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20,25-Diazacholesterol as an oral contraceptive for black-tailed prairie dog population management

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Abstract: Black-tailed prairie dog (*Cynomys ludovicianus*) colonies can become overcrowded, and the colonies, landscape, and people affected by them may benefit from controlled populations. Contraception is a method that may be useful, particularly where lethal control is inappropriate or illegal. We investigated if oral administration of 20,25-diazacholesterol (DiazaCon®), an inhibitor of cholesterol and reproductive steroid hormone production, could reduce reproductive success of treated black-tailed prairie dogs in a field trial. Ten treatments of approximately 45-mg DiazaCon per black-tailed prairie dog yielded a 47% reduction of young:adult ratios compared to control sites. Over a 3-month period, desmosterol, a cholesterol precursor used as an indicator of DiazaCon effects, was not detectable in any black-tailed prairie dogs trapped at control sites, whereas elevated levels were detectable in 33 of 35 blood samples from black-tailed prairie dogs trapped at treated sites. Average cholesterol levels were lower in treated animals than in control animals. DiazaCon administration may be a useful tool to control populations of black-tailed prairie dogs, especially in light of the desire for conservation while still managing populations.

Key words: 20,25-diazacholesterol, black-tailed prairie dog, cholesterol, contraception, *Cynomys ludovicianus*, DiazaCon®, Ornitrol®, population control, reproductive inhibition

Although the black-tailed prairie dog (*Cynomys ludovicianus*) is considered by some to be abundant, widespread, and a nuisance or pest species, the U.S. Fish and Wildlife Service concluded that it is warranted for listing as threatened under the Endangered Species Act of 1973, in large part due to increasing loss of habitat and the potential for plague to cause losses of entire colonies (U.S. Fish and Wildlife Service 2000). While not as numerous as they once were, black-tailed prairie dog colonies still require some form of population management. Conover and Decker (1991) found that prairie dogs were identified by some agricultural and wildlife professionals as causing the worst damage by any wildlife species in their state, contributing hundreds of thousands of dollars in damage annually to agriculture crops, earthen dams, airports, and golf courses. All states within the historic range of the black-tailed prairie dog classify the species as an agricultural pest and either allow or even require its eradication (Mulhern and Knowles 1995).

Prairie dogs, like many species, can also become troublesome in urban settings when their population exceeds the carrying capacity of the small areas in which they live. These animals cause conflict with residential neighbors as well as nearby farms. Lethal control often is less acceptable in urban settings than in agricultural settings for various reasons.

Contraception is a method that may be useful as an alternative to poison in managing and conserving black-tailed prairie dog populations. Overcrowding in colonies may contribute to disease spread, including plague infection, and disease can result in nearly 100% mortality in black-tailed prairie dogs (Raynor 1985). Contraception could be used to limit colony size and decrease overcrowding, thus potentially reducing disease spread. Plague epidemics cause a loss of genetic variability in black-tailed prairie dogs (Trudeau et al.

2004). Contraception could be used to manage population growth intermittently, allowing maintenance of genetic diversity by allowing individuals to breed in nontreatment years. Currently, several states comprising the historic

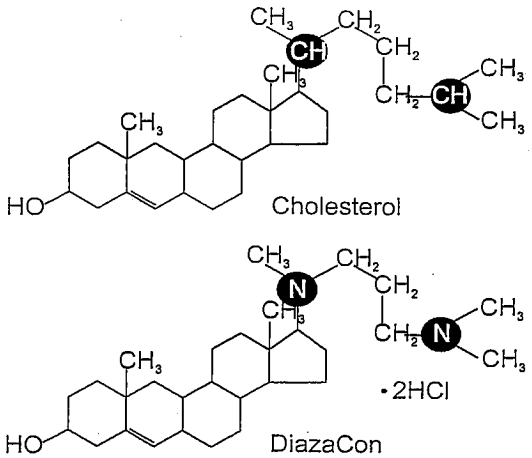

FIGURE 1. The structures of DiazaCon (20,25-diazacholesterol dihydrochloride) and cholesterol.

range of the black-tailed prairie dog have agreed to develop a plan to manage, maintain, and even enhance prairie dog populations and habitat, which may ultimately result in changes in regulations to limit control by poisoning (Van Pelt 1999, U.S. Fish and Wildlife Service 2000). Nonlethal control via contraception may be a viable option for population management of black-tailed prairie dogs in areas where lethal control by fumigation or poisoning is inappropriate or illegal. Fertility control of prairie dogs was successfully tested by Garret and Franklin (1983) using diethylstilbestrone (DES), a synthetic hormone that is used as an effective method of population management, but its use is limited by environmental concerns. Unlike DES, DiazaCon® would not be expected to act as estrogen in animals such as frogs that come in contact with it. Because DiazaCon can inhibit hormone production without the side effect of acting as a hormone itself, it may have fewer adverse effects on other animals in the environment.

Black-tailed prairie dogs were selected for this study due to their status as a species for which population control is desirable but most appropriately pursued through nonlethal techniques. Areas where urban sprawl encroaches upon black-tailed prairie dog habitat could benefit from slowed population growth via contraception (Hygnstrom and Virchow 1994). Farmers and ranchers may be more receptive to allowing existing black-tailed prairie dog colonies to remain on their lands if the population could be controlled such that the colony would not spread as quickly. In this study, we report successful inhibition of reproductive success of black-tailed prairie dogs through treatment under natural conditions in the field with oral bait containing DiazaCon.

DiazaCon (20,25-diazacholesterol dihydrochloride) is a cholesterol analog developed by G. D. Searle Co. (Figure 1). It was an effective drug for cholesterol reduction in human clinical trials (Counsell et al. 1962, 1965; Ranney and Cook 1965). However, some patients complained of an uncomfortable feeling and human trials were discontinued (Sachs and Wolfmann 1965). Because the compound effectively lowered cholesterol, G. D. Searle Co. decided to test it as a contraceptive in birds with the assumption that reducing cholesterol might make avian eggs infertile due to the large amounts of cholesterol they normally contain. Studies with pigeons in pens and in the field showed promising results of reduced reproductive success (Elder 1964; Wofford and Elder 1967; Schortemeyer and Beckwith 1968; Woulfe 1970 *a, b*). Experiments with blackbirds (Fringer and Granett 1970, Lacombe et al. 1986, Lacombe et al. 1987, Cyr and Lacombe 1992), grackles (Fringer and Granett 1970), sparrows (Sanders and Elder 1976, Mitchell et al. 1979), quail (Dam et al. 1979, Yoder 2000), and chickens (Cecil et al. 1979) showed reduction in fertility.

DiazaCon inhibits multiple enzymes required for the production of cholesterol (Emmons et al. 1982, Johnston et al. 2003) and cleavage of the cholesterol side-chain (Counsell et al. 1971). The inhibition of cholesterol production leads to an accumulation of cholesterol precursors, including desmosterol. The accumulation of desmosterol due to inhibition of double bond reduction at carbon 24 has been useful as a measurable effect of DiazaCon treatment (Johnston et al. 2003). Inhibiting side-chain cleavage of cholesterol causes a reduction in reproductive steroid hormone production by preventing the conversion of cholesterol to pregnenolone, a precursor to reproductive steroid hormones. In birds, inhibition of egg laying by DiazaCon is probably due to both a reduction in reproductive hormones and a reduction in cholesterol. In mammals, reduction of steroid reproductive hormone production provides contraceptive effects in both sexes.

Early safety and toxicity studies (W. E. Hambourger and T. B. Martinez, unpublished) done for the U.S. Food and Drug Administration (FDA) showed that a lethal dose of DiazaCon could not be achieved in dogs because the individuals regurgitated the drug long before a toxic level was reached. The intragastric 50%

lethal dose (LD_{50}) was 380 and 470 mg/kg in mice and rats, respectively. DiazaCon (100 mg/kg/day) fed to rats (n = 10) for 10 days (1000 mg/kg total dose) resulted in only 1 observed symptom of toxicity, i.e., muscle spasms in 1 individual (Nash, National Wildlife Research Center, unpublished). As some species are more sensitive to the effects of DiazaCon, it is suggested that DiazaCon treatment be considered primarily for species that require lower effective doses, helping to ensure safety and avoidance of nontarget hazards when applied in the field.

Only a few fertility studies have been conducted using DiazaCon as a reproductive inhibitor in mammals. One study indicated that an injection of 100–200 mg of DiazaCon per kg body mass in bandicoot rats (*Bandicota bengalensis*; Hikim and Chakraborty 1986, Hikim 1987) inhibited spermatogenesis. In mice, 28 doses per animal of 10, 20, or 30 mg/kg body weight (Singh and Chakravarty 2003) inhibited spermatogenesis without reports of toxicity. To date, no field trials of DiazaCon contraception of mammals have been reported in the literature.

Methods

Study area

We conducted this study during 2000 at a site in northern Colorado with 4 wards of black-tailed prairie dogs that were separated by roads, tall grassy areas, or marshes. Treatments were randomly assigned to these wards. Treated wards covered areas of 1.1 and 2.8 ha, while untreated (control) wards covered 0.4 and 1.8 ha. Occupied areas had sparse vegetation, mostly dry prairie grasses, at the beginning of the study. In the spring, some forbs, mostly bindweed (*Convolvulaceae*), appeared. Precipitation in February, March, April, and May was 0.38, 1.42, 0.83, and 1.49 water equivalent inches.

Bait preparation

Bait containing 0.25% DiazaCon (Avitrol Corporation, Tulsa, Oklahoma) was prepared using molasses-coated rolled oats. DiazaCon was dissolved in 70% ethanol to make a 100 mg/ml solution, and then 0.5 ml (50 mg) of the DiazaCon solution per ml of molasses was mixed to create a coating solution. The coating solution was then poured onto rolled oats at a ratio of 50 ml molasses to 950 g of oats in a Hobart mixer and mixed thoroughly. The volume of 70% ethanol was ignored in the calculations because evaporation was expected. Chemical analysis of 2 batches of bait that were prepared during the study showed that the actual concentrations of the baits were 0.25% (treatments 1–3) and 0.21% (treatments 4–10). Control bait was made in the same manner as treated bait, except that we used 70% ethanol rather than dissolved DiazaCon. High pressure liquid chromatography (HPLC) was used to determine levels of DiazaCon in the bait preparations by methods described previously (Johnston et al. 2001).

Bait application

We placed approximately 18-g of bait (containing 45 mg DiazaCon) per prairie dog (estimated) in small piles on the ground near burrow entrances on each of 10 days (February 9, 10, 14, 16, 18, 22, 23, 24, 29, and March 1). The amount we placed at each site varied slightly over the course of treatment as we made continued counts for population estimates. We placed spoonfuls of bait with about 12 g at the sites in the following average distribution: Site 0 received 37 spoonfuls, 1 at every 4 burrow entrances; Site 1 got 18 spoonfuls, 1 at every 1.5–2 burrow entrances; Site 2 got 32 spoonfuls, 1 at every 6 burrow entrances; and Site 3 received 23 spoonfuls, 1 at every 4 burrow entrances. Bait was placed at different entrances on different days. Baits were distributed in the morning to allow adequate time for bait consumption and were only distributed when there was no precipitation and it was warm enough to allow black-tailed prairie dogs to be active above ground during the day. In this study, 10 doses were used because a single high dose is of limited effectiveness (Schafer et al. 1977), and early studies in birds had used at least 10 treatments. Although fewer treatments may be effective, the purpose of the study was to determine whether DiazaCon would be effective in black-tailed prairie dogs, so a dosing regimen previously shown to be effective in other species was employed.

Visual sighting index

We used a population index based on visual observations for population estimates to determine the amount of bait to use and to indicate reproductive rates. We counted from a distance (empirically determined to not frighten prairie dogs) using binoculars. Because the wards were small, actual counts were made by scanning the entire ward. We used the highest count obtained (maximum number of animals in view while counting) as the index to evaluate populations. We made counts on 5 days for baiting number determinations (January 20; February 2, 8, 9, and 10) and 7 days for determining the young:adult ratio (May 9 and 24; June 1 [twice], 14, 28, and 29). The highest

number of young and the highest number of adults at a site were not necessarily obtained on the same day. We counted the wards in different orders on different days to reduce potential bias. Although we used the visual index to estimate the amount of bait to offer, the index was intended as a comparative measure and not as an actual measure of population.

Trapping and blood collection

We baited 2-door Tomahawk traps with oats or oats coated with molasses and wired the traps open for several days prior to capturing

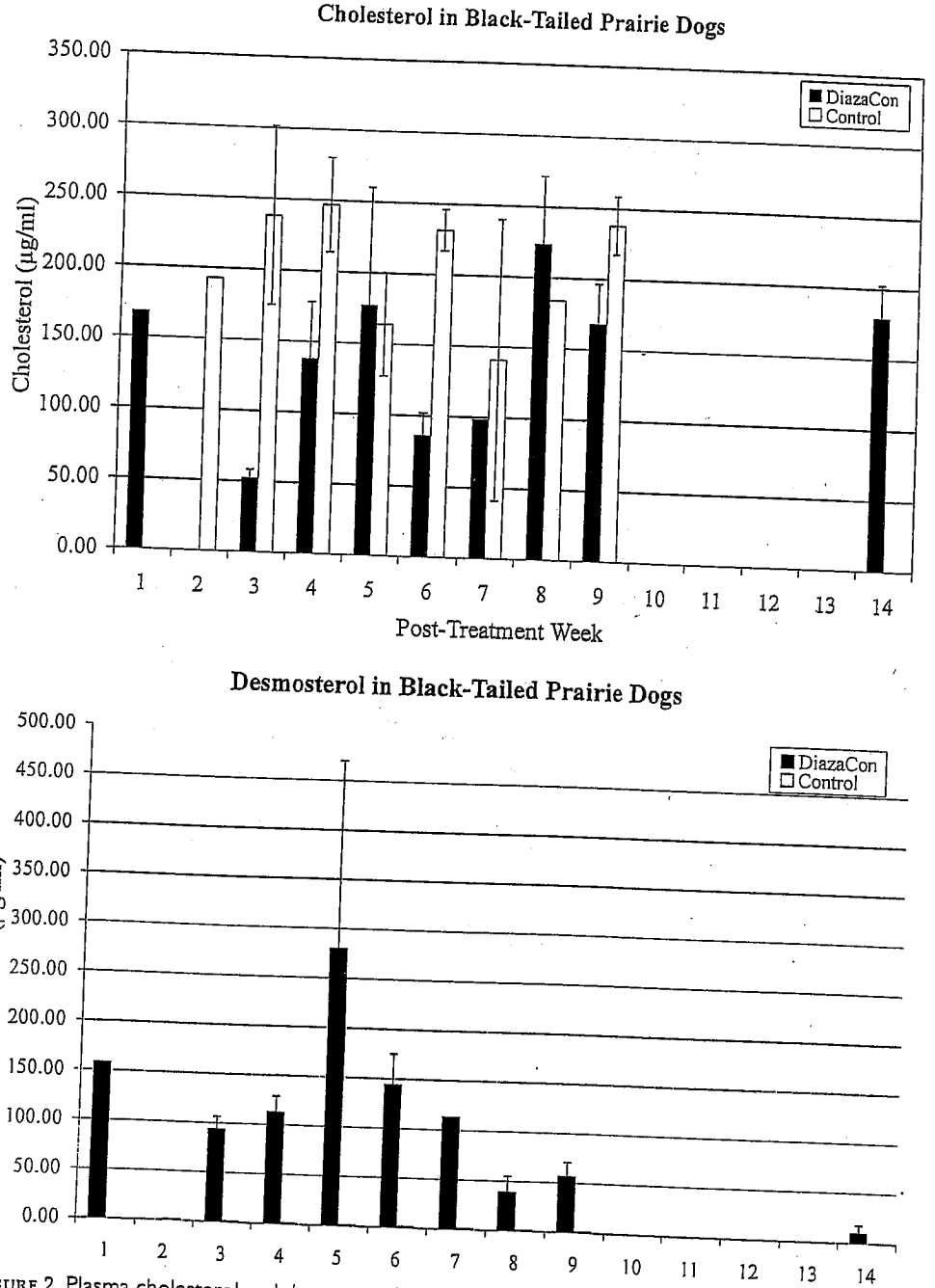

FIGURE 2. Plasma cholesterol and desmosterol of black-tailed prairie dogs trapped at 2 control and 2 DiazaCon-treated sites. Samples at the treated sites were collected on post-treatment weeks 1 (n = 1), 3 (n = 4), 4 (n = 9), 5 (n = 2), 6 (n = 4), 7 (n = 1), 8 (n = 4), 9 (n = 7), and 14 (n = 3). Samples from the control sites were collected on post-treatment weeks 2 (n = 1), 3 (n = 3), 4 (n = 3), 5 (n = 5), 6 (n = 5), 7 (n = 2), 8 (n = 1), and 9 (n = 12).

animals to acclimate the animals to the traps. We used 30, 8, 39, and 21 traps at Sites 0, 1, 2, and 3 respectively near burrow entrances. We ear-tagged each captured animal and dyed its fur to allow identification of repeat captures and allow identification of individuals for blood chemistry analyses. We checked traps frequently and left animals in traps typically for <1 hour. In rare cases where black-tailed prairie dogs were kept in the trap longer than an hour, we moved them to a location where they could be monitored and protected from adverse conditions. Weather permitting, we trapped 1–3 days per week through most of the study. To avoid placing additional stress on the animals, we did not trap when temperatures were <0°C or >27°C or when it was raining. We did not trap when baits were being distributed so that no animals would be kept from bait because they were in a trap. Traps were closed but left in the field when not in use. We released animals at the same location where they were trapped.

Quantification of cholesterol and desmosterol in blood plasma

After treatment, we tested most trapped animals, especially from treated sites, for cholesterol and desmosterol levels, except when logistical problems with the portable anesthesia equipment or capturing more animals prevented it. The number of animals we tested appears in the legend for Figure 2.

We trapped animals, anesthetized them with isoflurane, and clipped a claw into living tissue of each animal to allow blood to drip into a 1.5-ml microcentrifuge tube with ethylenediaminetetraacetic acid (EDTA). We immediately mixed collected blood with the EDTA by inversion. Plasma was collected by centrifugation of the blood for 10 minutes followed by removal of the plasma with a micro-pipettor. Plasma samples were stored frozen until evaluation of cholesterol and desmosterol levels. Each 100-µl plasma sample was analyzed by HPLC as previously described (Johnston et al. 2003).

Ray Sterner and Cherryl Tope handling a black-tailed prairie dog during trapping efforts on this study.

Data analysis

The 2 control sites had the largest and smallest populations, but the total population of the 2 control sites was about the same as the total population at the treated sites. No statistical analysis on numbers of young was performed because there were only 2 sites per treatment. We used analysis of variance to compare desmosterol and cholesterol results using StatView (SAS Institute Inc. 1998). We treated all samples as independent even though multiple samples were obtained from a few individuals. All samples were obtained post-treatment and pooled them for analysis independent of time elapsed since bait distribution.

Results

Pre-treatment population determinations

We made visual counts of each ward on 5 separate occasions prior to treatment, and 5 counts during treatment. Counting revealed 23 and 10 black-tailed prairie dogs at the 2 treatment sites and 9 and 27 black-tailed prairie dogs at the control sites. Although wards were near each other, over the course of the study we observed none of the animals trapped outside their wards, indicating no movement between wards.

Reproductive census

Following treatment, we made 7 counts for the reproductive index. The number of adults (single greatest number counted at each time) at the combined treatment sites ($n = 28$) was not appreciably different from the number of adults at the combined control sites ($n = 25$), but the number of juveniles at the treated sites ($n = 24$) was distinctly less than at control sites ($n = 42$). The ratio of juveniles:adults at the control site of 1.7 (9:5 and 33:20) compared to 0.9 (15:19 and 9:9) at the treated sites suggests a 47% reduction in reproductive success.

Cholesterol and desmosterol levels

Cholesterol levels were significantly lower in black-tailed prairie dogs from the DiazaCon-treated sites than from control sites. Average plasma cholesterol levels were 131 µg/ml ($n = 35$, SE = 14, range = 14–333) at the treated sites and 223 µg/ml ($n = 32$, SE = 12, range = 45–361) at the control sites ($P \leq 0.001$) during the 3-month period after baiting (Figure 2). During this period no individuals trapped at control sites had elevated desmosterol, whereas all but 2 black-tailed prairie dogs trapped at the treated sites had elevated desmosterol levels during the entire 3-month period. Black-tailed prairie dogs had mean desmosterol concentrations of 96 µg/ml (n=35, SE=14, high 470, and low 0) at

the treated sites, which was significantly higher ($P \leq 0.001$) than individuals at the control sites (all below the level of detection). Increased desmosterol was low but detectable more than 3 months after the last treatment. The lack of detectable desmosterol in plasma samples from the control sites provides further evidence that there was likely no movement between the wards.

Discussion

With a 47% reduction in the ratio of juveniles to adults, application of oral bait containing DiazaCon in the field resulted in an apparent reduction in reproductive success of black-tailed prairie dogs. This study suggests the potential for DiazaCon as a reproductive control agent for use in managing black-tailed prairie dogs. The breeding season for black-tailed prairie dogs in northern Colorado begins in mid-February and ends in mid-March, with peak breeding in February. Due to unforeseen delays in obtaining local permits, we did not start treatment until just before the breeding season began, and we did not give the last dose of DiazaCon until the peak of the breeding season was over. Taking the lateness of treatment into account, it is likely that the reduction in reproductive success would be improved with earlier treatment. Earlier treatment is particularly imporant for inhibiting male reproduction because sperm production requires approximately 60 days. Testosterone suppression is more likely to affect earlier stages of sperm development, leaving sperm already approaching maturation unaffected (Singh and Chakravarty 2003).

Treatment with DiazaCon bait for 10 days caused a decrease in cholesterol and desmosterol levels that lasts several months after treatment ends. This is especially apparent where the desmosterol level is still at an elevated, albeit low level 14 weeks post-treatment. It has not been established what levels of cholesterol and desmosterol correlate with inhibition of reproduction in black-tailed prairie dogs, but reproductive inhibition probably persisted for an extended period. Long-lasting effects may be an advantage if treatment begins earlier, providing more time for treatment to allow for missed days of baiting due to inclement weather. This study used 10 doses of DiazaCon bait, which resulted in a lower young:adult ratio that persisted for the year. Fewer doses may provide acceptable levels of reproductive inhibition while reducing application costs, but the actual number of treatments required has yet to be determined experimentally.

Oral DiazaCon bait is among only a handful of oral contraceptives tested successfully in the field for wildlife population management and is likely the only one that is not hormonal. Effective treatment can be achieved without the capture of animals or complications of darting. The ease of administration to large numbers of animals also makes DiazaCon use more economical than methods that require treating animals individually.

Possible effects (other than reduction in population) that may occur due to inhibition of breeding behaviors were not addressed in this study. The potential for effects on the population are presumed to be minimal, but the possibility of coterie disruption, abnormal movement, or other behavioral anomalies should be investigated so that any changes can be taken into account by managers considering use of the treatment. Likewise, studies of secondary nontargets need to be conducted to determine whether DiazaCon can pass through the food chain, and if so, at what concentrations. Cost and regulatory issues will need to be evaluated as well. DiazaCon was previously approved by the EPA for pigeon control under the name Ornitrol®, but the need for further studies to support continued registration caused the product to be withdrawn. Because this product was previously registered, it should be easier to do so again.

Use of DiazaCon could provide a nonlethal solution to manage black-tailed prairie dog populations in areas where population expansion is of concern. Locations where black-tailed prairie dogs are desirable but where high population densities cause starvation, disease, and increased pressure to move across roads would be prime candidates for reproductive control. Although appropriate delivery systems may require development, DiazaCon treatment will also likely be useful for other species for which nonlethal population management tools are needed, including other species of prairie dogs (*Cynomys* spp.), ground squirrels (*Spermophilus* spp.), woodchucks (*Marmota monax*), beavers (*Castor canadensis*), etc.

Depending on cost and effectiveness, DiazaCon reproduction inhibition may even be applicable to larger animals such as raccoons (*Procyon lotor*), feral horses (*Equus caballus*), and urban white-tailed deer (*Odocoileus virginianus*). Avian species are also potential targets for reproductive control using DiazaCon. In addition to public sentiment issues, safety issues can also play a role. In urban settings where accidental ingestion by humans is possible and locations where protected species may inadvertently be exposed, transient fertility control may be a safer alternative than lethal poisoning.

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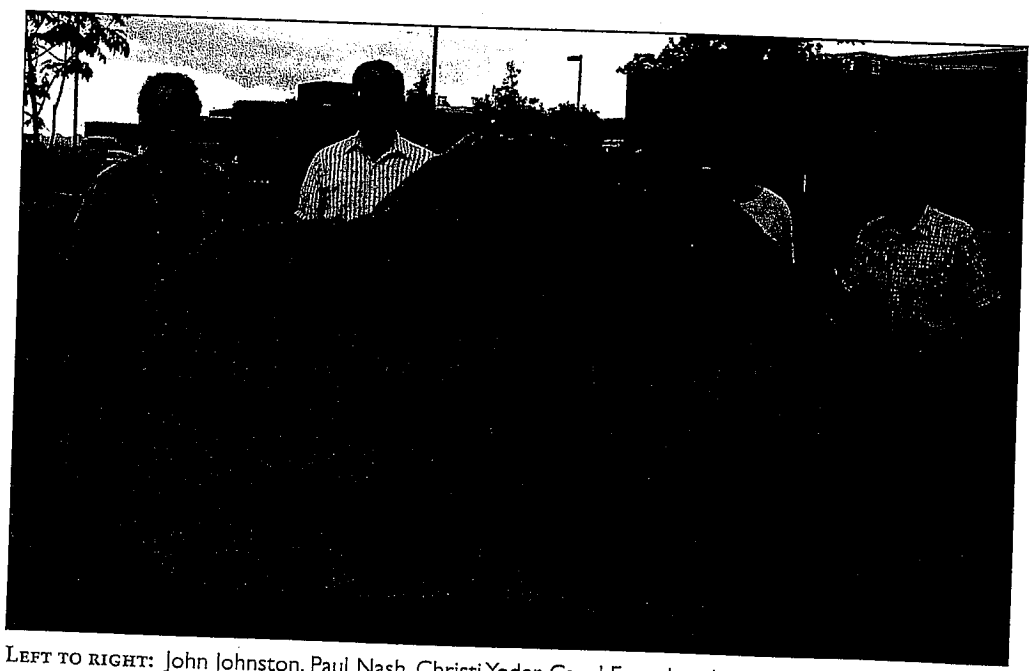
Carol A. Furcolow obtained her B.A. in microbiology from the University of Kansas and is a registered medical technologist. She has been at the National Wildlife Research Center since 1985, working in the analytical chemistry section. She is involved in many studies were she extracts analytes from tissues, soils, waters, baits, lures, etc. using various technologies such as liquid extraction, SPE, and microwave assisted reactions and quantifies them using HPLC, GC, and mass spectrometry.

Kimberly S. Bynum (not pictured) obtained her B.S. from Texas A&M University in 1993 and her Ph.D. in reproductive physiology from the University of Toledo in 2002. Currently residing in Ohio, she began at the National Wildlife Research Center in 2001 as part of the induced infertility team.

Christi A. Yoder obtained her associates degree from Bethany Lutheran College, her B.S. in zoology from Colorado State University in 1991, her M.S. in Wildlife Biology from Colorado State University in 2000, and her Ph.D. in physiology from Colorado State University in 2005. She has worked in a variety of positions studying wildlife and has been at the National Wildlife Research Center since 1994. She has been instrumental in the comparison of various wildlife contraceptives including, DiazaCon, several immunocontraceptives, and nicarbazin.

Lowell A. Miller obtained his B.A. in biology from Upland College in 1963, his M.S. in science/physiology and biophysics from Colorado State University in 1967, and his Ph.D. in physiology/Immunology from Colorado State University in 1989. He has been at the National Wildlife Research Center since 1992, functioning as the fertility control project leader for 11 years. He has been involved with the development of a variety of wildlife contraceptive methods including GnRH and zona pellucida immunocontraceptives and the oral bird contraceptive nicarbazin.

John J. Johnston obtained his B.S. degree in food science at Rutgers University in 1979, his Ph.D. in the toxicology program at University of Florida in 1986, and his M.B.A. at Colorado State University in 2000. He has been the chemistry project leader at the National Wildlife Research Center since 1994, overseeing metabolism/environmental fate, wildlife genetics, research and method development, exploratory chemistry, formulations chemistry, laboratory safety, analytical services, and QA/QC units.



Left to right: John Johnston, Paul Nash, Christi Yoder, Carol Furcolow, Lowell Miller.