2006

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C. A. Yoder
National Wildlife Research Center, christi.yoder@aphis.usda.gov

J. K. Graham
Colorado State University

L. A. Miller
National Wildlife Research Center

K. S. Bynum
National Wildlife Research Center

J. J. Johnston
National Wildlife Research Center

See next page for additional authors

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C. A. Yoder,*[1] J. K. Graham,† L. A. Miller,* K. S. Bynum,* J. J. Johnston,* and M. J. Goodall*

*National Wildlife Research Center, 4101 LaPorte Avenue, Fort Collins, CO 80521-2154;
and †Department of Biomedical Sciences/Physiology, Colorado State University, Fort Collins 80523

ABSTRACT Contraception may provide a useful nonlethal management tool to reduce wild bird populations. We tested the efficacy of nicarbazin (NCZ) as a contraceptive for waterfowl and assessed health effects of NCZ, using domestic mallards (*Anas platyrhynchos*) as a model for Canada geese (*Branta canadensis*). Mallards were given gelatin capsules containing 0, 8.5, 17.0, or 33.75 mg of NCZ/kg of BW perorally once daily for 14 d. Fecal 4,4′-dinitrocarbanilide (DNC) and fluorescein were evaluated as potential markers of plasma and egg DNC levels. Plasma, egg, and fecal DNC levels differed among treatment groups in a dose response relationship. There were no significant effects on the numbers of eggs laid per female per day, proportion of fertile eggs, proportion of eggs hatching, or egg yolk mottling. Hatchability was 0.55 ± 0.1 in the control group compared with 0.26 ± 0.1 in the 33.75 mg/kg of BW group. Degeneration of the vitelline membrane was evident at all treatment levels; severity was dose-related and greater in the outer vitelline membrane than the inner vitelline membrane. No significant health effects were observed for birds treated with NCZ. The heterophil:lymphocyte ratio was elevated during the treatment and posttreatment periods in all groups, indicating birds were experiencing stress due to handling. Fecal DNC levels did not correlate well with plasma DNC levels, likely due to NCZ being administered as a bolus dose rather than being fed ad libitum. Fluorescein correlated well with plasma DNC levels during the treatment period and can therefore be used successfully as a noninvasive marker to determine the approximate amount of NCZ a bird is consuming. As a contraceptive, NCZ likely would have minimal adverse health effects on the target animal, although field studies with the species of interest need to be conducted. Further research using higher NCZ levels needs to be conducted to determine whether NCZ can inhibit reproduction in waterfowl.

Key words: contraception, 4,4′-dinitrocarbanilide, nicarbazin, vitelline membrane, waterfowl

2006 Poultry Science 85:1275–1284

INTRODUCTION

Canada goose (*Branta canadensis*) populations are expanding, and nonmigratory populations are becoming more frequent in urban areas as development provides attractive, year round habitat (Forbes, 1993; Ankney, 1996; Gosser and Conover, 1999). This creates health and safety issues in urban areas as large numbers of geese in parks and golf courses damage grass, create hazards if they become aggressive (Conover and Chasko, 1985; Forbes, 1993), and deposit large amounts of fecal matter (Conover and Chasko, 1985; Fairaizl, 1992). In addition, geese are cause for concern at and around airports where bird-aircraft strikes occur, causing serious aircraft damage and potential loss of human life (Fairaizl, 1992). Hunting is not feasible in urban areas to control Canada goose populations (Conover and Chasko, 1985; Heusmann, 1999). There are few places that allow goose translocation, and annual roundups have met with public resistance in some areas. Contraception may provide an acceptable alternative to manage bird populations at levels that allow for the existence of geese and keep damage to socially acceptable levels (Stout et al., 1997).

Nicarbazin (**NCZ**) is an anticoccidial drug routinely used in the poultry industry at 125 ppm in feed to prevent coccidiosis in broiler chickens. It is an equimolar complex consisting of 4,4′-dinitrocarbinilide (**DNC**) and 2-hydroxy-4,6-dimethylpyrimidine (**HDP**). The function of HDP is to increase absorption of the material in the gut, whereas DNC is the active anticoccidial drug (Cuckler et al., 1955; Rogers et al., 1983). When fed to laying hens, NCZ impacts reproduction by either reducing hatchability of eggs or reducing rate of egg laying (Jones et al., 1990a; Hughes et al., 1991; Chapman, 1994). Nicarbazin is thought to affect integrity of the vitelline membrane (Britton and Hale, 1975; Cunningham, 1976; Chapman, 1994), allowing yolk and albumen to mix together (Cunningham, 1977), and by causing egg yolk mottling (Polin et al., 1957; Chapman, 1994). It can also affect pigmenta-

Received January 10, 2006.
Accepted February 25, 2006.
[1]Corresponding author: christi.yoder@aphis.usda.gov

tion of the eggshell (McLoughlin et al., 1957; Jones et al., 1990b; Hughes et al., 1991).

Although the target species for NCZ contraception is the Canada goose, the domestic mallard (*Anas platyrhynchos*) was used as a model species because mallards will produce more eggs than Canada geese if the eggs are removed to prevent the bird from incubating them. In addition, mallards are more suited to reproducing in a laboratory setting than Canada geese, and their reproductive cycle can be easily manipulated by controlling the light cycle.

Our objectives were to assess the potential of NCZ as a waterfowl contraceptive by feeding specific levels of NCZ to ducks and measuring DNC levels in plasma, whole egg, and feces. In addition, the number of eggs produced, egg fertility, and hatchability were also determined. Further objectives were to assess the effect of NCZ on egg quality by measuring egg weight, shell thickness, and degree of yolk mottling and by evaluating the vitelline membrane by scanning electron microscope (**SEM**) in order to determine a mechanism of action. Because adverse effects of NCZ have been reported in heat-stressed chickens (McDougald and McQuistion, 1980), we also assessed the effect of NCZ on bird health by measuring bird weight, hematocrit, counts of white blood cell types, and determining the heterophil:lymphocyte ratio (**H:L**). Fecal DNC levels as an indirect measure of plasma and egg DNC levels were evaluated to determine whether fecal DNC can accurately predict blood and egg DNC levels. Fecal fluorescein was also assessed as a field marker for bait intake and as an indirect measure of plasma and egg DNC levels.

MATERIALS AND METHODS

The experimental protocol was reviewed by the Colorado State University and National Wildlife Research Center's Animal Care and Use Committees and complied with the Animal Welfare Act. The experiment consisted of 4 treatment groups, each consisting of 16 breeding pairs of ducks (Whistling Wings Inc., Hanover, IL), treated with 1) 0 ppm of NCZ (Phibro Animal Health, Inc. Fairfield, NJ), 2) 125 ppm of NCZ (8.5 mg/kg of BW), 3) 250 ppm of NCZ (17.0 mg/kg of BW), and 4) 500 ppm of NCZ (33.75 mg/kg of BW), and only females were treated in this study. Ducks were randomly assigned to pairs and treatment groups, and pairs were randomly assigned to cages. All birds were 30 wk of age at the beginning of the study, and a 16L:8D light cycle was maintained throughout. Ducks were maintained on a game bird layer diet (Purina Mills Inc., St. Louis, MO) that included 3.25 to 4.25% calcium, 0.5% phosphorus, and 16% CP.

The NCZ dose levels were chosen based on previous studies of NCZ absorption in mallards at the National Wildlife Research Center that indicated doses greater than 8.4 mg/kg of BW would be necessary to achieve contraceptive effects (Yoder et al., 2005). Doses were formulated based on a 1.0 kg of average bird weight and were made by filling number 3 gelatin capsules (Torpac Inc., Fairfield, NJ) with 25% NCZ on wheat middlings and 0.05 mg of fluorescein per 15 mg of 25% NCZ. Capsules in the 125-ppm group contained 8.5 mg of pure NCZ and 0.11 mg of fluorescein per capsule. Capsules in the 250-ppm group contained 17.0 mg of pure NCZ and 0.23 mg of fluorescein per capsule. Capsules in the 500-ppm group contained 33.75 mg of pure NCZ and 0.45 mg of fluorescein per capsule. Females were given 1 gelatin capsule containing the appropriate amount of NCZ perorally once a day for 14 d. Control females were given empty number 3 gelatin capsules perorally.

Seven females from each group were randomly selected prior to onset of treatment for blood sampling approximately 3 h after receiving the daily NCZ dose. The same 7 females from each group were used for blood sampling throughout the study. A total of 3 mL of blood was obtained from the brachial vein once pretreatment, every 3 d during treatment, each of the first 4 d posttreatment, then once every 3 d until 14 d posttreatment. Two microhematocrit tubes per blood sample were filled and analyzed for hematocrit (Dein, 1986). Blood smears were made for analysis of differential counts of white blood cell types. Slides were prepared in Wright's buffer (VWR International, Aurora, CO), and differential counts were made using an oil immersion field. Each slide was counted twice, and the results were averaged. The remainder of the blood was centrifuged and plasma stored at –70°C until analysis of DNC concentration using HPLC (Primus et al., 2001).

Egg production and egg weight were monitored daily. For birds included in plasma DNC analysis, eggs laid the day of blood collection were also analyzed for DNC levels. Eggs laid the day prior to bleeding from these same birds were used for SEM analysis. Eggs laid the day after bleeding from these same birds were incubated and hatchability recorded. On the day of blood collection, eggs from birds not included in blood collection were incubated and hatchability recorded. All other eggs from birds not included in blood collections were discarded. Eggs that did not hatch were opened to determine fertility (Prince et al., 1968). Shell thickness was measured at 5 different locations using calipers and the results averaged. Eggs for SEM and DNC analysis were broken open and degree of mottling assessed. Mottling was assessed using the following scale: 0 = no mottling, 1 = mild mottling, 2 = moderate mottling, and 3 = severe mottling. Eggs were prepared for SEM analysis by removing a portion (approximately 1 cm^2) of vitelline membrane and washing the membrane with successive washes of saline until yolk no longer adhered to the membrane. The membrane was then fixed in 3% glutaraldehyde (G7651, Sigma Chemical Co., St. Louis, MO) and dried using a successive series of ethanol washes (25% to 100% ethanol). Finally, the membrane was immersed in hexamethyldisilazane (HMDS, 18605, Ted Pella Inc., Redding, CA) for 5 to 15 min and allowed to dry at room temperature. The remainder of the egg was homogenized in a blender and analyzed for DNC content by HPLC (Johnston et al., 2002).

Fecal samples (approximately 5 g) were obtained from each bird used for blood collections at the time of blood sampling and stored at –70°C for analysis of DNC content by HPLC (Stahl and Johnston, 2002). Fluorescein analysis was performed by homogenizing 1 g of fecal material in 2 mL of acetonitrile and analyzing the fluorescence on a Turner model 450 fluorometer. The excitation wavelength was set at 490 nm, and the emission wavelength was set at 535 nm. All birds were weighed once pretreatment and twice during treatment, on 11 and 14 d treatment.

Statistical Analyses

Dates were divided into 5 time periods among groups for all analyses except bird weight and fecal fluorescence as follows: 1) pretreatment (1 to 14 d pretreatment), 2) treatment 1 (1 to 7 d treatment), 3) treatment 2 (8 to 14 d treatment), 4) posttreatment 1 (1 to 3 d posttreatment), and 5) posttreatment 2 (4 to 14 d posttreatment). The mean proportion of fertile eggs was calculated by combining the number of eggs that hatched and the number of eggs that were fertile (defined either by the presence of an embryo or blastodisc) post expected hatching date, and dividing this number by total number of eggs set. The mean proportion of eggs that hatched was calculated by dividing the number of eggs that hatched by number of eggs set. Bird weights were grouped into either a pretreatment or treatment period. Fecal fluorescence was analyzed by treatment date. All data were analyzed as a mixed effects model (PROC MIXED, SAS Institute Inc., Cary, NC), and significance was defined as $P \leq 0.05$ for all analyses. Data were analyzed for treatment, time period, and treatment × time period effects. Means separations were carried out using PDMIX800 (Saxton, 1998).

Correlations were determined between fecal, plasma, and egg DNC levels, and between fecal fluorescence and plasma, egg, or fecal DNC levels. Correlations were determined between plasma or egg DNC levels and numbers of eggs laid, fertility, hatchability, egg weight, shell thickness, mottling score, or hematocrit.

RESULTS

Plasma and egg DNC levels differed among treatment groups and time periods (Table 1), and a significant treatment by period interaction existed. Peak plasma DNC levels were 1.36 ± 0.21, 1.74 ± 0.20, and 2.97 ± 0.18 μg/mL in the 125-, 250-, and 500-ppm groups, respectively. Peak egg DNC levels were 2.95 ± 0.44, 5.25 ± 0.68, and 7.22 ± 0.51 μg/g in the 125-, 250-, and 500-ppm groups, respectively. Fecal DNC levels differed among time periods but not among groups ($P = 0.2195$).

The proportion of fertile eggs and the proportion of eggs that hatched did not differ among treatment groups ($P = 0.5520$ and 0.1918, respectively) or time periods ($P = 0.2484$ and 0.0742, respectively). However, hatchability was 0.55 ± 0.10 in the control group compared to 0.26 ± 0.10 in the 500-ppm group. There was a significant period effect for the number of eggs laid per female per day (Table 2) but no treatment effect ($P = 0.6579$). Eggshell thickness did not differ among treatment groups ($P = 0.7184$), but there was a significant period effect, with eggs in post-treatment time periods exhibiting thinner shells. Egg yolk mottling scores did not differ among treatment groups ($P = 0.7729$), but there was a significant period effect, with eggs having higher mottling scores during treatment and posttreatment period 1. Egg weights differed among time periods with eggs weighing more during the posttreatment time periods, but did not differ among treatment groups ($P = 0.8889$). Period effects for eggs laid per female per day, shell thickness, yolk mottling, and egg weights were significant whether or not controls were included in the analysis, and the trends remained the same.

There was a significant period effect for bird weights and hematocrit (Table 2), but there was no significant difference among treatment groups ($P = 0.2308$ and 0.9100, respectively). Bird weights were lower during the treatment period. Hematocrit tended to increase over time, with the highest levels occurring during the posttreatment periods. Period effects for bird weight and hematocrit were significant whether or not controls were included in the analysis, and the trends remained the same.

There were significant period effects for eosinophil, basophil, heterophil, and monocyte counts, and H:L ratios (Table 3), but means did not differ among treatment groups ($P = 0.1075$, 0.3554, 0.9755, 0.7484, and 0.8633, respectively). Eosinophil and monocyte counts were lowest during both treatment periods, whereas basophil counts were lowest only during treatment period 1. Heterophil counts and the H:L ratio were lowest during pretreatment. Period effects were significant whether the control group was included in the analysis or not. Mean lymphocyte counts did not differ among treatment groups ($P = 0.9675$) or time periods ($P = 0.2085$).

Plasma DNC levels were positively correlated with egg DNC levels during treatment periods 1 and 2 and posttreatment periods 1 and 2, and with fecal DNC levels during treatment period 2 (Table 4). Egg DNC levels were positively correlated with fecal DNC levels during treatment period 1.

Fluorescence was positively correlated with plasma, egg, and fecal DNC levels overall (Table 5). Plasma DNC was positively correlated with fluorescence during treatment periods 1 and 2 and posttreatment period 1. Egg DNC was positively correlated with fluorescence during treatment period 1. Fecal DNC was positively correlated with fluorescence during treatment periods 1 and 2.

Shell thickness was positively correlated with plasma DNC at 125 and 500 ppm (Table 6). Egg weight was negatively correlated with plasma DNC at 250 and 500 ppm. Egg yolk mottling was positively correlated with egg DNC at 500 ppm. Fertility and hatchability were negatively correlated with egg DNC at 250 ppm. Bird weight was negatively correlated with egg DNC at 250 ppm.

Table 1. Plasma, egg, and fecal 4,4′-dinitrocarbanilide (DNC) levels for female mallards given 125 ppm (8.5 mg/kg of BW), 250 ppm (17.0 mg/kg of BW), or 500 ppm (33.75 mg/kg of BW) of nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000

		Plasma DNC (μg/mL)			Egg DNC (μg/g)			Fecal DNC (μg/g)		
Group	Period[1]	n	Mean	SE	n	Mean	SE	n	Mean	SE
0 ppm	PRE	7	0.00[a]	0.28	3	0.00[a,b]	0.68	7	0.30[a,b]	14.17
	TRT1	21	0.00[a]	0.18	7	0.01[a]	0.46	21	0.03[a]	8.18
	TRT2	14	0.00[a]	0.21	4	0.07[a]	0.56	14	0.03[a,b]	10.02
	POST1	21	0.00[a]	0.18	5	0.00[a]	0.55	20	0.00[a,b]	8.38
	POST2	28	0.00[a]	0.16	8	0.01[a]	0.44	14	0.00[a]	10.02
125 ppm	PRE	7	0.02[a]	0.27	6	0.00[a]	0.46	7	0.00[a,b]	13.26
	TRT1	21	1.31[b]	0.18	12	1.52[b,c]	0.37	21	24.70[b]	8.18
	TRT2	14	1.36[b]	0.21	8	2.95[d,e]	0.44	14	1.69[a,b]	10.02
	POST1	21	0.11[a]	0.18	7	2.34[c,d]	0.47	18	0.08[a]	8.84
	POST2	29	0.00[a]	0.16	10	0.35[a]	0.41	14	0.00[a,b]	10.02
250 ppm	PRE	7	0.00[a]	0.28	3	0.03[a,b]	0.68	7	0.00[a,b]	14.17
	TRT1	23	1.42[b]	0.17	8	0.85[a,b]	0.44	23	19.19[a,b]	7.82
	TRT2	16	1.74[b]	0.20	3	5.25[g]	0.68	16	3.79[a,b]	9.37
	POST1	24	0.18[a]	0.17	9	3.81[e,f,g]	0.41	22	6.52[a,b]	7.99
	POST2	31	0.00[a]	0.15	10	0.52[a,b]	0.38	16	0.00[a]	9.37
500 ppm	PRE	7	0.00[a]	0.28	2	0.05[a,b]	0.83	7	0.00[a,b]	14.17
	TRT1	21	2.97[c]	0.18	5	3.45[d,e,f]	0.55	21	53.28[c]	8.18
	TRT2	14	2.34[d]	0.21	6	7.22[h]	0.51	13	15.61[a,b]	10.40
	POST1	22	0.22[a]	0.18	8	4.63[f,g]	0.44	18	0.19[a,b]	8.60
	POST2	28	0.01[a]	0.16	9	0.18[a]	0.40	14	0.20[a]	10.02

[a–h]Means within columns within treatment groups with different superscripts are significantly different ($P < 0.05$). Means are from the LSMEANS option in PROC MIXED (SAS 9.1, Cary, NC).

[1]PRE = pretreatment d 1 to 14; TRT1 = treatment d 1 to 7; TRT2 = treatment d 8 to 14; POST1 = posttreatment d 1 to 3; POST2 = posttreatment d 4 to 14.

The outer and inner vitelline membranes (Figure 1) exhibited degenerative changes in a dose-related manner. Minor degenerative changes were observed in the outer vitelline membrane in the 125-ppm group, but the inner vitelline membrane did not have any discernible changes at 13 d treatment. Easily detectable changes in the outer vitelline membrane occurred in the 250-ppm group, and the inner membrane exhibited only minor changes by 1 d posttreatment. Dramatic changes were observed in the outer vitelline membrane in the 500-ppm group by 9 d treatment, such that it was difficult to ascertain which side of the membrane was being observed in some eggs (Figure 1). The inner vitelline membrane of the 500-ppm group exhibited only minor changes by 9 d treatment. Severe degradation of the outer vitelline membrane continued to occur through the end of the treatment period, with only very minor changes occurring in the inner vitelline membrane in the 500-ppm group. Although still easily noticeable, degradation in the outer vitelline membrane in the 500-ppm group became less severe during the first 3 d posttreatment.

DISCUSSION

When ducks were fed increasing amounts of NCZ, DNC levels in blood plasma, eggs, and feces exhibited a dose response relationship (Figure 2). Although there was little difference between 125 (8.5 mg/kg of BW) and 250

Table 2. Period effects across treatment groups for eggs laid/female per day, shell thickness, egg yolk mottling, egg weight, bird weight, and hematocrit in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000

	PRE[1]			TRT1			TRT2			POST1			POST2		
Item	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
Eggs laid/female/day	882	0.33[a]	0.03	441	0.37[a]	0.03	441	0.27[b]	0.03	189	0.33[a,b]	0.04	693	0.28[b]	0.03
Shell thickness (mm)	15	0.32[a]	0.01	33	0.31[a]	0.01	30	0.28[b]	0.01	29	0.22[c]	0.01	57	0.23[c]	0.01
Yolk mottling score[2]	15	0.00[a]	0.18	33	0.11[a]	0.12	28	0.51[b,c]	0.12	29	0.72[c]	0.12	57	0.40[b]	0.08
Egg weight (g)	293	43.03[a]	3.45	114	43.42[a,b]	3.49	117	43.90[a,b]	3.47	65	44.63[a,b]	3.52	197	44.93[b]	3.46
Bird weight[3] (g)	63	1005.9[a]	15.9				126	959.8[b]	11.2						
Hematocrit (%)	14	36.8[a]	1.2	66	38.9[a,b]	0.7	58	40.0[b]	0.7	86	39.7[b]	0.7	116	39.9[b]	0.6

[a–c]Means within rows with different superscripts significantly different ($P < 0.05$). Means are from the LSMEANS option in PROC MIXED (SAS 9.1, Cary, NC).

[1]PRE = pretreatment d 1 to 14; TRT1 = treatment d 1 to 7; TRT2 = treatment d 8 to 14; POST1 = posttreatment d 1 to 3; POST2 = posttreatment d 4 to 14.

[2]Yolk mottling scores were assigned using the following scale: 0 = no mottling, 1 = mild mottling, 2 = moderate mottling, and 3 = severe mottling.

[3]Birds were only weighed once during pretreatment, and on 11 and 14 d treatment.

Table 3. Period effects across treatment groups for differential counts of white blood cell types and heterophil:lymphocyte ratio (H:L) in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000[1]

	PRE			TRT1			TRT2			POST1			POST2		
Item	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
Eosinophils (%)	26	2.2[a,b]	0.3	89	1.8[b,c]	0.2	57	1.4[c]	0.2	87	2.4[a]	0.2	116	2.5[a]	0.2
Basophils (%)	26	2.6[a,b,c]	0.4	89	2.0[b]	0.3	57	2.6[c]	0.3	87	2.7[c]	0.3	116	3.1[a]	0.2
Heterophils (%)	26	57.1[a]	2.8	89	63.8[b]	1.8	57	64.5[b]	2.1	87	61.5[a,b]	1.8	116	59.5[a]	1.7
Monocytes (%)	26	2.3[a]	0.3	89	1.9[a]	0.2	57	0.8[b]	0.2	87	1.0[b]	0.2	116	2.1[a]	0.1
Lymphocytes[2] (%)	26	35.9[a]	2.6	89	30.5[b]	1.7	57	30.6[a,b]	1.9	87	32.4[a,b]	1.7	116	32.8[a,b]	1.6
H:L[3]	26	1.8[b]	0.3	89	2.6[a]	0.2	57	2.5[a]	0.2	87	2.3[a,b]	0.2	116	2.3[a]	0.2

[a–c]Means within rows with different superscripts are significantly different ($P < 0.05$).

[1]PRE = pretreatment d 1 to 14; TRT1 = treatment d 1 to 7; TRT2 = treatment d 8 to 14; POST1 = posttreatment d 1 to 3; POST2 = posttreatment d 4 to 14.

[2]No significant period effect on lymphocytes at $P < 0.05$.

[3]Heterophhil:lymphocyte ratio.

ppm (17.0 mg/kg of BW) in resulting plasma DNC levels, DNC levels for birds given 500 ppm NCZ (33.75 mg/kg of BW) were significantly higher. The peak DNC level at 500 ppm was 2.97 μg/mL in blood plasma during treatment period 1. Previous studies reported a minimum plasma level of 2.9 μg/mL is needed to observe reproductive effects in chickens (Jones et al., 1990b; Yoder et al., 2005). Results from this study show that a minimum level of 500 ppm (33.75 mg/kg of BW) is needed to affect reproduction in mallards, likely due to differences between species in absorption in the gut and metabolism of NCZ.

Peak plasma DNC levels were obtained in the first week of treatment, and peak egg DNC levels were not observed until the second week of treatment. Plasma DNC was nearly undetectable by 3 d posttreatment, whereas egg DNC levels did not fall below 1 μg/g until 14 d posttreatment. It appears that in order to affect egg hatchability, NCZ must be fed to waterfowl a minimum of 2 wk prior to the start of egg laying.

No significant treatment effect of NCZ on hatchability was found in this study, but this is probably due to the small sample size rather than a lack of NCZ effect. Power to detect differences ranged from 20 to 32% in this study, and sample sizes would need to be 4 to 6 times larger to achieve 90% power. The proportion of eggs that hatched overall in the control group was 0.55 ± 0.10 compared to 0.26 ± 0.10 in the 500-ppm treatment group. This is a 53% reduction in proportion of eggs hatching, which could be biologically significant depending on species of bird. There was no discernable difference in proportion of eggs hatching in the 125 ppm (0.49 ± 0.09) or 250 ppm (0.47 ± 0.11) groups compared with the control group. However, hatchability did exhibit a dose related decrease. The adverse effect of NCZ on hatchability of chicken eggs has been well documented. Levels as low as 20 ppm (20 mg/kg of feed) adversely affect reproduction (Jones et al., 1990a). Feeding 25 ppm (25 mg/kg of feed) for as few as 4 d reduced hatchability by 54% (Hughes et al., 1991). The results of this study indicate that the lowest dose that should be considered for further experimentation with waterfowl is 500 ppm (33.75 mg/kg of BW).

On average, fertility decreased by 13% overall in all treated groups as compared to the control group in this study. This is consistent with findings from previous studies showing a 10-50% decrease in fertility of eggs laid by hens treated with 6 to 700 ppm NCZ in feed (6 mg/kg of feed to 700 mg/kg of feed; Sherwood et al., 1956; Lucas, 1958). However, findings of adverse effects of NCZ on fertility in chickens are widely variable, and may depend on strain of chicken. Other studies found no effect of NCZ on fertility of eggs laid by hens treated with 100

Table 4. Correlations between plasma, egg, and fecal 4,4′-dinitrocarbanilide (DNC) levels in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000

		Plasma DNC		Egg DNC		Fecal DNC	
Item		n	r	n	r	n	r
Plasma DNC	TRT1[1]			31	0.68968*	85	0.14727
	TRT2			13	0.80085*	57	0.42740*
	POST1			28	0.42832*	79	0.00830
	POST2			26	0.69378*		
Egg DNC	TRT1	31	0.68968*			32	0.55967*
	TRT2	13	0.80085*			13	0.36485
	POST1	28	0.42832*			27	0.29918
	POST2	26	0.69378*				

[1]TRT1 = treatment d 1 to 7, TRT2 = treatment d 8 to 14, POST1 = posttreatment d 1 to 3, POST2 = posttreatment d 4 to 14.

*Significant correlation at $P < 0.05$.

Table 5. Correlations between fecal fluorescein fluorescence and plasma, egg, and fecal 4,4′-dintrocarbanilide (DNC) levels in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000

		Plasma DNC		Egg DNC		Fecal DNC	
		n	r	n	r	n	r
Fluorescence	TRT1[1]	27	0.50856*	10	0.82654*	27	0.55498*
	TRT2	33	0.65281*	8	0.58515	33	0.65570*
	POST1	38	0.47184*	13	–0.07880	38	–0.04169
	Overall	98	0.60843*	31	0.36513*	98	0.53327*

[1]TRT1 = treatment d 1 to 7; TRT2 = treatment d 8 to 14; POST1 = posttreatment d 1 to 3.

*Significant correlation at $P < 0.05$.

ppm of NCZ in feed (100 mg/kg of feed; Jones et al., 1990a; Hughes et al., 1991).

Although no statistically significant effect of NCZ on egg production was found, treatment groups had a greater percent decrease in egg production than did the control group. Egg production in the control group decreased by 13% from treatment period 1 to 2, whereas egg production decreased by 28, 43, and 26% in the 125-, 250-, and 500-ppm groups, respectively. Previous studies showed decreased egg production of 30 to 75% in layer hens fed 100 ppm (100 mg/kg of feed) NCZ in feed (Jones et al., 1990a,b; Hughes et al., 1991). A higher NCZ dose likely is needed to observe a significant effect on egg production in mallards.

No effect of NCZ on eggshell thickness was found in this study, in contrast to results of studies with chickens. However, the effect of NCZ on eggshell thickness in layer hens is variable and may depend on strain and age of chicken. Jones et al. (1990b) found 125 ppm of NCZ in feed (125 mg/kg of feed) decreased shell thickness in eggs laid by White Leghorn hens, whereas there was no effect on shell thickness of eggs laid by New Hampshire hens treated at the same level (McLoughlin et al., 1957). Increasing the NCZ content of feed to 400 ppm (400 mg/kg of feed) had no effect on shell thickness of eggs laid by New Hampshire chickens (Ott et al., 1956). It is possible that mallards did not receive a high enough NCZ dose to affect shell thickness or that their eggs are not susceptible to NCZ-related thinning.

Egg yolk mottling due to NCZ is a well-documented effect. Severity of mottling increases with increasing levels of NCZ in the feed (Polin et al., 1957; Jones et al., 1990b). Additionally, storage time exacerbates the mottling effect (Baker et al., 1957; Polin et al., 1958; Silvestrini et al., 1965). Because eggs in this study were opened within 1 to 2 d of laying, it is possible that the mottling may not have been severe enough to be seen at that stage. Polin (1957) reported that eggs from hens fed 125 ppm

Table 6. Correlations of reproductive parameters, egg quality parameters, and bird weight with plasma and egg 4,4′-dinitrocarbanilide (DNC) levels in female mallards given 125 ppm (8.5 mg/kg), 250 ppm (17.0 mg/kg), or 500 ppm (33.75 mg/kg) of nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000

		Plasma DNC		Egg DNC	
Group	Parameter	n	r	n	r
125 ppm	Egg shell thickness	41	0.41462*	44	0.07802
	Egg weight	41	–0.07271	44	–0.01706
	Mottling score[1]	41	–0.23310	43	0.13090
	Fertility	12	0.17481	11	0.24359
	Hatchability	12	0.39130	11	0.03818
	Bird weight	14	–0.40402	10	–0.33278
250 ppm	Egg shell thickness	27	0.34840	34	–0.25511
	Egg weight	28	–0.54232*	34	–0.13052
	Mottling score	27	0.34351	34	0.18166
	Fertility	11	–0.36510	8	–0.71261*
	Hatchability	11	–0.36510	8	–0.71261*
	Bird weight	15	–0.49440	6	–0.80802*
500 ppm	Egg shell thickness	23	0.70012*	31	0.18108
	Egg weight	23	–0.51691*	31	–0.18685
	Mottling score	23	–0.13185	31	0.40780*
	Fertility[2]	5	—	9	0.29445
	Hatchability	9	–0.18274	9	–0.42557
	Bird weight	14	–0.28385	5	–0.36042

[1]Yolk mottling scores were assigned using the following scale: 0 = no mottling, 1 = mild mottling, 2 = moderate mottling, and 3 = severe mottling.

[2]No correlation coefficients or P values for fertility and plasma DNC in the SAS (Cary, NC) output due to having no SEM for fertility.

*Significant correlation at $P < 0.05$.

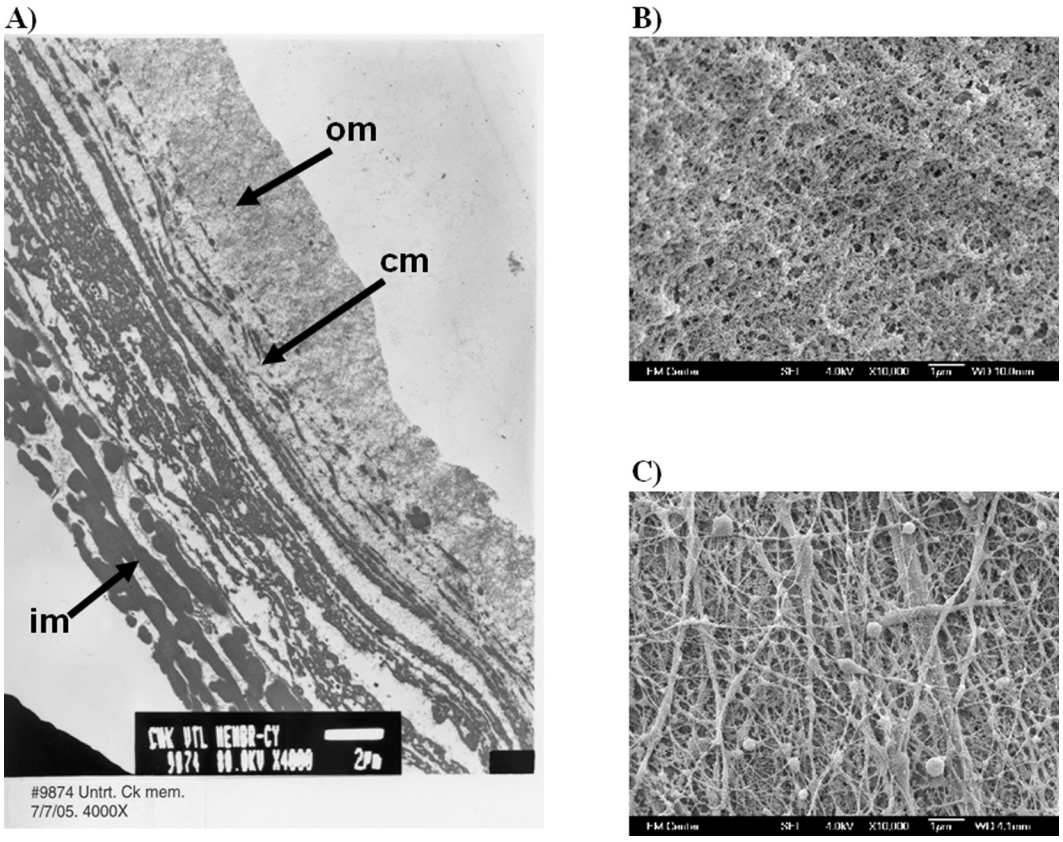

Figure 1. A) Transmission electron micrograph of a fresh chicken vitelline membrane (magnification 4000×; im = inner membrane; om = outer membrane; cm = continuous membrane); B) Scanning electron micrographs (SEM) of the inner vitelline membrane from eggs laid on treatment d 9 by mallards treated with 500 ppm (33.75 mg/kg of BW) of nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Colins, CO, May to June, 2000; C) SEM of the inner vitelline membranes from eggs laid on treatment d 9 by control mallards at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000.

of NCZ in feed (125 mg/kg of feed) had almost no mottling soon after laying, but that mottling increased after 5 d of storage in a cold room. However, at 500 ppm in this study, there was a significant positive correlation between yolk mottling scores and egg DNC levels. It is also likely that a higher level of NCZ would be needed to observe differences in mottling effects among treatment groups.

Authors of earlier studies speculated that mottling of the egg yolk was due to a change in the permeability of the vitelline membrane, which allowed yolk and albumen to mix (Polin, 1957; van Tienhoven et al., 1958; Cunningham, 1977). No electron microscopy of vitelline membranes was performed in any of these studies, so authors could only speculate as to the cause of the yolk mottling. The inner layer of the vitelline membrane is formed in the ovary (Bellairs et al., 1963; Wyburn et al., 1965), and the thin continuous and outer vitelline membrane layers are laid down in the infundibulum (Bellairs et al., 1963; Bain and Hall, 1969). The degenerative changes observed in this study in the vitelline membrane were primarily in the outer membrane. This suggests that there may be a factor in albumen due to treatment with NCZ that influences changes in the outer vitelline membrane.

An examination of follicles from ovaries of NCZ-treated chickens revealed no yolk damage (Baker et al., 1957; Polin, 1957; Mitchell and Stadelman, 1958; van Tienhoven et al., 1958), indicating that yolk damage due to NCZ occurs after the inner vitelline membrane is laid down. Some eggs within the oviduct showed yolk damage (Baker et al., 1957; Mitchell and Stadelman, 1958; van Tienhoven, 1958), although yolk damage became more apparent after a period of storage (Polin, 1957; van Tienhoven, 1958). This indicates that damage occurs sometime after deposition of the outer vitelline membrane, and at lower levels of NCZ, the vitelline membrane may require a period of exposure to albumen before damage is evident. A prior study on deposition of NCZ metabolites in the egg showed DNC is deposited primarily in yolk, and HDP is deposited in both yolk and albumen in a ratio of 3:1 (Cannavan et al., 2000). Thus, it seems likely that either HDP or DNC metabolites may be causing some of the changes associated with vitelline membrane damage.

The appearance of vitelline membranes in this study was similar in appearance to membranes from aged eggs (Kirunda and McKee, 2000). Britton (1973) suggested that NCZ accelerates the natural aging process, leading to an increased incidence of yolk mottling. Aging of eggs causes a decrease in vitelline membrane strength (Kirunda and McKee, 2000), partly due to a loss of 2 outer vitelline membrane proteins, VMO1 and VMO2 (Back, 1984; Schäfer et al., 1998), and degradation of one inner vitelline

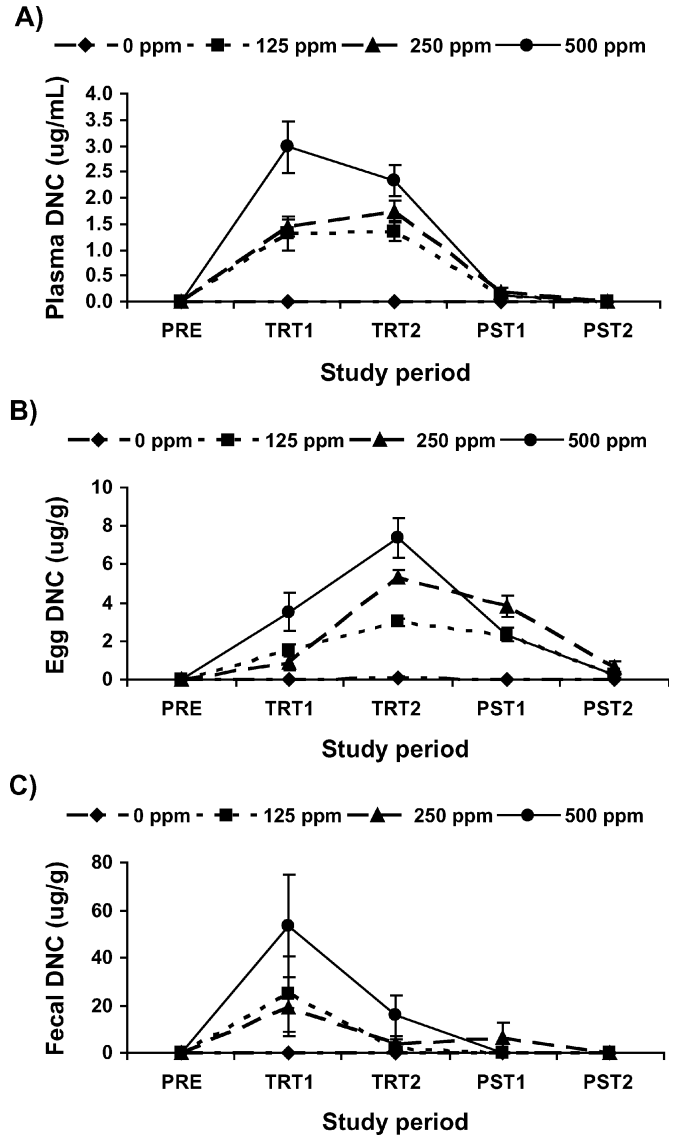

Figure 2. Levels of 4,4′-dinitrocarbanilide (DNC) in A) plasma, B) egg, and C) feces of female mallards given 125 ppm (8.5 mg/kg), 250 ppm (17.0 mg/kg), or 500 ppm (33.75 mg/kg) of nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000. PRE = 1 to 14 d pretreatment; TRT1 = 1 to 7 d treatment; TRT2 = 8 to 14 d treatment; PST1 = 1 to 3 d posttreatment; and PST2 = 4 to 14 d posttreatment.

membrane glycoprotein, GPII (Kido et al., 1975). Degradation of the vitelline membrane observed in this study may be due to the loss of the outer vitelline membrane proteins, VMO1 and VMO2. Electrophoretic studies need to be conducted to confirm this.

Period effects were noted in egg production, shell thickness, and egg mottling across treatment groups. This might have been due to the ducks entering a partial molt. The ducks had been laying eggs for 3 mo prior to the start of this experiment. Egg production consistently declined in all treatment groups from treatment period 1 to 2. Egg production increased in posttreatment period 1 in all groups, and continued to increase during posttreatment period 2 in the 500-ppm group. Egg production decreased during posttreatment period 2 to treatment period 2 levels in the 125-ppm group, decreased to the lowest level in the 250-ppm group, and decreased to pretreatment levels in the control group. By posttreatment period 2, egg production was higher than it had been during treatment in all groups but was still lower than pretreatment levels. This is consistent with what occurs in birds at the end of the laying year (Potts and Washburn, 1983; Grossman et al., 2000; Rodriguez-Navarro et al., 2002). Eggshell thickness also decreased in all treatment groups, reaching the lowest thickness during posttreatment period 1. Yolk mottling scores increased until posttreatment period 1 and then decreased. This may also have been due to reproductive senescence at the end of the laying year (Souza et al., 1994).

Period effects were also noted in bird weights. All treatment groups consistently lost weight throughout the treatment period. This may have been due to the stress of handling for dosing, blood sampling, and weighing. Control birds lost 2.6% of their pretreatment body weight, whereas treated birds lost 4.3 to 6.6% of their pretreatment body weight. The slightly higher weight loss in the treated groups may be due to NCZ itself. Nicarbazin reduces feed efficiency and weight gain in chickens (Bartov, 1989a; Sorribas et al., 1993). Bartov (1989b) found that NCZ significantly decreased weight gain and feed efficiency at 100 to 200 mg/kg of feed.

It is evident from the elevated H:L ratios during treatment and post-treatment periods that mallards were experiencing stress. Elevated H:L ratios are known to be associated with increased levels of stress in birds (Gross and Siegel, 1983; Vijayan and Rema, 1997; Kontecka et al., 1999). Because both the control and treated groups had elevated H:L ratios that were not significantly different, the stress was most likely due to repeated handling.

Fecal DNC levels were only significantly correlated with plasma DNC levels during treatment period 2, and therefore do not provide a good estimate of plasma DNC levels. To be a useful estimator, fecal DNC should also have been significantly correlated with plasma DNC during treatment period 1 and posttreatment period 1. During posttreatment period 2, DNC was undetectable in plasma and feces as DNC was ≥97% cleared from plasma by 3 d posttreatment. One reason why fecal DNC might not have been correlated with plasma DNC during these periods is that mallards were given a bolus dose rather than eating treated feed throughout the day. This likely results in more DNC being eliminated in feces quickly rather than an even elimination throughout the day. Because a single fecal sample was obtained at the time of blood sampling, it is possible that the sample we obtained contained lower amounts of DNC than an earlier or later fecal sample might have contained. A study should be conducted to test whether fecal DNC correlates better with plasma DNC when NCZ is fed ad libitum. This would provide a non-invasive way to assess plasma DNC levels in a field situation without having to capture the bird.

Egg DNC levels correlated well with plasma DNC levels, particularly by treatment period 2. This means un-

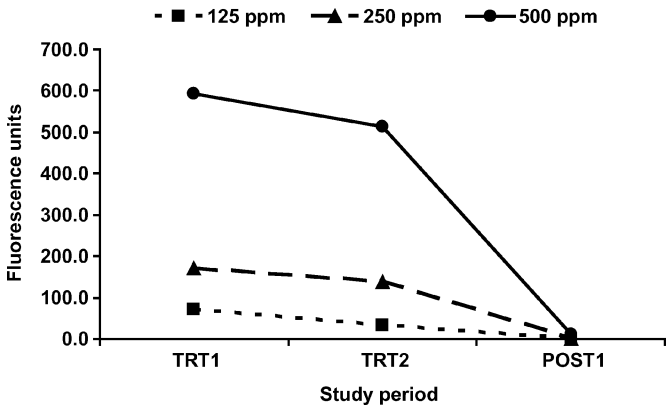

Figure 3. Fecal fluorescein levels of female mallards given 0.11 mg (125 ppm group), 0.23 mg (250 ppm group), or 0.45 mg (500 ppm group) of fluorescein perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, CO, May to June, 2000. TRT1 = 1 to 7 d treatment; TRT2 = 8 to 14 d treatment; and POST1 = 1 to 3 d post-treatment.

hatched eggs could be collected in the field and analyzed for DNC content to give a measure of NCZ in blood. However, this would only provide supporting information after treatment had taken place and would not allow a change in dosing protocol during the treatment period if DNC levels were too low to expect a contraceptive effect.

Fecal fluorescein levels were dose-related (Figure 3) and provided a reasonably good estimate of plasma DNC levels but not egg DNC levels. Because egg DNC levels lag behind plasma DNC levels, it is not expected that fluorescein would provide a good estimate of egg DNC levels. This technique would allow for collection of feces around nest sites for analysis of fluorescein and would give a general idea of how much NCZ each pair of geese ingested. It would not be possible to distinguish between male and female fecal material without genetic analysis. If feces were collected around bait stations, a change in dosing protocol during the treatment period could be made if fluorescein levels indicated low DNC levels. A handheld fluorometer could be used in the field for quick analysis.

As a contraceptive, NCZ may be ideal where waterfowl can be fed on a daily basis during egg laying. It is quickly cleared from the system and does not appear to produce any ill health effects at the levels investigated. Further laboratory studies with mallards should be done using 500 ppm (33.75 mg/kg of BW) as the starting dose and feeding treated feed rather than bolus administration of a capsule orally. This would simulate more closely what would be done in the field, and NCZ may be better absorbed if fed throughout the day rather than giving a bolus dose.

ACKNOWLEDGMENTS

We thank Kuffolk for providing funding, Phibro Animal Health for providing nicarbazin for this study, and the National Wildlife Research Center for providing the facilities. A. Tucker of Colorado State University provided the blood gas analyzer. R. Engeman of the National Wildlife Research Center assisted with the statistical analysis. K. Bentler, K. Crane, T. Felix, and V. Lamb of the National Wildlife Research Center provided technical assistance. K. Fagerstone provided comments on an earlier version of the manuscript.

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