion as resource availability;
- Diversity has not been shown to be a reliable predictor of species invasion under natural conditions at any spatial scale; and
- The same processes affecting species invasion are driving diversity.

On another level, wildlife can be construed to be locally overabundant when in fact they may be threatened or endangered. For example, there has been an increase in the population densities of the African elephant (*Loxodonta africana*) in many of its range countries due to the control of poaching and a ban on ivory trade. As another example, the common leopard (*Panthera pardus*) has taken refuge in tea gardens of Assam, India, and breeds rather well in this alternate habitat. African elephants and leopards are both endangered, yet overabundant in parts of Africa and India.

According to Caughley (1981), the criteria for overabundance of animals can be based upon the degree in which they:

- Threaten human life or livelihood,
- Are too numerous for their “own good,”

- Depress the densities of economically or aesthetically important species, and
- Cause ecosystem dysfunction.

The risk of zoonotic diseases such as avian influenza, rabies and tuberculosis gets compounded in the event of species overabundance. The influenza virus is now known to jump between 3 taxonomic groups: avian, human and swine. The overabundance of all the 3, in part, has resulted in the ongoing pandemic. The intra-specific spread of diseases among wildlife populations is no less daunting, since many species in some areas exceed their carrying capacity (K). Two-thirds of the one-horned rhinoceros (*Rhinoceros unicornis*) in the world survive in the grasslands of Kaziranga in India; more than 2000 rhinos in only 430 km^2 of the national park. The risk of having "all eggs in one basket" is accentuated by overabundance. There is also a glut of many captive wildlife species in zoos across the world, leading to constraints in housing and captive breeding of endangered species.

Native and exotic species alike, can impact human health, national and local economies, and ecosystems and ecosystem services (Davis 2009). Ecosystem services are defined as the conditions and processes through which natural ecosystems, and the species that make them up, sustain and fulfill human life (Daily 1997). Overabundance of free-ranging wildlife populations interferes with ecosystem services, and therefore is an important driver of human wildlife conflicts. The newly emerged discipline of wildlife damage management

is primarily concerned with the challenges of controlling overabundant species and their negative impacts. However, a diagnosis of wildlife overabundance can only be made when placed in a specific context (McShea et al. 1997).

There are many examples where overabundant wildlife populations have created conflicts with humans and other wildlife. For example, white-tailed deer (*Odocoileus virginanus*), Canada geese (*Branta canadensis*), African elephants (*Loxodonta africana*), and rhesus macaques (*Macaca mulatta*) have become problem species on a community, and in some cases on a landscape scale. In North America, white-tailed deer populations in some areas became overabundant in the mid-seventies. Increasing suburbanization and concurrent decreases in agricultural land use created large areas of predator-scarce habitat (Diamond 1992, McCullough et al. 1997). In South Carolina, because of expanding populations, white-tailed deer have been reported to cause an estimated $52.4 million dollars in damage to agricultural production in one year (Smathers et al. 1994). Conover (et al. 1995) reported an estimated 29,000 human injuries and 211 human fatalities each year as a result of 726,000 deer-vehicle accidents annually in the U.S. In 1991, there was a conservative estimate of over 538,000 deer deaths in 36 states as a result of deer-vehicle collisions (Lehnert and Bissonette 1997).

Overabundance of Canada geese is well documented in many areas of the U.S., with geese- inflicted damage to grain and forage crops. In addition, the presence of feeding geese on lawns, in parks, on golf courses and in backyards

has sparked ire among many residents of several U.S. cities (Conover and Chasko 1985). In Ghana and Zimbabwe, the African elephant long afforded protection from poaching and overexploitation, has created new conflicts. Elephant densities have swelled to such an extent that they freely roam communal lands, depredate crops and compete for scarce water resources (Lamarque et al. 2005). India has an overabundance of rhesus macaques and laws banning their export (Mandavalli 2006). This has led to high conflict levels in urban areas in recent times. In Italy, gray squirrel (*Sciurus carolinensis*) populations introduced to a broad-leaf forest patch in 1970, are now overabundant and likely to spread throughout Europe and Asia (Bertolino and Genovesi 2003).

Management programs that address conflicts caused by wildlife overabundance have four parts that include 1) problem identification, 2) ecology of the problem species, 3) application of control methods, and 4) evaluation of control efforts (Dolbeer et al. 1994). Problem definition refers to determining the species and numbers of wildlife causing a particular problem, the amount of loss or nature of the conflict, and other biological and social factors related to the problem. Understanding the ecology and life history of a species is important in wildlife damage management, especially in context of understanding cause of conflicts and potential solutions. Application of control methods utilizes an understanding of the ecology of a particular species to develop an appropriate management program to reduce conflict(s). Evaluation of control efforts helps to

assess the effectiveness of control methods in reducing or eliminating conflicts in a safe, humane, cost-effective, and socially acceptable manner.

Traditional techniques that have been used to reduce negative impacts associated with wildlife overabundance include 1) excluding problem wildlife, 2) habitat modification, 3) frightening problem wildlife, 4) repelling problem wildlife, 5) live trapping and removal of problem wildlife, 6) lethal methods, or 7) a combination of the above techniques (Hygnstrom et al. 1994).

Exclusion involves keeping problem wildlife out of an area using physical barriers. Examples include woven wire fences, electric fences, or any other barrier that prevents entry or access into an area that needs protection (Hygnstrom et al. 1994). For example, physical barriers have been used by land managers and conservation agencies in Australia to exclude feral cats (*Felis cattus*), red fox (*Vulpes vulpes*) and European rabbits (*Oryctolagus cuniculus*) (Moseby and Read 2006). All three species were introduced to Australia by Europeans and have successfully colonized much of mainland Australia. Fences are typically installed to reduce white-tailed deer damage in the U.S. and materials may include wire or plastic mesh, electrified high-tensile steel wire, and electrified polytape (VerCauteren et al. 2006)

Habitat modification as a means of reducing wildlife damage involves removing habitat components (e.g. food, shelter, and water) to make areas less desirable for problem wildlife species (Hygnstrom et al. 1994). For example, Canada geese require open areas to feed and can be discouraged by

landscaping an area with trees, bushes, hedges, boulders, or anything that geese would have difficulty seeing around. In addition, draining unwanted ponds that are adjacent to open areas (e.g. lawns) has been effective in making areas less inviting to geese (Conover 2002). In some cases, habitat modification may include enhancing habitat components in areas to attract wildlife away from sites that need protection. An example is planting food crops for white-tailed deer to "lure" them away from ornamental plantings and gardens in residential areas where they may cause damage.

Frightening can also be used to temporarily "scare" wildlife away from areas to reduce potential conflicts. A variety of frightening devices are used to move wildlife from local areas and include pyrotechnics, gas exploders, effigies, lights, lasers, reflectors, guard animals, bioacoustics and ultrasonic devices (Hygnstrom et al. 1994). These techniques are often more effective when used in an integrated system that incorporates multiple stimuli (Gilsdorf et al. 2002).

A variety of repellents have been used to keep wildlife away from protected areas. Repellents work by creating an aversion response based on taste, touch or smell (Hygnstrom et al. 1994). There are two general categories of repellents, primary and secondary. Primary repellents work with disruptive stimuli and affect normal behaviors of an animal. Secondary repellents work with aversive stimuli and affect occurrence of specific negative behaviors (Shivik et al. 2003). An example of a primary repellent is the use of predator odors, such as red fox or raccoon (*Procyon lotor*) urine smeared on butternuts, which repels

gray squirrels (Rosell 2001). An example of secondary repellents is the use of chili grease smeared on coir rope around the periphery of rice fields to deter elephants by conditioned taste aversion (Sitati and Walpole 2006).

Live-trapping involves removing problem wildlife by trapping and relocating to areas where conflicts are less likely to occur. There are pros and cons in trapping and relocating problem wildlife. For example, relocation of problem black bears (*Ursus americanus*) using culvert-traps to capture bears was used in northeastern Oregon as an alternative to lethal removal of bears that attacked sheep (Armistead et al. 1994). Although relocation costs did not differ from that of killing depredating bears, the former method had better social acceptance. Some state wildlife agencies (e.g. South Carolina), however, do not allow live-trapping and relocation of wildlife, especially problem wildlife. Concerns center around survivability of relocated wildlife, impacts of relocated wildlife on other species, and the potential for disease transmission (Cunningham 1996).

The use of lethal methods to control populations of overabundant wildlife involves killing animals by shooting, lethal traps, or poisoning. With game species, populations are regulated through recreational hunting. Public acceptance and stakeholder support of lethal methods to control wildlife overabundance is not universally accepted in some areas across the world. However, removal of some species using lethal means has a legitimate role in wildlife conservation (Treves and Naughton-Treves 2005). A case in point is the

hunting of white-tailed deer in North America, which provides recreation for sportsmen and revenue for conservation as well.

Several experimental approaches to address wildlife overabundance and associated conflicts are currently being tested and evaluated, one of which is the use of anti-fertility vaccines. The development of anti-fertility vaccines is an offshoot of similar technologies used in the prevention of infectious diseases (Tizard 2009). In one example, an antigen in the form of a protein, elicits an immune response when administered to a healthy animal, leading to the production of antibodies. This reaction is used to intercept critical steps in the production and secretion of sex hormones such as estrogen and testosterone. The net result is diminished reproduction or its cessation. This technology has been in vogue for several years and has been tested on a variety of species with varying success (Miller et al. 1998). Reversible sterilization of wildlife by holding them in permanent care facilities has proven to be a good management option for some species such as the African elephant and cheetah. However, the use of anti-fertility vaccines on free-ranging animals is often complicated by legal, biological, economic and ethical issues (Guynn 1993).

The goal of contraceptive vaccines can be categorized as either immunocontraception or immunoneutering. Immunocontraceptive vaccines aim to prevent fertilization of the oocyte by sperm or implantation of the fertilized egg while retaining sexual behavior patterns and competition in mating. Immunocontraception works on both sexes, but depending on the species, is

only used on one of the sexes. Immunocontraceptives (specifically GonaCon™) prevent production and/or maturation of gametes. This approach has gained acceptance by some for control of feral animal pests or native wildlife. Immunoneutering vaccines aim to prevent all sexual behaviors in both male and female animals, as well as control fertility. These outcomes are suitable for companion animals, livestock, and in some instances feral animals perceived as pests (Meeusen et al. 2007).

Antigens of the gametes (sperm and oocytes) have widely been targeted for prevention of fertilization in a variety of animals. A suite of over 20 sperm antigens have been identified and characterized, and may ultimately serve as potential vaccines in some animals. Most of these are surface proteins and include sperm antigens SP10, SP17, FA-1, LDH-C4, and PH-20 (Delves et al. 2002). However, side effects like autoimmune-mediated orchitis and lack of good results in contraception, have led to a focus on vaccinating the female with oocyte antigens.

Fertility levels in vaccinated females are generally reduced from levels of 75% to 80%, to levels of 25 to 30%, in a variety of species including mice (Lea et al. 2002), baboons (Stevens 1997), and guinea pigs (Tung et al. 1997). Among the oocyte antigens, a family of surface antigens from the zona pellucida (ZP) has been identified as providing effective immunocontraception (Meeusen et al. 2007). In 1988, Kirkpatrick et al. (1990) tried a failed human contraceptive called porcine zona pellucida (PZP) on wild horses on Assateague Island, off the

Maryland and Virginia coast in the U.S.. Zona pellucida proteins distilled from pig ovaries were injected into mares, and these foreign proteins prompted their immune systems to manufacture antibodies against the antigens. The antibodies latched onto the surface of newly ovulated mare oocytes, blocking sperm from entering and fertilizing the egg (Fox 2007). The vaccine was refined so that one inoculation rendered wild horses infertile for two years (Turner et al. 2007). SpayVac (Immuno Vaccine Technologies, Canada), a vaccine based on a crude PZP antigen preparation, has also been available for experimental wildlife population control.

The most studied and best characterized hormone used as a vaccine target has been luteinizing-hormone releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH). GnRH is the key hormone controlling reproductive function and development and is released from the hypothalamus. It is a simple 10-amino acid peptide that is found in all species of mammals, with variants identified in other organisms from lampreys to birds and fish. Immunoneutralization of this pivotal hormone of the pituitary-gonadal axis has been demonstrated to prevent reproductive function, provide contraception in mammals, control estrus behavior in females, and sexual aggression behavior in males (Meeusen et al. 2007). GonaCon™ is an example of an anti-GnRH vaccine developed by researchers at the USDA National Wildlife Research Center (NWRC) (Miller et al. 2004). In a recent (October 2009) and significant development, GonaCon™ was registered for use in white-tailed deer by the U.S.

Environmental Protection Agency (EPA), whose responsibility is to register products for use in wildlife. GonaCon™ is based on a peptide antigen mollusk hemocyanin carrier protein conjugate in an oil-based adjuvant (AdjuVac™). This formulation has the effect of making the skin of vaccinated animals test positive for *Mycobacterium avium*. The vaccine has proved to be effective in species such as deer (Gionfriddo et al. 2006), bison (Miller et al. 2004), wild horses (Killian et al. 2006), wild boar (Massei et al. 2008), feral cats (Levy et al. 2004), and California ground squirrels (Nash et al. 2004).

The goal of this research project study was to field test the vaccine GonaCon™ in Eastern gray squirrels (EGS). Specific objectives of the project were the following:

1. To evaluate the efficacy of GonaCon™ in reducing EGS fecundity in urban areas;
2. To determine appropriate metrics for evaluating the success of GonaCon™; and
3. To provide recommendations for the use of GonaCon™ as a potential tool for reducing EGS overabundance in urban areas.

CHAPTER TWO

FIELD TRIALS OF GONACON™ VACCINE IN EASTERN GRAY SQUIRRELS

Biology and Ecology of Eastern Gray Squirrels

The Eastern gray squirrel (*Sciurus carolinensis; Sciurus*, in the shadow of the tail*; carolinensis*, first identified in the Carolinas) is a common and prolific tree-dwelling rodent endemic to urban areas of the eastern United States (Thompson and Thompson 1980). It is one of several native species of tree squirrels found in North America (e.g. fox squirrel, *S.niger*; western gray squirrel, *S. griseus*; Abert's squirrel, *S. aberti*; Arizona gray squirrel, *S. arizonensis*; Mexican fox squirrel, *S. nayaritensis*; pine squirrel, *Tamisciurus hudsonicus*; and Douglas' squirrel, *T. douglasii*). The historic range of the Eastern gray squirrel (referred to in both singular and plural as EGS) was comprised of eastern North America, until its spread due to numerous introductions. Presently, outside of its historic range, the species is extant in California, Montana, Oregon, Oklahoma and Washington in the US. In North America it is also found in Quebec, New Brunswick, British Columbia, Manitoba, Nova Scotia, Ontario, and Saskatchewan in Canada (Robinson and Cowan 1954). EGS have also been introduced in Italy, England, Scotland, and Ireland (Lloyd 1983).

The EGS is a medium-sized tree squirrel that does not display sexual dimorphism in size or color (Koprowski 1994). Total body length is 380 – 525 mm and adult body mass ranges from 300 – 710 g (Schwartz and Schwartz 1981).

The dorsal pelage is dark to pale gray; the fur may be cinnamon colored on the hips, feet, and head; and the ventral side is white or gray, to buff or cinnamon (Flyger and Gates 1982). Ears are buff to gray or white, and the long, bushy tail is white to pale gray and 150 – 250 mm in length (Koprowski 1994). Both melanism and albinism are common in EGS (Steele and Koprowski 2001). The only natural sympatric congener of the EGS is the fox squirrel, which is 20% larger in body mass and brown to black in color (Koprowski 1994).

EGS prefer large tracts of dense, mature hardwoods, especially oaks (*Quercus* spp.) and hickories (*Carya* spp.), with an understory of smaller trees and shrubs. The fox squirrel, on the other hand, prefers open park-like stands of pine (*Pinus* spp.), mixed pine, and oak or oak-gum (*Nyssa* spp.) -cypress (*Taxodium* spp.) stands. Of the two, the EGS is by far the most abundant and it is numerous enough to manage for recreational hunting purposes (Yarrow and Yarrow 1999).

EGS feed heavily on nuts, flowers, and buds of nearly 24 oak species, 10 species of hickory and pecan (*Carya illinoensis*), walnuts (*Juglans* spp.), and American beech (*Fagus grandifolia*) when available (Nixon et al. 1968). Other important foods include fruits, seeds, buds, or flowers of maples (*Acer* spp.), mulberry (*Morus* spp.), hackberry (*Vaccinum* spp.), elms (*Ulmus* spp.), buckeye and horse chestnuts (*Aesculus* spp.), wild cherries (*Prunus* spp.), dogwoods (*Cornus* spp.), hawthorn (*Crataegus* spp.), black gum (*Nyssa sylvatica*), hazelnut (*Corylus* spp.), hop hornbeam (*Ostrya virginiana*), and gingko (*Gingko*

biloba) (Thompson and Thompson 1980). A variety of herbaceous species are also eaten; fungi are readily consumed in summer. Cultivated crops such as corn and wheat are also eaten. EGS are known to feed on insects, bones, bird eggs and nestlings, and frogs (Koprowski 1994). Cannibalism has also been reported (Holm 1976). Predators of EGS include rat snakes (*Elaphe* spp.); red-tailed (*Buteo jamaicensis*), red-shouldered (*Buteo lineatus*), marsh (*Circus cyaneus*), and Cooper's hawks (*Accipiter cooperii*); great horned (*Bubo virginianus*) and barred owls (*Strix varia)*; red (*Vulpes vulpes*) and gray (*Urocyon cinereoargenteus*) foxes; bobcats (*Felis rufus*); raccoons (*Procyon lotor*); house cats (*Felis catus*), and domestic dogs (*Canis lupus familiaris*). However, these predators do not limit EGS population growth in most areas. Since EGS habitat conditions are constantly in a state of flux, other factors regulate their reproductive rates. These factors make EGS population densities cyclical. Whenever an EGS population exceeds the carrying capacity of a forest stand, a mass movement and relocation of squirrels to other areas may take place. This exodus may involve thousands of EGS, many of which die during the journey (Yarrow and Yarrow 1999).

EGS typically have two breeding periods: the first between December and January, with litters produced between March and April (spring) (Gurnell 1983, 1987). The second breeding period is between May and June, with litters produced between July and August (summer) (Gurnell 1983, 1987). Female EGS can become sexually mature at 5.5 months of age (Smith and Barkalow 1967),

but most do not reproduce until after 1.25 years (Brauer and Dusing 1961). Gestation period is 44 days (Webley and Johnson 1983) and average litter size is 2 – 3 (Lurz et al. 2002). EGS may have 1, 2, or no litters during a single 10 – month period (Nixon and McClain 1975). Although males become sexually mature at 10 to 11 months of age (Kirkpatrick and Hoffman 1960), they undergo a semiannual cycle of testicular recrudescence and regression. This in turn impacts their sexual behavior and entire breeding seasons may sometimes be skipped (Webley et al. 1985). Spring-born males remain sexually active for 6–8 months, while summer-born males are sexually active for about 3 months. Both groups undergo sexual degeneration in the late summer months (Kirpatrick and Hoffman 1960). In the wild, EGS rarely live more than 6 years (Uhlig 1955), although their ecological longevity may be up to 9 years.

Problems Associated with Eastern Gray Squirrels

A tendency for wildlife species to show changes in their behavioral characteristics and population densities relative to urban areas is termed "synurbanization" (Parker and Nilon 2008). The EGS is a case in point. For instance, EGS in parks surrounded by greater levels of urbanization (more buildings and less trees) will exhibit higher population densities, increased rates of intraspecific aggression, increased activity levels, and reduced wariness (Parker and Nilon 2008). Consequently, high EGS densities often increase conflicts with humans as well as other wildlife.

The invasiveness and expansion of the EGS has caused problems to native fauna and humans in its extended range (Lurz et al. 2002, Lever 1994). Damage and death of hardwood trees by EGS, through bark stripping and gnawing, might be a result of territorial marking or agonistic gnawing behavior (Kenward and Parish 1986), and possibly also due to their high densities (Koprowski 2005). Densities of EGS are normally <3/ha in continuous woodlands (Barkalow et al. 1970), while EGS densities in small (<10 ha) woodlots can be 16/ha (Doebel and McGinnes 1974), and in urban parks can be > 21/ha (Manski et al. 1981**).**

EGS also impact the production of cash and orchard crops like walnuts, cherries, and pears, since they prefer to eat the nuts and fruits of these trees, as well as cache them. EGS are prone to travel power lines and short-out electrical transformers in urban areas causing power outages. They are known to enter buildings and houses, gnawing on electrical wires which increase the risks of fires, and build nests in attics destroying attic insulation. Other problems associated with high EGS densities include destruction of lawns associated with caching behavior; consumption of bird feeder food and damage to bird feeders; enlargement of bird house openings; predation on nestling songbirds; and damage to ornamental plants, planted seedlings, and fruits of planted shrubs and trees (Hygnstorm et al. 1994).

Current Techniques to Reduce Conflicts with Eastern Gray Squirrels

Exclusion

Exclusion involves keeping problem EGS out of an area using physical barriers. Examples may include woven wire fences, electric fences, or any other barrier that prevents entry or access into an area that needs protection (Hygnstrom et al. 1994). EGS can be prevented from climbing isolated trees and power poles by encircling them with a metal collar 1.8 m off the ground (Jackson 1994). Where EGS are entering buildings, a squirrel excluder can be improvised by mounting a 46-cm section of 10-cm plastic pipe over an opening (Jackson 1994). A one-way door can also be used over an opening to let squirrels out and prevent them from returning. Openings to buildings can also be closed using heavy 1.3-cm wire mesh. Custom-designed wire mesh fences topped with electrified wires have been effective in keeping EGS out of gardens or small orchards.

Habitat Modification

Habitat modification as a means of reducing EGS damage involves removing habitat components (e.g. food, shelter, or water) to make areas less desirable for EGS (Hygnstrom et al. 1994). Limbs and trees can be trimmed 1.8 to 2.4 m away from buildings to prevent EGS from jumping on to roofs (Jackson 1994). EGS can be kept away from bird feeders by tying an ear of corn away

from where they are causing problems. In some cases, agricultural producers have cleared trees near orchards to limit incursion and damage of orchard trees.

Repellents

A variety of repellents have been used to keep EGS away from protected areas. Repellents work by creating an aversion response based on taste, touch or smell (Hygnstrom et al. 1994). Ropel® is a taste repellent for EGS that can be applied to seeds, bulbs, and flowers; trees and shrubs; poles and fences; siding and outdoor furniture (Jackson 1994). Capsaicin is also a taste repellent, registered for use on maple sap collecting equipment. Polybutenes are sticky materials that can be applied to buildings, railings, downspouts, and other areas to keep EGS from climbing.

Trapping

Trapping can either be categorized as capturing live EGS or utilizing lethal traps that kill EGS. Live-trapping involves removing problem EGS by trapping and relocating to areas where conflicts are less unlikely to occur. A variety of traps can be used to catch EGS, including No. 0 or No. 1 leg hold traps, box traps, and cage traps (Jackson 1994). Glue boards, that are used to capture rats, have also been used to catch small EGS. Since EGS are classified as game species in most states, trapping permits may be required from state wildlife agencies to trap and release problem EGS. The South Carolina Department of

Natural Resources does not permit the trapping, translocation and releasing of EGS because of the stress placed on transported and released EGS, as well as potential impacts on resident EGS populations and concerns regarding the transmission of diseases. Snap traps used for rats can be used as lethal traps for small EGS. Effective baits to attract EGS to traps are slices of orange and apple, walnuts or pecans removed from the shell, and peanut butter (Jackson 1994). Other foods familiar to the EGS may also work well, such as corn or sunflower seeds.

Shooting

Where firearms are permitted, shooting is an effective control method to reduce EGS populations and associated problems. A shotgun with No. 6 shot or a .22-caliber rifle is suitable. However, state wildlife agency regulations and local ordinances need to be met, as well as the social acceptability of shooting EGS.

Other Methods

Often several control methods used simultaneously are more successful at reducing EGS conflicts than a single technique (Jackson 1994). For example, to remove EGS from an attic, they should be observed to determine their entry and exit portal. After this a combination of repellents and lights may be used to drive them out, followed by closing entry openings. Baited traps can also be used to capture any EGS that may have been accidentally closed in with

exclusion. This last step is important since “locked-in” EGS may cause damage when they try to chew their way out. Regardless of the technique(s) used, EGS damage in yards, gardens, forests, and orchards is often very difficult to control.

Immunocontraceptives and GonaCon™

Before the advent of GonaCon™, traditional immunocontraceptive research was restricted to the use of a vaccine made from zona pellucida extracted from the ovaries of pigs (*Sus scrofa*) and named porcine zona pellucida (PZP) (Miller et al. 1999). GonaCon™ is an immunocontraceptive vaccine that induces the immune system to generate antibodies to native (“self”) gonadotropin releasing hormone (GnRH). This is accomplished by conjugating GnRH to a foreign protein. Because the animal’s immune system has not been previously exposed to the foreign protein, it generates antibodies to both the foreign protein and to GnRH. The hypothalamus releases GnRH which then travels to the anterior pituitary, stimulating the release of leuteinizing hormone (LH), and to a lesser extent, follicle stimulating hormone (FSH). These two hormones then trigger the release of testosterone, estradiol, and progesterone from the testes or ovaries. Testosterone is necessary for breeding behavior and the production of sperm. Estradiol plays a crucial role in egg development and quality; whereas, progesterone is needed for ovulation and maintenance of pregnancy. Antibodies bind native GnRH as it leaves the hypothalamus, thus preventing it from binding to receptors in the anterior pituitary. As a result, no LH and little FSH is released

from the pituitary. Without the stimulus of LH and FSH, the testes and ovaries do not produce testosterone, progesterone, and produce little estradiol. Therefore no sperm or eggs are produced. Developed as a single-shot vaccine, GonaCon™ has been proven has been proven efficacious for ≥ 2 years in many pest species including white-tailed deer, domestic and feral pigs, bison, wild horses, cats and dogs (Miller et al. 2004). The GnRH immunocontraceptive vaccine has been successfully used on rats (*Rattus norvegicus*) and California ground squirrels (*Spermophilus beecheyi*) (Miller et al. 1997, Nash et al. 2004). Using only a single shot, its effects typically last ≥ 2 years which means it could render a rodent, like EGS, permanently infertile due to the short life span of rodents. There is no danger to non-targets since the vaccine is injected directly into the target animal. The vaccine consists of proteins; therefore, a secondary consumer is unlikely to be contracepted as proteins are broken down in the stomach. Although research continues on the development of an oral GonaCon™ immunocontraceptive, animals must currently be captured and injected by hand with a GonaCon™ vaccine.

Objectives and Methods

The purpose of this study was to field test the immunocontraceptive GonaCon™ as a potential vaccine to prevent reproduction in EGS in urban areas. Objectives of the study were the following:

1. To evaluate the efficacy of GonaCon™ in reducing EGS fecundity on the campus of Clemson University,
2. To evaluate the use and appropriateness of various metrics in determining the effectiveness of GonaCon™ in reducing EGS fecundity, and
3. To evaluate the use of GonaCon™ as a potential tool to reduce EGS overabundance in urban areas and provide recommendations for further research.

Study Area

Field trials examining the effects of GonaCon™ on EGS were conducted on Clemson University's (CU) main campus from March 2008 to June 2009. The CU campus, located in northwestern South Carolina, is composed of approximately 325 ha of teaching, research and administrative buildings interspersed with about 6600 trees (primarily oak, *Quercus* spp.; and hickory, *Carya* spp.), in addition to landscaping shrubs and bushes. Past estimates of EGS densities on CU's campus were higher (4.7 EGS/ha) than what has been reported in nonurban wooded habitats (0.6-3.8 EGS/ha) (Hein 1997). CU's

landscaping crew has documented over 100 mature trees killed, and an additional 100 trees severely damaged on CU's main campus by EGS for an estimated $ 1.3 million in damage (Carson, pers. comm.).

The study area consisted of 5.67 ha on CU's main campus (Figure 1), and was selected based on the following criteria:

- A visible overabundance of EGS,
- Ease of access to study animals, and
- Proximity to project research facilities at CU.

Eastern Gray Squirrel Trapping

EGS were captured on a 5.67 ha study site on Clemson University's main campus during four trapping sessions (TS1 = March – April 2008, TS2 = July 2008, TS3 = November 2008, and TS4 = May – June 2008) using a modified wooden box trap design (Mosby 1955). Forty wooden box traps (Table 1) were baited with a mixture of corn and oiled sunflower seeds during each of the four trapping sessions. The trap design (Mosby 1955) allowed a welded-mesh funnel and collar to be securely fastened to the front end of the trap. A slotted nylon capture cone made was tied on to the collar to facilitate EGS handling (Figure 2).

A total of 317 EGS (117 originals and 200 recaptures) were captured during the study. EGS were handled using the restraint cone and sexed, weighed, ear-tagged and implanted with a numbered microchip directly under the skin on the nape of all "originals", and then later identified as "recaptures" during

subsequent trapping sessions. EGS were vaccinated with GonaCon™ before the onset of their breeding season so they could be potentially contracepted before breeding began. On trapping, each EGS received either GonaCon™ or a sham control on the basis of toss of a coin for a randomized treatment design. The first trapping session (TS1) was conducted during March – April 2008 and 33 EGS were administered GonaCon™ and 22 a sham control. The ensuing breeding season of May – June 2008 was missed and the second trapping session (TS2) was conducted in July 2008. The third trapping session (TS3) was conducted in November 2008. The last session (TS4) corresponded with the May – June EGS breeding season. Thirty five EGS captured during TS4 were euthanized and necropsied for histological assessments.

Traps were set at dawn and remained open until dusk. Traps were checked at one hour intervals and all trapped EGS were processed in a timely manner (mean handling time = 10 minutes) and released at the trap site. Captured EGS normally moved into the restraining cone once the trap door was opened. If not, handlers used noise or aerosol cans of compressed air to move EGS into the cone. The cone worked well as a restraint device and EGS were easily handled, examined, treated and released.

Morphometric Data from Study Animals

EGS were sexed based on their external genitalia and presence of mammary glands. Lactation in females was assessed, as well as scrotal

pigmentation and testicular development in breeding males, as indicators of potential fertility. Presence or absence of lactation was determined by appearance of teats and milk secretions. Females were determined to be lactating if they had swollen teats with little or no hair covering them. Pigmented teats indicated that females had pups in the past. Testicular development was assessed on the basis of size in mm. Length and width of both control and treated EGS male scrotums were measured with digital calipers and scrotal size was used to assess age classes, in addition to being a potential metric for evaluating the effect of the vaccine. Furthermore, males were considered to be breeding if they had a gray or black pigmented scrotum with little hair covering the scrotum as well as enlarged testes. Males were considered to be non-breeding if they had a pink pigmented scrotum with hair regrowth evident and small flaccid testes (Pudney 1976, Webley et al. 1985, Ferryman et al. 2006).

A combination of pelage characteristics and body weights were used to assign an age class to individuals (Dimmick and Pelton 1996). Although scrotal pigmentation can be used in aging male EGS, the appearance of sub-adult and adult males with regressed testes can often be confusing (Hoffman and Kirkpatrick 1959). Skeletal and tooth characteristics, as well as dry weight of eye lens has been used to estimate EGS age, but these methods require euthanizing the animal. EGS age classes for the purpose of this study were defined as the following: juvenile: 0 – 6 months of age; sub-adult: 6 – 12 months of age; and adult: > 12 months of age.

All “original” and “recaptured” EGS were weighed using a digital weighing scale (Slater 1kg scale). The null hypothesis that body weights would not differ between treated and control EGS was tested.

Identification of Study Animals

EGS were ear-tagged for easy recognition. Self-piercing and uniquely numbered ear tags (Model 1005-1, National Band and Tag, Newport, KY) were applied with tag-pliers at the thickest part of the cartilage in the pinna of both ears. Ear-tag color codes used for identifications were the following; red = control female, white = treated female, yellow = control male, and blue = treated male.

Passive Integrated Transponder (PIT) microchip tags were inserted under the skin at the nape to serve as a second identifier in the event that ear tags were inadvertently pulled or fell out of EGS ears. Prior to insertion PIT tags, were scanned to verify that they worked and to record the number on data sheets (Table 3). The dorsal skin between the scapulas was pinched to form a “tent” and pit tags were delivered subcutaneously using a syringe and insertion needle. As the needle was withdrawn, the injection site was pinched off to ensure that the PIT tag would not fall out. EGS were then scanned to verify that PIT tags remained functional.

Estimation of Eastern Gray Squirrel Densities

Pooled capture-mark-recapture data were used to estimate EGS densities for each trapping session on the study site using the Lincoln-Peterson method (Gerhardt 2005). The sampling design for the basic capture–recapture model for estimating the size of a closed population involves randomly capturing EGS (n_0) from the population, tagging and releasing them, and later capturing a second EGS sample of size (n_1) and looking at the number of tagged EGS (m_1) in the second sample. The Lincoln–Peterson model was used to express the equation $N_{LP} = (n_1 x n_2)/m_2$. The assumption of a closed population in this density estimation was likely not violated, because EGS in this study had a high site fidelity based on high recapture rate in each of the trapping sessions.

Formulation of GonaCon™

The GnRH vaccine construct was developed by the USDA National Wildlife Research Center (NWRC) in Fort Collins, Colorado. The 10-amino acid GnRH peptide hormone was made immunogenic by coupling the peptide to a mollusk hemocyanin. The GnRH used in this study was synthesized at Macromolecular Resources, Colorado State University (Fort Collins, CO,) with the structure [p$^{\underline{\text{EHWSYGLRPG}}}$GC-SH]. The underlined amino acids represent the native GnRH molecule. A glycine was added at the C terminus as a spacer, and a cysteine was added to ensure consistent alignment of the peptide to the maleamide-activated mollusk protein. The aqueous-based GnRH conjugate was

combined in a 1:1 ratio by volume with a novel adjuvant (AdjuVac™), which is an oil-based adjuvant containing small quantities of killed *Mycobacterium avium.* GonaCon™ is supplied in refrigerated, 3ml, pre-loaded syringes (NWRC SOP BT 016.02).

GonaCon™ Treatment of Eastern Gray Squirrels

GonaCon™ was given by injection to 33 (17m, 16f), 23 (14m, 9f) and 11 (8m, 3f) EGS at a dosage rate of 0.4 ml containing 400 µg of GnRH-blue protein conjugate intramuscularly in the thigh during three trapping sessions (TS1, TS2, TS3). A sham injection containing 0.4 ml saline- AdjuVac™ was administered to 22 (16m, 6f), 20 (12m, 8f) and 8 (4m, 4f) control EGS during the same three sessions (NWRC SOP BT 004.01). EGS were randomized by the toss of a coin to receive either a GonaCon™ or a sham injection.

Methodology for Collecting and Processing Blood

A method of collecting blood from laboratory mice was modified for EGS (Hoff 2000). The saphenous vein, found on the caudal surface of the thigh, served as the site of veinpuncture and blood collection from EGS. After hair was removed from the area with the aid of clippers, the skin over the vein was prepped with isopropyl alcohol. The vein was then punctured using a 20 gauge needle and drops of blood were collected in serum separation tubes. Bleeding was then stopped by applying pressure. EGS blood samples were centrifuged to

separate serum and serum was stored in a freezer (-20° C) until assayed for steroid hormones and antibodies (NWRC SOP FP 030.00)

Scrotal Size Measurements

The length and width of scrotums were measured with digital calipers in both treated and control EGS males for the 3 trapping sessions (Table 4). The null hypothesis that scrotal size would not differ between treated and control males was tested.

Determination of Progesterone and Testosterone Concentrations

Radioimmunoassay (RIA) on EGS sera samples from treated and control females, and treated and control males, was performed to measure the progesterone and testosterone concentrations, respectively (NWRC SOP BT 025.00). The assays were performed using Coat-A-Count™ kits from Diagnostic Products Corporation (Los Angeles, CA). Repeat samples from EGS captured over at least two trapping sessions were used for assays to compare hormonal levels over time. For progesterone assays, treated females (n = 12) and control females (n = 7) were assessed for changes in progesterone concentrations (Table 6). Similarly, treated males (n = 7) and control males (n=7) were assessed for changes in testosterone concentrations (Table 5). The null hypothesis that testosterone concentrations would not differ between treated and control males

was tested. Similarly, the null hypothesis that progesterone concentrations would not differ between treated and control females was tested.

Detection of GnRH Antibodies

In order to detect GnRH antibodies from study animals over time, repeat samples from EGS captured over at least two trapping sessions were tested to detect antibodies to GonaCon™. Enzyme-linked immunosorbent assay (ELISA) on sera samples from treated females (n = 5) and control females (n = 4), and treated males (n = 6) and control males (n = 4) were performed (Table 7) (NWRC SOP BT 017.00). The ELISA assessed the immune response of EGS to the GnRH vaccine by detecting GnRH antibodies in EGS serum. Anti-rabbit IgG labeled with horseradish peroxidase was used to detect the quantity of bound antibody. The null hypothesis that antibody titers would not differ between treated and control EGS was tested.

Statistical Analysis

Descriptive statistics (mean ± S.E.) were calculated for continuous variables. Proportions or ratios were calculated for categorical variables in each group. Continuous variable means were compared over time between treatments and controls (testosterone and progesterone concentrations, scrotal size) using one-way repeated measures analysis of variance (ANOVA) followed by the pair wise t –test and trend analysis to generate graphs. Level of significance was set

at 0.05. Categorical variables (GnRH antibody titers) were compared over time and between treatments and controls using Fisher's exact test of proportions. All statistical calculations were performed using Proc GLM and Proc frequency of SAS.

Results

Eighty nine EGS were trapped on the 5.65 ha study site on CU's main campus from March-April 2008 (TS1: first trapping session). During July 2008 (TS2: second trapping session) 114 EGS were trapped, and in November 2008 (TS3: third trapping session) 79 EGS were trapped. During May-June 2009 (TS4: fourth trapping session) 35 EGS were trapped for necropsy examination. A total of 317 EGS were captured during the study (Table 1 and 2).

Eastern Gray Squirrel Densities

The Lincoln–Peterson model ($N_{LP} = (n_1 x n_2)/m_2$) was used to estimate ESG numbers on the study site where:

N_{LP} = EGS population estimate;

n_1 = Number of EGS originally trapped, tagged, and released;

n_2 = Number of EGS trapped subsequently with and without tags;

m_2 = Number of EGS trapped subsequently with original tags.

For TS1, N_{LP} = (65x42)/45 = 80 EGS on the study site, and density

(D) = 80/5.67 ha ≈ 14 EGS/ha

For TS2, N_{LP} = (43x73)/71 = 49 EGS on the study site, and density

(D) = 49/5.67 ha ≈ 9 EGS/ha

For TS2, N_{LP} = (19x64)/60 = 20 EGS on the study site, and density

(D) = 20/5.67 ha ≈ 4 EGS/ha

Mean (D) = 9 ± (2.89)

Body Weight Measurements

Body weight measurements were obtained from 33, 23, and 11 treated EGS; and 32, 20, and 8 control EGS over 3 trapping sessions (TS1, TS2, TS3), respectively. During TS1 the mean body weights (± SE) for treated and control EGS were 463.94 ± 12.37 and 455.31 ± 12.49 grams, respectively across all age classes and both sexes. These means were not significantly different (t = - 0.49, df = 63, p = 0.63). During TS2 mean body weights for treated and control EGS were 453.91± 16.46 and 448.00 ± 16.60 grams, respectively. These means were not significantly different (t = - 0.25, df = 40, p = 0.80). During TS3 the mean body weights for treated and control EGS were 451.00 ± 25.74 and 419.00 ± 27.22 grams, respectively. These means were not significantly different (t = - 0.86, df = 16, p = 0.40). Pooled data for control and treated EGS was used in the analysis above (Figure 9) since there is no sexual dimorphism in size.

Scrotal Size Measurements

Twenty one scrotal size measurements (length x breadth) collected over 3 trapping sessions (TS2, TS3, TS4) from 7 control and 7 treated EGS males were tested for differences. Scrotal size measurements were compared between treated and control males within each session and across all three sessions as well.

During TS2 mean scrotal size for treated males was 110.99 ± 96.68 mm and for control males 118.63 ± 127.39 mm. However, these means were not significantly different (t= 0.08, df = 8, p = 0.93).

During TS3 mean scrotal size for treated males was 45.61 ± 76.76 mm and for control males 109.16 ± 133.02 mm. However, these means were not significantly different (t= 76.76, df = 8, p = 0.56).

During TS4 mean scrotal size for treated males was 141.46 ± 76.76 mm and for control males 1101.75 ± 108.63 mm. These means were significantly different (t= 10.14, df = 8, p = 0.001) indicating a significant difference in scrotal size of treated and control EGS with a reduction in scrotal size being observed in treated males (Figure 10).

Hormone Concentrations

Testosterone Assays

Thirty three serum samples were collected over 3 trapping sessions (TS1, TS2, TS3) from 7 control and 7 treated EGS and analyzed using RIA. During

TS1, the mean testosterone concentration for treated males was 0.33 ± 0.14 ng/ml and for control males 0.34 ± 0.15 ng/ml. However, these means were not significantly different (t= 0.04, df = 15, p = 0.96).

During TS2, mean testosterone concentration for treated EGS males was 0.34 ± 0.13 ng/ml and for control males 0.28 ± 0.12 ng/ml. However, these means were not significantly different (t= -0.32, df = 15, p = 0.74). During TS3, mean testosterone concentration for treated EGS males was 0.45 ± 0.09 ng/ml and for control males 0.62 ± 0.13 ng/ml. However, these means were not significantly different (t= 1.02, df = 15, p = 0.32) There were no differences between testosterone concentrations of treated and control EGS by the third trapping session (Figure 11).

Progesterone Assays

Thirty serum samples were collected over 3 trapping sessions (TS1, TS2, TS3) from 12 treated and 7 control EGS females and analyzed using RIA. During TS1, mean progesterone concentration for treated females was 1.67 ± 0.93 ng/ml and for control females 1.26 ± 1.19 ng/ml. However, these means were not significantly different (t= - 0.29, df = 8, p = 0.78).

During TS2, mean progesterone concentration for treated females was 0.93 ± 1.69 ng/ml and for control females 1.86 ± 1.31 ng/ml. However, these means were not significantly different (t= 0.43, df = 8, p = 0.68). During TS3, mean progesterone concentration for control females was 4.64 ± 1.13 ng/ml.

There were no significant differences between the progesterone concentrations of treated and control EGS females by the third trapping session (Figure 12).

Antibody Titers

Seventy serum samples were collected over 3 trapping sessions (TS1, TS2, TS3) from 9 control and 7 treated EGS females, and 9 control and 7 treated EGS males. Samples were analyzed for the presence of active antibodies to GnRH using ELISA.

During TS1, the ratio of treated EGS with active antibodies was 1:8 and the ratio of control EGS with active antibodies was 0:11. These ratios were not significantly different ($\chi 2 = 1.286$, df = 1, $p = 0.256$). During TS2, the ratio of treated EGS with active antibodies was 2:7 and the ratio of control EGS with active antibodies was 12:6. These ratios were significantly different ($\chi 2 = 4.747$, df = 1, $p = 0.029$). During TS3, the ratio of treated EGS with active antibodies was 0:5 and the ratio of control EGS with active antibodies was 7:4. These ratios were significantly different ($\chi 2 = 5.656$, df = 1, $p = 0.017$).

ELISA showed significant differences between the antibody titers of treated and control EGS by the third session (Figure 13). However, 5 control animals, 3 females and 2 males, showed high antibody titer.

Injection Site Reactions and Mortalities

There were 4 injection site abscesses in treated and 2 in control EGS. The ratio of injections given to the occurrences of abscesses at the site of injection for treated EGS was 40:4 and 33:2 for control EGS. These ratios were not significantly different. ($\chi 2 = 0.3167$, df = 1, $p = 0.5736$) Two EGS died as a result of trap failure, and 3 animals died as a result of predation injuries, possibly from a raptor, during the period of study.

Discussion

The results of the controlled efficacy trial demonstrate that peak antibody titers of 1:12,800 were induced both in male and female EGS by a single injection of 400 µg of GonaCon™ when tested 2 months post-treatment. This is consistent with similar responses to GnRH in male cats (Levy et al. 2004), male dogs (Ladd et al. 1994), female white-tailed deer (Curtis et al. 2007), and female wild boar (Massei et al. 2008). Five control EGS showed high titer levels likely due to an inexplicable sampling error. The possibilities include a recording error, laboratory error, or the inadvertent vaccination of control EGS with GonaCon™. However, antibody titers are a good metric and an important pointer to the immunogenic success of GonaCon™ immunocontraception.

Hormonal assays proved to be inconclusive, because there were no significant differences in either progesterone or testosterone concentrations

between treated or control EGS. This likely is due to the fact that blood was not collected from EGS during the peak of breeding seasons.

The blood collection technique used in this study does not seem to be an appropriate one for hormonal assays that are colorimetric. Evidently, the EGS blood samples clotted rapidly, and this led to hemolysis when serum was separated. In the presence of hemolyzed serum, the concentration of a hormone or antibodies will be lowered and the results thereby affected. Therefore, a better blood sampling technique needs to be used.

The findings in 14 EGS males (7 treated and 7 control) in TS2, TS3 and TS4) indicated a significant reduction in scrotal size in GonaCon™ treated males by TS4, as compared to control males. Although there seemed a substantial difference in scrotal size in TS3 as well, this was not significant likely due to a small sample size or the large variances observed. The marked difference seen in TS4 was likely due to sustained vaccine effects that had, in all likelihood, caused a marked reduction in the scrotal size by TS4. This corresponds with differences in mean testicular weights of treated and control EGS on necropsy examination (Table 9). The reduction in scrotal size of treated males is an indicaton that GonaCon™ possibly caused immunological castration in male EGS, which is in agreement with an similar response seen in male dogs and male cats treated with a similar immunocontraceptive vaccine (Ladd et al. 1994, Levy 2004).

GonaCon™ did meet an important criterion of an ideal contraceptive vaccine, as it did not cause any significant differences in body weights between treated and control EGS. However, the vaccine did cause injection site reactions in 6 EGS as seen in other studies (Miller et al. 2008; Tizard 2009). This problem can likely be overcome when an improved vaccine formulation is designed by the manufacturer.

CHAPTER 3

HISTOLOGICAL CHANGES IN THE GONADS OF EASTERN GRAY SQUIRRELS VACCINATED WITH THE IMMUNOCONTRACEPTIVE GONACON™

Introduction

There have been occasional reports of adverse effects of vaccines in animals. Although often mild, these effects have included allergic reactions and the development of sarcomas in cats (Tizard 2009). The absence of harmful side effects is one important attribute of an ideal contraceptive. However, some studies have documented pathological impacts on the reproductive tract with the use of both immunocontraceptive vaccines and hormonal contraceptives. The Porcine vaccine zona pellucida (PZP) resulted in ovarian lesions in white-tailed deer (Curtis et al. 2007). Endomterial hyperplasia, hydrometra and uterine infections have occurred in melengestrol acetate (MGA) treated ungulates (Munson 2005).

GonaCon™ primarily blocks the entry of GnRH into the hypophsis of the pituitary gland, and thereby suppresses steroidogenesis, oogenesis, and spermatogenesis (Robbins 2004). The gonads are an important source of the sex hormones – testosterone in the male, and estrogen and progesterone in the female. It is probable that reversible or irreversible histological changes occur in the testes and ovaries as a result of GonaCon™ vaccination. A GnRH-KLH vaccine similar to GonaCon™ caused testicular atrophy in cats (Levy et al.

2004). However, testicular atrophy may be a desired effect as long as it does not negatively impact the health of the animal.

Histological changes in the testes and ovaries of 35 EGS were evaluated as metrics of effectiveness for GonaCon™ in EGS. Detailed necropsies of EGS were conducted to assess ovarian and testicular abnormalities, or other potential health concerns resulting from the vaccination (Table 1 and 2). Objectives of this study were the following:

1. To evaluate histological changes in testes and ovaries as a metric to determine the effectiveness of GonaCon™ in reducing EGS fecundity, and
2. To determine any potential side effects in EGS treated with either the GonaCon™ vaccine or sham control injections.

Materials and Methods

Reproductive Anatomy of Male Eastern Gray Squirrels

In adult EGS males, the testes lie in scrotal sacs on either side of the penis. The prostate is a single, elongated, compact gland located in the proximity of the urinary bladder and attached to the muscular part of the urethra (Allanson 1933). The seminal vesicles are small and adhere closely to the prostate. A pair of large spirally wound Cowper's glands are located at the sides of the rectum and lie embedded in the fascia of the thigh. A long thick duct passes from each to open into the bulb of the urethra. The penis is sharply bent backwards at its distal end. In sub-adult and juvenile male EGS, the testes lie subcutaneously on each

side of the penis. The accessory glands are small and the seminal vesicles and Cowper's glands are difficult to distinguish (Allanson 1933).

Reproductive Anatomy of Female Eastern Gray Squirrels

The female EGS has a duplex uterus with two cervices, no uterine body, and horns completely separated (Deanesley and Parkes 1933). The uterus is large and contains multiparous arteriopathies in the endometrium in the parous female. In the prepubertal female, the flattened cornua are small and threadlike. Externally, the surface of ovaries is similar for both prepubertal and parous females; however, the ovarian mass is greater in parous females (Nixon and McClain 1975). Internally, the ovaries of the EGS resemble those of Norway rats (*Rattus norvegicus*) or house mice (*Mus musculus*), with comparatively little interstitial tissue (Deanesley and Parkes 1933).

Study Area

Experimental field trials of GonaCon™ were conducted on Clemson University's (CU) main campus located in northwestern South Carolina. The campus is approximately 325 ha of teaching, research and administrative buildings interspersed with about 6600 trees (primarily oaks *Quercus* spp., and hickories *Carya* spp.), in addition shrubs and bushes used for landscaping. Densities of EGS are normally <3 EGS/ha (Barkalow et al. 1970). Using Lincoln-Peterson model, EGS density was estimated to be 9 EGS/ha on the study area.

Vaccine Formulation

Both male and female EGS were vaccinated with either GonaCon™ (treatment) or a sham-injection (control) prepared and supplied by the USDA National Wildlife Research Center (NWRC, Fort Collins, CO). Treated EGS were injected with 0.4 ml of GonaCon™ which contained 1000 micrograms GnRH-blue protein conjugate per ml; therefore, each 0.4 ml dose contained 400 µg GnRH-blue conjugate. Control EGS were injected with 0.4 ml saline-Adjuvac intramuscularly in the thigh.

Vaccination Protocol

Using a modified wooden box trap design (Mosby 1955), 99 EGS were captured from March-April 2008 (first trapping session = TS1). During July 2008 (second trapping session = TS2), 114 EGS were captured, and in November 2008 (third trapping season = TS3) 80 EGS were captured on the study. EGS were handled using a restraint cone, sexed, weighed, ear-tagged, and implanted with a microchip under the skin in the nape of all “originals” and read as “recaptures” on subsequent trapping.

Vaccination with GonaCon™ or sham-controls was conducted by intramuscular injection in the thigh of both male and female EGS. GonaCon™ was administered to 33, 23 and 11 EGS during the three respective trapping sessions (TS1, TS2, and TS3). EGS were released at the site of capture after treatment. Sham injections were administered intramuscularly in the thigh to 22,

20 and 8 EGS during the same three trapping sessions as treated EGS. EGS were randomized by the toss of a coin to receive either a GonaCon™ or a sham injection.

Eastern Gray Squirrel Necropsies

In April and May of 2009, 35 EGS were humanely euthanized by an overdose of halothane anesthesia (CU RS/SOP 300-04-02). Necropsy examinations were performed on all 35 EGS; 18 males (8 treated 10 control) and 17 females (8 treated 9 control) (Table 9 and 10). All necropsies were performed within 10 minutes after EGS were euthanized. Gross examinations and measurements included body weights, body condition, internal organs, weights of testes and ovaries, examination of injection sites, and documentation of any visible abnormalities. Ovaries, uteri and mammary glands were collected from females and fixed in 10% neutral buffered formalin (NBF) (Gugic et al. 2007). Testes and prostate glands were collected from males and fixed in modified Davidson's fluid (Latendresse et al. 2002). The pituitary gland was collected from both sexes and fixed in 10% NBF. Histological examination was conducted after tissues were embedded in paraffin, and stained with hematoxylin-eosin (Allanson 1933). Prepared histological slides were interpreted by a veterinary pathologist using an optical microscope (Nikon A2100 microscope equipped with DS-Ri1 color camera).

Terms of Reference

Seminiferous tubules in the testes are the specific site for the process of cellular differentiation to generate mature spermatozoa. Each testis contains many seminiferous tubules, which are connected at both ends to a collecting system called the rete testis. A degeneration of seminiferous tubules will disrupt the process of sperm production leading to infertility (Ogawa et al. 1997).

Sertoli cells or nurse cells line the seminiferous tubules and nurture spermatogenesis. The relationship between germ cells and Sertoli cells is important and obligatory (Griswold 1995). Evidence of cavity formation in the Sertoli cells with resultant shedding of immature sperm cells is termed vacuolation (Hild et al. 2001).

The interstitial cells (Leydig cells) in the testes secrete testosterone, are rich in lipid droplets, and have a cord-like arrangement. These cells provide spaces that improve cell secretion of hormones and facilitate transport into the blood (Hafez et al. 1997). In the event of testicular atrophy, a decrease in number and size of Leydig cells will occur, and testosterone production will be impaired as a result.

Statistical Analysis

Descriptive statistics (mean ± S.E.) were calculated and normally distributed data over time (organ weights and diameter) were compared using one-way repeated measures analysis of variance (ANOVA) using the general

linear models procedure (Proc GLM) of SAS software (Version 9.1). This was followed by a pair wise t –test and trend analysis to generate graphs. Level of significance was set at p = 0.05.

Results

Gross and histological examination were conducted on 8 treated males, 10 control males, 8 treated females, and 9 control female EGS. Mean wet testes weight of control EGS males was 4010 ± 704.64 mg and 336 ± 61.22 mg for treated males. There was a significant reduction in testes weights in treated males (t = 5.19, df = 8.12, p = 0.0008).

The proportion of treated EGS males with degeneration of seminiferous tubules was 1.0, and the proportion of control males with degeneration of seminiferous tubules was 0. These proportions were significantly different (χ^2 = 18.0, df = 1, p = 0.0001).

The proportion of treated EGS males with atrophy of Leydig cells was 1.0, and the proportion of control males with atrophy of Leydig cells was 0. These proportions were significantly different (χ^2 = 18.0, df = 1, p = 0.0001).

The proportion of treated EGS males with vacuolation of Sertoli cells was 1.0, and the proportion of control males with vacuolation of Sertoli cells was 0. These proportions were significantly different (χ^2 = 18.0, df = 1, p = 0.0001).

Mean wet weight of ovaries of control EGS females was 103 ± 25.96 mg and 98 ± 8.61 mg for treated EGS females and were not significantly different (t =

0.17, df = 9.73, p = 0.86). There were no significant differences in the diameter of the uterine horns and the length of the tract from the vagina to the ovaries between control and treated EGS females. There were no pregnancies found in either control or treated EGS females.

Representative cross sections of testes in control EGS males exhibited densely packed tubuli seminiferi with intact spermatogenesis (see arrow) and robust looking interstitial Leydig cells (Figure 3.A) In treated EGS males atrophic tubuli seminiferi and Leydig cells (see arrow) with degenerating spermatocytes are seen (Figure 3.B).

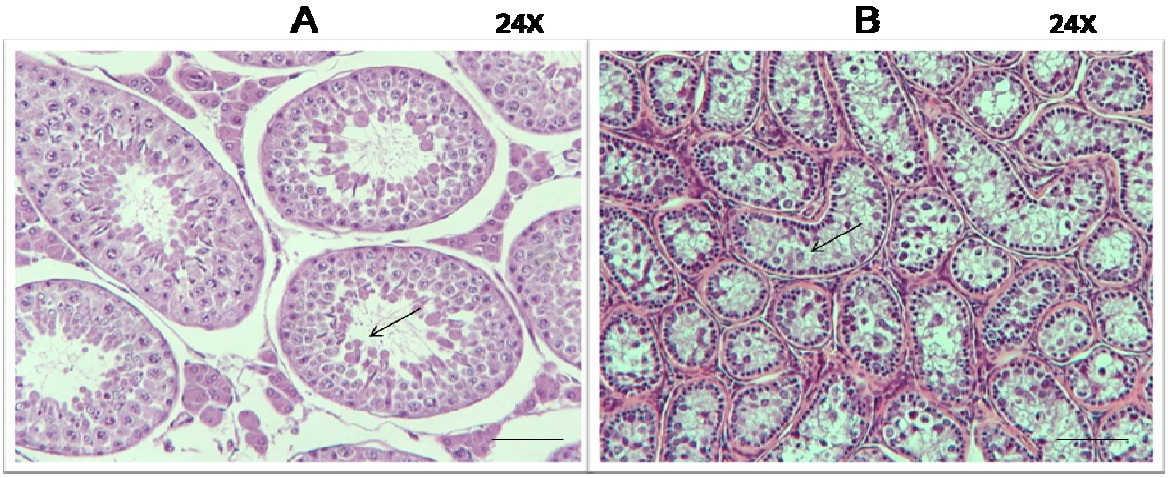

Figure 1. Cross-section of testes of control (A) and GonaCon^{TM} treated (B) EGS males (H&E stain, Bar = 90 µm).

Representative cross sections of epididymis in control EGS males exhibited a lumen filled with abundant mature spermatozoa (see arrow) (Figure 4.A). In treated EGS males lumen devoid of spermatozoa are seen (arrow) (Figure 4.B).

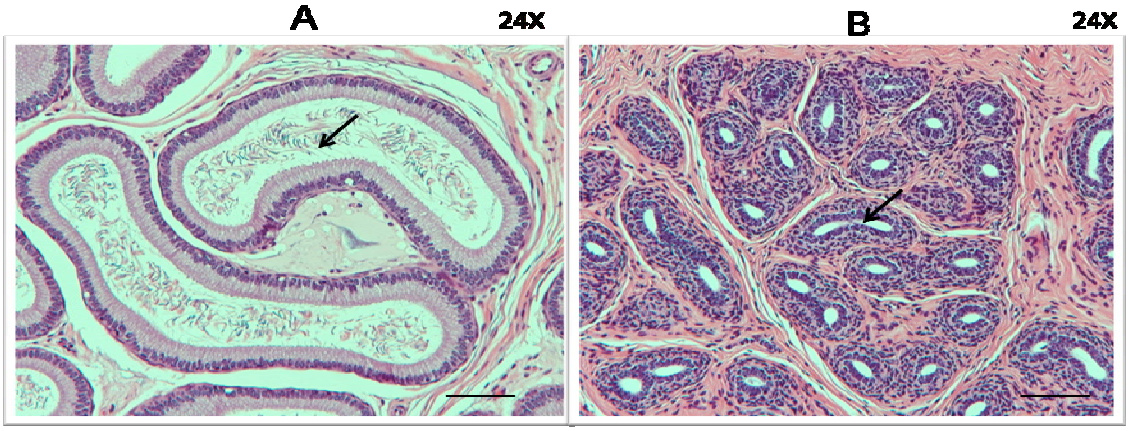

Figure 2. Cross-section of epididymis of control (A) and GonaCon™ treated (B) EGS males (H&E stain, Bar = 90 µm).

Representative cross sections of prostates in control EGS males exhibited normal glandular epithelium (see arrow) (Figure 5.A). In treated EGS males contracted dark glandular tissue and evidence of advanced atrophy is seen (arrow) (Figure 5.B).

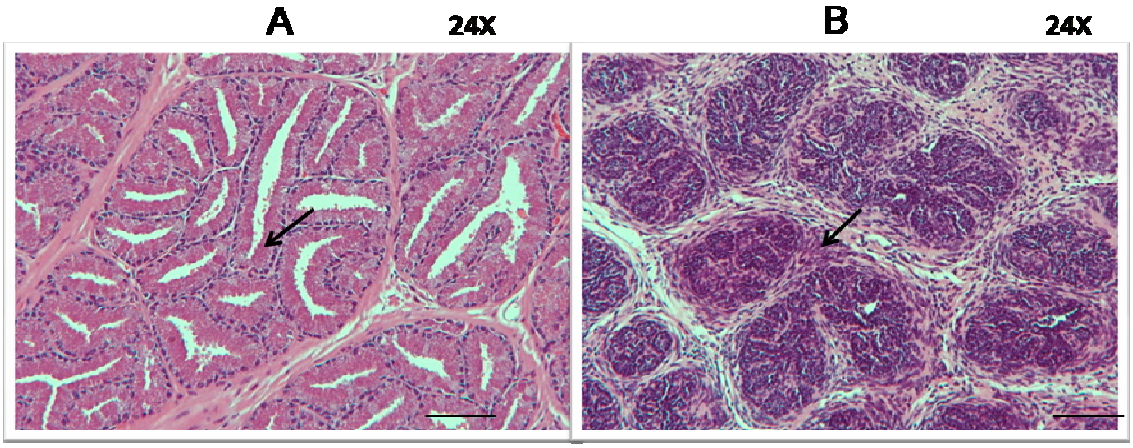

Figure 3. Cross-section of prostate of control (A) and GonaCon™ treated (B) EGS males (H&E stain, Bar = 90 µm).

Representative cross sections of ovaries in control and treated EGS females did not exhibit any observable differences (Figure 6)

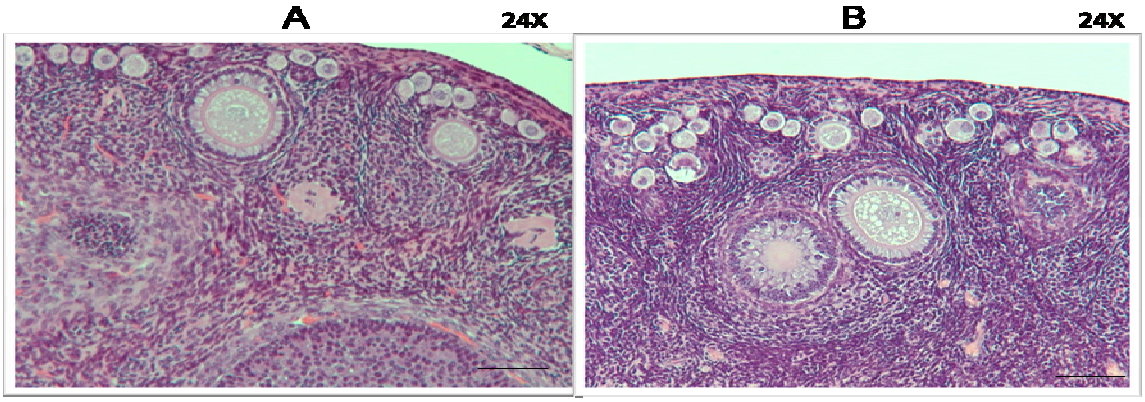

Figure 4. Cross-section of ovaries of control (A) and GonaCon™ treated (B) EGS females (H&E stain, Bar = 90 µm).

Representative cross sections of uterus in control and treated EGS females did not exhibit any observable differences (Figure 7).

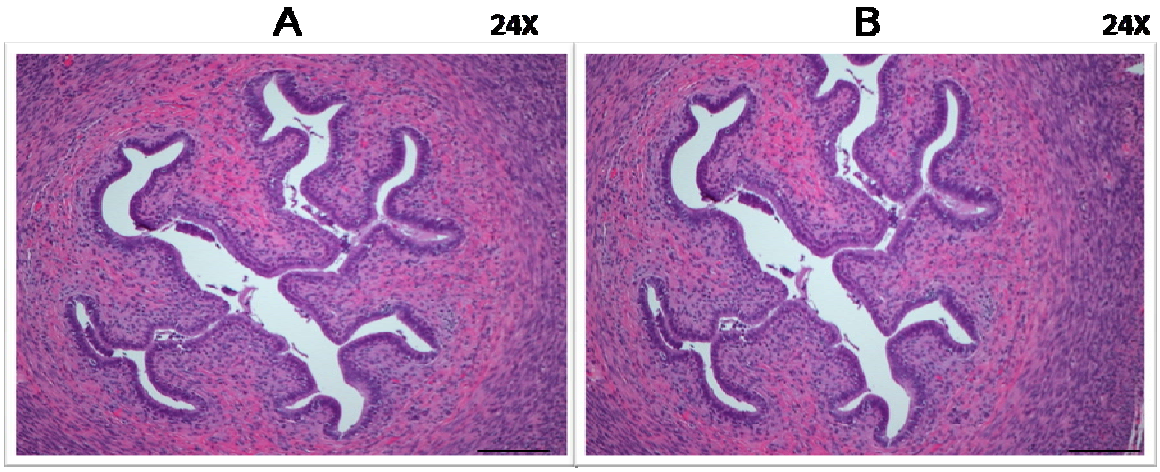

Figure 5. Cross-section of uterus of control (A) and GonaCon™ treated (B) EGS females (H&E stain, Bar = 90 µm).

Representative cross sections of pituitary gland in control and treated EGS of both sexes did not exhibit any observable differences (Figure 8).

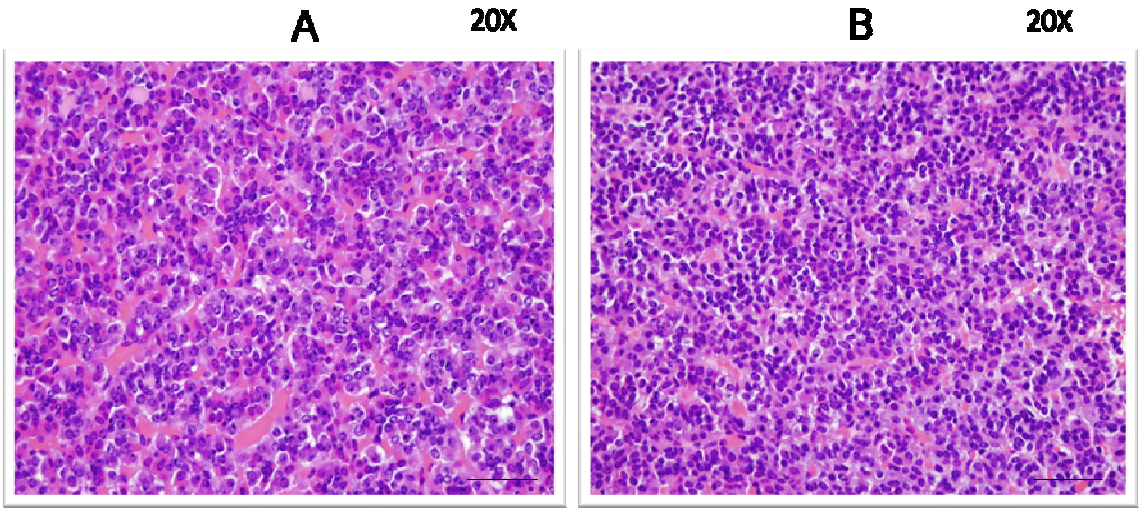

Figure 6. Cross-section of pituitary gland of control (A) and GonaCon™ treated (B) EGS males. (H&E stain, Bar = 90 µm).

Discussion

The findings in 18 necropsied EGS males (8 treated and 10 control) indicated a reduction in testes weight (90%) in GonaCon™ treated males, as compared to control males. There were also marked histological differences in the testes of treated males, as compared to control males. This suggests that GonaCon™ treated male EGS were immunoneutered, and exhibited physiologic traits similar to castrated males. These findings are consistent with studies in other animals, such as GonaCon™ treated male white-tailed deer, where testes size was also reduced as a result of anti-GnRH immunocontraception (Curtis et al. 2008, Pooler 2001, Killian et al. 2006).

Specific histological changes represented in comparisons of 8 treated EGS males and 10 control males that were necropsied included degeneration of

seminiferous tubules, atrophy of Leydig cells and vacuolated Sertoli cells. Degeneration of seminiferous tubules with concomitant diminished spermatogenesis observed in treated EGS males in this study, were also observed in similar studies in Sprague-Dawley rats (Jinshu et al. 2005), white-tailed deer (Curtis et al. 2008), dogs (Ladd et al. 1994), cats (Levy et al. 2004), and Scottish Suffolk-crossbred rams (Ferro et al. 2004). Atrophy of Leydig cells and vacuolated Sertoli cells observed in treated male EGS was also seen in male cats that received anti-GnRH vaccine (Levy et al. 2004).

Male EGS have one unique physiologic attribute that is germane to the question: whether marked histological changes that were observed in this study were permanent or transient? It is well documented that male EGS undergo a semiannual cycle of testicular recrudescence and regression and occasionally skip entire breeding seasons (Webley et al.1985). Consequently, it is difficult to preclude the possibility that the histological findings that imply a cessation of reproduction in GonaCon™ treated male EGS in this study were not due to the phenomenon of sexual quiescence.

No histological changes in uteri or ovaries were discerned in the 8 GonaCon™ treated EGS females on necropsy. Changes in hormonal levels over the estrous cycle of EGS results in morphological changes in the ovary, uterus, and vagina, all of which can be used to determine the stage of cycle (Davis et al. 2001). In other words, no histological changes observed in both GonaCon™ treated and control EGS females in this study could well conform to females that

were in anestrous or sexual rest, a normal physiologic process in the reproductive cycles of not only rodents but all mammalian females (Conaway 1971). On the other hand, it could well mean that the treated EGS females were in senescence. Several factors contribute to the variability in timing of reproductive senescence in rodents, including the species, environmental factors, and whether pregnancy actually occurred (Davis et al. 2001).

Even if GonaCon™ is effective only in male EGS, it might still help reduce or alleviate territorial marking and bark stripping, which seems to be a learned behavior in male EGS (Kenward and Parish 1986). Consequently, sterilization of male EGS with GonaCon™ may be a potential tool in preventing destructive behavior such as gnawing and girdling of trees which causes damage and economic losses in urban and suburban areas, like Clemson University's main campus.

There were no histological changes in the pituitary glands of EGS that received either GonaCon™ or sham control treatments as evidenced from necropsy examination of 35 EGS. The pituitary regulates other physiological processes in EGS, and it is important that these processes are not disrupted or compromised by GonaCon™. A study of active immunization against GnRH in pigs caused damage to cells in the hypothalamus other than those producing GnRH (an action called a by-stander effect) (Molenaar et al. 1993).

CHAPTER 4

CONCLUSIONS OF FIELD TRIALS OF GONACON™ IN EASTERN GRAY SQUIRRELS AND RECOMMENDATIONS

Results of this study indicate that a single-dose of 400 µg GonaCon™ vaccine injected in EGS induced GnRH-titer peaks suggestive of an immunologic response that may have inhibited reproduction in treated male and female EGS (n = 39:28). Examination of antibody titers, scrotal size, testicular weights; histological assessment of testes, epididymides, and prostates indicates that GonaCon™, in all likelihood, was successful in inhibiting breeding in male EGS. These results imply that GonaCon™, in this study, caused immunocastration in male EGS.

It is not clear whether GonaCon™ conferred sterility in female EGS in this study. However, based upon results of antibody titers in female EGS, it is evident that GonaCon™ was effective at the immunologic level. These results, however, cannot be extrapolated to imply that breeding was actually inhibited in female EGS in this study. Breeding behavioral observations of GonaCon™ treated female EGS were not recorded, and examination of potential parturition in GonaCon™ treated female EGS was not an objective of this study.

It is also important to examine the potential use of GonaCon™ for EGS in context of the 8 criteria developed by Becker and Katz (1997) for what is considered an ideal contraceptive agent. These criteria include reversibility, suitability for remote delivery, effectiveness of a single dose, effects on the food

chain, harmful side effects, effects on EGS social behavior, costs, and social acceptability.

Reversibility of immunocontraception restores breeding fitness to the target species. For some species, like raccoons and EGS, incriminated as pests in urban and suburban areas, the question of reversibility does not arise. However immunocastration, due to active immunization against Luteinizing Hormone-Releasing Hormone (LHRH) in dogs, was found to be reversible and could be dose dependent (Ladd et al. 1994). Further research is required to standardize the dosage rate at which GonaCon™ would cause irreversible infertility in EGS, if that is an objective of affected stakeholders in urban and suburban communities.

Suitability for remote delivery is an impediment for GonaCon™ use in EGS, as it can only be delivered at the present time by hand injection. Consequently, treatment of EGS with GonaCon™ can only be achieved by an intensive effort to trap EGS, which can be labor intensive, time-consuming, and costly. The potential difficulty of using GonaCon™ to control EGS reproduction and populations is compounded in urban and suburban areas, because EGS are often viewed with affection by a portion of the public, making control efforts problematic and controversial (Moore et al. 1997). Research at USDA's National Wildlife Research Center is currently underway to develop an oral immunocontraceptive which could be delivered without having to trap and handle EGS (Miller 1997).

An alternative to GonaCon™ may be a cholesterol analogue called DiazaCon™, which inhibits both cholesterol and reproductive hormone production. DiazaCon™ is delivered as bait by over coating a preferred food item (e.g. rolled oats for prairie dogs) for a period of 5 to 10 days, and the contraceptive effects last up to the length of a targeted species breeding season (Yoder et al. 2007). This product was found to be a potential tool for reducing fecundity in black-tailed prairie dogs (*Cynomys ludovicionus)* (Nash et al. 2007) and monk parakeets (*Myiopsitta monachus*) (Yoder et al. 2007).

In a recent (October 2009) and significant development in wildlife contraception, GonaCon™ was licensed by the US Environmental Protection Agency (EPA) for use in white-tailed deer at a single dose of 1000 µg (Miller, pers. comm.). It is anticipated that GonaCon™ will also meet the single dose administration criteria (Becker and Katz 1997) in EGS. Its efficacy as a single dose administration has proven effective in feral pigs (Massei et al. 2008) and feral cats (Levy et al. 2004). A long-lasting immune response to GonaCon™ depends on the retained antigen; therefore, a long-term study is required to determine if and when GnRH antibodies decline in EGS. If long-termed efficacy is not achieved, then GnRH would not be inhibited resulting in a reversal of steriodogenesis, spermatogenesis and oogenesis (Robbins et al. 2004).

The manufacturer of GonaCon™, the USDA National Wildlife Research Center, has determined that there is no danger associated with humans or wildlife consuming animals treated with GonaCon™. Both GonaCon™, and the

antibodies produced by an animal treated with GonaCon™, are proteins that once ingested, are broken down by stomach acids and enzymes. The USDA Food and Drug Administration (FDA) has also determined that there is little risk to humans if meat from deer and pigs treated with GonaCon™ was consumed (Fagerstone et al. 2008).

There were relatively few harmful side effects observed in EGS treated with GonaCon™ in this study. Of the 117 EGS treated in this study, 67 EGS (39 m, 28f) received GonaCon™ injections and 50 EGS (32m, 18f) the sham control injections. Six EGS, 4 treated (2 males, 2 females) and 2 controls (both males), developed injection site granulomas with moderate to severe sterile abscess formation. Consequently, there was a 7.62% incidence of injection site reactions in EGS. This was likely due to the water-in-oil emulsion present in the GonaCon™ formulation (Gupta et al. 1993), or the presence of the bacterium *Mycobaterium avium* in the adjuvant, which is necessary for single shot effectiveness (Perry et al. 2008). Besides the injection site reactions, there were no other serious side effects to GonaCon™ observed in EGS in this study.

In a separate study, that was conducted in concurrence with this study on the same study site, the effects of GonaCon™ on the social behavior of EGS was examined. Eighteen volunteer observers were trained to perform instantaneous focal sampling of EGS activity budgets over 10-minute sessions. Over 1150 sessions were recorded and analyzed for differences in EGS activity budgets between GonaCon™ treated and control EGS. Preliminary results of this

study showed that GonaCon™ significantly changed only one behavioral activity budget of EGS, that being significantly ($p = 0.05$) more self-grooming by GonaCon™ treated male EGS, as opposed to control male EGS (Etheredge, unpublished data). Additional research is needed to more accurately quantify and describe behavioral activity budgets of GonaCon™ treated and control EGS over multiple breeding seasons and years.

Economics is also an important consideration when evaluating the feasibility of wildlife contraception. Costs of GonaCon™ production and delivery, as well as who pays for these expenses, is an issue of debate (Kirkpatrick 2007). If GonaCon™ is proven to be effective as a wildlife contraceptive and registered for use in EGS, it will in all likelihood remain under the strict control and selective use of USDA Wildlife Services. Although costs of producing GonaCon™ by the USDA National Wildlife Research Center are not available, costs of delivery can be estimated based upon the effort involved in trapping and treating EGS in this study. For example, EGS density on Clemson University's main campus during this study was estimated to be 9 EGS/ha. With 567 ha of EGS habitat on Clemson University's main campus, the EGS population can be estimated to be approximately 5,103 squirrels. A previous study on immunocontraception in rodents concluded that over 90% of the population need to be sterilized to achieve the desired control (Moore et al. 1997). Based upon this study, approximately 4593 EGS would need to be treated with GonaCon™ to have an effect on controlling reproduction and consequently overabundance. Using the

best trapping success obtained in this study, the same effort to trap 90% of EGS on Clemson's campus would take 1000 days at a cost of $ 15/EGS. This assumes 2.1 hours/EGS to trap and treat with GonaCon™ at an hourly rate of $7.25/hour (2009 minimum wage). Thus, the labor costs alone for plausible success of GonaCon™ in control of EGS would be in the region of $ 70,000.

This hypothetical example illustrates the high costs of anti-fertility control in EGS, which does not include the costs of the vaccine itself. Costs may be reduced, and effectiveness enhanced, of treating EGS with GonaCon™ if EGS populations are reduced before initiating a contraceptive program. However, this integrated approach that includes population reduction, may not be socially acceptable within urban and suburban communities having high EGS populations. Other studies that have documented expenses associated with contraceptive programs concluded it would cost an estimated $25 to $500 to treat an individual deer, a wild horse, African elephant or even a captive kudu (Rutberg 2005). Costs of treating EGS with GonaCon™ in urban and suburban areas would in all likelihood have to be borne by affected individuals, municipalities and other stakeholders that would benefit from EGS population control.

Social acceptability of using GonaCon™ to control EGS numbers, as well as populations of other wildlife species, is another challenge wildlife managers face. A few animal rights groups maintain that wildlife contraception violates the reproductive rights of animals (Kirkpatrick 2007). The issue of alteration in

population genetics due to wildlife immunocontraception may not arise when small, isolated populations of pest species are concerned (Nettles 1997).

Longer study duration of up to 5 – 6 years, to coincide with the life span of EGS is critical to provide a frame of reference for year-round hormonal profiles in both sexes. In addition, a larger sample size of EGS is needed to better understand the disparity in GonaCon™ effectiveness observed in male and female EGS in this study. It would take a minimum of one year of continuous blood sampling in male and female EGS to establish baseline data for hormonal concentrations over a temporal scale of multiple breeding seasons.

Wildlife contraception remains a contentious issue and the use of anti-fertility vaccines for population control and reduction of wildlife damage management has both proponents and opponents. Some proponents are researchers and those affected by high EGS populations and subsequent damage in urban and suburban communities, as well as activists who seek non-lethal solutions to human–wildlife conflicts. Some opponents include wildlife biologists and managers, recreational hunters, and some animal welfare groups who oppose the use of wildlife contraception (Kirpatrick 2007). Despite the various perspectives on the use of contraceptives to control growth in wild animal populations, continued research is needed. Research to evaluate the use of GonaCon™ and other contraceptives can provide answers to questions that remain on effectiveness and efficacy, impacts on biology and behavior of target and non-target species, costs, and social acceptability. A key factor in the

sustained use of any anti-fertility vaccine is its margin of safety in the target species. It is important that the vaccine formulation used in this study be improved to avoid injection site reactions observed in EGS in this study.

Continued research should focus on collecting EGS blood samples of GonaCon™ treated and control EGS during peak breeding seasons. To determine the peak of EGS breeding seasons in local populations, baseline hormonal profiles of EGS are needed through at least one year of breeding seasons. This is important for future studies, since this study did not detect any significant differences in hormonal concentrations between GonaCon™ treated and control EGS. This was likely a result of peak breeding being missed when blood was collected. Consequently, collection of blood from EGS should coincide with peaks in breeding, as collection during post-breeding, to enhance the ability to detect differences in GonaCon™ treated and control EGS. A definitive method to determine occurrence of potential breeding, as well as potential parturition in GonaCon™ treated EGS, will provide conclusive evidence on the efficacy of GonaCon™ as a potential tool for immunocontraception in EGS.

Table 1. EGS trapped as “originals”.

	March- April 2008		Jul 2008		Nov 2008		
	T	C	T	C	T	C	Total
Male	17	16	14	12	8	4	71
Female	16	6	9	8	3	4	46
Total	33	22	23	20	11	8	117

Table 2. EGS trapped as “recaptures”.

	March- April 2008		Jul 2008		Nov 2008		May- June 2009		
	T	C	T	C	T	C	T	C	Total
Male	5	7	17	26	18	17	8	10	108
Female	3	19	15	13	5	20	8	9	92
Total	8	26	32	39	23	37	16	19	200

Table 3. EGS capture data sheet.

RESEARCH DATA SQUIRREL CONTRACEPTION RESEARCH PROJECT Clemson University Department of Forestry and Natural Resources Principal Investigator: Greg K. Yarrow Phone: 864 – 656 – 5334	Capture, Handling, Blood Collection and Vaccination Records	Project ID: QA-1534 Site: Date: Initials:

Trap#	Sex[1]	Age[2]	Weight (g)	Microchip Number	Color of Ear Tag Used[3]	Breeding Status	Blood Collected (ml)	Vaccine ID (Batch#)	Amount of Vaccine given (ml)	Handling Time[5]			Scrotal Size[6] (mm)	
										Start	End	Mt	L	B

[1]F = female, M = male, U = unknown
[2]A = adult, J = juvenile, U = unknown
[3]B = Blue, R = Red, G = Green, W = White
[4]LT = lactating, NLT = not lactating, P = pigmented scrotum, NP = not pigmented scrotum, T = descended testes, NT = not descended testes
[5]Time taken in minutes from start of handling to release of animal
[6]Scrotal size, length and breadth in mm
*last revised July 2008

Table 4. Mean scrotal size (mm) measurements of treated and control EGS males.

	Originals		Recaptures	
	Length (mm)	Breadth (mm)	Length (mm)	Breadth (mm)
Treated Males	20.41 ± 2.5	13.29 ± 1.89	15.07 ± 1.28	9.11 ± 0.95
Control Males	7.11 ± 0.26	5.50 ± 0.45	18.04 ± 2.31	11.73 ±1.76

Table 5. Mean testosterone (nmol/1) concentrations of treated and control EGS males (B = treated, Y = control).

No	Sex	Color of Ear Tag	Microchip Number	Treatment Status	Breeding Status	TESTOSTERONE (ng/ml)		
						Season 1	Season 2	Season 3
1	M	B-132	016-069-087	t	NPT	0.3	.	0.3
2	M	B-133	016-051-055	t	PT	0.2	0.2	0.2
3	M	B-134	016-076-638	t	NLT	0.2	0.6	1
4	M	B-135	016-086-001	t	NP, NT	.	0.5	0.8
5	M	B-137	016-054-004	t	NPT	0.4	.	0.3
6	M	B-139	016-039-883	t	NP, NT	0.1	.	0.4
7	M	B-191	016-064-337	t	PT	0.8	0.1	0.2
8	M	Y-118	016-095-873	c	PT	0.3	0.4	0.5
9	M	Y-119	016-064-825	c	PT	0.2	0.1	.
10	M	Y-120	016-101-082	c	PT	0.5	0.5	.
11	M	Y-125	019-012-327	c	PT	0.6	.	0.6
12	M	Y-126	016-099-082	c	PT	0.2	.	1.2
13	M	Y-127	019-051-563	c	PT	0.3	0.3	.
14	M	Y-128	016-085-082	c	PT	0.3	0.1	0.2

Table 6. Mean progesterone concentrations (nmol/1) concentrations of treated and control EGS females (W = treated, R = control).

No	Sex	Color of Ear Tag	Microchip Number	Treatment Status	Breeding Status	PROGESTERONE (ng/ml)		
						Season 1	Season 2	Season 3
1	F	W147	016-103-575	t	NLT	2.4	.	.
2	F	W149	016-044-608	t	NLT	1.1	.	.
3	F	W150	016-069-045	t	NLT	.	0.9	.
4	F	W151	016-089-573	t	NLT	2.3	.	.
5	F	W155	016-083-003	t	NLT	1.3	.	.
6	F	W157	016-084-556	t	NLT	1.2	.	.
7	F	W158	015-844-305	t	NLT	.	0.5	.
8	F	W160	015-792-356	t	NLT	1.8	.	.
9	F	W171	016-063-543	t	NLT	1.6	.	.
10	F	W178	019-023-068	t	NLT	1.1	1.4	.
11	F	W179	019-034-110	t	NLT	1.1	.	.
12	F	W180	019-025-256	t	NLT	2.8	.	.
13	F	R-102	016-076-278	c	NLT	1	.	1.6
14	F	R-104	016-088-882	c	NLT	0.9	.	2.1
15	F	R-106	016-082-602	c	NLT	1.1	1.9	1
16	F	R-107	016-050-802	c	NLT	2	0.9	.
17	F	R-111	016-095-798	c	NLT	0.9	3	1.1
18	F	R-114	016-083-358	c	NLT	0.7	0.6	17.4
19	F	R-168	019-036-348	c	NLT	2.2	2.9	.

Table 7. Measured antibody titers of treated and control EGS (R = control female, W = treated female, Y = control male, B = treated male).

						MEASURED TITERS		
No	Sex	Color of Ear Tag	Microchip Number	Treatment Status	Breeding Status	Season 1	Season 2	Season 3
1	F	R107	016-050-802	c	NLT	0	0	.
2	F	R114	016-095-798	c	NLT	0	0	0
3	F	R112	019-045-014	c	NLT	.	0	0
4	F	R49	019-046-542	c	NLT	0	0	0
5	F	W150	016-069-045	t	NLT	0	128000	.
6	F	W160	015-792-356	t	NLT	0	128000	.
7	F	W174	019-010-829	t	NLT	0	128000	.
8	F	W178	019-023-068	t	NLT	.	128000	.
9	F	W82	024-124-828	t	NLT		0	128000
10	M	Y127	016-085-082	c	NT	0	0	.
11	M	Y128	016-099-082	c	NT	.	0	0
12	M	Y64	019-032-012	c	NT	.	0	0
13	M	Y66	016-099-082	c	PT	.	2000	8000
14	M	B132	016-069-087	t	NT	0	128000	.
15	M	B133	016-051-055	t	PT	0	128000	.
16	M	B134	016-076-638	t	NT	0	128000	128000
17	M	B137	016-054-004	t	NT	0	.	128000
18	M	B139	016-039-883	t	NT	0	0	64000
19	M	B145	016-051-776	t	NPT	0	.	128000

Table 8. EGS males examined on necropsy (Y = control, B = treated).

Sex	Color of Ear Tag	Age	Weight (grams)	Treatment Status	Microchip Number	Status	Scrotal Size (mm)		Testes Weight (mg)
							L	B	
M	Y123	A	450	c	.			20	784.8
M	B135	A	610	t	016-086-001	PT	18.69	12.4	402.8
M	Y119	A	550	c	.	PT	30.43	20.61	788.63
M	.	A	550	t	016.041-341	NPNT	.	.	238
M	Y69	A	480	c	019-045-855	NPNT	.	.	147
M	B191	A	490	t	019-064-337	PT	14.84	8.88	512
M	Y129	A	620	c	016-050-529	PT	24.57	14.83	5767
M	Y125	A	520	c	.	PT	36.54	25	5713
M	Y124	A	470	c	016-097-558	PT	31.55	24.69	4641
M	B139	A	470	t	016-039-883	PT	18.81	11.36	401
M	Y64	A	510	c	019-032-012	PT	35.4	22.48	4134
M	B189	A	430	t	019-068-841	NPNT	.	.	178
M	Y127	A	450	c	019-051-563	PT	34.52	20.45	4130
M	Y117	A	470	c	016-077-309	PT	51.19	29.39	5027
M	B138	A	500	t	015-865-824	PT	10.05	3.18	579
M	B199	A	500	t	019-010-846	NPNT	.	.	266
M	B97	A	430	t	019-035-635	NPNT	.	.	182
M	Y121	A	540	c	.	PT	40.11	28.66	5742

Table 9. EGS females examined on necropsy.

Sex	Age	Weight (grams)	Treatment Status	Microchip Number	Breeding Status	Ovaries weight (mg)	Diameter of horn (mm)	Length of Tract (mm)
F	A	470	t	024-298-825	NLT	97	2	70
F	A	520	c	016-082-602	NLT	110	1.91	90
F	A	590	c	019-036-348	NLT	102	1.51	80
F	A	610	c	019-045-014	NLT	109	1.89	90
F	A	490	c	024-278-118	NLT	19	1.61	50
F	A	430	c	019-062-086	NLT	38	1.44	60
F	A	550	t	016-076-558	NLT	52	1.8	55
F	A	410	c	.	NLT	50	1.71	50
F	A	540	t	024-127-607	NLT	116	2.01	80
F	A	600	c	024-329-607	NLT	112	1.41	90
F	A	480	c	019-042-309	NLT	95	1.1	65
F	A	480	t	019-010-829	NLT	126	2.21	85
F	A	480	t	019-060-865	NLT	102	1.91	65
F	A	430	t	024-124-828	NLT	72	1.14	60
F	A	470	t	016-084-556	NLT	110	2.02	90
F	A	420	t	016-067-085	NLT	108	2	78
F	A	550	c	.	NLT	288	4	85

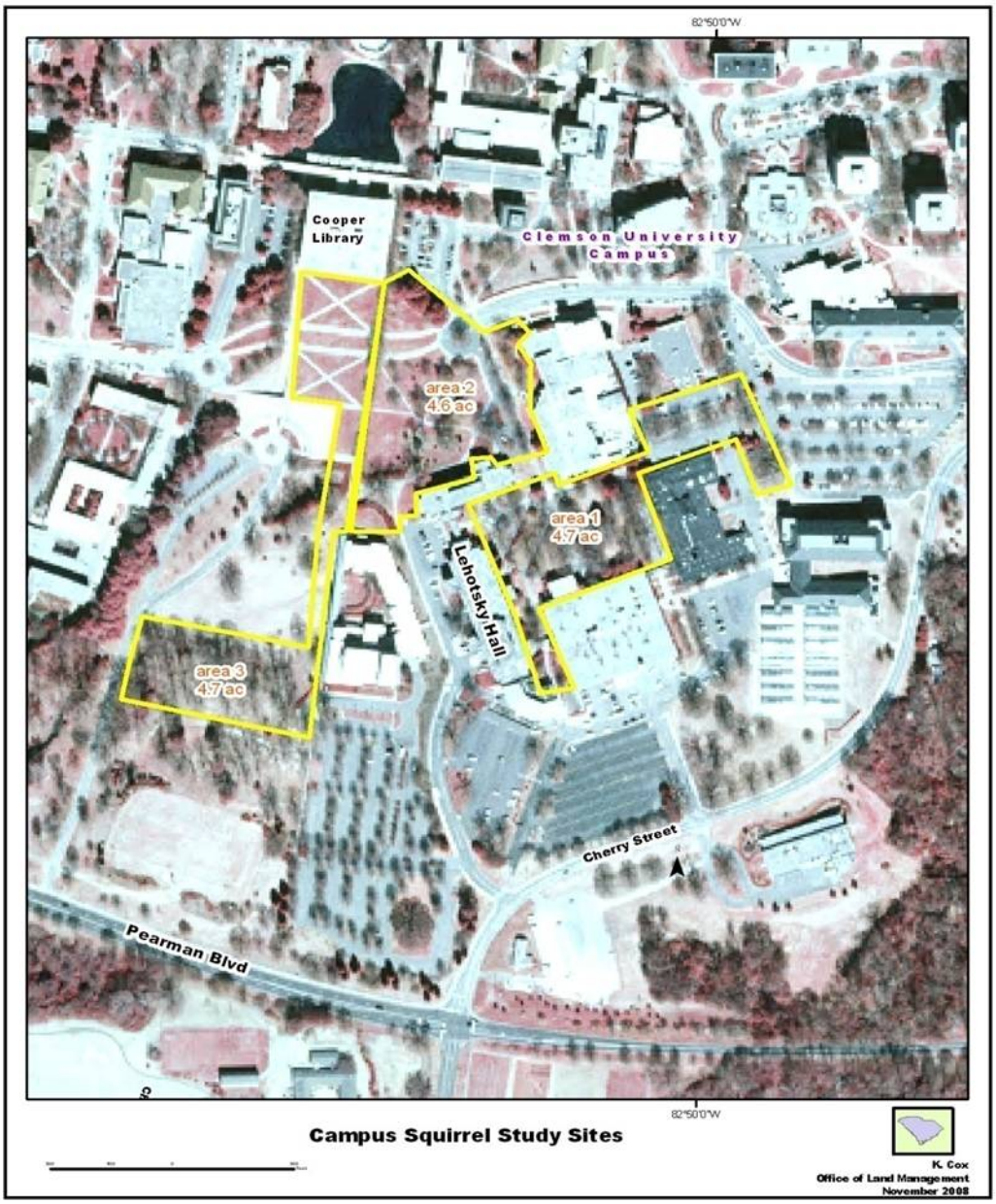

Figure 7. Map of EGS study area.

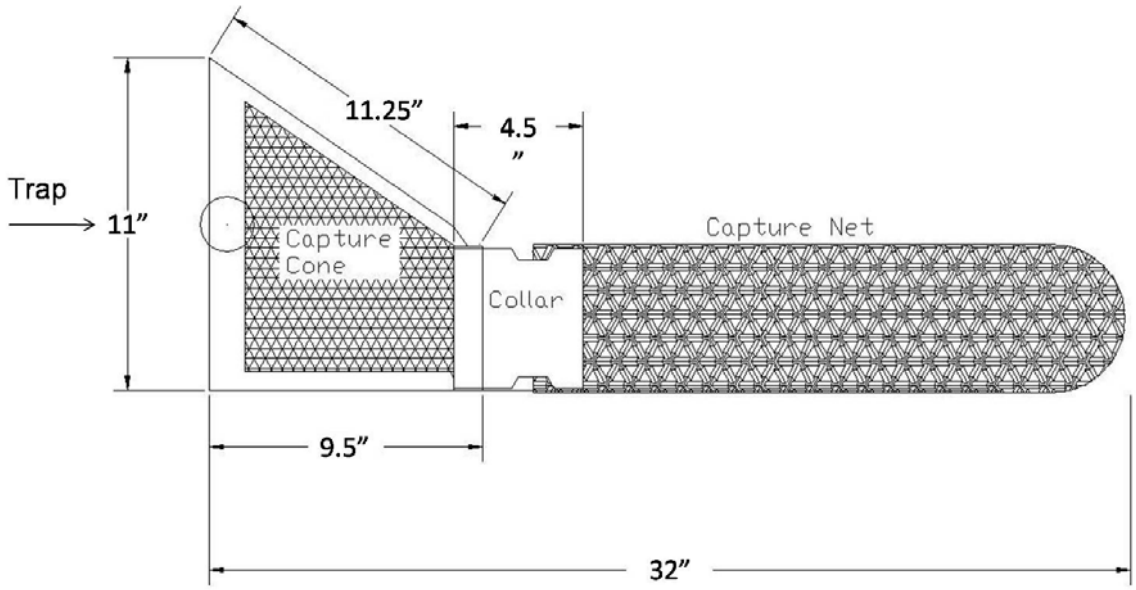

Figure 8. Modified trap design with capture cone

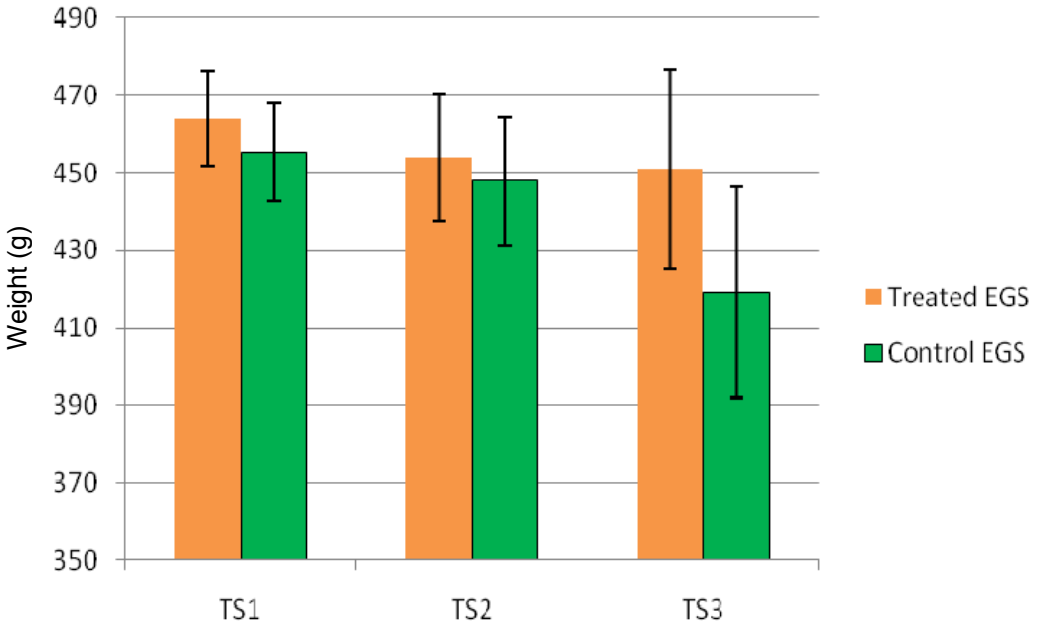

Figure 9. No significant differences in body weights of control and treated EGS in each trapping session TS1 ($p = 0.63$), TS2 ($p = 0.80$), TS3 ($p = 0.40$).

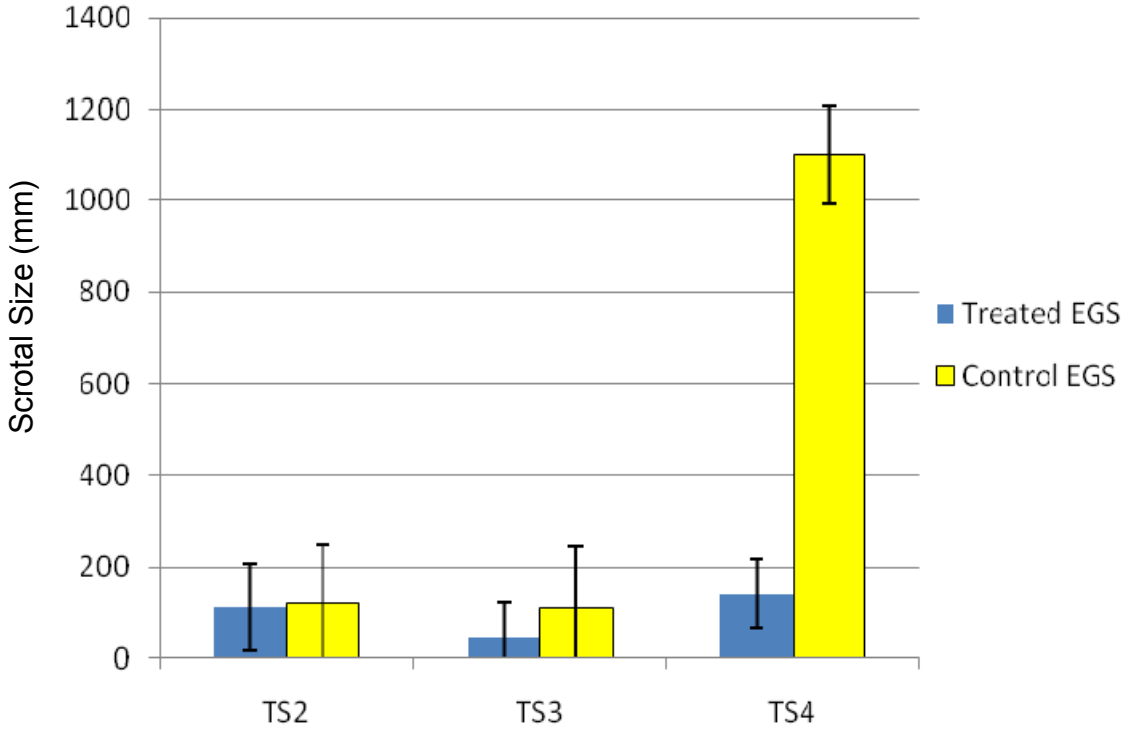

Figure 10. Significant differences in scrotal size of control and treated EGS males by the fourth trapping session TS4 ($p = 0.001$).

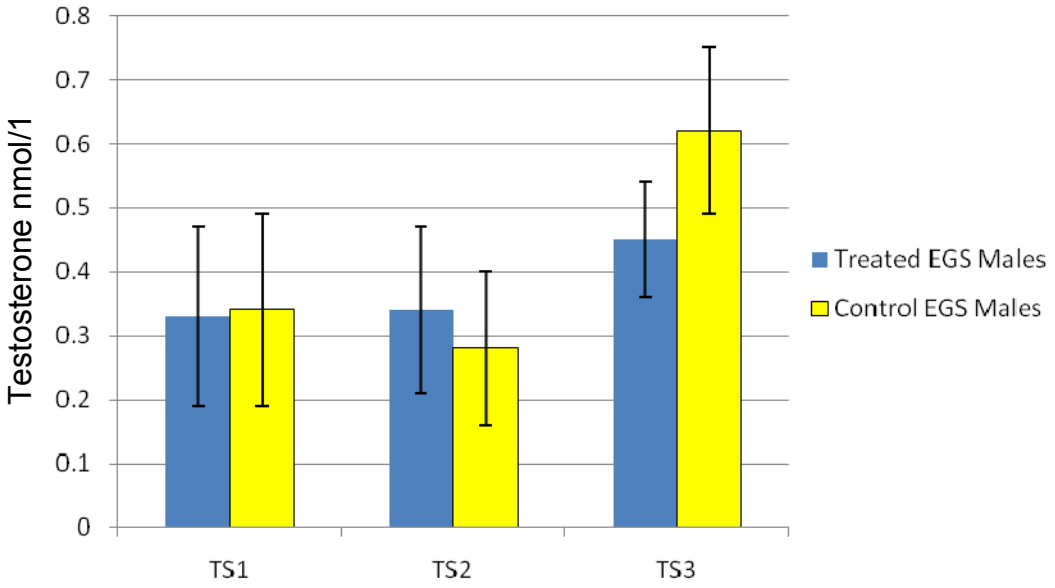

Figure 11. No significant differences in testosterone concentrations of control and treated EGS males by the third trapping session TS3 (p = 0.32).

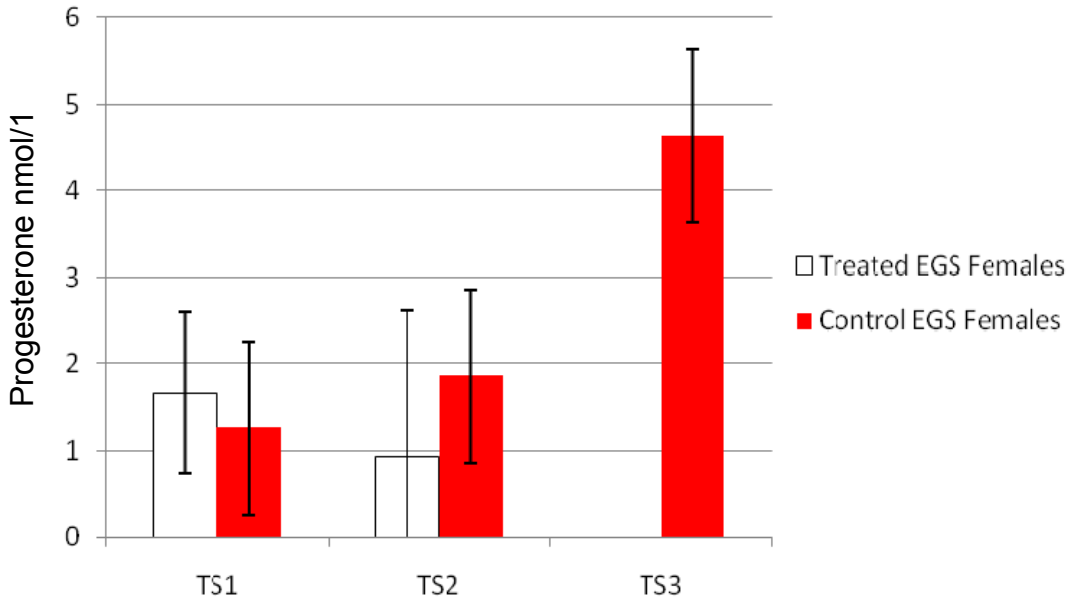

Figure 12. No significant differences in progesterone concentrations of control and treated EGS females by the second trapping session TS2 ($p = 0.68$).

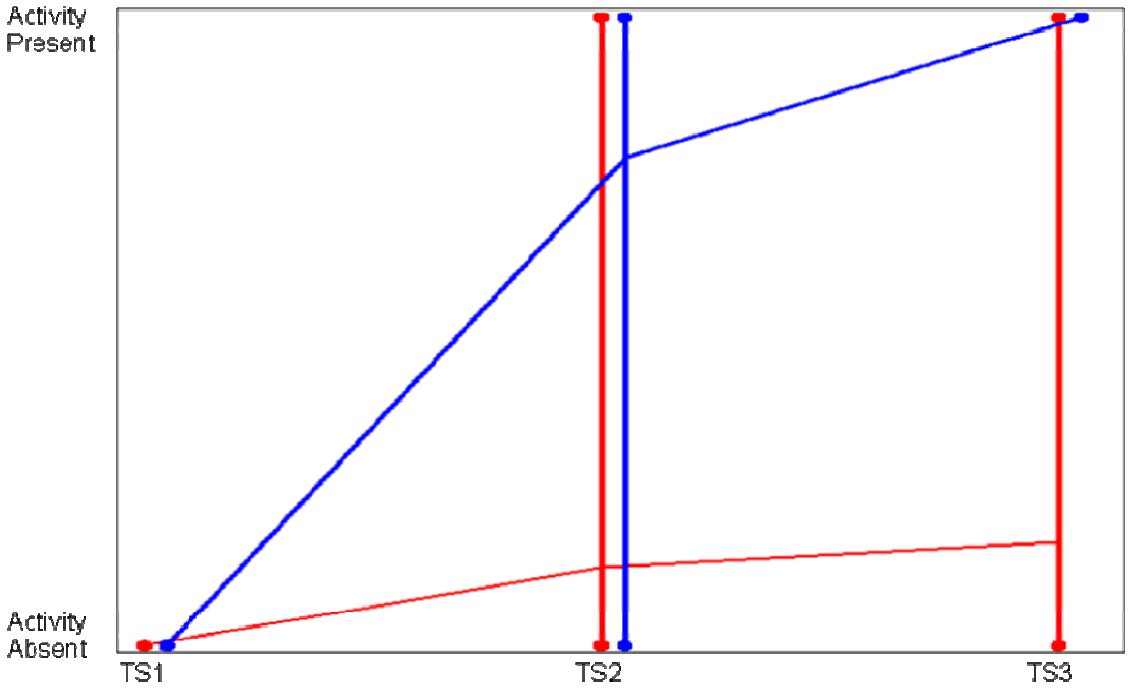

Figure 13. Significant differences in antibody titer activity of control EGS (red) and treated EGS (blue) by second trapping and third session, TS2 ($p = 0.029$) and TS3 ($p = 0.017$).

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