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DEVELOPMENT OF DIAZACON™ AS AN AVIAN CONTRACEPTIVE

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Abstract: Due to increasing human-wildlife conflicts with birds and growing opposition to lethal techniques, nonlethal methods need to be developed to help manage bird populations. DiazaCon™ is a promising oral contraceptive that acts by directly inhibiting the conversion of desmosterol to cholesterol. Because cholesterol is essential for the production of the steroid reproductive hormones testosterone, progesterone, and estradiol, DiazaCon™ also indirectly inhibits the formation of these hormones. These hormones are essential for sperm and egg production, and the production of egg yolk precursors in the liver. Because DiazaCon™ is cleared slowly from the liver, its contraceptive effects are long-lasting. Initial research with Coturnix quail (*Coturnix coturnix*) helped determine DiazaCon's mechanism of action. Further research showed efficacy in monk parakeets (*Myiopsitta monachus*) and mallards (*Anas platyrhynchos*). Brown-headed cowbirds (*Molothrus ater*), American crows (*Corvus brachyrhynchos*) and ring-necked doves (*Streptopelia risoria*) were also studied as potential candidates for DiazaCon™ contraception. Mallards and ring-necked doves were used as model species for Canada geese (*Branta canadensis*) and pigeons (*Columba livia*), respectively. DiazaCon™ application over a very short time results in long-lasting contraceptive effects, an advantage for target species, but a disadvantage for nontarget species. Care must be taken when delivering DiazaCon™ to determine what nontargets are present and how best to avoid them. Timing of delivery is also critical to ensure maximal reproductive effects, and information on the reproductive cycles of the species of interest is needed.

Key words: avian, bird, cholesterol, contraception, control, desmosterol, 20,25 diazacholesterol dihydrochloride, DiazaCon™, Ornitrol, reproduction

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INTRODUCTION

As human developments continue to grow, human-wildlife conflicts become more frequent. Health concerns exist where large numbers of birds congregate and foul the area with feces. Fecal material presents health hazards such as histoplasmosis (Stickley et al. 1987, Fischer 1995), salmonella (Smith et al. 2002, Vucemilo et

al. 2003), and E. coli (Morabito et al. 2001, Pennycott et al. 2002). Safety concerns exist at airports where birds congregate on or near runways (Dolbeer et al. 2000, Sodhi 2002). Birds cause agricultural damage to grain crops (Bullard and York 1996, Avery et al. 1998, Blackwell et al. 2003), fruits and vegetables (Tobin et al. 1991, Brugger et al. 1993, Cummings et al. 1998), livestock feed

(Mason et al. 1985, Glahn et al. 1991, Van-Niekerk 2003), and fish at aquaculture facilities (Glahn et al. 2002, Dorr et al. 2004). Breeding activities of some species causes damage to various structures (Evans et al. 1984, Stemmerman 1988), and birds may become aggressive during breeding. Birds roosting in urban areas disturb neighbors with noise. Because there is growing opposition to lethal control methods, particularly in urban areas, acceptable nonlethal methods must be developed to help in the management of overpopulated bird species. Contraception is one generally acceptable nonlethal method that can be used in conjunction with other control methods (Messmer et al. 1997, Stout et al. 1997).

There are several characteristics that are desirable in any wildlife contraceptive agent. It should last for an entire breeding season and require a minimal number of days of treatment. Ideally, it should be reversible so as not to permanently remove individuals from the gene pool. Oral administration is desirable to eliminate the cost and handling stress associated with capture of animals. Contraceptives should be safe for the environment and have a minimal impact on nontarget species. Finally, contraceptives should be relatively inexpensive.

A number of contraceptives have been tested in birds, but several produced unacceptable results. Surgical sterilization was used for Canada geese, but this method requires that geese be captured and anesthetized, and is prohibitively expensive (Converse and Kennelly 1994). Mestranol (Wentworth et al. 1968, Sturtevant 1970), α -chlorohydrin (Aire and Olusanya 1980), and triethylenemelamine (Messersmith 1971, Bhat and Maiti 1989) could permanently sterilize adults and young being fed crop milk. Research on DiazaCon™ (20,25 diazacholesterol dihydrochloride) as an avian contraceptive first occurred in the late

1960s and early 1970s, but produced conflicting results.

HISTORICAL DIAZACON™ RESEARCH

Originally, DiazaCon™ was investigated as a cholesterol-lowering agent for humans (Sachs and Wolfman 1965). Human trials were suspended when males receiving the compound complained of an “uncomfortable feeling”. Research was then conducted to determine if DiazaCon™ could lower the cholesterol content in egg yolks of eggs to be consumed by humans (Singh et al. 1972, Dam et al. 1979, Cecil et al. 1981).

Next, DiazaCon™ was considered as a potential contraceptive for feral pigeons, and was registered for such use under the trade name Ornitrol (Schortemeyer and Beckwith 1970, Sturtevant and Wentworth 1970). Research continued on species such as red-winged blackbirds (*Agelaius phoeniceus*) (Lacombe et al. 1986, 1987), house sparrows (*Passer domesticus*) (Sanders and Elder 1976, Mitchell et al. 1979), grackles (*Quiscalus quiscula*) (Fringer and Granett 1970), Japanese quail (Powell 1966), and parakeets (probably *Melopsittacus undulates*) (Powell 1966). These studies sometimes presented conflicting results, with one showing efficacy and another showing little effect on the same species. Limited conclusions could be drawn from these studies because very little physiological work was conducted to determine how the compound worked. The National Wildlife Research Center (NWRC) in Fort Collins, Colorado initiated research in 1997 with Coturnix quail to determine DiazaCon’s mode of action (Yoder et al. 2004).

COTURNIX QUAIL STUDY

Coturnix quail were used as an avian model species because their reproductive cycles are easily manipulated in the laboratory and they have a high reproductive rate. Quail received an average of 100

mg/kg DiazaCon™ in feed per bird per day for 12-14 days. Testosterone, progesterone, cholesterol, desmosterol, egg production, fertility, and hatchability were monitored. Initially, an enzymatic test was used to monitor cholesterol concentrations, but this test was not specific enough to distinguish between cholesterol and desmosterol. A high performance liquid chromatography (HPLC) method was developed to more accurately monitor cholesterol and desmosterol concentrations (Johnston et al. 2001).

The HPLC results showed a 40% decrease in cholesterol concentrations in male treated birds. Desmosterol concentrations in treated birds rose to approximately 230 µg/mL compared to 7 µg/mL in control birds. Treated males were paired with untreated females to determine fertility. Fertility decreased by 41% and hatchability by 35%, and both remained suppressed for ≥ 3 months. Decreased fertility and hatchability were correlated with a 37% decrease in testosterone concentrations.

Treated female quail experienced a 52% decrease in cholesterol concentrations.

Desmosterol levels in treated birds rose to approximately 280 µg/mL compared to 30 µg/mL in control birds. Egg production was decreased by 85%, fertility of laid eggs by 70%, and hatchability by 100%. Egg production, fertility, and hatchability remained suppressed for ≥ 3 months, and were correlated with a 42% decrease in progesterone concentrations.

MECHANISM OF ACTION

DiazaCon™ is structurally similar to cholesterol (Figure 1), and may competitively inhibit enzymes in the cholesterol biosynthesis pathway (Dietert and Scallen 1969, Counsell et al. 1971, Emmons et al. 1982). Because DiazaCon™ mimics cholesterol, it can competitively bind to enzyme substrates, with nitrogen substitutions in the 20 and 25 positions interfering with enzyme action. Desmosterol is converted to cholesterol by the Δ_{24} reductase enzyme. The quail study concluded that DiazaCon™ acted to inhibit Δ_{24} reductase activity (Figure 2), which decreased cholesterol and increased desmosterol concentrations.

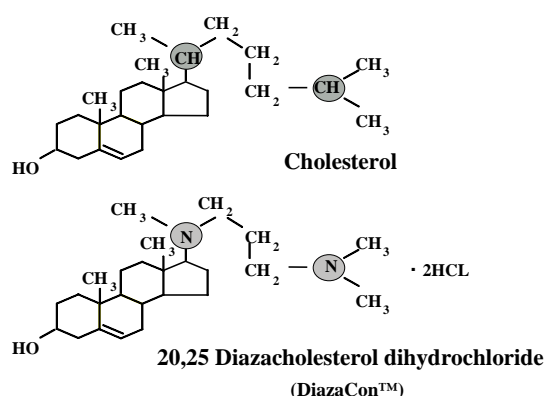


Figure 1. The chemical structure of cholesterol and DiazaCon™ (20,25 diazacholesterol dihydrochloride).

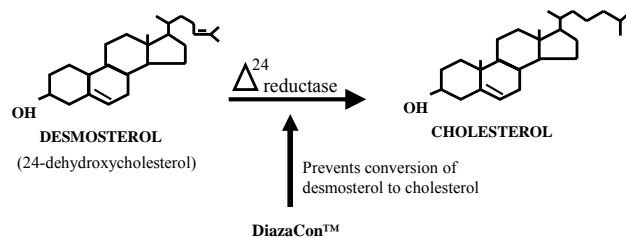


Figure 2. Inhibition of the conversion of desmosterol into cholesterol by DiazCon™.

Reductions in cholesterol concentration cause reductions in steroid reproductive hormone concentrations, which in turn decreases sperm and egg production. The side chain of cholesterol is cleaved to form the steroid hormone pregnenolone, the precursor to the steroid reproductive hormones testosterone, progesterone, and estradiol (Figure 3). Testosterone is needed for sperm formation and breeding behavior. Progesterone plays a role in ovulation and

oviposition. Estradiol stimulates the production of egg yolk precursors, such as vitellogenin and very low density lipoprotein, in the liver, and is also needed in some songbirds for singing behavior. Although not investigated in the quail study, DiazCon™ may also inhibit the P₄₅₀ side chain cleavage enzyme responsible for cleaving cholesterol's side chain to form pregnenolone (Figure 4).

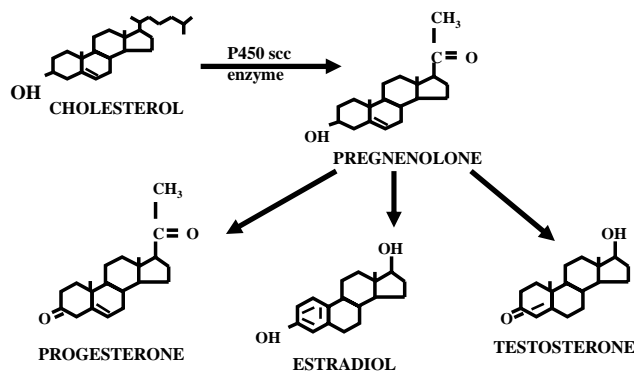


Figure 3. Synthesis of steroid reproductive hormones from cholesterol.

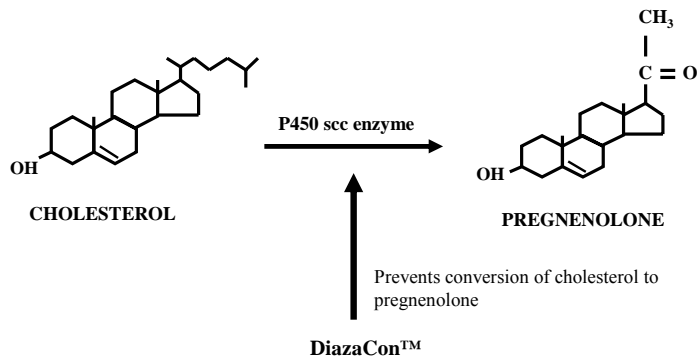


Figure 4. Inhibition of conversion of cholesterol into pregnenolone by DiazaCon™.

Radiolabel studies in pigeons show DiazaCon™ has a half-life of 28 days (Ranney 1968). Approximately half of orally administered DiazaCon™ is cleared in the feces within 24 hours. The remainder is primarily stored in the liver and cleared slowly over time. Steroids are not water soluble, and therefore must first be conjugated in the liver to make them water soluble, allowing them to be cleared by kidney excretion. This conjugation occurs predominately at the D ring of the steroid structure. DiazaCon™ is no longer water soluble once it enters the body, and is stored in fat like steroids. Because DiazaCon™ has nitrogen molecules present near the D ring, the liver cannot conjugate it effectively. The primary means of excretion from the body is most likely via leakage of lipid soluble DiazaCon™ into the blood stream. This accounts for DiazaCon's long-lasting reproductive effects.

ADDITIONAL SPECIES STUDIED TO DATE

Ring-necked Doves

Because Diaza-Con™ appeared to have potential as an avian contraceptive, a study on ring-necked doves was conducted during 2002 at NWRC (Yoder et al. unpublished data). Doves were used as a model for pigeons because they required less space to house. DiazaCon™ was coated onto a mixture of hulled seeds (millet, thistle, sunflower seed hearts, peanut hearts) at rates of 0.03% (w/w), 0.05%, and 0.1%, and fed *ad libitum* for 14 days. Based on consumption data, doves received an average dose of 14.0, 25.7, and 52.5 mg/kg per bird per day in the 0.03%, 0.05%, and 0.1% groups, respectively.

Cholesterol concentrations decreased by 48%, 43%, and 46% in the 0.03%, 0.05%, and 0.1% groups, respectively. Desmosterol concentrations increased to 3800-5500 µg/mL in treated doves compared to 20 µg/mL in control doves. By 15 weeks post-treatment, the changes observed in desmosterol and cholesterol concentrations had diminished so that no difference was observed between treated and control groups. No effect on reproduction was observed during the treatment period. A

complete cessation of egg laying was observed during the treatment period in the control group and 2 treatment groups. This was likely due to 2 factors. Doves have very high inherent cholesterol concentrations. Even though cholesterol was significantly reduced in the treatment groups, it was not sufficient to affect reproduction. Additionally, dove reproductive hormone concentrations are strongly regulated by visual cues (O'Connell et al. 1981, Cheng 1986). Any decrease in breeding behaviors in the treatment groups also likely caused a decrease in breeding behaviors in the control group also because all birds were housed in the same room.

Brown-headed Cowbirds

A laboratory study on brown-headed cowbirds was conducted in 2003 at NWRC (Bynum et al. unpublished data). DiazaCon™ was coated onto a mixture of hulled seeds (millet, thistle, sunflower seed hearts, peanut hearts) at rates of 0.0078% (w/w), 0.0156%, and 0.03125%. Treated seed was fed *ad libitum* for 14 days. The actual doses received were 8.5, 18.7, and 52.2 mg/kg per bird per day in the 0.0078%, 0.0156%, and 0.03125% groups respectively. Although reproduction could not be monitored in the laboratory setting, cholesterol and desmosterol concentrations were measured. Cholesterol concentrations decreased by 85%, 86%, and 81% in the 0.0078%, 0.0156%, and 0.03125% groups respectively. Desmosterol concentrations increased to 245-380 µg/mL in treated birds compared to 50 µg/mL in the control birds. Cholesterol concentrations remained suppressed for ≥ 1 month.

Monk Parakeets

Several laboratory studies using monk parakeets were conducted in 2003 and 2004 at NWRC's Florida field station (Yoder et al. unpublished data). An initial gavage

study to identify the target dose for further studies was conducted using 50, 75, and 100 mg/kg doses of DiazaCon™. Cholesterol concentrations decreased by 81%, 85%, and 86% in the 50, 75, and 100 mg/kg groups, respectively. Desmosterol concentrations increased to 600-760 µg/mL in treated birds compared to 5 µg/mL in control birds. Cholesterol concentrations were suppressed for ≥ 12 weeks. The target dose was determined to be 50 mg/kg. Next, another oral gavage study investigated differences in cholesterol and desmosterol concentrations based on the number of days of treatment by comparing 5 and 10 days of treatment. Cholesterol concentrations decreased by 67% in both treatment groups. Desmosterol concentrations increased to 850-950 µg/mL in treated birds compared to 4 µg/mL in control birds. Cholesterol concentrations were suppressed for ≥ 10 weeks. This study showed 5-7 days of treatment were adequate to reduce cholesterol concentrations. *Ad libitum* studies proved captive pairs of monk parakeets would eat adequate amounts of DiazaCon™ coated sunflower seeds over 5 days to prevent successful reproduction. Birds in the *ad libitum* study ingested an average dose of 33.7 mg/kg per bird per day. Egg production was decreased in treated birds by 59% over a 2 month period, and no eggs hatched in the treatment group. Monk parakeets are ideal candidates for field application of contraceptive techniques because of their low dispersal rates, and their willingness to take sunflower bait from feeding stations.

American Crows

Two studies on American crows were conducted during 2003 at NWRC (Yoder et al. unpublished data). Crows were gavaged with 50, 75, or 100 mg/kg DiazaCon™ per bird per day for 14 days. Cholesterol concentrations decreased by 46%, 47%, and 52% in the 50, 75, and 100 mg/kg groups

respectively. Desmosterol concentrations increased to 170-200 µg/mL in treated crows compared to 70 µg/mL in control crows. Cholesterol concentrations were suppressed for ≥ 1 month. Based on the results, crows were fed scrambled eggs treated with DiazaCon™ at a target dose of 50 mg/kg per bird per day for 14 days. Crows received an average dose of 43.0 mg/kg per bird per day, and cholesterol decreased by 47%. Desmosterol concentrations increased to 140 µg/mL in treated crows compared to 6 µg/mL in control crows. Cholesterol and desmosterol concentrations were recovered by 5 weeks post-treatment.

In 2005, a preliminary field study in California was conducted to assess various capture and monitoring techniques, and bait attractiveness (Gionfriddo et al. unpublished data). This study showed the difficulty in monitoring crow reproduction. Because nests are relatively inaccessible, close monitoring of nests would be difficult and expensive, making an accurate assessment of DiazaCon™ efficacy difficult. The bait tested was not attractive to crows, and none was consumed. It would be impossible to target only resident crows in areas where winter roosts occur due to the large number of migrants. Contraception alone would be ineffective in reducing wintering roost sizes because crows disperse during breeding. It is unknown how large an area wintering crows disperse over during the breeding season.

Mallards

A free feeding study using mallards as a model species for Canada geese was conducted during 2004 at NWRC (Yoder et al. unpublished data). Mallards were fed DiazaCon™ treated waterfowl pellets (0.1% DiazaCon™ w/w) *ad libitum* for either 6 consecutive days or on 5 days over a 10 day period. The doses received were 67.6 mg/kg

per bird per day for the 6 consecutive day group and 63.8 mg/kg per bird per day for the 5 dose group. Cholesterol concentrations decreased by 72% in the group receiving 5 doses over 10 days, and by 74% in the group receiving 6 consecutive doses. Desmosterol concentrations increased to approximately 285 µg/mL in treated birds compared to 1 µg/mL in control birds. Egg production was decreased by 97%, and hatchability by 100% in the group receiving 5 doses over 10 days. In the group receiving 6 consecutive doses, egg production was decreased by 94% and hatchability by 100%. Cholesterol concentrations remained suppressed for ≥ 10 weeks. This study proved it isn't necessary for birds to consume DiazaCon™ bait daily for efficacy.

ADVANTAGES OF DIAZACON™

The short application period of DiazaCon™ is advantageous from an operational standpoint. Research showed DiazaCon™ may be applied for as few as 5 days with efficacy. In addition, DiazaCon™ need not be consumed on a daily basis to affect reproduction. Several doses over a period of time are as effective as the same number of doses on consecutive days. This is important because birds may not visit a bait site on a daily basis.

A single DiazaCon™ dose will not produce reproductive effects. Absorption of DiazaCon™ may be controlled through a receptor-mediated mechanism. Once the receptors are full, the rest of the DiazaCon™ passes through the gut and is excreted in the feces. Recycling of receptors is necessary to allow more DiazaCon™ to be absorbed in subsequent doses. An acute dose is likely cleared from the system without much being stored in the liver. Chronic dosing allows a level of DiazaCon™ to build up in the liver.

Another advantage of DiazaCon™ is the length of time the reproductive effects last.

Prior research showed DiazaCon™ effects last ≥ 3 months in some species, enough time to preclude renesting within a breeding season. This allows flexibility in baiting programs. Bait can be applied, for example, prior to the greening up of grass for Canada geese. Geese are more likely to eat bait at this stage because they do not have other highly desirable food sources readily available to them. Birds not easily accessible once they begin breeding can be treated prior to breeding. This eliminates the need to search out nests for egg oiling and addling programs.

DiazaCon™ affects both male and female reproduction, which presents an advantage over a contraceptive that would affect only one sex. In situations where only males are treated, extra-pair copulations by untreated males still result in successful reproduction by females. However, if both males and females are treated, extra-pair copulations are not an issue. In situations where it is desirable to only treat one sex, DiazaCon™ could be selectively applied provided you had access to the desired sex. For example, males arriving on territories earlier than females could be treated selectively. Conversely, if females tend to congregate in large flocks prior to breeding, they could be treated selectively, allowing males to maintain breeding territories.

The effects of DiazaCon™ are reversible, which is a highly desirable trait in a wildlife contraceptive because the treated animals can be returned to the gene pool the following season. If a nontarget species were to eat enough DiazaCon™ bait to prevent reproduction, that animal would not be permanently sterilized.

DISADVANTAGES OF DIAZACON™

Although the long-lasting reproductive effect of DiazaCon is an advantage for target species, it is a disadvantage for nontarget species. A single acute dose has no

reproductive effects (Woulfe 1967), but several repeated doses have reproductive consequences. It is critical to know what species are in the baiting area in order to develop appropriate baits and better plan baiting operations. DiazaCon affects mammals as well as birds, so they must be considered when determining what nontarget hazards are present. Some hazards to songbirds can be mitigated by using bait that is too large for smaller birds to consume. Selective placement of bait could further eliminate hazards. For example, placing bait off the ground could eliminate hazards to domestic dogs. Work on secondary hazards has yet to be conducted. However, a predator eating a single treated bird is unlikely to be affected because chronic dosing is required for contraception (Woulfe 1967).

Bait development is important to increase palatability of DiazaCon™. Previous studies have coated DiazaCon™ onto the bait preferred by the target species. Because DiazaCon™ is bitter, use of masking agents or encapsulation is needed to increase consumption. Allowing DiazaCon™ to be coated onto bait, such as whole corn or seeds, is an advantage because bait will be readily recognizable as food by target species. Another method is to incorporate DiazaCon™ into extruded bait. While this would increase palatability, it would likely require more prebaiting to acclimate birds to a new food source.

Toxicity was noted in DiazaCon™ treated pigeons (Elder 1964, Sturtevant and Wentworth 1970), quail (Powell 1966, Yoder et al. 2004), cowbirds (Bynum, NWRC, personal communication), and monk parakeets (Avery, NWRC, personal communication.). These effects include listlessness, weight loss, difficulty breathing, loss of muscle control, and death. However, studies on red-winged blackbirds (Lacombe et al. 1986), house sparrows (Mitchell et al.

1979), crows, and mallards did not show any toxic effects.

It is critical that dose response studies be performed in the laboratory on the species of interest prior to field application of DiazaCon™. Absorption of DiazaCon™ will differ among species, with some being more sensitive to DiazaCon™ than others. Although reproduction of many wild species cannot be monitored in the laboratory, cholesterol and desmosterol concentrations can be used as markers of efficacy (Johnston et al. 2003, Yoder et al. 2004). This allows a safety range to be identified so that a target dose can be chosen to maximize contraceptive effects while minimizing the risk of toxicity.

MANAGEMENT IMPLICATIONS

It is important to consider the breeding biology of the species of interest to effectively administer DiazaCon™. Increasing day lengths cause an increase in testicular size. Once the testes are reproductively mature, it takes 2-5 days for maturation of a germ cell into a sperm cell capable of fertilization. DiazaCon™ should be given to males ≥ 3 weeks before the first copulation to prevent sperm maturation.

A similar increase in ovarian size occurs in females in response to increased day lengths. As day length increases, there is a slow, gradual deposition of yolk in the follicle. A rapid follicular growth occurs 6-11 days prior to ovulation. It takes approximately 24 hours for an ovulated egg to traverse the reproductive tract and be laid. DiazaCon™ should be applied to females ≥ 2 weeks prior to laying the first egg to prevent ovulation. In order to prevent follicular maturation, DiazaCon™ should be applied ≥ 1 month prior to the onset of egg laying.

DiazaCon™ is not an appropriate tool for non-seasonal breeders. Because of its cholesterol lowering effects, it is essential to

limit the length of treatment. Continuous use of DiazaCon™ throughout the year could potentially cause other health effects during non-breeding. Because DiazaCon™ has only suppressed cholesterol for 3-4 months in most species studied, it is ideal for use during a single breeding season.

Contraception is a tool best used as a proactive measure, or in conjunction with other control techniques. Because population effects would not be noticeable for several years, it is desirable to first reduce the population to an acceptable concentration, and then maintain the population with contraception. If problems are widespread over a large area, logistical problems exist. Cooperation over much of the area is needed in order to be successful. Field studies will be conducted in the future with the goal of registering DiazaCon™ as an avian contraceptive. Other species to be considered as candidates for DiazaCon™ include gulls and ring-necked parakeets. DiazaCon™ is a promising contraceptive that can be added to the tools already used to manage problem avian populations.

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