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Population Ecology

Efficacy and Health Effects of a Wildlife Immunocontraceptive Vaccine on Fox Squirrels

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ABSTRACT Continued range expansion of introduced eastern fox squirrels (*Sciurus niger*) in the western United States could lead to widespread damage to agricultural crops, facility infrastructures, and displacement of the native western gray squirrel (*S. griseus*). Because traditional management alternatives may not be feasible in many areas, public interest in the use of immunocontraceptive to control local populations has increased. We evaluated the efficacy of GonaCon™ immunocontraceptive vaccine for controlling eastern fox squirrel reproduction in Davis, California. We administered GonaCon to 33 male and 26 female fox squirrels, and a control substance to 33 males and 24 females. We subsequently compared the reproductive status, health, and serum concentrations of testosterone and progesterone of our treated and control populations. In our treated population, we also measured serum concentrations of antibodies to gonadotropin-releasing hormone (GnRH). To determine potential side effects of the vaccine, we recorded body weight and body condition of all animals, examined injection sites during each recapture, and observed the treated squirrels in the field for signs of discomfort and impaired mobility. Over 17 months, none of the recaptured GonaCon-treated females ($n = 20$) reproduced, compared to 12 of 15 control females. Treated males and females developed sufficient antibodies to GnRH to suppress reproduction, suggesting that GonaCon has the potential to be 100% effective in inhibiting reproduction in both sexes. We also observed a reduction in physical signs of reproductive activity for males ($P < 0.001$) and in hormone levels of both females ($P < 0.001$) and males ($P < 0.001$). Control and GonaCon-treated animals did not differ in body weight but vaccinated squirrels had poorer body condition scores and exhibited severe injection site abscesses. The abscesses may have been caused by the GnRH conjugate used in GonaCon. In our study, GonaCon was effective in reducing eastern fox squirrel reproduction. Changes in the conjugate or its preparation may reduce the severity of associated injection site reactions.

KEY WORDS California, eastern fox squirrel, GonaCon, immunocontraception, population control, reproduction, *Sciurus niger*, wildlife contraception, wildlife damage.

The eastern fox squirrel (*Sciurus niger*; fox squirrel), native to the eastern United States, was introduced to the western United States during the last century (Byrne 1979). Fox squirrels are now abundant in many urban and suburban areas in California, and isolated populations reside in Oregon and Washington. Their distribution was expanding at a rate of about 7 km/year near Los Angeles (King 2004) and approximately 3 km/year in Davis, California (Krause et al. 2010).

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Fox squirrels are considered pests where they occur. They may depredate gardens and orchard trees (Salmon et al. 2006) and raid backyard bird feeders, and their gnawing may cause extensive damage, including killing tree limbs and damaging wooden and plastic structures (Krause et al. 2010). Fox squirrel range expansion throughout the western United States could lead to widespread damage to agricultural crops such as nuts, avocados, and citrus (Salmon et al. 2006), damage to facility structures (e.g., sprinkler heads, motor vehicles, electrical wiring; Krause et al. 2010), and displacement of the native western gray squirrel (*S. griseus*; Muchlinski et al. 2009). Feasible methods for controlling fox squirrel populations vary by location. No poisons are registered for use on tree squirrels in the United States, and although control in rural areas probably is best achieved through hunting and trapping, these options generally are prohibited or restricted in urban and suburban areas.

Urban populations may have greater annual survival rates than rural populations. As such, urban populations may serve as immigration sources for surrounding areas (McCleery et al. 2008). Controlling populations within urban areas could also reduce densities in surrounding areas. Although habitat modifications (e.g., removal of oak trees [*Quercus* spp.]) may be effective, stakeholders may consider tree removal unpopular for aesthetic or ecological reasons. Contraception may offer stakeholders a feasible option for controlling fox squirrel populations in urban and suburban areas.

Increased interest by the public and wildlife managers in contraception as a potential alternative approach to regulate reproduction in overabundant wildlife populations has fueled research on contraceptive methods (Fagerstone et al. 2010). One promising contraceptive method for application to wildlife populations is the use of an immunocontraceptive vaccine against gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone is an essential hormone in the reproductive pathway leading to gametogenesis; GnRH-based immunocontraceptive vaccines are formulated by conjugating GnRH to a large, foreign, immunogenic protein and then combining it with a powerful adjuvant in an emulsion. When injected into an animal, this vaccine results in a persistent immune response that includes prolonged production of antibodies to GnRH. The exact mechanism for inducing infertility is not yet certain (Molenaar et al. 1993), but the presence of high anti-GnRH antibody titers is correlated with infertility in a variety of species (Fagerstone et al. 2010). Numerous GnRH vaccines have been developed and successfully used for decades in physiological research to interrupt the hormonal pathway that controls ovulation (Powers et al. 2007), but their use was not feasible for free-ranging wildlife because their short duration of action created the need for multiple treatments, and because they contained the controversial Freund's complete adjuvant, which can cause severe injection site reactions (Fagerstone et al. 2010, Gray and Cameron 2010).

The GonaCon™ immunocontraceptive vaccine was a promising GnRH-based wildlife contraceptive agent developed by the United States Department of Agriculture National Wildlife Research Center (NWRC; Fort Collins, CO) and registered by the United States Environmental Protection Agency in 2009 for use on adult female white-tailed deer (*Odocoileus virginianus*). The vaccine consisted of mammalian GnRH conjugated to a carrier protein (either keyhole limpet [*Megathura crenulata*] hemocyanin [GnRH-KLH formulation; Pierce Endogen, Rockford, IL] or *Concholepas concholepas* hemocyanin [GnRH-blue]) and emulsified in an adjuvant developed by NWRC (Adjuvac™) that was not associated with the severe deleterious effects caused by Freund's complete adjuvant. Although no studies have been conducted to determine exactly how GonaCon elicits a strong immune response, several components of the GonaCon vaccine are designed to increase the strength and extend the duration of the immune response, resulting in an effective single-dose vaccine that lasts up to 5 years.

Since its development, GonaCon has been used to induce temporary infertility in many mammals, including white-tailed deer (Miller et al. 2008*b*, Gionfriddo et al. 2011*a*), mule deer (*O. hemionus*; Perry et al. 2006), domestic cats (*Felis catus*; Levy et al. 2011), California ground squirrels (*Otospermophilus beecheyi*; Nash et al. 2004), Norway rats (*Rattus norvegicus*; Miller et al. 1997), feral pigs (*Sus scrofa*; Killian et al. 2006), eastern gray squirrels (*Sciurus carolinensis*; Pai 2009), and black-tailed prairie dogs (*Cynomys ludovicianus*; Yoder and Miller 2011). Although the safety and efficacy of GonaCon have been documented in a variety of species, less is known about the types and severity of side effects. Because the vaccine shuts down the hormonal pathway leading to gametogenesis, it reduces blood serum concentrations of progesterone and testosterone, testicle size (Nash et al. 2004, Killian et al. 2005, Gionfriddo et al. 2011*b*), and sperm number and motility (Levy et al. 2004).

The GonaCon vaccine did not affect weight or blood chemistry in black-tailed prairie dogs (Yoder and Miller 2011), and had no effect on time budgets, social rank, or blood parameters in feral pigs (Massei et al. 2008). However, some species have demonstrated altered breeding behaviors. Female elk (*Cervus elaphus nelsoni*) treated with GonaCon exhibited extended precopulatory behavior (Powers et al. 2011) and female white-tailed deer had fewer estrous events (Miller et al. 2000). Many adjuvants and vaccines cause injection site reactions, including pain, local inflammation, swelling, tissue necrosis, granulomas, ulcers, and sterile abscesses (Aquilar and Rodriguez 2007). These reactions generally were directly related to the effectiveness of the vaccine.

Some components of the GonaCon vaccine have been linked to injection site reactions including granulomas, sterile abscesses, and cysts (World Health Organization 1976, Miller et al. 2008*a*), so researchers testing GonaCon have specifically looked for such reactions. No studies have found reactions that may compromise the animals' health, but several species have exhibited palpable but not externally visible injection site reactions, with relatively severe reactions in only a few individuals (Miller et al. 2008*a*). Severe reactions have been found in 3 species including domestic dogs (*Canis familiaris*; Griffin et al. 2005), captive coyotes (*C. latrans*), and captive elk, but these were all associated with cross-reactions from having been given a previous vaccine (Miller et al. 2008*a*). Although side effects from GonaCon generally appear to be minor, they can be widespread and these studies illustrate that the severity of side effects can vary among individuals and species.

Any study of contraception presupposes an understanding of reproductive ecology, and although this is well known for fox squirrels in rural areas within its native range, it has not been described where the species has been introduced. Fox squirrels usually breed twice annually (winter: Nov–Feb with a peak in Dec and Jan; summer: Apr–Jul, with a peak in late

Jun; Allen 1942, Brown and Yeager 1945, Moore 1957, Koprowski 1994), although summer breeding does not always occur (McCloskey and Vohs 1971, Weigl et al. 1989). The proportion of females breeding during a given reproductive season may vary from 0.10 to 0.83 (McCloskey and Vohs 1971, Harnishfeger et al. 1978, Hansen and Nixon 1985, Weigl et al. 1989, Herkert et al. 1992), but annual proportions in rural populations have exceeded 1.0 because some females reproduce twice per year (e.g., 1.3 in 1 rural population after some females were experimentally removed [Hansen and Nixon 1985] and 1.2 in an unmanipulated urban population [McCleery 2009]). Whether these observed high annual proportions are also typical of urban settings is not clear.

To conduct our assessment of the efficacy of GonaCon as a mechanism for reproductive control of fox squirrels in an urban setting, we first studied the reproductive ecology of fox squirrels in a recently occupied suburban area (Davis, California). We then tested the efficacy of GonaCon on males and females in this population, and compared ancillary responses such as body condition, body weight, and behavior and injection site reactions in GonaCon-treated animals against those observed in animals given a sham treatment (injection with adjuvant but lacking GonaCon).

STUDY AREA

We field tested GonaCon on 2 subpopulations of fox squirrels that inhabited 2-ha areas located approximately 1.5 km apart on the University of California, Davis (UCD) campus (38.54°N, 121.75°W). We treated the 2 sites as independent because over the course of our study we observed no movements of any of the squirrels we studied between sites. The first study site, Orchard Park, was located in a housing complex near the northwest end of campus. The second study site, Mrak, was located among academic buildings near the southeast end of campus. The habitats at both study sites were typical of most North American university campuses, exhibiting a mosaic of mowed lawns, paved paths, and buildings, with scattered mature trees. Dominant tree species included oaks, pines (*Pinus* spp.), and Japanese zelkova (*Zelkova serrate*). Because human foot traffic was common at both sites, squirrels were well habituated to people.

METHODS

Trapping and Handling

We captured fox squirrels using Tomahawk live traps (Models 204 and 604.5; Tomahawk Live Trap Co., Tomahawk, WI), which we covered with canvas to reduce visual stimuli. We placed traps in shaded locations and pre-baited them with whole walnuts for 1–5 days. Trapping occurred over 2–4 days; we opened traps at sunrise and closed them 1–2 hours before sunset, except when conditions required early closing of traps because of inclement weather. We checked open traps at ≤90-minute intervals. We trapped for 9 seasons from winter 2008 through winter 2010. To mark as many squirrels as possible at the beginning of our study, we conducted extended trapping sessions during the first winter season for a total of 20 days between 8 November and 22 December. In subsequent seasons, we trapped a mean of 7 days per season (winter, 10–17 Dec; spring, 20–26 Mar and/or 8–23 May; summer, 7–31 Jul; fall, 16 Oct–1 Nov).

We removed animals from traps with cone-shaped cloth handling bags (Koprowski 2002). We recorded body weight using spring scales (accuracy 10 g) and scored body condition as 1, 2, or 3 based on a tactile assessment of fat over the ribs, vertebrae, and pelvic bones: 1 = animals in poor condition with vertebrae, ribs, and pelvic bones readily palpable; 2 = animals with palpable vertebrae and ribs but not pelvic bones; 3 = only the vertebrae readily palpable. The senior author trained all technicians to minimize error. Although weight and body condition are related, we assessed both because weight can be affected by reproductive activity such as pregnancy, lactation, and sperm production. We determined the sex and reproductive status of each animal by visual examination of external genitalia. Lactating females had enlarged nipples, often with a surrounding hairless area. Beginning in summer 2010, we also noted if a female had an enlarged vulva, an indicator of estrus. We considered males to be reproductively active when they had enlarged testes and a black-pigmented scrotum lacking hair posteriorly. We easily observed these characteristics when animals were not in a trap. We measured length and width of the scrotal sac (containing both testicles) with a ruler during handling and determined the cross-sectional area for comparisons between treatment groups. Because converting these to a volume measurement would have amplified differences, this approach was conservative. For those animals whose testes were not descended, we gently manipulated them into the scrotal sac prior to measurement. During our trapping efforts, we found that male squirrels often developed enlarged prostate glands that were palpable through the skin. We began recording these occurrences at the time of treatment (summer 2009).

We classified animals as juvenile, subadult, or adult (McCloskey 1977). We classified juveniles (≤6 months) by weight (<500 g) or by the presence of small testes in males. We examined, marked, and released juvenile squirrels at their capture sites, but we did not include them in this study. Subadults (6–12 months) and adults (>12 months) weighed ≥500 g and/or had developed testes. Subadult male fox squirrels typically have a partially furred scrotum that is gray to brown, whereas the scrotum typically is hairless and black in adult males (McCloskey 1977). These criteria proved somewhat unreliable, however, because the scrotum evidently became less pigmented during periods of testicular quiescence (Ferryman et al. 2006). Thus, we treated all newly trapped male squirrels weighing ≥500 g and with developed testes as adults; the only males defined as subadults in this study were animals we recaptured with a known history (i.e., those first trapped as juveniles).

We permanently marked all captured squirrels with uniquely numbered tags in each ear (National Band and Tag Co., Newport, KY; Model 1005-1). We also dyed fur with individually unique patterns using Nyanzol D (Albanil

Dyestuffs International, Jersey City, NJ) for field recognition at a distance (Melchior and Iwen 1965). We re-applied fur dye as necessary after spring and fall molting events.

We noted the presence of external parasites, abrasions, or other injuries when handling animals, and all trapping and handling followed the guidelines of the American Society of Mammalogists (Sikes et al. 2011). Study protocols were approved by the Institutional Animal Care and Use Committees at the University of California, Davis (#15056) and the National Wildlife Research Center (#1633).

Field Observations

The senior author and trained undergraduate interns observed squirrels from January 2009 to October 2010. During approximately 1,000 hours observing squirrels at each site, we recorded data on reproductive condition, the presence of impaired walking ability, and the presence of visible injection-site reactions. Observers searched for marked individuals in an area extending approximately 100 m outside the perimeter of each study site. Because squirrels were habituated to humans, observers occasionally approached within 10 m of squirrels to confirm reproductive status. We identified individual squirrels with binoculars by their fur dye patterns. We recorded reproductive status of females when the venter was clearly visible; we could observe nipples swollen from lactation from a distance of <30 m with binoculars. We noted movements as impaired or normal whenever we observed squirrels walking or climbing for at least 5 minutes; observers described any impaired movements they observed.

Reproductive Ecology

Based on field observations from wild populations, we classified winter (Dec–Feb) and summer (Jun–Aug) as breeding seasons, with intervening spring and fall as non-breeding seasons. We considered adult females to be reproductively active if they had enlarged vulvae during the breeding season or were observed to be lactating during or after the breeding season; both conditions were readily observed with binoculars. We considered adult females to be reproductively inactive if we never (with ≥ 2 observations) observed them in reproductive condition.

To evaluate reproductive responses to our contraception treatment, we calculated the proportion of females reproductively active during winter 2008 and summer 2009 and annually prior to treatment. During each season and annually subsequent to treatment, we also calculated the proportion of each treated group reproductively active. Because we anticipated that the proportion of GonaCon-treated females exhibiting reproductive activity might be zero, we also calculated the proportion of all females (i.e., both treatments) reproductively active.

Evaluation of Contraceptive Vaccine Efficacy and Health Effects

During summer, fall, and winter 2009, we assigned all trapped subadult and adult squirrels alternately within sex and site either to the GonaCon (Mrak, 18 males, 14 females; Orchard Park, 15 males, 12 females) or sham treatment (Mrak, 18 and 14; Orchard Park, 15 and 10). We gave each squirrel a single 0.4-ml injection of either GonaCon (400 μg of GnRH-blue protein conjugate/ml emulsified in AdjuVac) or sham treatment (saline solution and AdjuVac) into the deep muscle tissue of the right or left thigh. Because we had trapped at both sites since November 2008, we knew ages of most squirrels at the time of treatment. During treatment, we captured 6 new adult males.

We visually assessed the efficacy of GonaCon treatment including reproductive activity for 2 seasons prior to treatment (winter 2008–2009, summer 2009) and 3 seasons after treatment (winter 2009–2010, summer 2010, winter 2010–2011). Visual evidence included lactation or swollen vulvae for females and enlarged scrotal size and presence of a palpable prostate for males. We also completed hormonal and antibody assays from blood samples (≤ 3 ml) taken from the saphenous vein from each squirrel immediately before the injection was given and then again during each recapture event (but separated by ≥ 14 days). We also took blood samples from subadult and adult untreated squirrels caught during 4 trapping periods after we applied treatments (spring, summer, fall, and winter 2010). We refrigerated samples for 4–16 hours, after which we extracted serum and stored it at -80° C until we could ship it on dry ice to NWRC. There, blood serum samples were analyzed for progesterone and testosterone concentrations using a direct, competitive, solid phase, radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA; Siemens Coat-A-Count Total Testosterone In-vitro Diagnostic Test Kit and Siemens Coat-A-Count Progesterone In-vitro Diagnostic Test Kit) according to manufacturer's directions and for anti-GnRH antibodies using an enzyme-linked immunosorbent assay (ELISA; Muller et al. 1997).

We assumed that progesterone concentrations during pregnancy followed the same patterns reported in other mammals including eastern gray squirrels (Tait et al. 1981), increasing during pregnancy and reaching a peak around day 35 of the 44-day gestation period, after which they decline (Tait et al. 1981). Thus, the mean progesterone concentration was dependent upon both the number of females that were pregnant and their stage of pregnancy. We expected low progesterone concentrations in all females during the non-breeding season and elevated progesterone concentrations in sham females (because of pregnancies) during the breeding season. Because testosterone concentrations are elevated whenever males are in breeding condition, which could occur throughout the year (e.g., unlike progesterone which is contingent upon pregnancy), we expected that GonaCon-treated males would have lower testosterone concentrations than sham males throughout the post-treatment period of the study.

In the ELISA, we used sham and untreated animals to establish a baseline for comparison against GonaCon-treated animals. Squirrel blood serum was serially diluted from 1:1,000 to 1:128,000. Although this test has not been validated for fox squirrels, we infer from other species (Miller et al. 2000, Levy et al. 2004, Yoder and Miller 2011) that this should span the range of concentrations indicative of reproductive suppression; in either case, elevated levels of anti-GnRH antibodies in treated animals should indicate infertility in both sexes.

We monitored all animals for ancillary health effects. During handling procedures, we thoroughly checked both hind legs for injection site reactions each time we captured an animal. We noted scars, abscesses, or any other abnormalities. To determine whether abscesses were sterile or infected, we cultured 1 abscess during winter 2009 and 2 more in spring 2010. One sham animal died during handling; we donated the squirrel to the University of California, Davis Museum of Wildlife and Fish Biology and asked the preparer to note any abnormalities near the injection site.

Statistical Analyses

We conducted all analyses in R (R Development Core Team 2011). Analyses included 2 primary types of data. For analyses with repeated but unbalanced measures for individuals (e.g., progesterone levels, scrotal size, animal weight, and body condition), we applied linear mixed-effects models. In contrast, for models with a single outcome per individual (e.g., evidence of female fertility, presence of palpable prostate glands, presence of nipple or scrotal pigmentation, limping, testosterone levels, and injection site reactions), we applied Fisher's exact tests to compare GonaCon-treated and sham groups.

We analyzed the linear mixed-effects models containing continuous response variables (progesterone levels, scrotal size, and weight) using lmer in R package lme4 (Bates 2005). For all of these models, we treated squirrel identification (ID), squirrel ID × state, and squirrel ID × season as random factors, whereas we treated sex (male or female), season (breeding or non-breeding), time (trapping session), state (pre- or post-treatment), and site (Orchard Park or Mrak) as fixed effects. We chose our optimal fixed-effects structure for each mixed-effects model following the approach outlined by Zuur et al. (2009) in which we started with the full model (all fixed effects fully crossed), then used Akaike's Information Criterion (AIC, $\Delta AIC < 2.0$) and iterative (reverse stepwise) likelihood ratio tests ($P < 0.05$) to determine the optimal fixed structure. We log-transformed the response variable when necessary to meet assumption of homoscedasticity. We used restricted maximum likelihood methods for all final models. Because debate is ongoing concerning the denominator degrees of freedom in mixed models and subsequent calculation of P-values (Bolker et al. 2008), the lme4 package does not provide denominator degrees of freedom or P-values. For lmer models in this study, we assessed the significance of fixed variables with P-values of likelihood ratio tests comparing models with and without each fixed variable. Variables were included if P-value < 0.05.

For analysis of progesterone, we excluded data from winter 2009 because of a nearly complete absence of breeding during this season (S. K. Krause, UCD, personal observation). Our full model tested progesterone levels of female squirrels after treatment and included fully crossed fixed effects of treatment, season, and site. We did not include pre-treatment data in the analysis because we had collected pre-treatment data only during the breeding season. Because the full model for scrotal size (containing treatment, state, site, and season fully crossed) would not converge, we tested for scrotal size differences separately in pre- and post-treatment periods, including the fully crossed fixed effects for treatment, season, and site. Our full model for squirrel body weight included the fixed effects of treatment, state, site, and sex.

Because the response variable for body condition was ordinal rather than continuous, we could not use the mixed-modeling approach outlined above. Instead, we used a cumulative link mixed model fitted with Laplace approximation (clmm2 in package ordinal). We treated squirrel ID as a random factor. Our full model included fully crossed fixed effects of treatment, time, and site. As recommended by Christensen (2011) and consistent with our lme4 models, we identified our final model using iterative likelihood ratio tests. We present the t-values and P-values provided by the clmm2 package and the P-values associated with the likelihood ratio tests for effects not present in the final model.

For all Fisher's exact tests, we compared the outcomes of a given response between treatment groups (GonaCon vs. sham). For female fertility, we classified each female as reproductively active or inactive. We used separate tests for females before and after treatment. For the presence of palpable prostate glands in males, we compared males that had such a condition at any time post-treatment against those males that did not; we only had post-treatment data on presence of prostate glands. We only collected data on pigmentation in scrotum and nipples post-treatment; we classified individuals as having lost pigmentation if they lacked pigmentation at any time after treatment.

We were unable to analyze plasma testosterone concentration with linear mixed models because the abundance of zero values in GonaCon-treated animals violated the assumptions of a mixed model. Therefore, we calculated the mean of all post-treatment observations for each individual and assigned each individual to 1 of 2 categories of mean testosterone (>0.1 or ≤ 0.1, where 0.1 is the lowest detectable testosterone concentration). We compared these groups with Fisher's exact test.

RESULTS

Reproductive Ecology

The reproductive ecology of fox squirrels on the UCD campus was similar to native populations. We inferred through peaks in capture rates of juveniles that the UCD fox squirrels have 2 annual breeding seasons occurring in December–February and June–August; we caught juveniles during all seasons, but they comprised a greater proportion of the captured population during summer and winter (with the exception of winter 2009; Table 1). As expected for animals with a long breeding season, juvenile body weights varied within trapping periods, suggesting a range of ages (Table 1). Mean body weights of juveniles were lowest during spring

Table 1. Percentage of captured fox squirrels (*Sciurus niger*) that were juveniles in each season at the University of California, Davis, USA. Also provided is the mean (range) of juvenile body weight (g). No range is available for spring and summer 2010 because we only captured a single juvenile in these seasons.

Trapping season	Percentage juveniles (total captures)	Body weight (g)
Winter 2008	27.6 (145)	449 (290–520)
Spring 2009	6.2 (113)	346 (290–420)
Summer 2009	20.8 (96)	487 (415–560)
Fall 2009	9.9 (81)	343 (155–530)
Winter 2009	16.0 (100)	479 (365–590)
Spring 2010	0.6 (168)	300
Summer 2010	1.0 (100)	440
Fall 2010	5.4 (74)	343 (235–450)
Winter 2010	28.0 (93)	453 (360–530)

and fall (Table 1), suggesting that juveniles were younger at these times.

Consistent with our inference of 2 breeding peaks, we noted enlarged vulvae during summer ($n = 15$) and winter ($n = 8$) but never in spring or fall. For each breeding season, the proportion of sham females with enlarged vulvae or lactating ranged from 0.14 to 1.00 and was lowest in winter 2009 (Table 2). Prior to treatment, at least 6 of 32 adult females were reproductively active during both annual seasons, whereas after treatment at least 5 of 15 adult sham females were reproductively active in more than 1 season (2 individuals were reproductively active in all 3 post-treatment seasons).

Efficacy of GonaCon

Visual observations, hormone levels, and antibody titers suggested that GonaCon was highly effective at inhibiting reproduction in fox squirrels. Visual observations indicated that although the proportion of reproductively active females was similar in both treatment and sham groups prior to treatment, no GonaCon-treated squirrels exhibited visual evidence of fertility (Table 2). For males, mean scrotal size before treatment did not differ between treatment groups (Table 3). After treatment, GonaCon-treated males had smaller scrota than sham animals (320 mm^2 vs. 948 mm^2; Table 3). This effect was more pronounced during breeding seasons (Fig. 1). The treatment × site interaction (Table 3) likely reflected a slightly larger pre-treatment scrotal size in sham males at Orchard Park (947 mm^2) than Mrak (824 mm^2). We considered the presence of a palpable prostate gland an indicator of breeding condition. The GonaCon-treated males were 92% less likely than sham males to exhibit this trait (Table 4). In sham animals, enlarged prostate glands were more common during the 2 breeding seasons (summer 2010, 96% of 23 animals; winter 2010, 90% of 10 animals). We recorded fewer sham animals with enlarged prostate glands during winter 2009 (29% of 17 animals) when the proportion of reproductively active females was lowest, and during the non-breeding seasons (fall 2009, 0% of 9; early spring 2010, 6% of 18; fall 2010, 50% of 14).

The hormone levels we recorded supported the above observations. Progesterone levels were lower in GonaCon-treated than in sham females (1.02 ng/ml and 1.72 ng/ml, respectively; with the greatest difference occurring during the breeding season (1.11 ng/ml and 2.99 ng/ml, respectively; Fig. 2; Table 3). During the breeding season, GonaCon-treated females had progesterone levels similar to those of all females during the non-breeding season (1.11 ng/ml and 1.01 ng/ml, respectively). In males, testosterone concentrations varied seasonally, peaking during the breeding seasons (Fig. 2). GonaCon-treated animals were more likely to have low plasma testosterone concentrations (mean $\leq$0.1 ng/ml; Table 4; Fig. 2); after treatment, mean testosterone concentration in GonaCon-treated males was 0.03 ng/ml ($n = 26$, SE $= 0.005$). Sham animals had a mean of 0.70 ng/ml ($n = 28$, SE $= 0.094$).

Finally, immediately prior to treatment no study animals had anti-GnRH antibodies, but within 2 weeks of GonaCon treatment these were elevated and most individuals remained at or above 1:128,000 throughout the study (Table 5). Antibody titers in most squirrels remained high throughout the 17-month study, although 5 of 49 individuals had low enough antibodies at some point during the study that reproduction might have been possible (see Table S1, available online at www.onlinelibrary.wiley.com). However, none of these individuals had titers that remained low throughout the study, and none exhibited other signs of reproduction. Consequently, we were unable to identify the threshold antibody titer needed for effective vaccination.

Table 2. Number of reproductively active female fox squirrels (*Sciurus niger*; RAF) in each treatment group by season before and after treatment with control material or GonaCon™ at the University of California, Davis campus, 2008–2010.

Reproductive season	Control		GonaCon		*P*-value[a]
	n	RAF	*n*	RAF	
Winter 2008	11	7	8	3	
Summer 2009	15	9	16	6	
Total pre-treatment[b]	16	12	16	7	0.150
Winter 2009	14	2	16	0	
Summer 2010	11	11	15	0	
Winter 2010	7	6	10	0	
Total post-treatment[b]	15	12	20	0	$\leq$0.001

[a] Fisher's exact test.
[b] Note that total values are not simple sums because some animals were observed in multiple seasons.

Overall Health and Condition

Body condition (Table 6) was lower in GonaCon-treated than in control animals. Body weight as a function of GonaCon treatment trended lower for GonaCon-treated individuals although it fell short of statistical significance (treatment:state $P = 0.062$; Table 3; Fig. 3). Reduced body weights were attributed to lower weights in females after treatment (Table 3; Fig. 3). Most GonaCon-treated males lost scrotal pigmentation; this effect was not observed in any control animals (Table 4). In females, both treatment groups exhibited similar levels of nipple pigmentation after treatment (Table 4).

Table 3. Summary of results of linear mixed effects models for a study of fox squirrels (*Sciurus niger*) on the University of California, Davis campus 2008–2010. The full model included all fixed effects shown: the optimal model contained all fixed effects shown with *t*-values. For each model, the intercept estimate provides the reference and estimate values for other fixed effects represent deviation from the estimate for the listed parameter. All models included a random effect for squirrel identification (ID) to account for repeated measures of individuals. State is a categorical variable indicating pre- or post-treatment.

Response variable and random effects	Fixed effect	Parameter	Estimate	SE	*t*	LRT[a]	*P*[b]
Scrotal size	(Intercept)		907.17	71.37	12.71		
(pre-treatment)	Season	Non-breeding	−166.19	106.47	−1.56	5.43	0.066
Random = season|ID	Site	Orchard	−50.53	109.92	−0.46	7.75	0.021
	Season:site	Non-breeding:Orchard	454.63	173.22	2.63	5.42	0.020
	Treatment					1.97	0.161
	Treatment:season					1.45	0.228
	Treatment:site					0.11	0.744
	Treatment:season:site					3.26	0.071
Scrotal size	(Intercept)		5.81	0.11	51.57		
(post-treatment, log-transformed)	Treatment	Sham	0.62	0.16	4.00	68.53	≤0.001
Random = season|ID	Season	Non-breeding	−0.08	0.10	−0.81	18.65	≤0.001
	Site	Orchard	−0.20	0.14	−1.46	5.42	0.067
	Treatment:season	Sham:Non-breeding	0.52	0.14	3.78	13.54	≤0.001
	Treatment:site	Sham:Orchard	0.46	0.20	2.31	5.39	0.020
	Season:site					0.17	0.677
	Treatment:season:site					1.39	0.238
Progesterone (log-transformed)	(Intercept)		0.08	0.16	0.52		
Random = season|ID	Treatment	Sham	0.79	0.22	3.57	27.10	≤0.001
	Season	Non-breeding	−0.37	0.17	−2.19	33.80	≤0.001
	Site	Orchard	−0.23	0.23	−1.02	5.49	0.241
	Treatment:season	Sham:Non-breeding	−0.42	0.24	−1.75	15.59	≤0.001
	Treatment:site	Sham:Orchard	0.36	0.40	0.92	4.98	0.083
	Season:site	Non-breeding:Orchard	0.41	0.24	1.68	4.86	0.088
	Treatment:season:site	Sham:Non-breeding:Orchard	−0.91	0.42	−2.15	4.59	0.032
Body weight	(Intercept)		668.74	10.98	60.92		
Random = state|ID	Treatment	Sham	49.45	15.05	3.29	13.55	0.009
	State	Pre	27.87	8.60	3.24	24.68	≤0.001
	Sex	Male	33.49	13.35	2.51	24.68	≤0.001
	Site	Orchard	−31.27	9.69	−3.23	10.43	0.001
	Treatment:state	Sham:pre	−27.41	11.73	−2.34	5.56	0.062
	Treatment:sex	Sham:male	−39.73	19.41	−2.05	6.77	0.034
	State:sex	Sham:pre:male	−48.68	10.61	−4.59	21.30	≤0.001
	Treatment:state:sex	Sham:pre:male	29.53	14.95	1.98	4.00	0.046
	Treatment:site					2.19	0.139
	Site:state					0.94	0.331
	Site:sex					0.00	0.952
	Treatment:site:sex					1.56	0.212
	Treatment:site:state					0.84	0.361
	State:site:sex					0.12	0.762
	Treatment:site:state:sex					0.43	0.511

[a] Likelihood ratio test.
[b] *P*-values were calculated using likelihood ratio tests comparing models with and without the variable in question.

We began to encounter individuals with abscesses at or very close to the injection site shortly after treatment (fall–winter 2009). Initially, these abscesses appeared sterile; we cultured 1 and found no bacteria. By spring 2010, some appeared infected so we cultured 2 more, both of which yielded bacteria (*Escherichia coli*). Overall, most (44/50 = 87%) recaptured GonaCon-treated animals had ≥1 externally detectable abscess after treatment, whereas no sham animals ever had a detectable abscess, although a small number (5/44 = 11%) of sham animals had small scars near the injection sites (Table 4). Abscesses persisted throughout the study. Often, squirrels had multiple abscesses near the injection site. This reaction was likely caused by fluid accumulating in subcutaneous pockets in the leg. Affected individuals had a mean of 1.6 abscesses (range 1–4) with a mean surface area of 228 mm^2 (range 15–1,253 mm^2); total abscess area (per individual) averaged 353 mm^2 (range 20–1,300 mm^2). Field personnel frequently noted open abscesses exuding pus. During post-treatment field observations, GonaCon-treated squirrels were more likely than control squirrels to limp or walk stiffly (Table 4). Finally, 1 sham animal that died during handling lacked any externally visible injection site reaction, but pus-filled nodules were found in the muscles near the injection site when the animal was examined internally.

DISCUSSION

The fox squirrels we studied exhibited a bimodal reproductive cycle similar to native populations in the eastern United States (Koprowski 1994). The GonaCon vaccine we evaluated was successful in suppressing reproductive activity in male and female squirrels. We found a difference in body condition and a trend towards lower weight of GonaCon-treated versus sham animals and an increase in rate of abscesses in GonaCon-treated squirrels.

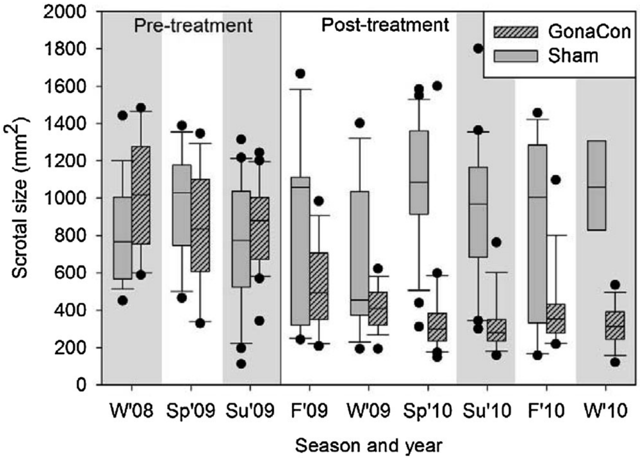

Figure 1. Mean cross-sectional area of the scrotal sac, showing 1 standard error and 95% confidence limits, for GonaCon-treated and sham (control) eastern fox squirrels (*Sciurus niger*) before and after treatment at the University of California, Davis, USA, 2008–2010. Solid circles indicate outliers. Shaded areas indicate breeding seasons (breeding failed during winter 2009). Season notations include winter (W), spring (Sp), summer (Su), and fall (F) and are followed by the last two digits of the year.

The reproductive biology of the fox squirrels we studied did not differ from other populations. McCleery (2009) suggested that urban fox squirrel populations may grow more rapidly than rural ones (McCleery 2009). Some urban populations in California are expanding rapidly (King 2004, Krause et al. 2010), and have displaced native western gray squirrels in at least 1 urban park (Muchlinski et al. 2009). The fox squirrels we studied exhibited annual proportions of reproductively active females (0.78) within the range (0.10–0.83) reported for rural populations.

At the population level, the effects of contraception may be offset through reproductive compensation by untreated females (Davis and Pech 2002). For example, density-dependent behaviors in which treated (hence, non-reproductive) females do not participate, such as competition for nesting sites and the reduced number of juveniles competing for resources, may facilitate increased reproductive success by untreated females (Davis and Pech 2002). Ricefield rats (*Rattus argentiventer*) exhibited reproductive compensation when 75% of a population was sterilized, but not when only 25–50% was sterilized (Jacob et al. 2004); house mice (*Mus musculus*) exhibited compensation when 67% of a population was sterilized (Chambers et al. 1999). Survival of juvenile and sterile female European rabbits (*Oryctolagus cuniculus*) increased with decreasing population density, but with 60–80% sterility rates, abundance of rabbits still declined (Twigg and Williams 1999).

Table 4. Characteristics of sham (control) and GonaCon^{TM}-treated fox squirrels (*Sciurus niger*) at the University of California, Davis campus 2008–2010. Positive indicates the number of individuals captured with the indicated condition.

	Sham		GonaCon		
	n	Positive	*n*	Positive	*P*-value[a]
Palpable prostate	25	24	26	2	≤0.001
Testosterone >0.1 ng/ml	27	27	23	5	≤0.001
Scrotal pigmentation	26	25	25	0	≤0.001
Nipple pigmentation	20	7	17	2	0.137
Abscess or scar	44	5	50	44	≤0.001
Impaired walking	44	7	47	17	0.034

[a] Fisher exact test.

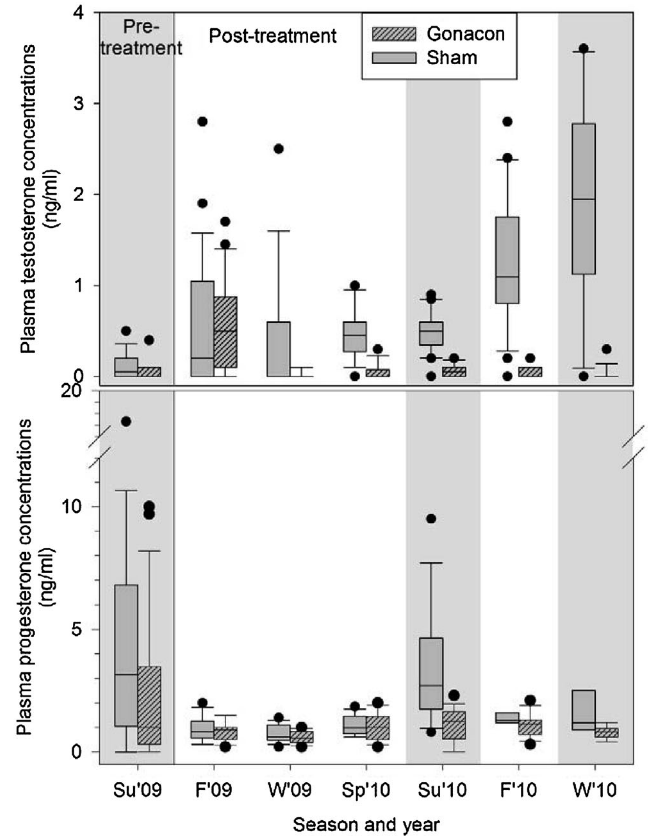

Figure 2. Mean plasma testosterone (males) and progesterone (female) concentrations, showing 1 standard error and 95% confidence limits, of GonaCon-treated and sham (control) eastern fox squirrels (*Sciurus niger*) before and after treatment at the University of California, Davis, USA, 2008–2010. Solid circles indicate outliers. Shaded areas indicate breeding seasons (breeding failed during winter 2009). Season notations include winter (W), spring (Sp), summer (Su), and fall (F) and are followed by the last two digits of the year.

We observed that with a 50% vaccination rate, the proportion of reproductively active control females increased from 1.00 prior to treatment to 1.23 after treatment. Whether this increase represented reproductive compensation or intrinsic variation in the reproductive rate of squirrels merits further investigation. A similar response was documented for female eastern fox squirrels in which a hunted population had a greater annual proportion of reproductively active females than a similar, unhunted population (1.33 vs. 0.89; Herkert et al. 1992). Given that fox squirrels are able to increase reproductive output and that reproductive compensation in the presence of sterile individuals has been documented for other species, the increased proportion of reproductively active females after treatment could be compensatory. Future contraceptive studies should be designed to evaluate what type of change, if any, occurs in the reproductive rate of untreated females.

Table 5. Summary of the number of fox squirrels (*Sciurus niger*) by sex with anti-gonadotropin-releasing hormone (anti-GnRH) antibody titers during 8 sampling periods spanning 17 months after application of GonaCon^{TM} immunocontraceptive vaccine treatment at the University of California, Davis campus, 2008–2010. Vaccinations began in July 2008 and we measured response titers as early as 2 weeks later and during each subsequent season. Su, summer; F, fall; W, winter; Sp, spring; March and May samples in 2010 are distinguished here as $Sp10^1$ and $Sp10^2$, respectively.

Anti-GnRH titer	Su09 (M/F)	F09 (M/F)	W09 (M/F)	$Sp10^{1a}$ (M/F)	$Sp10^{2b}$ (M/F)	Su10 (M/F)	F10 (M/F)	W10 (M/F)
≤1:32,000	1/0	0/2	0/0	1/0	0/1	0/1	1/0	3/0
1:64,000	2/1	0/0	0/0	1/0	3/0	0/2	0/2	1/0
≥1:128,000	7/2	14/8	21/15	18/16	16/14	19/12	15/10	13/11

Although we were unable to determine the anti-GnRH threshold necessary to inhibit reproduction in fox squirrels, the lack of reproduction indicated that the levels used in our study were sufficient to have an effect. Most (96%) squirrels maintained titer levels above 1:64,000, a threshold known to inhibit reproduction in white-tailed deer (Miller et al. 2000). GonaCon has suppressed reproduction for up to 5 years in female domestic cats (Levy et al. 2011) and white-tailed deer (Miller et al. 2008*b*), and the persistence of high antibody titers at the conclusion of this study suggests that the vaccine may inhibit reproduction for more than 2 years. Studies on more closely related species such as eastern gray squirrels (Pai 2009) and California ground squirrels (Nash et al. 2004) have also shown high rates of effectiveness but also failed to track the vaccine's long-term contraceptive efficacy. These results are promising for implementation of control measures for fox squirrel populations (Gray and Cameron 2010), but further studies are needed to determine the duration of the contraceptive effects of GonaCon.

Although none of our GonaCon-treated fox squirrels remained fertile, we observed variation in individual immune responses, as reported elsewhere (Nettles 1997, Cooper and Herbert 2001, Herbert and Trigg 2005, Powers et al. 2011). Specifically, 2 males presented slightly enlarged prostate glands, an indication that seminal fluids were being produced, and 4 individuals had anti-GnRH titers below 1:64,000, a fertility threshold in some species (Miller et al. 2000). Nettles (1997) suggested that individual variation in response to immunocontraceptive vaccines could increase the proportion of genetic non-responders (those with weak immune systems) in the population, through relatively high rates of reproduction among non-responding individuals, thereby causing adverse effects on the overall health of the population. These proposed ill effects would occur only if 1) the vaccine is 100% effective (or nearly so) in animals that actually respond to it, 2) non-response is genetically based, 3) no immigration occurs, and 4) such effects are not diluted or masked by other, stronger selective pressures.

The GonaCon treatments resulted in reduced scrotal size in UCD fox squirrels, similar to the reduced testis size documented in white-tailed deer (Killian et al. 2005, Curtis et al. 2008, Gionfriddo et al. 2011*b*), feral pigs (Killian et al. 2006), domestic cats (Levy et al. 2004), and eastern gray squirrels (Pai et al. 2011). Pai et al. (2011) documented testicular, prostatic, and epididymal atrophy in GonaCon-treated eastern gray squirrels, as well as atrophy of tubuli and prostatic glandular lumen. We believe that the fox squirrels in our study exhibited similar atrophy in these structures, but we did not test this directly. These side effects were expected and do not appear to pose health risks for the animals.

In contrast to previous studies, we documented a poorer condition and a trend towards lower body weight in GonaCon-treated squirrels. The reduced treatment female body weight likely reflects the fact that GonaCon-treated females did not become pregnant. Reduced body weight may

Table 6. Summary of a mixed effects model for body condition (ordinal response variable) of fox squirrels (*Sciurus niger*) captured at the University of California, Davis campus 2008–2010 after treatment with GonaCon^{TM}.

Fixed effect[a]	Parameter	Estimate	SE	Z	LRT[b]	P-value[c]
Treatment	Sham	0.58	0.26	2.24		0.025
Time	Winter '09	−1.21	0.51	−2.37		0.018
	Spring '10	−0.34	0.44	−0.77		0.440
	Summer '10	−1.56	0.51	−3.05		0.002
	Fall '10	−1.56	0.57	−2.72		0.007
	Winter '10	−2.47	0.58	−4.27		≤0.001
Site	Orchard	−0.85	0.75	−1.13		0.257
Time:site	Winter '09:Orchard	2.90	0.92	3.16		0.002
	Spring '10:Orchard	0.56	0.82	0.68		0.498
	Summer '10:Orchard	0.45	0.89	0.51		0.611
	Fall '10:Orchard	0.16	0.97	0.16		0.870
	Winter '10:Orchard	2.09	1.00	2.09		0.037
Treatment:time					−9.46	0.09
Treatment:site					−0.01	0.913
Treatment:time:site					−3.49	0.624

[a] These results are from clmm2 command in ordinal package in R. The full model included all fixed effects shown. The optimal model contained all fixed effects shown with a Z. Squirrel identification (ID) was treated as a random factor.
[b] LRT = Likelihood ratio test.
[c] P-values were calculated by clmm2 (based on Z-values) or using likelihood ratio tests comparing models with and without the variable in question.

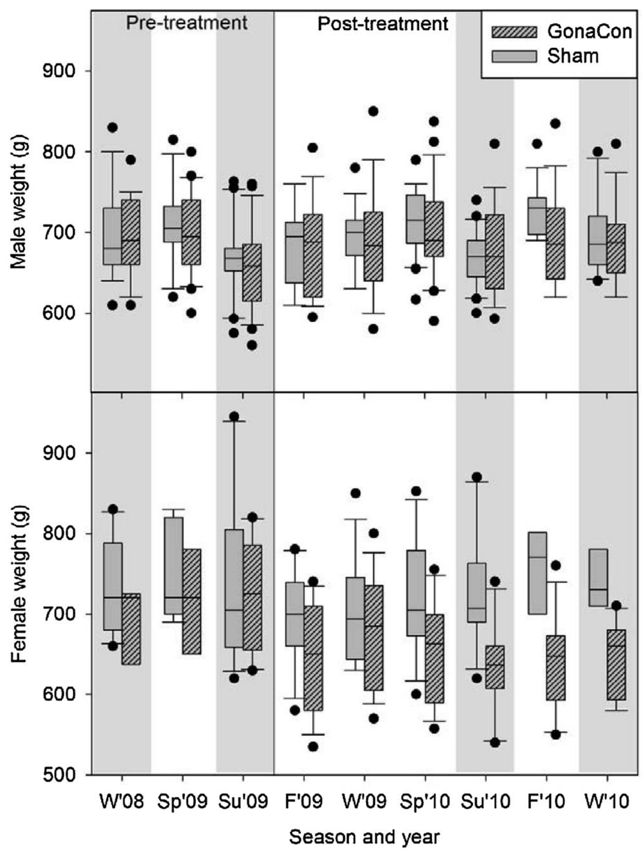

Figure 3. Mean body weights, showing 1 standard error and 95% confidence limits, of GonaCon-treated and sham (control) eastern fox squirrels (*Sciurus niger*) before and after treatment at the University of California, Davis, USA, 2008–2010. Solid circles indicate outliers. Shaded areas indicate breeding seasons (breeding failed during winter 2009). Season notations include winter (W), spring (Sp), summer (Su), and fall (F) and are followed by the last two digits of the year.

not adversely affect general health, but lower body condition may be of greater concern. Although further research is needed to determine the cause of the reduced body condition, 1 possibility is that the presence of abscesses added weight to the animal but reduced body condition as the animal directed resources to the abscess.

Although the severity of the injection site reactions we observed was unusual, localized reactions to vaccination are common, and their occurrence may be an essential manifestation of the strong immune response needed to achieve contraception (see Schijns 2000). At least 2 characteristics of GonaCon contribute to the occurrence of reactions at injection sites. AdjuVac (the adjuvant used in GonaCon) contains very small amounts of the ubiquitous bacterium *Mycobacterium avium* (Miller et al. 2008*a*), which is known to cause injection site reactions (Broderson 1989). In addition, the water-in-oil emulsion used in GonaCon is known to cause inflammation and granulomas at injection sites (Straw et al. 1985). The fact that both sham and GonaCon treatments shared these 2 characteristics raises the question of why severe reactions were found only in GonaCon-treated fox squirrels in our study.

The fundamental difference between GonaCon and the sham material was the presence in GonaCon of the conjugated GnRH-mollusk protein molecule. The mollusk protein is known to be highly immunogenic because of its large size and being recognized by the immune system as non-self because mollusks are phylogenetically distant to mammals (Miller et al. 2008*b*). Thus, we believe that some feature of the conjugate molecule (or perhaps an unknown chemical residue from the conjugation process) was responsible for the injection site reactions observed in GonaCon-treated squirrels in our study. The conjugate could have also been responsible for the common injection site reactions observed in other species; for free-ranging, adult female white-tailed deer, Gionfriddo et al. (2011*b*) reported the occurrence of abscesses at necropsy in 94% (17 of 18) of GonaCon-treated and 82% (9 of 11) of sham animals. The sham material used in that study was a mixture of AdjuVac and mollusk stabilizing buffer; perhaps an undetected residue of the latter ingredient contributed to the severity of the injection site reactions in fox squirrels at UCD.

Most studies on GonaCon reported minor injection site reactions. However, Griffin et al. (2005) found persistent and severe reactions characterized by swelling, secondary infection, and pain in all 3 domestic dogs tested with an earlier formulation of the vaccine (the GnRH-KLH formulation). No sham material was tested and the cause of the reactions was unknown. We tested the GnRH-blue formulation of the vaccine and ours is the first study to report persistent and severe reactions in nearly all GonaCon-treated individuals, with open draining wounds and secondary infections associated with limb stiffness and limping.

In other studies using the GnRH-blue formulation, injection site reactions were common and persistent but relatively minor (white-tailed deer, Gionfriddo et al. 2011*b*; coyotes and horses (*Equus caballus*), Miller et al., 2008*a*) and were detected only because researchers were specifically looking for such reactions (Miller et al. 2008*a*). The relatively severe injection site reactions observed in fox squirrels at UCD suggest that this species may be more sensitive to the factors that induce such reactions than other species tested, and further efforts should aim to refine the formulation to reduce the severity of injection site reactions in fox squirrels and other sensitive species.

MANAGEMENT IMPLICATIONS

Increasing populations of non-native fox squirrels may constitute an emerging pest problem. Invasive populations not only retain the reproductive patterns of those in their native range, with 2 annual breeding seasons, but survival may be greater in urban versus rural areas where natural predators may be lacking. Wildlife managers and stakeholders wanting to mitigate the impacts of invasive fox squirrel populations should consider all available management tools. The use of immunocontraception may become an increasingly important viable option in urban areas where traditional damage control strategies such as hunting and habitat modification are unacceptable. We documented that reproduction in relatively small and closed populations of fox squirrels may be effectively controlled by GonaCon. The use of GonaCon in urban areas exhibiting conditions similar to our study may afford wildlife managers with a viable non-lethal control option. However, further research is still needed to resolve concerns with injection site reactions we observed in our study.

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