

Project Plan

Field Assessment of the Effectiveness of DiazaCon™ on Reducing Gray Squirrel Reproduction and Population

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Introduction

The Eastern gray squirrel (*Sciurus carolinensis*) (EGS) is one of the most common wildlife species in urban and suburban communities within the eastern United States. Due to their relative adaptability and lack of predators in urban environments, their numbers have increased in communities across their natural range. With this increase in numbers there has also been a corresponding increase in human-squirrel conflicts. Most notably is the damage EGS are causing to trees and shrubs in managed landscape settings. Gnawing and stripping of tree bark by EGS has become a serious problem in communities where EGS populations are unusually high. For example, recent observations by landscaping crews on Clemson University's main campus have documented over 100 mature trees killed directly by EGS gnawing and bark stripping, and an additional 100 trees severely damaged by EGS (Bill Carson, personal communication). According to an outside arborist consultant, the estimated loss of one mature tree from EGS on Clemson's campus can be \$13,275.00. Using these estimates, damage caused by EGS on Clemson's campus may exceed \$1.3 million dollars. These findings are also consistent with other studies that have shown significant tree losses due to bark stripping by EGS (Lurz et al. 2001). Unusually high EGS populations in urban environments are also known to damage ornamental shrubs and herbaceous plantings, short out electrical power transformers, cause structural damage to buildings, and increase the likelihood of transmittable diseases and parasites among local squirrel populations.

Efforts to control EGS numbers and their associated damage have been limited to exclusion techniques, habitat modification, repellents, trapping, shooting, and recreational hunting. Unfortunately in some situations, these alternatives are not effective, practical, or socially acceptable as tools for controlling EGS numbers and associated problems that they cause in urban and suburban communities. Recent work

by Pai (2009) to evaluate the effectiveness of the immunocontraceptive GonaCon™ in reducing reproduction in a small group (317) of EGS on the campus of Clemson University demonstrated that the contraceptive was effective in preventing reproduction in both male and female squirrels. However, the cost of capturing and vaccinating individual EGS with GonaCon™ was \$15/squirrel and proved to be cost prohibitive, especially when administering on a large scale basis like the entire campus of Clemson University (325 ha).

This project proposes to field test the immunocontraptive DiazaCon™ in reducing EGS reproduction and damage on the entire campus of Clemson University. Like GonaCon™, DiazaCon™ has been shown to be effective at inhibiting reproduction in a variety of birds and mammals. An additional benefit of DiazaCon™ is that it is an oral bait so it does not require capturing EGS; therefore, it may be a cost-effective method of controlling EGS reproduction and damage on Clemson University's campus.

How DiazaCon™ Works (USDA National Wildlife Research Center)

DiazaCon™ is an oral contraceptive that inhibits the production of cholesterol. Its chemical structure is identical to cholesterol, with the exception of two nitrogen substitutions at the 20th and 25th hydrocarbons. Because of this substitution, the liver cannot effectively eliminate DiazaCon™ as it does cholesterol, and consequently DiazaCon™ persists in the liver over several months. The side chain of cholesterol is cleaved to form the precursor hormone pregnenolone, which is the parent compound for all steroid hormones. In the body, pregnenolone is altered to form testosterone, estradiol, and progesterone. These hormones are necessary for sperm and egg production. The nitrogen substitutions at the 20th and 25th hydrocarbon prevent the cleavage of cholesterol from forming pregnenolone. As a result, plasma cholesterol concentrations decrease and desmosterol concentrations increase. Desmosterol is the immediate precursor to cholesterol in the cholesterol synthesis pathway and cannot be transformed into pregnenolone. Without sufficient cholesterol, the body "protects" itself by conserving the existing cholesterol for functions necessary for survival. Consequently, because reproduction is not necessary for an individual animal to survive, the reproductive system is shut down.

DiazaCon™ has been tested on a variety of birds and small mammals and shows promise as an oral contraceptive. Preliminary laboratory tests using DiazaCon™ for contraception in EGS is promising, and work continues at the USDA National Wildlife

Research Center. A treatment period of 5-10 days is generally required, and depending on the species, the contraceptive effects can last up to four months. Because DiazaCon™ is an oral bait, there are potential risks to non-target animals that may feed on the treated bait. These risks can be partially mitigated by the type of bait used, placement of bait, and the design of bait stations (use of one that only allows the targeted animals to feed). DiazaCon™ may also be associated with some secondary effects because of its persistence in the liver; however, research is currently being conducted to determine the magnitude of the effect. Unlike other immunocontraceptives like GonaCon™, animals do not have to be captured to deliver DiazaCon™ which offers benefits in terms of associated logistics and costs (Table 1).

Table 1. Comparison of GonaCon™ and DiazaCon™

	GonaCon™	DiazaCon™
Delivery mode	Injection	Oral
Length of treatment	Once	5-10 days
Longevity of effects	2-4 years	≥ 4 months
Sex affected	Both	Both
Reversibility	Usually*	Yes
Animal capture required?	Yes	No
Toxicity/health effects	Minor	Only with overdose
Non-target hazards	No	Yes
Secondary effects	No	Yes

*May last the lifetime in shorter lived species, making contraception permanent.

Project Objective(s)

- To determine peak breeding seasons for EGS on the campus of Clemson University.
- To evaluate the effectiveness of DiazaCon™ in reducing EGS reproduction on the campus of Clemson University.

Methods

Clemson University's main campus, located in Clemson, South Carolina, has been selected as the study site to evaluate the effectiveness of DiazaCon™ in reducing eastern gray squirrel (EGS) reproduction. Clemson University's main campus is approximately 800 acres of teaching, research and administrative buildings, interspersed with mature hardwoods (primarily *Quercus* spp., *Carya* spp.) and landscaping shrubs. Currently, estimates of EGS populations on Clemson's campus are extremely high (9 squirrels/acre),

and tree damage by EGS is significant. Carrying capacity for EGS in most habitat types across the southeastern United States ranges from 1-5 squirrels/acre (Waters 2006).

In the first year of the study, EGS will be trapped monthly (March 2011-February 2012) according to SOP FP031.00, and blood samples will be collected from 5-10 males, and 5-10 females to determine breeding periods for EGS on campus. Within 2 hours of capture, each animal will be assigned an identification number that corresponds to its trap number (SOP ACCO 002.00) and have blood collected from the lateral femoral vein according to SOP FP030.00. Plasma will be analyzed for progesterone and testosterone levels using radioimmunoassay (RIA) according to SOP BT025.00. By analyzing monthly EGS progesterone and testosterone levels, we will be able to determine peak breeding season(s) of EGS on Clemson's campus. This information is imperative for establishing the proper time frame for dispensing the DiazaCon™ contraceptive to EGS on Clemson University's campus. In order for DiazaCon™ to be effective, the contraceptive needs to be available to male EGS one month before peak testosterone levels are achieved (Yoder, pers.communication). EGS will be trapped during a one week period each month using wooden box traps baited with sunflower seeds according to SOP FP031.00. Trapping sites will be pre-baited one week prior to trapping. Traps will be open from sunrise until 10 a.m., at which time they will be locked and placed upside down against a tree. Traps will be reopened at 3 p.m. and remain open until sunset. At sunset, traps will be locked and placed upside down against a tree. While traps are in use they will be checked at two hour intervals to minimize potential stress on EGS. During the first year of the study up to 10 male and 10 female EGS per month will be euthanized with a lethal dose of carbon dioxide (SOP ACCO008.00)(American Veterinary Medical Association (AVMA) 2001) in an effort to reduce EGS numbers on Clemson University's campus before delivery of DiazaCon™ during years 2 and 3 of the study. In the fall of year one of the study, bait stations containing sunflower seeds will be mounted to select trees across Clemson University's campus. At the site of each bait station, cameras will be installed to determine whether or not non-targets can access the seeds.

In years 2 and 3 of the study (March 2012-February 2014), bait stations specific for EGS (designed and developed in Europe) will be mounted to select trees distributed across the treatment study area. Pre-baiting with sunflower seeds will occur for 2 weeks to acclimate EGS to the bait stations. High oil content sunflower seeds will be used as bait in feeding stations. After the 2 week baiting period, DiazaCon™ coated sunflower seeds will be placed in feeding stations (15 across campus) for 2 weeks. DiazaCon™ bait will be

manufactured according to SOP BT030.00 to contain 0.23% DiazaCon™ (w/w). In addition, the bait will also contain Rhodamine B, an inert, nontoxic, and persistent dye. The dye will serve as a biomarker on the teeth to determine which EGS have consumed treated DiazaCon™ bait (Fisher 1999). Bait will be placed in feeding stations early in the morning. Prior to sunset, feeding stations will be closed to reduce the potential feeding of southern flying squirrels which are primarily nocturnal. The amount of bait placed in each station, and the amount of bait left in each station at the end of the 2 week period will be recorded. DiazaCon™ will be administered for 2-3 weeks at the end of both March and October, 2012 and 2013; four weeks prior to spring and winter breeding seasons.

In late spring of years 2 and 3 of the study (March 2012- February 2014), to evaluate the effectiveness of DiazaCon™ on reducing EGS reproduction, 100 EGS will be trapped in treated areas according to SOP FP031.00, be assigned an identification number that corresponds to its trap number (SOP ACCO 002.00), have blood samples taken (SOP FP030.00), and be ear-tagged and micro-chipped. Blood samples will also be collected from female EGS post-breeding to determine pregnancy. Blood will be collected from the lateral or femoral vein according to SOP FP030.00. Plasma will be analyzed for progesterone and testosterone concentrations using radioimmunoassay (RIA) according to SOP BT025.00. Plasma samples will also be analyzed for desmosterol and non-esterified cholesterol by the Analytical Chemistry Project, using the procedures outlined in "Determination of Desmosterol and Free Cholesterol in Prairie Dog, Rat, and Brown Tree Snake Plasma" (method 98A; Johnston et al. 2003). All plasma samples will be stored at -20 to -70° C until analysis.

Additional measures of fertility will be evidenced by lactation in females and scrotal pigmentation in breeding male EGS. Lactation will be assessed according to the appearance of teats and presence of milk. Breeding male EGS will typically have a gray or black pigmented scrotum with little hair covering and enlarged testes. Non-breeding males typically have a pink pigmented scrotum with hair re-growth evident and small flaccid testes (Pudney 1976, Webley et al. 1985, Ferryman et al. 2006). Another technique we will be using to gauge the effectiveness of DiazaCon™ is remote camera peepers. This non-invasive tool will allow us to see whether DiazaCon™ tagged female EGS have any pups in their nests or dens.

Throughout 2011-2014, GonaCon™ treated ear and micro-chipped EGS that are trapped according to SOP FP031.00, will have blood samples collected from them (SOP

FP 030.00) to evaluate the ongoing effectiveness of GonaCon™ in reducing reproduction in EGS. Antibody titers and hormonal levels will be measured as an index of fertility.

At the end of the study, 40 marked and 40 unmarked EGS will be trapped (SOP FP031.00) and euthanized (SOP ACCO008.00) for a comparative analysis of treated and non-treated reproductive tissues and reproductive organs. The ovaries will be examined histologically for evidence of corpora lutea scars. Briefly, ovaries will be fixed in formalin and serially sectioned at 6-10 μ . Sections will be stained with hematoxylin and eosin, and examined for corpora lutea (Nixon and McClain 1975). Histological examination of reproductive organs in both males and female EGS will also be conducted to determine the effectiveness of DiazaCon™ as described by Pai (2009).

Age Determination and Marking

A combination of pelage characteristics and body weight should be used to more accurately assign an age class to individuals. Although scrotal appearance can be used to aid in aging male gray squirrels, the appearance of subadult and adult males with regressed testes can often be confusing (Hoffman and Kirkpatrick 1956). Skeletal and tooth characteristics can be used to accurately age gray squirrels, but these methods require euthanizing the animal. Age classes for the purpose of this study are defined as follows: *juvenile* – 0-6 months of age, *subadult* – 6-12 months of age, and *adult* - > 12 months of age.

The appearance of scrotum or teats can be used to help separate gray squirrels into these age classes. Juvenile males (3-6 months) will have hair covering the scrotum with only light pigmentation. Subadult males (6-12 months of age) will have furred and pigmented scrotum. Adult males (> 1 year) will have a furless, pigmented scrotum. The scrotum may become less pigmented during periods of testicular quiescence (Ferryman et al. 2006). Non-breeding, juvenile females will have very small, pink teats that can only be seen by moving the fur. Adult, breeding females will have teats that can easily be seen through the fur, and may have small pigmented areas of gray/black (Dimmick and Pelton 1996). In lactating females, most of the hair will be absent from the teats.

Tail pelage characteristics can also be used to separate gray squirrels into age classes (Sharp 1958, Dimmick and Pelton 1996). Juveniles do not have short secondary hairs appressed (laying flat) along the ventral side of the tailbone, making the outline of the tailbone easily visible. In addition, there are 2-3 dark bands running through the reddish fur parallel to the tailbone on either side. These bands are most

easily seen in the mid-region of the tail. Subadults have short secondary hairs appressed along the lower third of the ventral side of the tail. The outline of the tailbone is still visible. Like juveniles, subadults have 2-3 dark bands running parallel to either side of the tail. Adults have short secondary hairs appressed along the entire length of the ventral side of the tail, obscuring the outline of the tailbone. The dark, parallel bands present in juveniles and subadults are not visible in adults.

During the winter months, pelage can also be used to assign age classes when the fur in the rump region is separated and laid flat (Barrier and Barkalow 1967, Dimmick and Pelton 1996). The area just adjacent to the mid-dorsal stripe must be used as all age classes have black-tipped hairs in the mid-dorsal stripe regardless of season. Juveniles will have no yellow pre-basal band in the black underfur and guard hairs will be tipped with black (Figure 3). Subadults will have either no yellow pre-basal band in the underfur or an indistinct pre-basal band. Guard hairs will be tipped with white. Adults will have a distinct yellow pre-basal band in the underfur and white-tipped guard hairs. During summer months, all age classes have black-tipped guard hairs.

The body weight of an adult EGS is typically between 396.2-710.0 g (Uhlig 1955, Koprowski 1994, Dimmick and Pelton 1996). Squirrels will be weighed using a spring scale according to SOP FP 029.00.

EGS will be ear-tagged for easy recognition. Ear tags will be positioned such that they are attached where the cartilage is the thickest. Pliers will be used to attach ear tags, and numbers will be positioned dorsally. In addition, PIT tags may also be inserted to serve as a second identifier in case ear tags are lost. Prior to inserting the PIT tag, we will scan the tag to make sure it is working properly and also verify the number. We will then pinch the dorsal skin between the scapula to form a "tent", place the needle at the base of the tent $\leq 45^\circ$ from the dorsal midline, insert it until the needle tip can be felt against the skin on the side opposite the injection site, and then depress the plunger to insert the PIT tag, making sure the needle has not gone all the way through the skin. As the needle is withdrawn, we will use our fingers to pinch off the injection site, ensuring that the PIT tag does not fall out. Finally we will scan the animal to verify the PIT tag was inserted properly.

Gray Squirrel Density Estimates

Although density estimates are not an accurate metric to assess the effects of contraceptive treatments, they are important parameters to document during the study.

Prior to initiation of DiazaCon™ contraceptive treatments, EGS population densities will be estimated on Clemson University's main campus using the Lincoln-Peterson Index mark-recapture method. Between April and August, 2011, a total of 50 EGS will be randomly captured using box traps according to SOP FP031.00. Captured EGS will be ear-tagged for easy recognition and then released. A second sample of 50 EGS will be trapped between October, 2011 and February, 2012. During the second sampling, population densities will be estimated by calculating the ratio of recaptured animals to the total number of individuals captured during the second trapping session.

Secondary DiazaCon™ Exposure to Non-Targets

Feeding station design significantly reduces potential ingestion of DiazaCon™ treated bait by non-target animals (Yoder et al. 2005). Studies have shown that ingestion of DiazaCon™ over a long period of time can result in increased DiazaCon™ levels in the liver (Yoder et al. 2005) so there are concerns regarding possible secondary effects to hawks (primarily red-tailed) whose predation on EGS on Clemson University's main campus have been observed. To obtain baseline blood parameter levels in hawks, red-tailed and/or red-shouldered hawks will be trapped according to SOP FP032.00, and assigned an identification number that corresponds to its trap number (SOP ACCO 002.00). Blood will be drawn from 5-10 male and 5-10 female hawks prior to dispensing DiazaCon™ to EGS on Clemson University's campus. The guidelines in SOP ENTOX302.21.03 for drawing blood from the brachial vein in the wing will be followed. Once DiazaCon™ is administered as oral bait to EGS on campus, in years 2 and 3, hawks (red-tailed and/or red-shouldered) will be trapped according to SOP FP032.00, assigned an identification number that corresponds to its trap number (SOP ACCO 002.00), and blood will be drawn from 5-10 male and 5-10 female hawks for a post exposure assessment of hawk DiazaCon™ levels. The guidelines in SOP ENT302.21.03 for drawing blood from the brachial vein in the wing will be followed. Plasma will be analyzed for DiazaCon™ concentrations using radioimmunoassay (RIA) according to SOP BT025.00 for comparison to the aforementioned pre-treatment hawk sampling. Even though the contraceptive effects of DiazaCon™ are reversible, research has shown effects to some avian species which may include reduced egg production, fertility, and hatchability (Yoder et al. 2004 and Yoder et al. 2005).

Statistical Analyses

Descriptive statistics (mean \pm S.E.) will be calculated for continuous variables and proportions or ratios will be calculated for categorical variables in each group. Continuous variable means will be compared over time between treatments and controls (e.g. 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 will be set at 0.05. Categorical variables (desmosterol and cholesterol levels) will be compared over time between treatments and controls using Fisher's exact test of proportions. All statistical calculations will be performed using Proc GLM and Proc frequency of SAS.

List Number and Title of Standard Operating Procedures (SOPs)

AC/CO 002.00	Animal Handling to Maintain Secure Identification
AC/CO 008.00	Euthanasia with CO ₂
BT017.00	ELISA Procedure to Assess Antibody Titers in Plasma or Serum
BT 025.00	Use of Radioimmunoassay (RIA) to Assess Reproductive Hormone Concentrations in Plasma or Serum
BT 026.00	Purification of Antibodies From Plasma or Serum Using the Pierce NAb Spin Kit
BT 030.00	Manufacture of 0.23% DiazaCon™ (w/w) rolled oat bait for prairie dogs
ENTOX 302.21.03	Nestling Bald Eagle Handling, Measuring, Blood and Feather Collection
FP 029.00	Use of a Spring Scale for Body Mass Measurements
FP 030.00	Collection and Processing of Blood Samples from Prairie Dogs and Similar Size Mammals
FP 031.00	Trapping Eastern Gray Squirrels on Clemson University's Campus
FP 032.00	Trapping Hawks on Clemson University's Campus

List of Records to be Maintained

- Aging records
- Animal weights
- Antibody purification records
- Breeding status records
- Density estimates
- Distance sampling records
- Euthanasia records
- DiazaCon™ manufacturing records

Ovary/corpora lutea examination records
 RIA records and results
 Sexing records
 Squirrel blood chain of custody records
 Squirrel blood collection records
 Squirrel vaccination records
 Statistical records
 Tagging (ear tags and PIT tags) records
 Capture-Mark-Recapture records
 Trapping records

Permits/Certifications Required

Clemson University Animal Use Protocol (AUP)
 USFWS Section 7 Consult
 SC Pesticide Regulatory
 SCDNR Scientific Collecting Permit
 Zoonotic & Hantavirus Training
 Enrollment in Medical Surveillance Program

Cooperators

- Dr. Christi Yoder, Reproductive Physiologist, USDA Wildlife Services, Fort Collins, CO
- Mr. Noel Myers, South Carolina State Director, USDA Wildlife Services.
- Mr. Tom Swaynham, Regional Wildlife Biologist, South Carolina Department of Natural Resources
- Mr. Derrell Shipes, Chief of Statewide Projects, Research & Surveys with the South Carolina Department of Natural Resources

Time-frame

Year 1 (March 2011 – February 2012)

Initial gray squirrel population survey campus-wide

Conduct monthly hormonal profile to determine potential shifts in breeding seasons

Collection of blood from hawks to establish baseline DiazaCon™ levels for hawks -
 October

Place squirrel feeding stations, baited with sunflower seeds, across campus. Cameras will be installed to determine whether or not non-targets can access the seeds

Year 2 (March 2012 – February 2013)

Build 15 squirrel feeding stations and place across campus

Pre-bait & bait feeding stations – March

Post-treatment capture, mark and collect blood from squirrels

Post-treatment capture and collect blood samples from hawks for a post-exposure
 assessment of hawk DiazaCon™ levels

Pre-bait & bait feeding stations – October

Post-treatment capture, mark and collect blood from squirrels
 Post-treatment capture and collect blood samples from hawks for a post-exposure
 assessment of hawk DiazaCon™ levels
 Conduct population estimation
 Analyze first year's data
 Submit first year project report

Year 3 (March 2013 – February 2014)

Pre-bait & bait feeding stations – March
 Post-treatment capture, mark and collect blood from squirrels
 Post-treatment capture and collect blood samples from hawks for a post-exposure
 assessment of hawk DiazaCon™ levels
 Pre-bait & bait feeding stations – October
 Post-treatment capture, mark and collect blood from squirrels
 Post-treatment capture and collect blood samples from hawks for a post-exposure
 assessment of hawk DiazaCon™ levels
 Conduct population estimation
 Analyze second year's data

Year 4 (March 2014 – May 2014)

Analyze all project data
 Completion of Ph.D. dissertation and publications
 Submit final project report

Deliverable Products and Expected Outcomes

- Reduced reproduction in gray squirrels, reduce gray squirrel numbers, reduction in damage at Clemson University.
- A protocol for maintaining gray squirrel populations at a sustainable level that minimizes damage on campus.
- Technical report and Ph.D. Dissertation
- 1 professional journal article.
- Select presentations.
- Final report to Clemson University, USDA Wildlife Services, and SC Department of Natural Resources.

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