

DISSERTATION

NICARBAZIN FOR CONTRACEPTION OF CANADA GEESE: LABORATORY
AND FIELD STUDIES

Submitted by

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Biomedical Sciences

In partial fulfillment of the requirements

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ABSTRACT OF DISSERTATION
NICARBAZIN FOR CONTRACEPTION OF CANADA GEESE: LABORATORY
AND FIELD STUDIES

Increasing numbers of resident Canada geese (*Branta canadensis*) and opposition to lethal control measures have resulted in a greater need to develop nonlethal techniques to manage bird populations. Contraception may provide a useful nonlethal management tool to reduce wild bird populations. Nicarbazin (NCZ) is an anticoccidial drug routinely used in the poultry industry that can negatively impact reproduction by reducing egg production, egg weight, and egg hatchability. We conducted studies to develop NCZ as a contraceptive for Canada geese.

The molecular mechanisms by which nicarbazin affects reproduction are unknown. Lipoprotein lipase, vitellogenin, transglutaminase, and calcium are all involved in egg formation and embryogenesis. Therefore, in vitro assays were used to evaluate four potential mechanisms of action of NCZ on egg formation and embryogenesis. First, an assay was conducted to determine if NCZ increases lipoprotein lipase activity. Second, vitellogenin phosphorylation was evaluated to determine if NCZ acts as a vitellogenin phosphatase. Transglutaminase activity was measured to determine if NCZ inhibits transglutaminase activity. Finally, bull sperm were used as a model to determine if specific channel-mediated calcium uptake can be blocked by NCZ. Nicarbazin increased the activity of lipoprotein lipase in vitro at 3.9 and 7.8 μg nicarbazin/mL. Nicarbazin

increased intracellular calcium levels in bull sperm, suggesting it also acts as a calcium ionophore. The portion of the NCZ molecule responsible for the increase in intracellular calcium is 2-hydroxy-4,6-dimethylpyrimidine (HDP). Nicarbazin affected vitellogenin phosphorylation, but only at a concentration many times higher than expected plasma values. Nicarbazin also inhibited transglutaminase activity in vitro. While the 4,4'-dinitrocarbanilide (DNC) portion of the NCZ molecule inhibited transglutaminase activity, the HDP portion increased transglutaminase activity. All of these assays were conducted in vitro, therefore these results should be viewed as preliminary findings to aid in directing further research on the effect of NCZ on reproduction in vivo. Because nicarbazin increases lipoprotein lipase activity and acts as a calcium ionophore, future experiments should investigate these effects in particular.

We also conducted three laboratory studies using domestic mallards (*Anas platyrhynchos*) as a model for Canada geese to determine effect of NCZ on waterfowl reproduction. In the first study, mallards were given gelatin capsules containing 0, 8.5, 17.0, or 33.75 mg/kg body weight NCZ perorally once daily for 14 d (n = 16/group). Fecal 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/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 to 0.26 ± 0.1 in the 33.75 mg/kg body weight group. Degeneration of the vitelline membrane was evident at

all treatment levels, with severity being 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 post-treatment 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 non-invasive marker to determine the approximate amount of NCZ a bird is consuming.

A second trial tested the effect of the method of NCZ delivery on plasma DNC levels. Mallards were given 34 mg NCZ/kg body weight daily for 12 d either by gavage with a corn oil suspension (n = 7), gavage with a water suspension (n = 7), peroral administration of a capsule (n = 6), or feeding a 500 mg NCZ/kg feed pelleted bait *ad libitum* (n = 7). The method of delivery significantly affected plasma DNC levels, with the highest levels in the corn oil suspension group, and the lowest levels in the pelleted bait group. This is likely due to decreased availability of NCZ in a pellet compared to gavage with a suspension or capsule. Mallards receiving 34.2 mg NCZ/kg BW when fed cracked corn coated with NCZ daily for 14 d had higher plasma DNC levels than those obtained by liquid gavage, capsule, or pelleted NCZ bait.

In the third laboratory study, corn treated with 0 (n = 12 females, 5 males), 750 (n = 12 females), 1000 (n = 13 females, 5 males), or 1500 (n = 13 females) mg NCZ/kg feed

was fed *ad libitum* for 14 d. Plasma and egg DNC levels differed among treatment groups in a dose response relationship, but did not differ between sexes. Nicarbazine caused a decrease in egg weight, but there was no effect of NCZ on the numbers of eggs laid per female per day. Nicarbazine did not significantly impact bird health.

We tested the efficacy of NCZ to reduce hatchability of Canada goose eggs at 2 sites in northern Colorado, a reclaimed gravel pit and a water holding pond at a utility facility. Cracked corn coated with 1,320 mg NCZ/kg corn was offered daily for 32-50 d during 2 consecutive treatment years. Control data was collected from the gravel pit site the year prior to baiting (2000), and only data from that site was used to analyze the effect of NCZ on hatchability. Nicarbazine reduced hatchability by 56.3% (n = 35) and 49.4 % (n = 29) the first (2001) and second (2002) year of treatment, respectively. In 2000, 42.9% of nests at the gravel pit site had hatchability \leq 75% compared to 94.3% and 86.2% in 2001 and 2002, respectively. There were no nests in which none of the eggs hatched at the gravel pit site in 2000 compared to 28.6% and 27.6% of nests in 2001 and 2002, respectively. Egg DNC levels were negatively correlated with hatchability, fertility, clutch sizes, and stage of embryonic development. Eggshell thickness was positively correlated with egg DNC levels. Egg DNC levels tended to be higher the closer the nest was to a bait site. Nests located farther from bait sites were associated with higher hatchability, overall fertility, and more advanced stages of embryonic development. Nests located \leq 70 m from a bait site had \leq 45% hatchability. Eggshells were thinner the farther a nest was from a bait site. The population models show yearly

use of NCZ and culling once every 3 yr is sufficient to maintain the population within 100 geese of the starting population for ≥ 20 yr. Longer periods between culling result in only being able to maintain the population within 100 geese of the starting population for ≤ 10 yr. It is recommended that contraception be used yearly, and culling programs be implemented once every 3 yr to maintain the population at a given size while reducing the frequency of culling programs. Recently, NCZ was registered as OvoControlG® as a contraceptive agent for urban Canada geese.

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TABLE OF CONTENTS

LITERATURE REVIEW	1
Nicarbazin and its effect on coccidiosis	1
Adverse effects of nicarbazin on reproduction	4
Shell bleaching	4
Yolk mottling	6
Egg production	9
Hatchability and fertility	11
Egg formation	13
Very low density lipoprotein	13
Vitellogenin	15
Vitelline membrane	17
Changes in the egg during incubation	19
Changes in the egg during aging	20
Urban Canada goose management	21
References	24
 IN VITRO ASSAYS	 47
Methods	50
Lipoprotein lipase assay	50
Vitellogenin phosphorylation assay	52
Transglutaminase assay	55
Calcium channel assay	57
Statistical analyses	59
Lipoprotein lipase assay	59
Vitellogenin phosphorylation assay	59
Transglutaminase assay	60
Calcium channel assay	60
Results	61
Discussion	62
References	67
Tables	74
 GAVAGE LABORATORY STUDY	 78
Methods	80
Statistical analyses	83
Results	84
Discussion	87
References	97
Tables	106
Figures	115
 AD LIBITUM STUDY	 119
Methods	121
Comparison of delivery methods	121

Reproductive trial	122
Statistical analyses	124
Results	125
Delivery methods	125
Reproductive trial	125
Discussion	127
Delivery methods	127
Reproductive trial	129
References	132
Tables	136
Figures	142
COLORADO FIELD STUDY	144
Study area	146
Methods	146
Results	152
Discussion	155
Management implications	161
References	162
Tables	167
Figures	171
CONCLUSIONS	174

CHAPTER 1

LITERATURE REVIEW

Nicarbazin and Its Effect on Coccidiosis

Nicarbazin (NCZ) is a coccidiostat routinely used in the poultry industry since the 1950s to control protozoan cecal and intestinal infections by *Eimeria* species in broiler chickens (Chapman, 1994). Coccidial infections in poultry prevent efficient weight gain and increase bird mortality, which reduces profits for the poultry industry. The cost of coccidiosis in Great Britain in 1995 was approximately 60 million dollars, primarily due to the effects of coccidiosis on reducing weight gain and feed efficiency, and increasing mortality rate (Williams, 1999). Treating coccidiosis permits broiler chickens to utilize feed more efficiently (Ott et al., 1956a; Newberne and Buck, 1957; Ott et al., 1958), reducing the costs required to raise them for market.

Nicarbazin is used either alone or in conjunction with other coccidiostats, particularly ionophores to treat coccidiosis, and using NCZ followed by two or more anti-coccidial products during the growing phase provides greater protection against a wider variety of *Eimeria* species (Chapman, 1994). Generally, it is recommended that NCZ be used in the starter rations for broiler chicks at 0.0125% until 21 days of age, then another coccidiostat is used for the growing period (Williams, 1999).

In the 1950s, substituted carbanilide compounds were investigated for their potential anti-parasitic activity. Among the compounds tested, 4,4'-dinitrocarbanilide (DNC) was found to be an effective anticoccidial. However, DNC was poorly absorbed

into the blood from the chicken's gut, and to improve absorption, researchers complexed DNC with 2-hydroxy-4,6-dimethylpyrimidine (HDP). The NCZ complex is believed to be formed via hydrogen bonding between DNC and HDP (Cuckler et al., 1955; Rogers et al., 1983). This complex, now known as NCZ, improved the anticoccidial activity of DNC tenfold (Cuckler et al., 1955), whereas simply administering DNC and HDP together in a mixture was ineffective.

The increased anticoccidial activity of NCZ is believed to be due to the formation of smaller DNC crystals in the gut (Rogers et al., 1983). Because HDP is water soluble, the NCZ complex dissociates when exposed to the aqueous environment in the gut. Although DNC crystals tend to aggregate in aqueous environments, HDP prevents DNC from aggregating in larger crystals by keeping the DNC crystals more separated than if DNC is fed alone (Rogers et al., 1983). The average diameter of wetted DNC crystals formed from DNC alone is 0.53 μm compared to an average diameter of 0.11 μm obtained from the wetted NCZ complex. (Rogers et al., 1983). Because of the smaller size, the DNC crystals have a larger surface area, resulting in greater absorption.

Radio-labeling studies showed that HDP was poorly metabolized by birds, and 90% of it was rapidly excreted in the urine (Wells, 1999). In contrast, DNC is excreted primarily in the feces, and is excreted at a much slower rate than HDP. Tissue concentrations of DNC are highest in the liver and kidney and lowest in the plasma and muscle, whereas HDP concentrations are similar in all tissues, including blood plasma (Nessel, 1977; Wells, 1999). Three metabolites of DNC have been identified, one of which was only found in fecal material (Manthey, 1986). To date, no attempt has been made to identify metabolites of HDP because of its rapid excretion rate. Chickens fed

125 mg NCZ/kg feed for 7 days exhibited peak plasma HDP concentration of 2.1 mg/kg on day two versus 3.8 mg/kg on day four for DNC (Nessel, 1977; Wells, 1999).

Chickens fed 0.01-0.08% NCZ for eight weeks exhibited undetectable plasma HDP by 20 hours post-treatment, and the only detectable tissue HDP was in the liver (Porter and Gilfillian, 1955). However, this study employed a spectrophotometric method to identify HDP, and showed a high degree of intra-assay variability. Porter and Gilfillian (1955) also reported DNC was mostly cleared from plasma, liver, and muscle by 48 hours post-treatment. Clark et al. (1956) confirmed that chickens fed 0.015 or 0.06% NCZ for seven days had only traces of DNC and DNC metabolites in the tissues two and four days after NCZ withdrawal, respectively. Chickens fed 0.0125% NCZ for three days cleared HDP and most of the DNC from the plasma, liver, kidney, and muscle by five days post-treatment, although trace amounts of DNC were still present in the liver (Merck and Lilley, 1986; Wells, 1999). In the egg, DNC is found primarily in the yolk, and is cleared by 12 days post-treatment. The HDP component is found both in albumen and yolk in a 3:1 ratio, and is undetectable three days after NCZ withdrawal (Cannavan et al., 2000). Differences in rates for DNC and HDP clearance between studies may be due in part to different chicken breeds or strains used, and assay methodologies used in each study.

Treating chickens with NCZ affects asexual reproduction of *Eimeria* by preventing the development of second generation schizonts and the number of oocysts shed, and decreases the pathogenic effect of *Eimeria* infection (Cuckler and Malanga, 1956). Second generation schizonts from NCZ treated chickens exhibit stunted growth and appeared abnormal (Ball, 1959; Ball et al., 1997). Although NCZ primarily acts on second generation schizonts, it may also affect earlier stages of *Eimeria* (McLoughlin and

Wehr, 1960; McDougald and Galloway, 1973; Strout and Ouellette, 1973; Ryley and Wilson, 1976). Nicarbazin may have some effect within the intestinal tract, but systemic absorption is necessary for anticoccidial activity (Waletsky and Probst, 1957).

There has been debate over whether NCZ is a coccidiostat or a coccidiocide. Most authors agree that it is a coccidiostat (McLoughlin and Wehr, 1960; McDougald and Galloway, 1973; Wilson, 1976), but some found NCZ to act more like a coccidiocide (Ball et al., 1997). Although the exact molecular mechanism by which NCZ controls coccidiosis is unknown, *in vitro* studies provide clues. Wang (1978) found DNC caused chicken erythrocytes to leak potassium ions. The DNC component may also affect energy production by mitochondria. Energy-dependent transhydrogenase activity and the accumulation of calcium ions in rat liver mitochondria are inhibited by DNC (Wang, 1978), and reduction of succinate-linked nicotinamide adenine dinucleotide (NAD⁺) is inhibited by DNC in beef heart mitochondria (Dougherty, 1974).

Adverse Effects of Nicarbazin on Reproduction

Shell Bleaching. Accidental delivery of NCZ to layer hens can occur, particularly when feed rations for broilers and layers are mixed in the same mill. Depending on the dose received, a suite of reproductive effects are observed in layer hens. The most immediate effect is on egg quality, with egg shell bleaching being the most obvious. Susceptibility to shell bleaching depends to some extent on the breed of hen (Schwartz et al., 1975), as well as dose and length of time NCZ is administered. Shell bleaching in Hisex hens fed either 50 or 100 ppm NCZ in the feed occurred with an egg yolk DNC level of 1.17 $\mu\text{g/g}$ (Jones et al., 1990a), whereas only 0.95 $\mu\text{g/g}$ DNC in yolk was needed

to observe bleaching effects in Hubbard hens fed NCZ at the same levels (Jones et al., 1990b).

Baker et al. (1957) found 90 ppm NCZ in feed affected shell bleaching in heavy breed hens, such as Barred and White Plymouth Rock hens, more than lighter breeds such as White Leghorns. New Hampshire hens were affected by as little as 30 ppm NCZ in feed, with increasing depigmentation as levels of NCZ were increased to 125 ppm (Ott et al., 1956b; McLoughlin et al., 1957). Shell depigmentation in these hens was observed as early as two days after initiation of treatment, and recovery began at three days post-treatment. White Rock hens exhibited considerable individual variability, with some hens laying normally pigmented eggs even at 700 ppm NCZ in the feed (Sherwood et al., 1956a). However, most White Rock hens exhibited shell depigmentation two to three days into treatment with 50 and 100 ppm NCZ in feed, and pigmentation returned to normal three days after cessation of treatment. The Arbor Acres line of hens is susceptible to shell depigmentation at 25 ppm NCZ in the feed, showing signs of bleaching as early as two days into treatment (Hughes et al., 1991). Complete bleaching of shells occurred three days after treatment initiation in Arbor Acres hens fed 100 ppm NCZ in feed (Hughes et al., 1991). All eggs returned to normal pigmentation within six to eight days of drug withdrawal.

Brown shell color is due to protoporphyrin deposition in the uterus (Warren and Conrad, 1942). The glandular tissue of the uterus is capable of converting δ -amino-levulenic acid into porphyrin *in vitro* (Polin, 1957a). Nicarbazin had no effect on porphyrin synthesis in the uterus, although there was a decrease in shell protoporphyrin concentration (Polin, 1959). Protoporphyrin concentrations in shells from hens fed 50

ppm NCZ in the feed dropped 58% from 85 γ /g to 35 γ /g. Protoporphyrin concentrations in shells from hens fed 100 ppm NCZ in the feed dropped 71% from 59 γ /g to 17 γ /g. There was a linear relationship between percentage of NCZ in the feed and percentage decrease of shell protoporphyrin concentration (Polin, 1959).

To determine which component of NCZ was responsible for the eggshell bleaching effect, Polin et al. (1959) injected hens with 20-80 mg of DNC, HDP, or NCZ intramuscularly. The DNC component caused shell depigmentation, whereas HDP did not. Nicarbazin was effective at a lower dose than DNC, but the bleaching effect lasted longer with DNC, indicating NCZ was absorbed and excreted faster than DNC alone.

Nicarbazin had no effect on the activities of heme synthetase, ALA dehydratase, or porphyrinogens (Schwartz et al., 1975). In contrast to Polin et al. (1959), Schwartz et al. (1975) found NCZ only caused shell bleaching when administered orally. Both brown egg layers and blue-green egg layers were affected by an average daily dose of 40 mg NCZ/bird. Porphyrin concentrations decreased in egg shells, uterus, and feces, whereas it increased two to four fold in red blood cells (Schwartz et al., 1975).

Yolk Mottling. Egg yolk mottling is the egg quality factor that most affects consumer acceptance of table eggs. Although natural mottling as high as 43% has been reported (Polin et al., 1957), the incidence of mottling increases with NCZ treatment. A minimum level of 50 ppm NCZ in feed is needed to induce mottling (Polin et al., 1957), and almost 100% of eggs are affected at 125 ppm NCZ in feed (Baker et al., 1957). The degree and incidence of mottling increases with increased storage time (Baker et al., 1957; Polin et al., 1958; Silvestrini et al., 1965; Jones et al., 1990c). There is much individual variability for hens among days (Polin et al., 1957), with treated hens laying

severely mottled eggs one day, and eggs with little mottling the next. At 100 ppm NCZ in feed, 81% of eggs were mottled and egg DNC levels were 22.2 $\mu\text{g/g}$, whereas only 35% of eggs were mottled at 50 ppm NCZ in feed and egg DNC levels were 11.7 $\mu\text{g/g}$ (Jones et al., 1990c).

Various studies have been undertaken to determine what differences exist between eggs from control hens and mottled eggs from NCZ-treated hens. Mottled yolks were 3.5% heavier than control yolks (Polin, 1957b), contained more water (Baker et al., 1956; Polin, 1957b; Silvestrini et al., 1961), and comprised a greater percentage of the total egg (Polin, 1957b). Additionally, mottled yolks had a more alkaline pH than control yolks (Baker et al., 1956). Albumen was detected in mottled yolks (Baker et al., 1956; Silvestrini et al., 1960), and egg yolk was found in the albumen of mottled eggs (Polin, 1957b). An increase of ovalbumin occurred in mottled yolks, with as much as 20% of the yolk consisting of ovalbumin in severe cases (Silvestrini et al., 1965; McCready and Roland, 1973).

Lipovitellin and lipovitellenin content is lower in mottled yolks, whereas livetin content is higher (Silvestrini et al., 1961). Lipovitellin content decreased as mottling increased, whereas lipovitellenin content only decreased in yolks from extremely mottled eggs (Silvestrini et al., 1965). In contrast, McCready and Roland (1973) found an increase in lipovitellenin content in mottled yolks and a slight decrease in livetin content. They reported these changes are similar to what occurs in mottling due to calcium deficiency.

Egg yolk mottling may be the result of changes to the vitelline membrane, causing it to become weaker and more permeable, and allowing yolk and albumen to intermix.

Mottled yolks show a decrease in yolk solids (Cunningham, 1976), and exhibit an increase in albumen (Cunningham, 1977), resulting in an overall reduction in albumen moisture. The percentages of fat, protein, and ash are also reduced in mottled yolks (Cunningham, 1976), but these components increased in the albumen (Cunningham, 1977), with fat content being four times higher. Ions such as calcium, phosphorus, and iron are reduced in mottled yolks (Cunningham, 1976), and are higher in egg albumen (Cunningham, 1977). Additionally, mottled yolks exhibit a more alkaline pH, have a higher carbohydrate content, and contain two egg white proteins believed to be ovalbumin and conalbumin (Cunningham, 1976). However, there is no difference in cholesterol content between mottled yolks and normal yolks (Cunningham, 1976). The protein composition of the albumen from eggs having mottled yolks appears to be unchanged (Cunningham, 1977).

The exact nature of the change to the vitelline membrane caused by NCZ treatment is not known. No difference in vitelline membrane strength was found between eggs from NCZ-treated hens and control hens, although this was likely due to the large variability among eggs within treatment groups (van Tienhoven et al., 1958). The moisture content of the vitelline membrane increased with storage and degree of mottling in eggs from treated hens (Britton, 1973). Protein content decreased as the level of mottling increased (Britton, 1973), although the amino acid composition was the same for eggs from NCZ-treated and control hens (Britton and Hale, 1975). The vitelline membranes of fresh eggs from NCZ-treated hens had a higher lipid content than eggs from control birds, but the lipid content was lower in NCZ-treated eggs after storage (Britton, 1973), and no differences were found in ash content (Britton, 1973). No gross

chemical changes in the vitelline membrane were observed when the degree of mottling was the same for natural versus NCZ-induced mottling (Britton, 1973). Britton (1973) suggested changes due to NCZ occur first in the vitelline membrane, and this is followed by yolk mottling. He further suggested that NCZ itself does not induce changes which would not occur naturally, but rather increases the rate and incidence at which those changes occur.

Damage to the egg yolk appears to occur after ovulation, as no mottling was found in developing ova (Baker et al., 1957; Polin, 1957b; Mitchell and Stadelman, 1958; van Tienhoven et al., 1958). Some mottling occurs to yolks while in the oviduct when birds are fed 30 to 125 ppm NCZ in feed (Baker et al., 1957; Mitchell and Stadelman, 1958; van Tienhoven et al., 1958), although Polin (1957b) reported no mottling in eggs in either the magnum or the uterus for birds fed 200 ppm NCZ in the feed. Freshly laid eggs showed no signs of mottling at 125 ppm NCZ in the feed, but the incidence of mottling increased after five days of storage (Polin, 1957b).

Egg Production. The effect of NCZ on egg production may be due to a failure of ova to mature (Baker et al., 1957). The effect of NCZ on egg production is highly variable and is dependent on the age at which treatment was initiated, as well as breed or strain of chicken involved (Weiss, 1957). New Hampshire chicks fed 125 or 200 ppm NCZ in feed from day one of age to maturity had no decline in egg production, whereas mature hens fed 80 ppm NCZ in feed had decreased egg production lasting three to four weeks post-treatment (Ott et al., 1956a,b).

Doses as low as 25 ppm NCZ in feed fed for ≥ 4 days decreased egg production in an Arbor Acres line of hens, with recovery occurring eight days post-treatment (Hughes

et al., 1991). White Rock hens fed 70 ppm NCZ in feed had only a slight decrease in egg production (Sherwood et al., 1956b). Heavy breeds, such as Barred and White Plymouth Rock, had decreased egg production at 90 ppm NCZ in feed, whereas White Leghorns needed 125 ppm NCZ in feed to decrease egg production (Baker et al., 1957). In contrast, Jones et al. (1990c) found White Leghorns hens fed 50 ppm NCZ in feed had an 11% decrease in egg production, and a peak egg DNC level of 11.7 $\mu\text{g/g}$. Hens in the same study fed 100 ppm NCZ in feed had a 13% reduction in egg production, and a peak egg DNC level of 22.2 $\mu\text{g/g}$ (Jones et al., 1990c). Feeding 100 ppm NCZ in feed to Hubbard hens had no effect on egg production (Jones et al., 1990b), whereas feeding the same level to Hisex hens caused a 67% reduction (Jones et al., 1990a). Feed levels of 125 ppm NCZ reduced egg production in Arbor Acres hens (Jones et al., 1990b), New Hampshire hens (McLoughlin et al., 1957), White Leghorn hens (Lucas, 1958) and White Rock hens (Sherwood et al., 1956a). Hisex hens ceased laying eggs after being fed 375 ppm NCZ in feed (Luck, 1979). White Rock hens fed 700 ppm NCZ in feed ceased laying eggs after ten days of treatment, but began laying eggs ≥ 10 days post-treatment (Sherwood et al., 1956a).

Necropsy of hens fed a ration containing 90 ppm NCZ revealed that the largest follicle was absent with no signs of recent ovulation or atresia (Baker et al., 1957). Luck (1979) also found ovaries without a follicular hierarchy, and less well-developed oviducts in laying hens treated with 375 ppm NCZ in feed. However, NCZ did not affect LH levels or pituitary responsiveness to LHRH, but NCZ treatment decreased the sensitivity of the chicken hypothalamus to exogenous progesterone, suggesting that egg production is decreased because yolk deposition in the follicles is prevented (Luck, 1979). White

Leghorns fed 400 to 700 ppm NCZ in feed exhibited reduced egg production concomitant with a two fold rise in plasma cholesterol concentrations within two weeks after treatment began, supporting Luck's hypothesis (Weiss, 1979).

Hatchability and Fertility. Although it is generally accepted that NCZ has no effect on fertility, data are conflicting, and the effect appears strain dependent. Nicarbazin did not affect fertility in New Hampshire chickens fed 100, 200, or 700 ppm NCZ in feed (Ott et al., 1955; Ott et al., 1956b), and feeding a range of NCZ in feed of 10 to 125 ppm had no effect on the fertility of Hisex or Hubbard chickens (Jones et al., 1990a,b) or an Arbor Acres line of chickens (Jones et al., 1990b; Hughes et al., 1991). In contrast, Lucas (1958) observed a 50% reduction in the fertility of White Leghorn pullets fed 125 ppm NCZ in feed, but not cockerels. The effect on males was confirmed by van Tienhoven et al. (1957) who found that feeding 125 ppm NCZ in feed to White Leghorn males had no effects on sperm concentration, percentage dead or abnormal sperm, or egg hatchability. A range of 6 to 700 ppm NCZ in feed gave inconsistent results on the fertility of White Rock chickens (Sherwood et al., 1956a), whereas a subsequent study showed no effect of 70 ppm NCZ in feed (Sherwood et al., 1956b).

The level of NCZ needed to reduce hatchability, and the degree to which hatchability is affected, is also strain dependent. Slight decreases in egg hatchability were observed for eggs from White Rock hens fed 6 ppm NCZ in feed (Sherwood et al., 1956a), and for New Hampshire hens fed 10 ppm NCZ in feed (Ott et al., 1956b). Feeding 20 ppm NCZ in feed for ≥ 10 days reduced egg hatchability 20% in White Rock hens (Sherwood et al., 1956a,b), 29% in Hubbard hens (Jones et al., 1990b), but 61% in Hisex hens (Jones et al., 1990a). A level of 25 ppm feed NCZ in feed fed ≥ 4 days

reduced egg hatchability 63% in an Arbor Acres line of hens (Hughes et al., 1991). Peak egg DNC levels for Hisex eggs were 5.97 $\mu\text{g/g}$ with 20 ppm NCZ in feed (Jones et al., 1990a), and 4.5 $\mu\text{g/g}$ in Arbor Acres eggs at 25 ppm NCZ in feed (Hughes et al., 1991).

Feeding 50 ppm NCZ in feed reduced egg hatchability by 42% in Hubbard hens (Jones et al., 1990b), 50% in White Rock hens (Sherwood et al., 1956a), 60% in New Hampshire hens (Ott et al., 1956a), 76% in an Arbor Acres line of hens (Hughes et al., 1991), and 100% in Hisex hens (Jones et al., 1990a). This reduction in egg hatchability was associated with peak egg DNC levels of 7.6 $\mu\text{g/g}$ in Arbor Acres eggs (Hughes et al., 1991), and 13.3 $\mu\text{g/g}$ in Hisex eggs (Jones et al., 1990a). A 66% decrease in hatchability was achieved by feeding 70 ppm NCZ in feed to White Rock hens (Sherwood et al., 1956b).

A feed level of 100 ppm decreased egg hatchability 60% in New Hampshire hens (Ott et al., 1956a), 83% in Hubbard hens (Jones et al., 1990b), and 100% in Hisex (Jones et al., 1990a) and Arbor Acres hens (Hughes et al., 1991). Peak egg DNC levels were 11.8 $\mu\text{g/g}$ in Arbor Acres eggs (Hughes et al., 1991), and 15.7 $\mu\text{g/g}$ in Hisex eggs (Jones et al., 1990a). Jones et al. (1990b) reported a level of 6 $\mu\text{g DNC/g egg}$ was needed to significantly affect egg hatchability. On average, 125 ppm NCZ in feed produced a 39% decrease in hatchability in eggs from White Leghorn hens (Lucas, 1958), 50% in White Rock hens (Sherwood et al., 1956a), and 100% in Arbor Acres hens (Jones et al., 1990b). Feeding 200 to 400 ppm NCZ in feed produced an average 89% reduction in hatchability in New Hampshire hens (Ott et al., 1956a), whereas 700 ppm completely suppressed hatchability (Ott et al., 1956a,b). Nicarbazine increases embryonic mortality during the first week of incubation, affecting hatchability (Lucas, 1958). White Rock embryos from

hens fed NCZ at 50 ppm in feed had 40% mortality, 50% mortality at 125 ppm, and 100% mortality at 700 ppm (Sherwood et al., 1956a).

Egg Formation

Egg yolk consists of both white and yellow yolk. Yellow yolk spheres consist of soluble yolk globules 50 to 100 μm in diameter containing small particles, composed of low density lipoprotein (Burley and Vadehra, 1989). Whole yolk also contains insoluble yolk globules that contain more phospholipids than soluble yolk globules. Approximately one quarter of egg yolk consists of yolk granules that contain the vitellogenin scission products phosvitin and lipovitellin (Burley and Vadehra, 1989).

Very Low Density Lipoprotein (VLDL). The main constituent of egg yolk is very low density lipoprotein (VLDL). Although non-laying hens and roosters have serum VLDL, it is only a major serum component in laying hens (Burley et al., 1984), and its production in the liver is induced by estrogen (Hillyard et al., 1956; Kudzma et al., 1979). As a precursor to yolk lipoproteins, VLDL contains most of the yolk lipids. These lipids are primarily derived from the diet, but can also be synthesized from fat reserves, carbohydrates, and other metabolites in the liver (Leveille et al., 1975). Dietary lipids are emulsified with bile acids and absorbed through microvilli into the intestinal wall where they are coated with protein (Bensadoun and Rothfeld, 1972). Because birds do not have a well developed lymphatic system, portomicrons enter the liver via blood capillaries where the lipids are further modified and deposited into secretory vesicles as VLDL particles (Janero and Lane, 1983).

Very low density lipoprotein consists of two main components, apoB and apoVLDL-II. Each VLDL particle contains approximately 46 apoVLDL-II particles for

every apoB particle (Walzem et al., 1999). ApoVLDL-II, also called apoII (Blue and Williams, 1981), has no mammalian counterpart (Jackson et al., 1977) and is the precursor to apovitellenin I in the yolk (Dugaiczky et al., 1981). ApoVLDL-II is encoded for by its own gene rather than arising as a degradation product from a larger protein (Wiskocil et al., 1981). Synthesis of apoVLDL-II on the rough endoplasmic reticulum of liver cells is induced by estrogen in laying hens (Chan et al., 1976; Blue and Williams, 1981; Lin and Chan, 1982). ApoVLDL-II provides the VLDL particles some resistance to degradation by lipoprotein lipase (Griffin et al., 1982; Schneider et al., 1990).

Avian apoB is homologous to mammalian apoB (Chapman et al., 1977), and is synthesized primarily in the endoplasmic reticulum of liver cells, although truncated forms are also synthesized in the intestines and kidneys (Blue et al., 1980; Siuta-Mangano et al., 1982). Unlike apoVLDL-II, apoB is also synthesized by non-laying hens and roosters, however, estrogen causes a four to six fold increase in synthesis (Williams, 1979; Capony and Williams, 1980; Nadin-Davis et al., 1980; Kirchgessner et al., 1987).

The VLDL particles of laying hens differ from those of non-laying hens and are targeted to the egg yolk. Particles must be smaller than 44 nm in diameter to pass through the basal lamina of the granulosa cell layer (Evans et al., 1979; Griffin and Perry, 1985; Walzem et al., 1999). Laying hen VLDL particles are about half the size of non-laying hen VLDL particles (Chapman et al., 1977; Griffin and Perry, 1985). This allows them to pass through the intracellular spaces in the granulosa cell layer while excluding non-laying hen VLDL particles (Jordanov and Boyadjieva-Michailova, 1974).

The VLDL particles in laying hens contain a high proportion of phospholipids, specifically triacylglycerols, and a low proportion of cholesteryl esters (Griffin and Perry,

1985; Walzem et al., 1999). Lipoprotein lipase breaks VLDL into intermediate density and low density lipoproteins, which contain a lower proportion of triacylglycerols needed for embryonic development (Walzem et al., 1999). To prevent VLDL degradation by lipoprotein lipase prior to deposition in the yolk, VLDL particles in laying hens contain a high proportion of apoVLDL-II and a low proportion of apoC-II, a lipoprotein lipase activator (Griffin et al., 1982; Griffin and Perry, 1985). Once past the granulosa cell layer, VLDL particles bind to a 95 kDa receptor that recognizes the apoB component, and the receptor complex is endocytosed (Perry et al., 1984; Nimpf et al., 1988; Bujo et al., 1997).

Vitellogenin. Vitellogenin is the other component of egg yolk. Three forms of vitellogenin have been found in hens to date, with VTGII being the most abundant (Wang et al., 1983). Vitellogenin is synthesized in the liver in response to stimulation by estrone or estradiol 17β (Green, 1980; Shapiro, 1982; Wallace, 1985), and has a half life of approximately one day (Redshaw and Follett, 1976). The cells that estradiol targets in the liver are the same parenchymal cells that normally produce serum albumen (Williams et al., 1978). The plasma of roosters contains only 4-8 ng/mL vitellogenin (Blue and Williams, 1981), whereas the plasma of laying hens contains 10-25 mg/mL (Redshaw and Follett, 1976).

In the presence of calcium, vitellogenin forms a dimer (MW 5.4×10^5). These dimers are comprised of monomers side by side connected by calcium bridges (Hermsdorf et al., 1979). Each monomer consists of one lipovitellin and two phosvitin polypeptides (Deely et al., 1975; Christmann et al., 1977). Phosvitin is a glycosylated protein containing a high number of serine residues, most of which are phosphorylated.

The phosphates confer a negative charge which allows phosvitin to easily bind calcium and iron (Allerton and Perlmann, 1965; Clark, 1970; Taborsky, 1980). Because of this, vitellogenin is the main carrier of calcium and iron to egg yolk (Morgan, 1975; Grunder et al., 1980; Lopez-Berjes et al., 1981).

Once synthesized, vitellogenin undergoes several post-translational modifications. These include the addition of carbohydrate, phosphorylation of serine residues (Wang and Williams, 1982), attachment of calcium to phosphates, and the formation of dimer configurations. Upon release into the blood stream, vitellogenin passes out of the capillaries surrounding the oocyte (Perry et al., 1978a,b), through the granulosa cell layer, and reaches the oolemma. Prior to yolk deposition, the granulosa cell layer separates to allow the passage of large particles between cells (Evans et al., 1979).

Vitellogenin binds the same 95 kDa receptor that recognizes VLDL (George et al., 1987; Stifani et al., 1990; Barber et al., 1991). Maximal binding of vitellogenin to its receptor occurs at a pH of 6.0 (Yusko et al., 1981). Clusters of receptor bound vitellogenin induce the formation of clathrin coated pits that become engulfed by the oolemma to become clathrin coated vesicles (Wyburn et al., 1965; Schjeide et al., 1969; Tucciarone and Lanclos, 1981). These endosomes then fuse to become yolk globules. Within 20 minutes of being endocytosed, vitellogenin is split into lipovitellin and phosvitin (Griffin et al., 1984), which are then incorporated into yolk granules (Burley and Vadehra, 1989). The lipid portion of the yolk, mostly phosphatidylcholine and phosphatidylethanolamine, is primarily associated with the lipovitellin fraction (Ohlendorf et al., 1977).

Vitelline Membrane. The vitelline membrane is comprised of an outer and inner layer separated by a thin, continuous membrane (Bellairs et al., 1963; Bain and Hall, 1970; Debruyne and Stockx, 1978). The inner layer is deposited prior to ovulation in the follicle (Wyburn et al., 1965), whereas the continuous and outer layers are deposited in the infundibulum (Bellairs et al., 1963; Bain and Hall, 1969). The outer layer is 3.0-8.5 μm thick and consists of several sublayers (Bellairs et al., 1963). Each sublayer is made up of fibrils 15-300 nm thick with a granular substance occurring between the fibrils. In the outermost sublayer, the fibrils combine to form bundles.

The outer vitelline layer consists of ovomucin, lysozyme, outer vitelline membrane protein I (VMOI), outer vitelline membrane protein II (VMOII), and lectin. Ovomucin is the most abundant protein in the outer layer (MacDonnell et al., 1951; Back et al., 1982; Kido and Doi, 1988). Although the albumen also contains ovomucin, the ovomucin here contains different sulfated glycopeptides than the ovomucin contained in the vitelline membrane (Kato et al., 1982). The ovomucin in the outer layer of the vitelline membrane has proportionately more β -ovomucin than α -ovomucin (Kido et al., 1975; Debruyne and Stockx, 1982; Kato et al., 1982), and provides a framework to support the rest of the outer layer proteins.

Lysozyme is the second most abundant protein in the outer layer of the vitelline membrane (Back et al., 1982; DeBoeck and Stockx, 1982; Kido and Doi, 1988). It exhibits similar enzymatic activity as albumen lysozyme (DeBoeck and Stockx, 1982), and likely has both antibacterial and structural integrity roles in the outer layer. Although VMOI is the third most abundant protein in the outer layer, little else is known about it (Back et al., 1982; DeBoeck and Stockx, 1982; Kido and Doi, 1988). Similarly, the

function of VMOII is unknown (Back and Burley, 1985; Burley and Back, 1987; Kido et al., 1992). Lectin may assist in protection against micro-organisms such as yeast (Rutherford and Cook, 1981). The continuous membrane is 50-100 nm thick and granular in nature, but its function is unknown (Bellairs et al., 1963).

The inner layer, also known as the perivitelline layer, is analogous to the mammalian zona pellucida (Bakst and Howarth, 1977; Takeuchi et al., 1999). It is 1.0-3.5 μm thick, and is comprised of a network of thick, cylindrical fibers 0.2-0.6 μm in diameter (Bellairs et al., 1963). Each fiber is made up of fibrils 25-30 nm thick. Micovillous projections in the blastodisc region may play a role in sperm attachment and penetration (Bakst, 1978; Bramwell and Howarth, 1992; Snell and White, 1996).

The inner layer is made up of three glycoproteins: GPI, GPII, and GPIII (Kido et al., 1975; Kido et al., 1976; Kido et al., 1977; Back et al., 1982). The existence of GPIII is controversial, but it may be related to ovomucin (Debruyne and Stockx, 1982; Kido and Doi, 1988). Kido and Doi (1988) also reported finding a fourth, insoluble glycoprotein in the inner layer.

Hydrophobic in nature, GPI is synthesized by the granulosa cells (Pan et al., 2001; Sasanami et al., 2002) under the control of testosterone (Pan et al., 2001) and FSH (Sasanami et al., 1999; Pan et al., 2003). It is homologous to mammalian ZP3 or ZPC (Waclawek et al., 1998; Takeuchi et al., 1999; Pan et al., 2000) and binds GPII to form the perivitelline layer (Ohtsuki et al., 2004). In contrast, GPII is synthesized in the liver under the control of estradiol, is homologous to mammalian ZP1 or ZPB (Bausek et al., 2000; Sasanami et al., 2003), and may play a role in the structural integrity of the inner vitelline membrane layer.

Changes in the Egg During Incubation

Several important changes must occur in the egg during the early days of incubation for successful embryonic development to occur. Eggs opened during the first few days of incubation exhibited a decrease in albumen height, indicating that thinning of thick albumen is occurring (Benton and Brake, 1996). Albumen viscosity is greatest at oviposition (Walsh, 1993), and helps keep the yolk from coming into contact with the eggshell, reducing the risk of embryonic contamination by bacteria (Board and Fuller, 1974). Once the egg is laid, the role of albumen is to provide the embryo with water for subembryonic fluid, protein, and other nutrients (Burley and Vadehra, 1989; Stern, 1991; Benton and Brake, 1996).

Albumen thinning is necessary to permit adequate gas diffusion inside the egg and nutrient availability to the embryo (Meuer and Baumann, 1988; Burley and Vadehra, 1989). Thinning of the albumen is related to and occurs with a concomitant rise in albumen pH (Benton and Brake, 1996). The pH of the albumen at oviposition is approximately 7.6 (Stern, 1991), but it rapidly rises to 9.0-9.5 within 48 hours of incubation (Sadler, 1955; Stern, 1991). As albumen pH increases, it reaches the isoelectric point of lysozyme, and this may influence the egg white thinning by decreasing electrostatic interactions between lysozyme and ovomucin (Cotterill and Winter, 1955; Sauveur, 1976; Miller et al., 1982). Additionally, the rise in pH provides a 1000 fold pH gradient across the vitelline membrane (Stern, 1991) that may be necessary for normal embryogenesis to occur (Benton and Brake, 1996). The pH gradient may be essential for certain transport functions during early incubation (Benton and Brake, 1996)

because the vitelline membrane is an asymmetrical ion-exchange membrane (Rymen and Stockx, 1974).

Prolonged exposure of the embryo to an alkaline pH is detrimental (Funk and Biellier, 1944; Sadler, 1955; Arora and Kosin, 1966), and during the first four days of incubation, the vitelline membrane and thick albumen, also called the chalaziferous sac or membrane, protect the embryo from the alkaline environment of the thin albumen (Sadler, 1955; Fromm, 1964). The vitelline membrane gradually begins to dissipate, and by 72 hours of incubation, it has completely disappeared from the area over the embryo. From this point until 96 hours of incubation, the chalaziferous sac helps protect the embryo, but it too gradually disappears. By 96 hours of incubation, the embryo is fully enclosed in the amniotic and chorionic sacs, which then protects the embryo from the alkaline environment of albumen (Sadler, 1955).

Changes in the Egg During Aging

Several changes occur in egg albumen during the normal aging process when eggs are stored. The viscosity of the albumen decreases with storage time (Hurnik et al., 1978; Walsh, 1993), and this is accompanied by a decrease in albumen height (Lapão et al., 1999; Silversides and Budgell, 2004). Concomitant with these changes is the increase in albumen pH (Lapão et al., 1999; Tona et al., 2002; Silversides and Budgell, 2004). During the aging process, β -ovomucin is released from the lysozyme-ovomucin complex, causing the formation of insoluble α -ovomucin aggregates (Kato and Sato, 1972; Kato et al., 1981). A change in the interaction between lysozyme and ovomucin has been proposed as the mechanism for egg white thinning (Cotterill and Winter, 1955; Sauveur, 1976; Miller et al., 1982).

The vitelline membrane also undergoes changes during storage. Some of these changes include a decrease in vitelline membrane strength (Kirunda and McKee, 2000) accompanied by a decrease in vitelline membrane weight (Trziszka and Smolińska, 1982). This weight loss may be accounted for by the loss of VMOI and VMOII from the outer vitelline membrane (Back, 1984; Schäfer et al., 1998). Additionally, GPII of the inner vitelline membrane degrades into its subunits (Kido et al., 1975). These changes can be partially prevented by preventing the pH increase in the albumen (Fromm, 1967; Burley and Vadehra, 1989).

Urban Canada Goose Management

Canada goose (*Branta canadensis*) populations are expanding, and nonmigratory populations are becoming more frequent in populated 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, become aggressive (Conover and Chasko, 1985; Forbes, 1993), and deposit large amounts of fecal matter (Conover and Chasko, 1985; Fairaizl 1992). Fecal material may contaminate drinking water supplies (Conover and Chasko, 1985), and cause over-fertilization of lakes (Manny et al., 1975). 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 a feasible alternative in urban areas (Conover and Chasko, 1985; Heusmann, 1999), and the use of special hunting seasons has had limited success in controlling these resident goose populations (Heusmann, 1999). Currently, there are few places that allow goose translocation, and annual roundups have met with public

resistance in some areas. Therefore, contraception may provide an acceptable alternative to help manage bird populations at levels that minimize damage but allow for the existence of geese (Forbes, 1993; Stout et al., 1997).

Several things should be considered when developing contraceptives for use in Canada geese. A primary concern is that the contraceptive does not significantly affect nontarget animals. This can be controlled in part by bait design; for example, making the bait larger than most songbirds can consume will prohibit these birds from consuming the bait. In addition, because geese nest earlier than most nontarget songbirds, and due to its rapid clearance rate, NCZ should be relatively safe for nontarget songbirds.

Ideally, a contraceptive should have minimal effect on reproductive hormones of the target animal to permit normal breeding behaviors. This is less of a concern from a biological standpoint than it is from a sociological standpoint, because birders often enjoy watching the courtship behaviors of waterfowl (Bjerke et al., 2004). To this effect, Luck (1979) found plasma LH concentrations and the ability of the pituitary to respond to LHRH to be similar for control and NCZ-treated birds. Therefore, it seems likely NCZ will have a minimal impact on reproductive behaviors.

Because geese can renest if their first nest fails (Mowbray et al., 2002), a contraceptive for geese must either contracept for the entire breeding season, or decrease egg hatchability to be effective. Preventing egg hatching would likely preclude renesting by geese because they would incubate the nest until the expected hatching date or beyond, making it unlikely that they would start another clutch during that season (Converse and Kennelly, 1994; Christens et al., 1995). Because NCZ does not affect egg laying but prevents egg hatching, it fulfills these criteria (Jones et al. 1990b). However,

if migratory geese consume treated bait only a couple of times during migration, the contraceptive effects of NCZ will not persist, and normal breeding for these birds can occur. In addition, to be practical in the field, a contraceptive must be easy to deliver, and require a minimal amount of time to apply. This can be accomplished with NCZ by placing bait in normal loafing areas near nesting grounds utilized by geese. Finally, a contraceptive must be cost effective.

Current nonlethal control methods for Canada geese include egg oiling or addling (e.g. shaking the egg). These methods can be very effective in controlling goose populations, and require intense effort for only a few days each season (Forbes, 1993; Christens et al., 1995). However, the timing of oiling eggs is critical to its success, as is the availability of trained personnel. Successful egg oiling programs often involve several applications to prevent egg hatching for late-nesting and re-nesting birds (Blackwell et al., 2000). Utilizing the public to incorporate oiling programs can be effective if people are trained in locating nests. However, adequate personnel may not always be available, and occasionally egg oiling programs are viewed negatively by the public. Nicarbazine could be useful in these situations because it would require minimal amounts of time and personnel to place bait daily at a site. Additionally, the public has a more positive perception of this type of control effort because they don't see nests being destroyed.

It needs to be emphasized, however, that contraception is not a magic bullet. Contraception used alone, particularly in areas where the goose population is already too large, will be ineffective in reducing the population size quickly (Watola, 2003). Instead, contraception should be viewed as a tool to be used in conjunction with other control

methods as part of an integrated pest management plan. Reducing goose populations to an acceptable level through round-ups followed by maintenance with contraception will likely be more efficacious than either method used alone. In addition, other management techniques to help reduce damage, such as hazing birds and bird repellents, should also be incorporated into management plans (Colorado Division of Wildlife, 2003).

Studies to determine the efficacy of NCZ as a contraceptive agent for Canada geese were conducted at the National Wildlife Research Center. Initial laboratory studies utilized mallards (*Anas platyrhynchos*) as a model for Canada geese because they reproduce more readily in a laboratory setting and lay more eggs than Canada geese if eggs are removed daily. The first mallard laboratory study was conducted as a gavage study to determine a target NCZ dose for further research, and to assess health effects of NCZ treatment on waterfowl. Based on the results of the first study, a second mallard study was conducted to determine the effects of NCZ on reproduction when fed *ad libitum* in feed daily for 14 days. *In vitro* experiments were also conducted to assess the molecular mechanism of action of nicarbazin on reducing reproduction. Results of the laboratory studies were used to design and conduct a multi-year field study on the effects of NCZ on Canada goose reproduction in two populations of free-ranging geese in Colorado.

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CHAPTER 2

IN VITRO ASSAYS

Nicarbazin (**NCZ**) is a coccidiostat routinely used in the poultry industry since the 1950s to control protozoan cecal and intestinal infections by *Eimeria* species in broiler chickens. It is an equimolar complex consisting of 4,4'-dinitrocarbanilide (**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 coccidiostat (Cuckler et al., 1955; Rogers et al., 1983). When fed to laying hens, NCZ impacts reproduction by reducing egg production, egg weight, and egg hatchability (Jones et al., 1990a; Hughes et al., 1991; Chapman, 1994).

Although the mechanisms by which NCZ reduces egg production and egg weight are unknown, NCZ may be preventing ova from maturing (Baker et al., 1957). Necropsy of hens fed a ration containing 90 ppm NCZ revealed that the largest follicle was absent from the ovary with no signs of recent ovulation or atresia (Baker et al., 1957). Luck (1979) also found ovaries without a follicular hierarchy, and less well-developed oviducts in laying hens treated with 375 ppm NCZ in their feed. Although NCZ did not affect luteinizing hormone levels or pituitary responsiveness to luteinizing hormone releasing hormone, it decreased the sensitivity of the chicken hypothalamus to exogenous progesterone (Luck, 1979). Luck (1979) suggested that egg production is decreased because yolk deposition in the follicles is prevented. White Leghorn hens fed 400 to 700

ppm NCZ in feed exhibited reduced egg production concomitant with a two fold rise in plasma cholesterol concentrations, supporting Luck's hypothesis (Weiss, 1979).

Egg yolk is comprised of very low density lipoprotein (**VLDL**) and vitellogenin (**VTG**), both of which are produced in the liver in response to estrogen stimulation (Hillyard et al., 1956; Kudzma et al., 1979; Green, 1980; Shapiro, 1982; Wallace, 1985). The main constituent of egg yolk is VLDL. Although non-laying hens and roosters have small amounts of serum VLDL, it is a major serum component in laying hens (Burley et al., 1984). One component of VLDL, apoVLDL-II, provides the VLDL particles some resistance to degradation by lipoprotein lipase (**LL**) (Griffin et al., 1982; Schneider et al., 1990). In addition, laying hen VLDL particles contain less apoC-II, a LL activator (Griffin et al., 1982; Griffin and Perry, 1985).

Vitellogenin is comprised of one lipovitellin and two phosvitin polypeptides (Deely et al., 1975; Chistmann et al., 1977). One of the post-translational modifications VTG undergoes is the phosphorylation of serine residues on phosvitin (Wang and Williams, 1982). The phosphates confer a negative charge that allows phosvitin to bind calcium and iron (Allerton and Perlmann, 1965; Clark, 1970; Taborsky, 1980). Because of this, VTG is the main carrier of calcium and iron to the egg yolk (Morgan, 1975; Grunder et al., 1980; Lopez-Berjes et al., 1981). Dephosphorylation of these serine residues on phosvitin prevents the uptake of VTG into the follicle (Miller et al., 1982). Hens treated with 400 ppm NCZ in their feed exhibit reduced calcium binding by calcium binding protein and blood hypercalcemia (Bar and Hurwitz, 1971). This indicates that although VTG is produced, it is altered by NCZ somehow, preventing it from binding calcium.

Upon release into the blood stream, VLDL and VTG pass out of the capillaries surrounding the oocyte (Perry et al., 1978a,b), and through the granulosa cell layer of the oocyte. Once they reach the oolemma, they bind to the same 95 kDa receptor (George et al., 1987; Stifani et al., 1990; Barber et al., 1991). Clusters of occupied receptors induce the formation of clathrin coated pits that are engulfed by the oolemma to become clathrin coated vesicles (Wyburn et al., 1965; Schjeide et al., 1969). Transglutaminase assists in the formation of clathrin coated pits, and inhibition of the enzyme prevents uptake of VTG (Tucciarone and Lanclos, 1981).

The molecular mechanism by which NCZ reduces egg hatchability is also unknown. However, NCZ may change the permeability of the vitelline membrane, creating an unfavorable environment for embryonic development (Polin, 1957; van Tienhoven et al., 1958; Cunningham, 1977). Laying hens fed NCZ produce eggs with mottled yolks (Baker et al., 1957; Polin et al., 1957; Jones et al., 1990b). Mottled yolks show a decrease in yolk solids (Cunningham, 1976), and exhibit an increase in albumen (Cunningham, 1977). In addition, yolk components such as fat, protein, ash, calcium, phosphorus, and iron decrease in mottled yolks, but increase in the albumen of eggs with mottled yolks (Cunningham, 1976; Cunningham, 1977).

We hypothesized that if NCZ increases LL activity, it could cause the degradation of VLDL in the blood prior to its reaching the egg. Because VLDL is the major component of egg yolk, egg weight and egg production would decrease as a result. Phosphorylation of serine residues on VTG are necessary for calcium and iron binding, and binding to the 95 kDa receptor. Therefore, we hypothesized that if NCZ acts as a phosphatase, it would prevent VTG from binding to the receptor, thus reducing egg

weight and production. Additionally, there would be less calcium and iron available to the embryo, which could impact egg hatchability. We hypothesized that if NCZ inhibited transglutaminase activity, clathrin coated pits could not form and uptake of yolk components would not occur, resulting in reduced egg weight and production. We also hypothesized that if NCZ acts as a calcium channel blocker, it could disrupt crucial ion gradients needed for proper egg formation and embryogenesis.

We chose to focus on the mechanisms described in the previous paragraph as potential targets of nicarbazin. The objective of this study was to determine the molecular mechanisms by which NCZ affects egg hatchability and egg production. We accomplished this by testing 4 hypotheses as follows: 1) NCZ increases LL activity; 2) NCZ acts as a VTG phosphatase; 3) NCZ inhibits TG activity; 4) NCZ acts as a calcium channel blocker.

METHODS

Lipoprotein Lipase Assay

The LL assay was based on the principal that LL will cleave dibutylfluorescein (**DBF**), releasing fluorescein which can then be measured in a spectrofluorometer (Del Prado et al., 1994). Dibutylfluorescein was prepared as described previously by Del Prado et al. (1994). Briefly, 10 mL pyridine (P4036, Sigma Chemical Co., St. Louis, MO), 30 mL butyric anhydride (150540, Sigma Chemical Co., St. Louis, MO), and 10 mg fluorescein (F6377, Sigma Chemical Co., St. Louis, MO) were mixed at 23°C for 8 min, and then incubated in the dark for 24 h at room temperature. To this mixture was added 30 mL 100% ethanol (111000200, Pharmco Products, Inc., Brookfield, CT), and

the mixture was incubated at -20°C for 23 h. The mixture was thawed at 23°C for 15 min, then mixed on a vortex mixer for 15 min to break up the crystals.

Solvent was removed using a vacuum flask and 5.5 cm, grade 362 filter paper (F2215-55, Baxter). The filtrate was washed with 95% ethanol until the solvent ran clear, and the DBF was stored in the dark at 4°C. A DBF stock solution was made by dissolving 10 mg DBF in 50 mL ethylene glycol monomethyl ether (**EGME**; E2632, Sigma Chemical Co., St. Louis, MO). A DBF working solution was made by mixing 5 mL DBF stock solution with 100 mL low potassium phosphate buffer (291 mOsm, pH = 7.09).

Disposable 12 x 75 mm borosilicate glass tubes (60825-913, VWR International, Aurora, CO) were used for the assay. To each test tube was added 1 mL DBF working solution, and 15 µg LL (1 µg LL/µL Dulbecco's PBS). In two separate tubes, 10 µL of LL inhibitors, AA861 (0.03M; A3711, Sigma Chemical Co., St. Louis, MO) or nordihydroguaiaretic acid (0.1M; N5023, Sigma Chemical Co., St. Louis, MO) in EGME, were added as negative controls. To 1 of 4 other tubes was added 10 µL of either 1, 2, 4, or 8 µg NCZ (Phibro Animal Health, Inc., Fairfield, NJ)/10 µL dimethyl sulfoxide (**DMSO**; D5879, Sigma Chemical Co., St. Louis, MO). Two tubes containing either 10 µL DMSO or EGME served as controls for the NCZ or inhibitor tubes, respectively. A test tube with only DBF and LL served as a positive control. A test tube with DBF and no LL served as a blank to monitor background fluorescence. The solution in each test tube was mixed briefly on a vortex mixer, then incubated in a water bath at 37°C.

The spectrofluorometer was zeroed first using a blank tube consisting of DBF working solution only, and the gain was set to 1. The excitation wavelength was set at

490 nm and the emission wavelength was set at 535 nm. Tubes were removed after 1 min of incubation, and the amount of fluorescein released was determined by measuring fluorescence with a Turner model 450 spectrofluorometer. After obtaining readings, the test tubes were returned to the water bath. Tubes were removed for subsequent readings at 2 min intervals until 11 min of incubation time had passed. The experiment was replicated 5 times.

Vitellogenin Phosphorylation Assay

Phosphorylation of VTG was assessed using a purchased phosphoprotein stain (Molecular Probes, Inc., Eugene, OR). Plasma samples were obtained by drawing 3 mL of blood from the brachial vein of laying and non-laying chickens. The plasma of non-laying chickens was used as a negative control. Positive controls consisted of laying hen plasma only or laying hen plasma plus 10 μ L DMSO. Just prior to starting the assay, fresh NCZ, DNC (390151, Aldrich Chemical Co., Milwaukee, WI), and HDP (22588-6, Aldrich Chemical Co., Milwaukee, WI) solutions were made. To 100 μ L laying hen plasma was added 10 μ L of either 1, 2, 4, or 8 μ g NCZ/10 μ L DMSO; 1, 2, 4, or 8 μ g DNC/10 μ L DMSO; or 1, 2, 4, or 8 μ g HDP/10 μ L water. Samples were mixed and incubated at 4°C for 30 min. After incubation, all plasma samples were diluted 1:100 in PBS (P4417, Sigma Chemical Co., St. Louis, MO). A standards solution was prepared by mixing 2 μ L PeppermintStick standard (P33350, Molecular Probes, Inc., Eugene, OR) with 38 μ L ultra pure water.

A 1X SDS-Tris-glycine running buffer was made by adding 70 mL 10X SDS-glycine (161-0732, Bio-Rad Laboratories, Hercules, CA) to 630 mL ultra pure water. Fixing solution consisted of 100 mL methanol (A433P-4, Fisher Scientific, Fair Lawn,

NJ), 20 mL acetic acid (45726, Sigma-Aldrich, St. Louis, MO), and 80 mL deionized water. A Pro-Q Diamond destaining solution was made by mixing 187.5 mL deionized water, 50 mL acetonitrile (494445, Sigma-Aldrich, St. Louis, MO), and 12.5 mL 1M sodium acetate (110191, Aldrich Chemical Co., Milwaukee, WI).

Sample buffer (3X, 20 μ L; 87703S, New England BioLabs, Inc., Ipswich, MA) was added to 40 μ L plasma dilutions and to the standards solution. Samples were mixed briefly and centrifuged for 5 sec at 8 to 10 x G. Samples were heated for 5 min at 95°C, then centrifuged again for 5 sec at 8 to 10 x G. A 4-20% Tris-glycine-SDS minigel (81002-006, VWR International, Aurora, CO) was covered with 1X SDS-Tris-glycine running buffer. Each well was loaded with 50 μ L sample and the plasma proteins were separated by gel electrophoresis at 150V for 90 min.

The gel was removed from the electrophoresis apparatus, covered with 100 mL fixing solution, and incubated by gently agitating at 23°C for 30 min. The gel was washed twice by covering it with 100 mL ultra pure water and gently agitating at 23°C for 10 min. The gel was then covered with 50 mL Pro-Q Diamond phosphoprotein stain (P33300, Molecular Probes, Inc., Eugene, OR) and incubated in the dark with gentle agitation at 23°C for 2 h. The phosphoprotein stain was removed, and 80 mL Pro-Q Diamond destaining solution was added to the gel. The gel was incubated in the dark with gentle agitation at 23°C for 1 h. The destaining step was repeated once.

Images of the gel were produced on an Epichemi3 Darkroom 2UV benchtop transilluminator (UVP Bioimaging Systems, Ultraviolet Products Ltd., Cambridge, UK) using an ethidium bromide filter (excitation = 365 nm, emission = 570 to 640 nm).

Digital images were analyzed by densitometry using Scion Image for Windows (Scion Corporation, 2001). The experiment was replicated 5 times.

A western blot was used to confirm the presence and position of VTG on the gel. Briefly, plasma samples from a laying hen and from a male were diluted 1:100 in PBS and applied to a 4-20% Tris-glycine-SDS minigel with sample buffer. Proteins were separated for 90 min at 150V using SDS-PAGE. Proteins were transferred to a nitrocellulose membrane in transfer buffer for 60 min at 100V. The membrane was blocked for 30 min at 23°C with gentle agitation using blocking buffer consisting of Tris buffered saline (TBS) and 5% milk powder. The membrane was then incubated with 1:1000 rabbit anti-VTG antibody (Dr. David Williams, Pharmacological Sciences, SUNY, Stony Brook, NY) in blocking solution for 2 h at 23°C with gentle agitation. The membrane was washed once with TBS containing 0.05% Tween 20 (v/v; P1379, Sigma Chemical Co., St. Louis, MO), and twice with TBS.

The membrane was then incubated with alkaline phosphatase labeled goat anti-rabbit IgG antibody (1:1000; A7778, Sigma Chemical Co., St. Louis, MO) in blocking buffer for 60 min at 23°C with gentle agitation. The membrane was washed once with TBS-Tween 20, and twice with TBS. Color was developed by incubating the membrane in alkaline phosphatase substrate (pH 9.5; B5655, Sigma Chemical Co., St. Louis, MO) containing 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.3 mg/mL Nitro blue tetrazolium, 100 mM Tris buffer, and 5 mM magnesium chloride. The reaction was stopped after 10 min by washing the membrane in deionized water.

Transglutaminase Assay

Transglutaminase activity was assessed using an assay previously described by Lilley et al. (1997). The assay measures the protein crosslinking activity of transglutaminase based upon incorporation of biotin-labeled casein into unlabeled casein that is bound to microtiter plates.

Casein was biotinylated using a procedure previously described for labeling antibodies with biotin (Harlow and Lane, 1988). A 0.1M sodium borate buffer was prepared by dissolving 7 g boric acid (B6768, Sigma Chemical Co., St. Louis, MO) and 10 g sodium tetraborate (B0127, Sigma Chemical Co., St. Louis, MO) in 1 L deionized water, and titrating the solution to pH 8.8. A 3 mg/mL N',N'-dimethylcasein (C9801, Sigma Chemical Co., St. Louis, MO) solution was prepared in 0.1M sodium borate buffer. A 3 mg/mL N-hydroxysuccinimide biotin (H1759, Sigma Chemical Co., St. Louis, MO) solution was prepared in DMSO.

The casein and biotin solutions were combined in a 9:1 casein:biotin ratio and incubated at 23°C for 4 h. Ammonium chloride (1M; A4515, Sigma Chemical Co., St. Louis, MO) was added to the biotin ester solution at a rate of 20 µL per 250 µg biotin ester. The solution was incubated for 10 min at 23°C, and then dialyzed against PBS overnight in #3 Spectra/Por dialysis tubing (132724, Spectrum Medical Industries, Los Angeles, CA). The biotinylated casein was stored at -70°C until use.

Flat bottom 96 well microtiter plates (3455, Thermo LabSystems, Franklin, MA) were coated with 50 ng/well N,N'-dimethylcasein in 50 mM sodium carbonate buffer (C3041, Sigma Chemical Co., St. Louis, MO) at pH 9.8 (100 µL/well) and incubated at

37°C for 1 h. Plates were washed twice with PBS containing 0.05% Tween 80 (v/v; P8074, Sigma Chemical Co., St. Louis, MO), and twice with deionized water.

Plates were blocked with 300 μ L/well BSA (1 mg/mL) in 50 mM sodium carbonate buffer for 30 min at 23°C. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 100 mM Tris-HCl (pH 8.5; T3253, Sigma Chemical Co., St. Louis, MO). Each plate was then incubated overnight at 37°C with 100 μ L/well 100 mM Tris-HCl containing 5 mM calcium chloride (C4901, Sigma Chemical Co., St. Louis, MO), 10 mM dithiothreitol (D0632, Sigma-Aldrich, St. Louis, MO), 37.5 mM putrescine (D13208, Aldrich Chemical Co., Milwaukee, WI), and 0.25% transglutaminase (w:v; T5398, Sigma Chemical Co., St. Louis, MO). Plates were removed from the incubator and washed twice with PBS-Tween 80, twice with deionized water, and once with 100 mM Tris-HCl.

To each plate was added 100 μ L/well 100mM Tris-HCl containing 5 mM calcium chloride, 10 mM dithiothreitol, 0.75 μ g/mL biotinylated casein, and 0.5% transglutaminase (w:v). In addition, 10 μ L/well of Tris-HCl, DMSO, 1:200 goat anti-transglutaminase antibody (T7066, Sigma Chemical Co., St. Louis, MO), NCZ, DNC, or HDP were added to the appropriate wells. The NCZ and DNC solutions consisted of either 1, 2, 4, or 8 μ g NCZ or DNC/10 μ L DMSO, and the HDP solutions consisted of either 1, 2, 4, or 8 μ g HDP/10 μ L water. All NCZ, DNC, and HDP solutions were made just prior to starting the assay. Each plate had 6 wells per treatment group. Wells containing only Tris-HCl were used as negative controls. Plates were incubated for 1 h at 37°C. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 100 mM Tris-HCl.

A 1:625 dilution of extravidin peroxidase (E2886, Sigma Chemical Co., St. Louis, MO) was added to each well (100 μ L/well), and plates were incubated at 37°C for 45 min. Plates were washed twice with PBS-Tween 80, twice with deionized water, and once with 0.05M phosphate-citrate buffer (pH 5.0; P9305, Sigma Chemical Co., St. Louis, MO) containing 0.014% hydrogen peroxide.

A 3,3',5,5'-tetramethylbenzidine (T3405, Sigma Chemical Co., St. Louis, MO) solution was made by dissolving one 3,3',5,5'-tetramethylbenzidine tablet per 10 mL 0.05M phosphate-citrate buffer. The 3,3',5,5'-tetramethylbenzidine solution was added to each well (100 μ L/well) and color was allowed to develop. The reaction was terminated after 2 to 3 min using 100 μ L/well 2M sulfuric acid. Plates were read at 450 nm on an Ultramark Microplate Imaging System (170-9500, Bio-Rad Laboratories, Hercules, CA). The experiment was replicated 5 times, with 1 plate per replication.

Calcium Channel Assay

Bull sperm were used as a model for this experiment because sperm contain L-type calcium channels similar to those found in avian follicular cells (Schwartz et al., 1989; Goodwin et al., 2000). In addition, millions of sperm can be obtained without extensive purification, which can alter a cell's membrane function. A large influx of intracellular calcium through calcium channels occurs in sperm during capacitation, and this influx can be induced in vitro by incubating sperm with progesterone (Kobori et al., 2000). The influx of intracellular calcium can be monitored using flow cytometry.

Bull tyrodes solution was made by dissolving 5.69 g sodium chloride (S7653, Sigma Chemical Co., St. Louis, MO), 0.23 g potassium chloride (P3911, Sigma Chemical Co., St. Louis, MO), 0.04 g sodium phosphate (S0876, Sigma Chemical Co., St. Louis,

MO), 2.09 g sodium bicarbonate (S5761, Sigma Chemical Co., St. Louis, MO), 0.29 g calcium chloride dehydrate (C5080, Sigma Chemical Co., St. Louis, MO), and 0.08 g magnesium chloride hexahydrate (M2670, Sigma Chemical Co., St. Louis, MO) in nanopure water. Bull Tyrode's albumin-lactate-pyruvate diluent (TALP) was made by dissolving 0.0022 g sodium pyruvate (P2256, Sigma Chemical Co., St. Louis, MO), 0.368 mL sodium lactate (L1375, Sigma Chemical Co., St. Louis, MO), 0.09 g glucose (G7528, Sigma Chemical Co., St. Louis, MO), 0.238 g HEPES (H3375, Sigma Chemical Co., St. Louis, MO), and 0.3g BSA (A2153, Sigma Chemical Co., St. Louis, MO) in 100 mL bull tyrodes solution.

Bull sperm were diluted to 50×10^6 cells/mL in bull TALP, and 2 mL of the diluted sperm were added to each sample tube. Sperm in all the sample tubes except the control tubes were stained with 10 μ M Fluo-3 AM (an intracellular calcium indicator; F1241, Invitrogen, Carlsbad, CA) and 5 μ M propidium iodide (a stain to detect dead cells; P1304MP, Invitrogen, Carlsbad, CA). There were 3 control tubes consisting of Fluo-3 AM stain only, propidium iodide stain only, and both stains.

To each sample tube, 20 μ L of either DMSO, 80 μ M nifedipine (calcium channel inhibitor; N7634, Sigma Chemical Co., St. Louis, MO), 4.75 μ M A23187 (calcium ionophore; C5149, Sigma-Aldrich, St. Louis, MO), NCZ, DNC, or HDP solutions were added. The NCZ solutions consisted of 1, 2, 4, or 8 μ g NCZ/20 μ L DMSO. The DNC solution consisted of 8 μ g DNC/20 μ L DMSO. The HDP solution consisted of 8 μ g HDP/20 μ L water.

Tubes were mixed briefly using a vortex mixer, then incubated for 20 min at 23°C in the dark. A 0.5 mL subsample was analyzed on an Epics V flow cytometer (Coulter

Electronics, Miami, FL), with the argon laser tuned to 488 nm to excite both Fluo-3 AM and propidium iodide. The filter setup included a 457-505 nm laser blocker; a 550 nm dichroic beam splitter; a 525-560 nm band-pass filter to detect Fluo-3 AM; and a 610 nm long-pass filter to detect propidium iodide. To the remainder of the samples was added 800 μ L of 40 μ M progesterone in DMSO. The samples were mixed briefly on a vortex mixer, and then were incubated at 37°C for 1 h. Subsamples (0.5 mL) were taken every 15 min during the hour of incubation for analysis on the flow cytometer. This experiment was replicated 5 times.

Statistical Analysis

Lipoprotein Lipase Assay. The absorbance value for the blank test tube was subtracted from the absorbance for all other tubes in the same time period. The difference between the absorbance of the LL positive control and the DMSO control was subtracted from all test tubes containing DMSO in the same time period. The difference between the absorbance of the LL positive control and the EGME control was subtracted from all test tubes containing EGME in the same time period. The adjusted absorbances were used to standardize the data by calculating a percent of the LL positive control. Absorbances for each tube were divided by the absorbance for the LL positive control in the same time period and the result was multiplied by 100 to obtain a percent of the positive control. The standardized percentages were analyzed as a mixed effects model (PROC MIXED; SAS Institute, Inc., 2003), and significance was defined as $P \leq 0.05$. Means separations were carried out using PDMIX800 (Saxton, 1998).

Vitellogenin Phosphorylation Assay. To obtain a mean background value for each gel, the mean optical density of the area of the gel corresponding to the VTG band was

averaged for the non-laying chicken plasma and PeppermintStick standard lanes. The mean background value was subtracted from the mean optical density of the VTG bands for each gel to create an adjusted density. The adjusted density for each VTG band was compared to the adjusted density of the VTG band for laying chicken plasma on the same gel to obtain a percentage of the control. The percentages of the control were analyzed by ANOVA (PROC GLM; SAS Institute, Inc., 2003), and significance was defined as $P \leq 0.05$. Means were separated using the least significant difference (LSD; SAS Institute, Inc., 2003).

Transglutaminase Assay. The absorbances of the blank wells were averaged and the mean absorbance was subtracted from the absorbance of each well to eliminate background fluorescence. For each plate, all 6 wells in each treatment group were averaged. The average absorbance for each treatment group was divided by the average absorbance for the DMSO control group for that plate. The result was multiplied by 100 to obtain a percentage of the DMSO control. The percentages of the DMSO control were analyzed by ANOVA (PROC GLM; SAS Institute, Inc., 2003), and significance was defined as $P \leq 0.05$. Means were separated using the least significant difference (LSD; SAS Institute, Inc., 2003).

Calcium Channel Assay. Data were standardized by calculating a percent of the DMSO control for the percent of cells with low intracellular calcium, the percent of cells with high intracellular calcium, and the percent of dead cells. The percent of cells with low intracellular calcium in each group was divided by the percent of cells with low intracellular calcium in the DMSO control group for the same time period. The result was multiplied by 100 to obtain a percentage of the DMSO control. The same procedure

was used to calculate a percent of the DMSO control for the percent of cells with high intracellular calcium and the percent of dead cells. The standardized percentages were analyzed as a mixed effects model (PROC MIXED; SAS Institute, Inc., 2003), and significance was defined as $P \leq 0.05$. Means separations were carried out using PDMIX800 (Saxton, 1998).

RESULTS

Nicarbazin significantly increased LL activity (Table 2.1). However, LL activity decreased over time ($P < 0.0001$, $n = 30$), with most of the change occurring during the first 3 min of incubation. A significant treatment by period interaction also existed ($P < 0.0001$, $n = 30$). Changes in LL activity during the remainder of the incubation period were slight. Both AA861 and NDGA inhibited LL activity, giving 100% and 89% inhibition, respectively.

Vitellogenin phosphorylation differed among treatments (Table 2.2). Whereas, DMSO decreased the amount of VTG phosphorylation by 19.5% as compared to the control, NCZ and DNC were not significantly different from the control or from DMSO. Although treatment with 1, 2, and 4 μg HDP decreased the amount of VTG phosphorylation as compared to the control, they were not different from DMSO.

Transglutaminase activity also differed among treatments (Table 2.3). While HDP tended to increase the activity of TG by 30 to 40%, both NCZ and DNC tended to decrease the activity of TG by 30 to 61.5%. The anti-TG antibody inhibited TG activity by 66%.

As shown in Table 2.4, there was a significant treatment effect on the percentages of cells having high intracellular calcium ($P = 0.0360$). There was no effect on the

percentages of cells having low intracellular calcium ($P = 0.4737$). The percentage of cells having low intracellular calcium tended to increase over time ($P < 0.0001$), whereas the percentage of cells having high intracellular calcium tended to decrease over time ($P < 0.0001$). There was a significant treatment by time interaction for the percentage of cells having high intracellular calcium ($P < 0.0001$; Table 2.4). Nifedipine and A23187 induced the highest percentages of dead cells ($P < 0.0001$). The percentage of dead cells in the NCZ, DNC, and HDP groups were not different from the controls with and without DMSO. As expected, the percentage of dead cells increased over time ($P < 0.0001$).

DISCUSSION

Nicarbazin increased the activity of LL in vitro in the 4 and 8 μg treatment groups. The total assay volume in each test tube was 1.025 mL, giving a concentration of 3.9 $\mu\text{g/mL}$ and 7.8 $\mu\text{g/mL}$ in the 4 and 8 μg NCZ treatment groups respectively. The entire NCZ molecule has a molecular weight of 426.38, whereas the DNC portion has a molecular weight of 292.25 (Wells, 1999), which is 68.5% of the NCZ molecule. Therefore, 4 μg NCZ contains 2.74 μg DNC and 8 μg NCZ contains 5.48 μg DNC. The concentration of DNC in the assay was therefore 2.67 and 5.35 $\mu\text{g/mL}$ in the 4 and 8 μg NCZ treatment groups respectively. These values are within the range expected in the plasma of waterfowl fed NCZ treated bait at 31 to 49 mg/kg BW NCZ. A study of mallards fed at these dose levels showed peak plasma DNC levels were 2.7 to 5.4 $\mu\text{g/mL}$ (Chapter 4).

Chickens fed 400 mg NCZ/kg feed had peak plasma DNC levels of approximately 3 $\mu\text{g/mL}$ and a 71% reduction in egg production (Ott et al., 1956). Several studies found feeding 125 mg NCZ/kg feed reduced egg production significantly (Baker et al., 1957;

McLoughlin et al., 1957; Jones et al., 1990c). A comparative gavage study showed treatment of chickens with NCZ at 125 ppm produced a peak plasma DNC level of 2.9 µg/mL (Yoder et al., 2005). These plasma DNC levels are comparable to the concentrations used in the in vitro assay.

The increased activity of LL due to NCZ likely causes premature degradation of VLDL while in the blood, resulting in a decrease of lipid being deposited into the yolk, thereby decreasing overall egg weight and production. Baker et al. (1957) suggested NCZ may prevent ova from maturing. Necropsy of hens fed a ration containing 90 ppm NCZ revealed that the largest follicle was absent with no signs of recent ovulation or atresia (Baker et al., 1957). Luck (1979) also found ovaries without a follicular hierarchy, and less well-developed oviducts in laying hens treated with 375 ppm NCZ in feed.

Nicarbazin did not affect luteinizing hormone levels or pituitary responsiveness to luteinizing hormone releasing hormone, but decreased the sensitivity of the chicken hypothalamus to exogenous progesterone (Luck, 1979). Luck (1979) suggested that egg production is decreased because yolk deposition in the follicles is prevented. White Leghorns fed 400 to 700 ppm NCZ in feed exhibited reduced egg production concomitant with a two fold rise in plasma cholesterol concentrations, supporting Luck's hypothesis (Weiss, 1979). These studies support our conclusion that the increased LL activity due to NCZ causes premature degradation of VLDL. Future studies should investigate the effect of NCZ treatment on the activity of LL in vivo.

Although a statistically significant effect of NCZ on phosphorylation of VTG was found, this effect is probably not biologically significant. The total assay volume of

plasma plus treatment was 0.11 mL. If DNC comprises 68.5% of the NCZ molecule, then HDP must comprise 31.5% of the NCZ molecule. Using these figures, the concentrations of DNC and HDP in this assay ranged from 9.1 to 72.7 $\mu\text{g}/\text{mL}$ in the DNC and HDP groups. The concentration of DNC in the NCZ groups ranged from 6.2 to 49.8 $\mu\text{g}/\text{mL}$, and the concentration of HDP in the NCZ groups ranged from 2.8 to 22.9 $\mu\text{g}/\text{mL}$. This is many times higher than what would be expected in plasma. A decrease in VTG phosphorylation was caused by DMSO by itself. Although the NCZ and DNC groups had VTG with a greater degree of phosphorylation than the DMSO group, they were not significantly different from the DMSO group. The HDP group appeared to have no effect except at the 8 μg level.

There was a very large amount of VTG on the gels which might make it difficult to detect small changes in phosphorylation. The assay could be rerun with plasma diluted at least 1:1000 in PBS. However, such small changes would not likely be biologically significant. A more appropriate experiment would be to treat laying hens with NCZ and compare the phosphorylation of VTG from the plasma of treated and control hens.

Nicarbazin had an inhibitory effect on transglutaminase in vitro. The portion of the NCZ molecule that appears to be responsible for this effect is DNC. Both DNC and NCZ decreased transglutaminase activity compared to the DMSO control, whereas HDP increased transglutaminase activity.

The total assay volume used in each well was 0.11 mL. Again, the concentrations of DNC and HDP ranged from 9.1 to 72.7 $\mu\text{g}/\text{mL}$ in the DNC and HDP groups, much higher than what would be expected in plasma. The concentration of DNC in the NCZ groups ranged from 6.2 to 49.8 $\mu\text{g}/\text{mL}$, and the concentration of HDP in the NCZ groups

ranged from 2.8 to 22.9 $\mu\text{g}/\text{mL}$. The same inhibitory effect might not occur at lower levels. Only the 4 and 8 μg NCZ and DNC groups produced a decrease in transglutaminase activity similar to the anti-transglutaminase antibody. We chose to use the higher concentrations of NCZ, DNC, and HDP for this experiment due to the difficulty of accurately measuring such small quantities of NCZ, DNC, and HDP.

The total assay volume used in the calcium assays was 2.82 mL. The concentrations of DNC used ranged from 0.2 to 1.9 $\mu\text{g}/\text{mL}$ in the NCZ groups, and was 2.8 $\mu\text{g}/\text{mL}$ in the DNC group. The concentrations of DNC in the DNC and 8 μg NCZ groups are comparable to what is expected in plasma, whereas the concentrations in the remaining NCZ groups are lower than expected plasma values. The concentrations of HDP ranged from 0.1 to 0.9 $\mu\text{g}/\text{mL}$ in the NCZ groups, and 2.8 $\mu\text{g}/\text{mL}$ in the HDP group. The concentration of HDP in the 8 μg NCZ group is close to expected plasma values, whereas the concentrations in the remaining NCZ groups are lower than expected. The concentration in the HDP group is higher than expected. Wells (1999) reported a range of HDP concentrations from 1.07 to 2.07 $\mu\text{g}/\text{mL}$ in chickens fed 125 ppm NCZ for 7 d.

The effects of NCZ on intracellular calcium levels occurred within the first 15 min of incubation. No significant effects on intracellular calcium levels were observed after 30 min of incubation. The HDP group consistently had a greater percentage of sperm cells with high intracellular calcium than the DMSO control, indicating it is acting as an ionophore. However, as has already been pointed out, the concentration of HDP used in that group is slightly higher than expected plasma values. Fifteen minutes after the addition of progesterone, the NCZ groups had a higher percentage of cells with high intracellular calcium than the DMSO control. The percentage of cells with high

intracellular calcium was comparable in the NCZ and HDP groups. Because the concentrations of HDP in the NCZ groups were lower than expected plasma values, it seems reasonable to conclude that NCZ acts as an ionophore. The portion of the NCZ molecule responsible for ionophore activity is HDP. As compared to the DMSO control, the DNC group had comparatively fewer sperm cells with high intracellular calcium, indicating it may act as a weak calcium channel blocker.

The apparent activity of NCZ as an ionophore may help explain damage to the vitelline membrane in NCZ treated hens that lead to egg yolk mottling and a reduction in egg hatchability. As an ionophore, NCZ could insert itself into the vitelline membrane, making the membrane more permeable. Evidence that vitelline membranes from NCZ treated hens are more permeable was shown by Cunningham (1976, 1977). Cunningham (1976, 1977) found mottled yolks from NCZ treated hens exhibited a decrease in yolk solids, and an increase in egg albumen. The percentages of fat, protein, ash, calcium, phosphorus, and iron are also reduced in mottled yolks (Cunningham, 1976), but these components increased in the egg albumen (Cunningham, 1977). Mottled yolks also contain the egg white proteins ovalbumin and conalbumin (Cunningham, 1976). In addition, vitelline membranes from NCZ treated hens show degeneration at the microscopic level (Chapter 3).

Although these assays examined the effects of NCZ in vitro, they provide some clues as to the mechanism by which NCZ affects reproduction. One of the main effects of NCZ on reproduction is to increase the activity of LL, thereby decreasing the amount of VLDL deposited into the follicle. The other main effect is the activity of NCZ as an ionophore to increase the permeability of the vitelline membrane. These assays should be

viewed as preliminary studies to aid in directing further research on the effect of NCZ on reproduction in vivo.

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Table 2.1. Effect across time periods of addition of 10 μ L of either 1, 2, 4, or 8 μ g/10 μ L nicarbazin (NCZ) in DMSO, lipoprotein lipase (LL) inhibitors AA861 (0.03M) and nordihydroguaiaretic acid (NDGA; 0.1M) in ethylene glycol monomethyl ether (EGME), or DMSO to test tubes containing 1 mL dibutylfluorescein (DBF) and 15 μ g LL on LL activity in vitro after 1, 3, 5, 7, 9, and 11 min of incubation at 37°C.

Treatment	<i>n</i>	Mean percent of DMSO control ¹
DMSO control	30	100.0 ^c
0.03M AA861	30	-17.7 ^c
0.1M NDGA	30	10.9 ^d
1 μ g NCZ	30	107.3 ^c
2 μ g NCZ	30	108.6 ^c
4 μ g NCZ	30	169.4 ^b
8 μ g NCZ	30	233.4 ^a
SEM	8.4	

¹Means within the column with different subscripts are significantly different ($P \leq 0.05$).

Table 2.2. Effect of addition of 10 μ L of either 1, 2, 4, or 8 μ g/10 μ L nicarbazin (NCZ) in DMSO, 1, 2, 4, or 8 μ g/10 μ L 4,4'-dinitrocarbanilide (DNC) in DMSO, 1, 2, 4, or 8 μ g/10 μ L 4,6-dimethylpyrimidine (HDP) in water, or DMSO to 100 μ L chicken plasma on phosphorylation of vitellogenin. Plasma was diluted 1:100 in PBS and proteins were separated on a 4-20% Tris-glycine minigel for 90 min at 150V. Phosphoproteins were stained with Pro-Q Diamond phosphoprotein stain (33300, Molecular Probes, Eugene, OR).

Treatment	<i>n</i>	Mean percent of laying hen control ¹
Laying control	12	100.0 ^a
DMSO	5	80.5 ^{b, c, d}
1 μ g NCZ	5	93.6 ^{a, b}
2 μ g NCZ	5	86.3 ^{a, b, c, d}
4 μ g NCZ	5	92.6 ^{a, b, c}
8 μ g NCZ	5	89.3 ^{a, b, c}
1 μ g DNC	5	83.3 ^{b, c, d}
2 μ g DNC	5	88.0 ^{a, b, c, d}
4 μ g DNC	5	90.6 ^{a, b, c}
8 μ g DNC	5	92.9 ^{a, b, c}
1 μ g HDP	5	79.9 ^{b, c, d}
2 μ g HDP	5	79.2 ^{c, d}
4 μ g HDP	5	75.3 ^d
8 μ g HDP	5	93.1 ^{a, b, c}
SEM	4.9	

¹Means within the column with different subscripts are significantly different ($P \leq 0.05$).

Table 2.3. Effect of addition of 10 μ L of either 1, 2, 4, or 8 μ g/10 μ L nicarbazin (NCZ) in DMSO, 4,4'-dinitrocarbanilide (DNC) in DMSO, 4,6-dimethylpyrimidine (HDP) in water, 1:200 goat anti-transglutaminase antibody in PBS, or DMSO to microtiter plates containing 100 μ L/well Tris-HCl solution consisting of 5 mM calcium chloride, 10 mM dithiotheitol, 0.75 μ g/mL biotinylated casein, and 0.5% transglutaminase (w:v) on transglutaminase (TG) activity in vitro.

Treatment	<i>n</i>	Mean percent of DMSO control ¹
DMSO control	5	100.0 ^b
Anti-TG antibody	5	33.6 ^c
1 μ g NCZ	5	70.2 ^c
2 μ g NCZ	5	55.6 ^{c, d}
4 μ g NCZ	5	38.5 ^{d, e}
8 μ g NCZ	5	43.2 ^{d, e}
1 μ g DNC	5	65.0 ^c
2 μ g DNC	5	55.1 ^{c, d}
4 μ g DNC	5	51.0 ^{c, d, e}
8 μ g DNC	5	51.9 ^{c, d, e}
1 μ g HDP	5	141.1 ^a
2 μ g HDP	5	131.2 ^a
4 μ g HDP	5	132.4 ^a
8 μ g HDP	5	135.2 ^a
SEM	7.2	

¹Means within the column with different subscripts are significantly different ($P \leq 0.05$).

Table 2.4. Effect of addition of 20 μ L of either 1, 2, 4, or 8 μ g/20 μ L nicarbazin (NCZ) in DMSO, 8 μ g/20 μ L 4,4'-dinitrocarbanilide (DNC) in DMSO, 8 μ g/20 μ L 4,6-dimethylpyrimidine (HDP) in water, 80 μ M nifedipine (calcium channel inhibitor), 4.75 μ M A23187 (calcium ionophore), or DMSO to test tubes containing 2 mL bull sperm in TALP (50×10^6 cells/mL) stained with 10 μ M Fluo-3 AM and 5 μ M propidium iodide on the percentage of sperm cells having high intracellular calcium 15, 30, 45, and 60 min after the addition of 800 μ L 40 μ M progesterone and incubation at 37°C.

Treatment	0 minutes		15 minutes		30 minutes		45 minutes		60 minutes	
	<i>n</i>	mean ^{1, 2}	<i>n</i>	mean ^{1, 2}	<i>n</i>	mean ^{1, 2}	<i>n</i>	mean ^{1, 2}	<i>n</i>	mean ^{1, 2}
DMSO control	5	100.0 ^{defghijk}	5	100.0 ^{defghijk}	5	100.0 ^{defghijk}	5	100.0 ^{defghijk}	5	100.0 ^{defghijk}
Nifedipine	5	97.8 ^{defghijk}	5	60.0 ^{jk}	5	63.2 ^{jk}	5	56.7 ^{jk}	4	53.1 ^{jk}
A23187	5	237.5 ^b	5	297.5 ^a	5	132.4 ^{cdefgh}	5	94.9 ^{defghijk}	4	43.6 ^k
1 μ g NCZ	5	143.3 ^{cdf}	5	113.1 ^{cdefghij}	5	103.4 ^{cdefghijk}	5	91.7 ^{eghijkl}	5	108.5 ^{cdefghilk}
2 μ g NCZ	5	142.7 ^{cdef}	5	127.2 ^{cdefghi}	5	99.9 ^{defghijk}	5	94.3 ^{defghijk}	5	118.3 ^{cdefghij}
4 μ g NCZ	5	134.9 ^{cde}	5	84.3 ^{fghijkl}	5	92.6 ^{defghijk}	5	77.5 ^{ghijk}	5	107.8 ^{cdefghijk}
8 μ g NCZ	5	131.9 ^{cdefg}	5	112.8 ^{cdefghij}	5	94.4 ^{defghijk}	5	71.5 ^{hijk}	5	92.4 ^{defghijk}
8 μ g DNC	4	114.0 ^{cdefghij}	4	86.1 ^{defghijk}	4	65.0 ^{ijk}	4	79.1 ^{defghijk}	4	93.7 ^{defghijk}
8 μ g HDP	4	167.5 ^c	4	114.1 ^{cdefghij}	4	106.6 ^{defghijk}	4	103.4 ^{defghijk}	4	103.3 ^{defghijk}
SEM		22.5								

¹Means are percent of the DMSO control.

²Means within columns with the different subscripts are significantly different ($P \leq 0.05$).

CHAPTER 3

GAVAGE LABORATORY STUDY

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 induces 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 while keeping 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 treat and 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). Nicarbazine 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 pigmentation of the egg shell (McLoughlin et al., 1957; Jones et al., 1990a; 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 produce more eggs than Canada geese if 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, degree of yolk mottling, and 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 McQuiston, 1980), we also assessed the effect of NCZ on bird health by measuring bird weight, hematocrit, oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), bicarbonate levels (HCO_3^-), saturated oxygen

levels (sO_2), blood pH, 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.

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 NCZ (Phibro Animal Health, Inc., Fairfield, NJ), 2) 125 ppm NCZ (8.5 mg/kg BW), 3) 250 ppm NCZ (17.0 mg/kg BW), and 4) 500 ppm NCZ (33.75 mg/kg 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 BW would be necessary to achieve contraceptive effects (Yoder et al., 2005). Doses were formulated based on a 1.0 kg 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 fluorescein per 15 mg 25% NCZ. Capsules in the 125 ppm group

contained 8.5 mg pure NCZ and 0.11 mg fluorescein per capsule. Capsules in the 250 ppm group contained 17.0 mg pure NCZ and 0.23 mg fluorescein per capsule. Capsules in the 500 ppm group contained 33.75 mg pure NCZ and 0.45 mg 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 post-treatment, then once every 3 d until 14 d post-treatment. 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 remaining blood was placed on ice (approximately 30 to 45 min) until blood gas analysis of a 0.2 mL subsample using a Radiometer ABL5 (Radiometer America, Inc., Westlake, OH). 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 cm²) 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 & Johnston, 2002). Fluorescein analysis was performed by homogenizing 1 g fecal material in 2 mL 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 d 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) post-treatment 1 (1 to 3 d post-treatment), and 5) post-treatment 2 (4 to 14 d post-treatment). 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., 2003), and significance was defined as $P \leq 0.05$ for all analyses. Data were analyzed for treatment, time period, and treatment by 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, hematocrit, blood pH, pCO₂, pO₂, sO₂, or HCO₃⁻.

RESULTS

Plasma and egg DNC levels differed among treatment groups and time periods (Table 3.1), 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 $\mu\text{g/mL}$ 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 $\mu\text{g/g}$ 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 $P = 0.1918$ respectively) or time periods ($P = 0.2484$ and $P = 0.0742$ 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 3.2), but no treatment effect ($P = 0.6579$). Egg shell 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 post-treatment period 1. Egg weights differed among time periods with eggs weighing more during the post-treatment 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 no significant difference among treatment groups ($P = 0.2308$, $P = 0.7907$, $P = 0.3409$, and $P = 0.9100$ respectively), but there was a significant period

effect for bird weights, pO_2 , HCO_3^- , and hematocrit (Table 3.3). 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 and treatment group effects for pCO_2 and sO_2 (Table 3.3, Table 3.4). There was a significant treatment group effect for pH, but there were no significant period effects ($P = 0.2817$; Table 3.4). Bird weights were lower during the treatment period. Hematocrit, pO_2 , and sO_2 tended to increase over time, with the highest levels occurring during the post-treatment periods. Bicarbonate (HCO_3^-) levels decreased during the treatment period, returning to pretreatment levels during post-treatment. The lowest levels of pCO_2 occurred during pretreatment, and the highest levels occurred during treatment period 1.

There were significant period effects for eosinophil, basophil, heterophil, and monocyte counts, and H:L ratios (Table 3.5), but means did not differ among treatment groups ($P = 0.1075$, $P = 0.3554$, $P = 0.9755$, $P = 0.7484$, and $P = 0.8633$, respectively). Eosinophil and monocyte counts were lowest during both treatment periods, whereas basophil counts were lowest only during treatment period 1. Heterophil counts and H:L ratios were lowest during pretreatment. Mean lymphocyte counts did not differ among treatment groups ($P = 0.9675$) or time periods ($P = 0.2085$). Period effects were significant whether the control group was included in the analysis or not.

Plasma DNC levels were positively correlated with egg DNC levels during treatment periods 1 and 2 and post-treatment periods 1 and 2, and with fecal DNC levels during treatment period 2 (Table 3.6). 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 3.7). Plasma DNC was positively correlated with fluorescence during treatment periods 1 and 2 and post-treatment 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 3.8). 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 3.9). Blood pH and HCO_3^- were negatively correlated with plasma DNC at 250 and 500 ppm. At 500 ppm, pCO_2 was positively correlated with plasma DNC and sO_2 was negatively correlated with plasma DNC. At 250 ppm, pCO_2 was positively correlated with egg DNC.

The outer and inner vitelline membranes (Figure 3.1) 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 post-treatment. 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 3.1). 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 post-treatment.

DISCUSSION

When ducks were fed increasing amounts of NCZ, DNC levels in blood plasma, eggs, and feces exhibited a dose response relationship (Figure 3.2). Although there was little difference between 125 (8.5 mg/kg BW) and 250 ppm (17.0 mg/kg BW) in resulting plasma DNC levels, DNC levels for birds given 500 ppm NCZ (33.75 mg/kg BW) were significantly higher. The peak DNC level at 500 ppm was 2.97 $\mu\text{g}/\text{mL}$ in blood plasma during treatment period 1. Previous studies reported a minimum plasma level of 2.9 $\mu\text{g}/\text{mL}$ is needed to observe reproductive effects in chickens (Jones et al., 1990a; Yoder et al., 2005). Results from this study show that a minimum level of 500 ppm (33.75 mg/kg 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, while peak egg DNC levels were not observed until the second week of treatment (Figure 3.3). Plasma DNC was nearly undetectable by 3 d post-treatment, whereas egg DNC levels did not fall below 1 $\mu\text{g}/\text{g}$ until 14 d post-treatment (Figure 3.3). 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 to achieve 90% power, sample sizes would need to be 4 to 6 times larger than those in this study. 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 to 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 feed) adversely affect reproduction (Jones et al. 1990b). Feeding 25 ppm (25 mg/kg 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 BW).

The decrease in hatchability in the 500 ppm group may partly be due to a change in pH gradient across the vitelline membrane. Nicarbazine causes egg yolk to become more alkaline (Baker et al. 1956, Cunningham 1976), but albumen pH remains unchanged (Cunningham 1977). During natural egg aging, yolk pH changes little but albumen pH changes from 7.6 to approximately 9.5 (Lapão et al. 1999, Silversides and Budgell 2004). It has been postulated that in order for embryos to develop, a pH gradient must exist across the vitelline membrane with the albumen being alkaline and the yolk neutral to slightly acidic (Gillespie and McHanwell 1987, Meuer and Baumann 1988, Meijerhof

1994). Without this gradient, normal embryogenesis does not occur (Benton and Brake 1996). The change of egg yolk to an alkaline pH with NCZ treatment is likely due to the DNC component which is present in yolk (Cannavan et al. 2000).

On average, fertility decreased by 13% overall in all the 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 feed to 700 mg/kg 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 ppm NCZ in feed (100 mg/kg feed) (Jones et al. 1990b, 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 treatment period 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 feed) NCZ in feed (Jones et al. 1990a, Jones et al. 1990b, 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. (1990a) found 125 ppm NCZ in feed (125 mg/kg 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 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 nicarbazin in the feed (Polin et al. 1957, Jones et al. 1990a). Additionally, storage time exacerbates the mottling effect (Baker et al. 1957, Silvestrini et al. 1965, Polin et al. 1958). 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 NCZ in feed (125 mg/kg 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 1 inner vitelline 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.

Age induced changes in albumen include decreased lysozyme content (Evans et al. 1958), decreased albumen height and viscosity (Lapão et al. 1999, Silversides and Budgell 2004), and increased pH (Lapão et al. 1999, Tona et al. 2002, Silversides and Budgell 2004). Egg white thinning due to aging has been partly contributed to a change in the interaction of lysozyme and ovomucin (Cotterill and Winter 1955).

Several hypotheses have been advanced to explain thinning of egg white with aging. These include a dissociation of lysozyme and ovomucin due to changes in pH, ionic strength, or both (Cotterill and Winter 1955, Sauveur 1976, Miller et al. 1982). Breakdown of egg white during aging may also be due to reduction of disulfide bonds in ovomucin (MacDonnell et al. 1951, Beveridge and Nakai 1975), or disturbance of *O*-linked glycoside bonds (Kato et al. 1979). Similar mechanisms could be occurring in the vitelline membrane, causing it to become more permeable as suggested by Feeny et al. (1956).

Both albumen and the vitelline membrane contain ovomucin and lysozyme (Burley and Vadehra 1989). Ovomucin is responsible for the gel-like structure of thick egg white (Hayakawa et al. 1983) and provides a framework for other proteins in the outer vitelline membrane (Burley and Vadehra 1989). Lysozyme plays a structural role in albumen with the highest concentrations in fresh eggs occurring in thick whites (Kato et al. 1978), and comprises 60% of the outer vitelline membrane (Back et al. 1982). Either HDP or a DNC

metabolite could be disrupting the disulfide or *O*-linked glycoside bonds, or less likely, the ionic strength of the albumen. Although HDP has been detected in albumen, DNC itself has not (Cannavan et al. 2000). However, no studies have been performed to determine whether DNC metabolites are compartmentalized in the egg and where they might be located.

Period effects were noted in egg production, shell thickness, and egg mottling across treatment groups. This may 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 treatment period 2. Egg production increased in post-treatment period 1 in all groups, and continued to increase during post-treatment period 2 in the 500 ppm group. Egg production decreased during post-treatment 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 post-treatment 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). Egg shell thickness also decreased in all treatment groups, reaching the lowest thickness during post-treatment period 1. Yolk mottling scores increased until post-treatment 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).

Additional period effects were noted in hematocrit and the blood gas parameters (pO_2 , HCO_3^- , pCO_2 , and sO_2) across treatment groups. Hematocrit, pO_2 , and sO_2 tended

to increase over time, and $p\text{CO}_2$ was generally lowest during pretreatment and then increased over time. Bicarbonate levels decreased until treatment period 2 and then began to increase. Although it has been suggested that HCO_3^- may be related to shell strength, no strong correlations between HCO_3^- and shell strength have been found (Hamilton and Thompson, 1980; Hamilton 1981). It is possible that a metabolic alkalosis was occurring, but this seems unlikely because pH did not change significantly, and the effect was observed in all treatment groups. The most likely explanation for these period effects is that we became more efficient over time in transporting blood samples to the blood gas analyzer, which was about 10 min away. During pretreatment we took all blood samples at the same time to the blood gas analyzer, which meant that some samples were on ice for up to 2 hr. Subsequent to this, we brought blood samples to the analyzer in 2 or 3 groupings, which meant that samples were not on ice for more than 30 to 45 min. This may have given more accurate results because there was less time for metabolic processes to occur in the blood. Low levels of $p\text{O}_2$ and $s\text{O}_2$, and high levels of $p\text{CO}_2$ were likely observed in this study because an airtight syringe was not used for blood collection.

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. Nicarbazine 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 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 post-treatment period 1. During post-treatment period 2, DNC was undetectable in plasma and feces; DNC was $\geq 97\%$ cleared from plasma by 3 d post-treatment. 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 unhatched eggs could be collected in the field and analyzed for DNC content to give a measure of NCZ in the 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 3.4), and provided a reasonably good estimate of plasma DNC levels, but not egg DNC levels. Because egg DNC levels lag behind plasma DNC levels (Figure 3.3), 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 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.

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Table 3.1. Plasma, egg, and fecal 4,4'-dinitrocarbanilide (DNC) levels for female mallards given 0 ppm, 125 ppm (8.5 mg/kg BW), 250 ppm (17.0 mg/kg BW), or 500 ppm (33.75 mg/kg BW) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

Group	Period ¹	Plasma DNC (µg/mL) ²			Egg DNC (µg/g) ²			Fecal DNC (µg/g) ³		
		<i>n</i>	\bar{x}^4	SE	<i>n</i>	\bar{x}^4	SE	<i>n</i>	\bar{x}^4	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

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3, POST2 = post-treatment days 4 to 14.

² $P = < 0.0001$ for treatment, period, and treatment by period effects.

³ $P = 0.2195$ for treatment, $P = 0.0002$ for period, and $P = 0.2422$ for treatment by period effects.

⁴Means 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).

Table 3.2. Period effects across treatment groups for eggs laid/female per day, shell thickness, egg yolk mottling, and egg weight in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

	PRE ¹			TRT1			TRT2			POST1			POST2		
	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	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 ³	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

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3, POST2 = post-treatment days 4 to 14.

²Means within rows with different superscripts significantly different ($P < 0.05$). Means are from the LSMEANS option in PROC MIXED (SAS 9.1).

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

Table 3.3. Period effects across treatment groups for bird weights, partial pressure of oxygen (pO₂), partial pressure of carbon dioxide (pCO₂), blood pH, saturated oxygen (sO₂) and bicarbonate (HCO₃⁻) levels in female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

	PRE ¹			TRT1			TRT2			POST1			POST2		
	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE
Bird weight (g) ³	63	1005.9 ^a	15.9				126	959.8 ^b	11.2						
pO ₂ (mm Hg)	26	39.5 ^a	2.8	85	40.0 ^a	1.6	58	47.7 ^b	1.9	83	54.0 ^c	1.7	111	50.4 ^{b,c}	1.5
HCO ₃ ⁻ (mmol/L)	26	20.5 ^a	0.7	85	19.1 ^b	0.5	58	18.0 ^c	0.5	83	19.5 ^{a,b}	0.5	111	20.1 ^a	0.5
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
pCO ₂ (mm Hg)	26	73.1 ^a	3.3	85	92.6 ^b	2.0	58	80.8 ^c	2.3	83	87.4 ^d	2.0	111	85.3 ^{c,d}	1.8
sO ₂ (%)	26	45.2 ^a	3.3	85	41.8 ^a	2.0	58	56.7 ^b	2.3	83	64.1 ^c	2.0	111	60.5 ^{b,c}	1.9

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3, POST2 = post-treatment days 4 to 14.

²Means within rows with different superscripts are significantly different ($P < 0.05$). Means are from the LSMEANS option in PROC MIXED (SAS 9.1).

³Birds were only weighed once during pretreatment, and on treatment days 11 and 14.

Table 3.4. Partial pressure of carbon dioxide (pCO₂), saturated oxygen (sO₂), and blood pH by treatment group for female mallards given 125 ppm (8.5 mg/kg BW), 250 ppm (17.0 mg/kg BW), or 500 ppm (33.75 mg/kg BW) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

Group	pCO ₂ (mm Hg)			sO ₂ (%)			pH		
	<i>n</i>	\bar{x}	SE	<i>n</i>	\bar{x}	SE	<i>n</i>	\bar{x}	SE
0 ppm	87	88.1 ^a	2.8	87	52.0 ^a	2.7	87	6.99 ^a	0.05
125 ppm	90	76.8 ^b	2.7	90	59.8 ^b	2.6	90	6.85 ^b	0.04
250 ppm	101	85.1 ^a	2.6	101	53.3 ^{a,b}	2.5	101	7.00 ^a	0.04
500 ppm	86	85.3 ^a	2.7	86	49.6 ^a	2.6	86	6.94 ^{a,b}	0.04

¹Means within columns with different superscripts are significantly different ($P < 0.05$). Means are from the LSMEANS option in PROC MIXED (SAS 9.1).

Table 3.5. Period effects across treatment groups for differential counts of female mallards given nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

	PRE ¹			TRT1			TRT2			POST1			POST2		
	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	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 (%)	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	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

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3, POST2 = post-treatment days 4 to 14.

²Means within rows with different superscripts are significantly different ($P < 0.05$).

Table 3.6. 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, Colorado, USA, May to June, 2000

		Plasma DNC			Egg DNC			Fecal DNC		
		<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
Plasma DNC	TRT1 ¹				31	0.68968	< 0.0001	85	0.14727	0.1786
	TRT2				13	0.80085	0.0010	57	0.42740	0.0009
	POST1				28	0.42832	0.0230	79	0.00830	0.9421
	POST2				26	0.69378	< 0.0001			
Egg DNC	TRT1	31	0.68968	< 0.0001				32	0.55967	0.0009
	TRT2	13	0.80085	0.0010				13	0.36485	0.2203
	POST1	28	0.42832	0.0230				27	0.29918	0.1295
	POST2	26	0.69378	< 0.0001						

¹TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3, POST2 = post-treatment days 4 to 14.

Table 3.7. Correlations between fecal fluorescein fluorescence and 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, Colorado, USA, May to June, 2000

		Plasma DNC			Egg DNC			Fecal DNC		
		<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
Fluorescence	TRT1 ¹	27	0.50856	0.0068	10	0.82654	0.0032	27	0.55498	0.0027
	TRT2	33	0.65281	<0.0001	8	0.58515	0.1276	33	0.65570	<0.0001
	POST1	38	0.47184	0.0028	13	-0.07880	0.7980	38	-0.04169	0.8037
	Overall	98	0.60843	<0.0001	31	0.36513	0.0434	98	0.53327	<0.0001

¹TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = post-treatment days 1 to 3.

Table 3.8. Correlations between reproductive and egg quality parameters and 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) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

Group	Parameter	Plasma DNC			Egg DNC		
		<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
125 ppm	Eggs laid/female/day	35	-0.20626	0.2345	23	0.28289	0.1909
	Egg shell thickness	41	0.41462	0.0070	44	0.07802	0.6147
	Egg weight	41	-0.07271	0.6514	44	-0.01706	0.9125
	Mottling score ¹	41	-0.23310	0.1425	43	0.13090	0.4028
	Fertility	12	0.17481	0.5869	11	0.24359	0.4704
	Hatchability	12	0.39130	0.2084	11	0.03818	0.9113
250 ppm	Eggs laid/female/day	39	-0.16565	0.3136	23	0.16672	0.4471
	Egg shell thickness	27	0.34840	0.0749	34	-0.25511	0.1454
	Egg weight	28	-0.54232	0.0029	34	-0.13052	0.4619
	Mottling score	27	0.34351	0.0794	34	0.18166	0.3039
	Fertility	11	-0.36510	0.2696	8	-0.71261	0.0473
	Hatchability	11	-0.36510	0.2696	8	-0.71261	0.0473
500 ppm	Eggs laid/female/day	35	-0.13834	0.4281	17	0.17787	0.4946
	Egg shell thickness	23	0.70012	0.0002	31	0.18108	0.3296
	Egg weight	23	-0.51691	0.0115	31	-0.18685	0.3142
	Mottling score	23	-0.13185	0.5487	31	0.40780	0.0228
	Fertility ²	5			9	0.29445	0.4418
	Hatchability	9	-0.18274	0.6379	9	-0.42557	0.2535

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

²No correlation coefficients or *P* values for fertility and plasma DNC in the SAS output due to having no SEM for fertility.

Table 3.9. Correlations between health parameters and 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) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000

Group	Parameter ¹	Plasma DNC			Egg DNC		
		<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
125 ppm	Bird weight	14	-0.40402	0.1519	10	-0.33278	0.3474
	Blood pH	90	0.03867	0.7175	39	0.12219	0.4587
	sO ₂	90	-0.18138	0.0871	39	-0.00873	0.9579
	pCO ₂	90	0.01156	0.9139	39	0.26563	0.1022
	HCO ₃ ⁻	90	-0.12072	0.2570	39	-0.04681	0.7772
250 ppm	Bird weight	15	-0.49440	0.0610	6	-0.80802	0.0517
	Blood pH	100	-0.28903	0.0035	25	-0.15320	0.4647
	sO ₂	100	-0.12329	0.2217	25	-0.17511	0.4025
	pCO ₂	100	0.02608	0.7968	25	0.39198	0.0526
	HCO ₃ ⁻	100	-0.28172	0.0045	25	0.34835	0.0879
500 ppm	Bird weight	14	-0.28385	0.3254	5	-0.36042	0.5512
	Blood pH	85	-0.28543	0.0081	21	0.16228	0.4822
	sO ₂	85	-0.28257	0.0088	21	0.01002	0.9656
	pCO ₂	85	0.30893	0.0040	21	0.06361	0.7842
	HCO ₃ ⁻	84	-0.24180	0.0267	21	0.39996	0.0724

¹sO₂ = saturated oxygen; pCO₂ = partial pressure of carbon dioxide; HCO₃⁻ = bicarbonate.

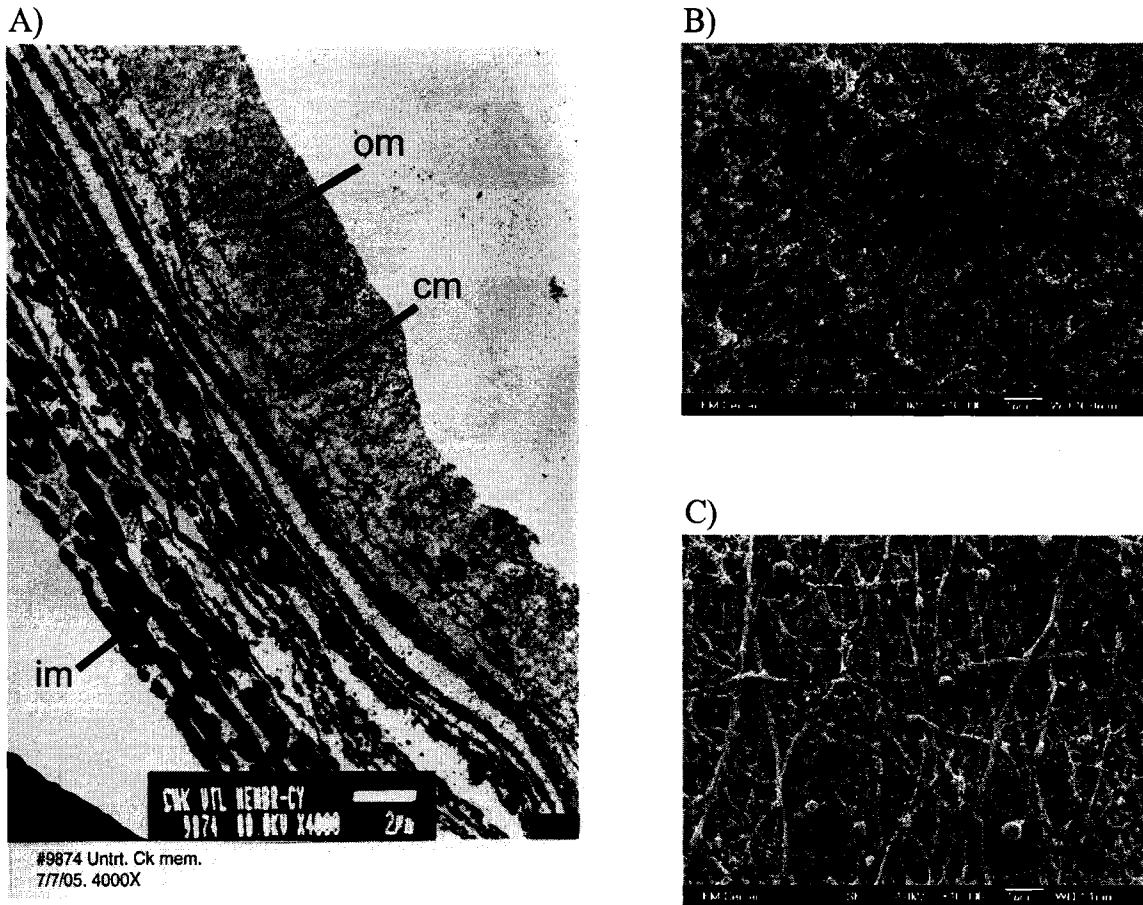


Figure 3.1. A) Transmission electron micrograph (TEM) of a fresh chicken vitelline membrane (magnification 4000X; im = inner membrane; om = outer membrane; cm = continuous membrane); B) Scanning electron micrographs (SEM) of the inner vitelline membrane from eggs laid on treatment day nine by mallards treated with 500 ppm (33.75 mg/kg BW) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000; C) Scanning electron micrographs (SEM) of the inner vitelline membranes from eggs laid on treatment day nine by control mallards at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000.

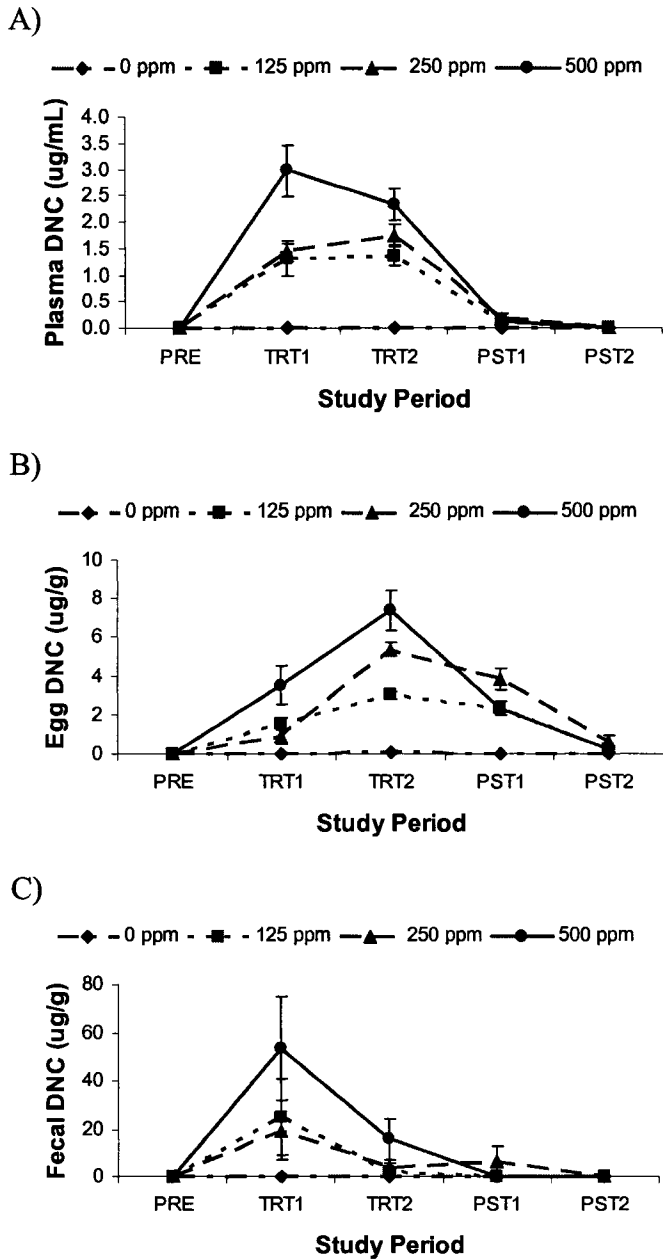


Figure 3.2. Levels of 4,4'-dinitrocarbanilide (DNC) in A) plasma, B) egg, and C) feces of female mallards given 125 ppm (8.5 mg/kg), 250 ppm (17.0 mg/kg), or 500 ppm (33.75 mg/kg) nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000. Note that PRE = 1 to 14 d pretreatment, TRT1 = 1 to 7 d treatment, TRT2 = 8 to 14 d treatment, PST1 = 1 to 3 d post-treatment, and PST2 = 4 to 14 d post-treatment.

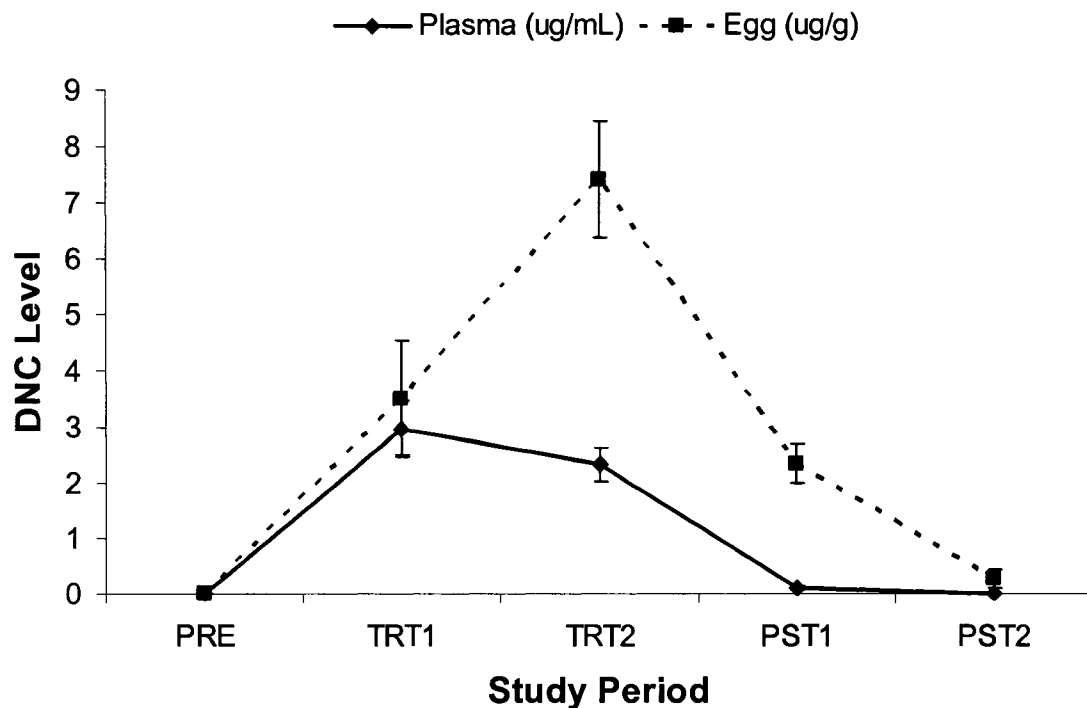


Figure 3.3. Time to peak egg and plasma 4,4'-dinitrocarbanilide (DNC) levels and time for egg and plasma 4,4'-dinitrocarbanilide (DNC) levels to return to undetectable levels in female mallards treated with 500 ppm nicarbazin perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000. Note that PRE = 1 to 14 d pretreatment, TRT1 = 1 to 7 d treatment, TRT2 = 8 to 14 d treatment, PST1 = 1 to 3 d post-treatment, and PST2 = 4 to 14 d post-treatment.

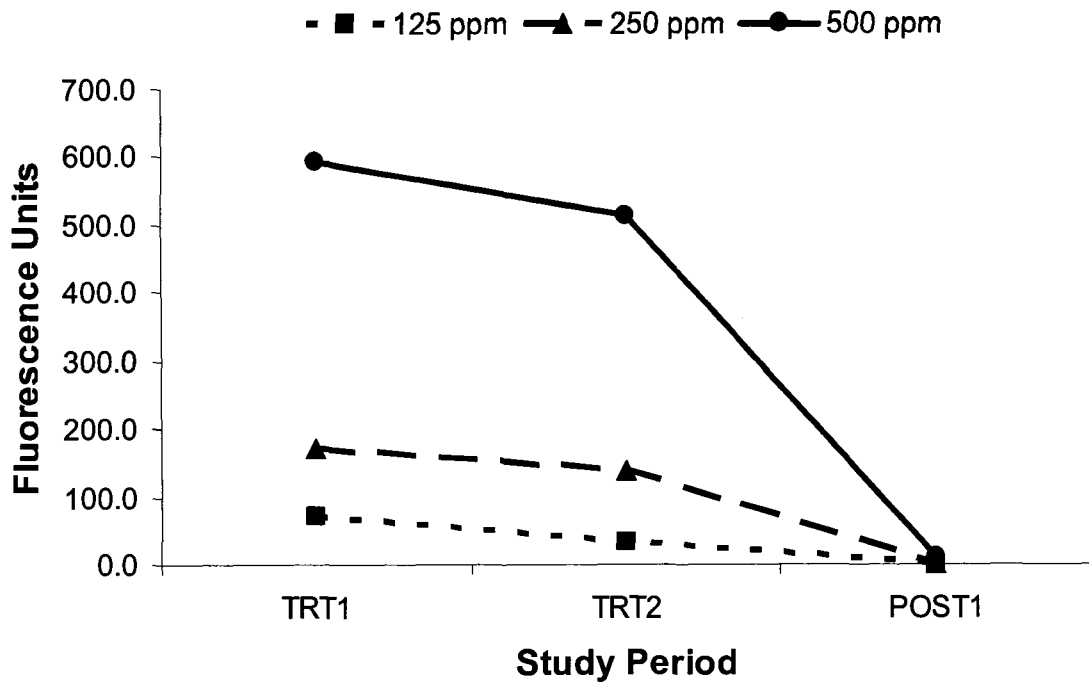


Figure 3.4. Fecal fluorescein levels of female mallards given 0.11 mg (125 ppm group), 0.23 mg (250 ppm group), or 0.45 mg (500 ppm group) fluorescein perorally once daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2000. Note the TRT1 = 1 to 7 d treatment, TRT2 = 8 to 14 d treatment, and POST1 = 1 to 3 d post-treatment.

CHAPTER 4

AD LIBITUM STUDY

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 causes health and safety issues in urban areas because 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). Fecal material may contaminate drinking water supplies (Conover and Chasko, 1985), and cause over-fertilization of lakes (Manny et al., 1975). 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 a feasible alternative in urban areas (Conover and Chasko, 1985; Heusmann, 1999), and the use of special hunting seasons has had limited success in controlling these resident goose populations (Heusmann, 1999). There are few places that allow goose translocation, and annual roundups have met with public resistance in some areas. Current nonlethal control methods for Canada geese include egg oiling or addling (e.g. shaking the egg). However, these methods require each nest to be located, resulting in several days of intense effort each season. In areas where nests are not easily accessible or adequate personnel are unavailable, contraception may be a reasonable alternative requiring fewer man-hours to implement. Therefore, contraception may

provide an acceptable alternative to help manage goose populations at levels that minimize damage but allow for the existence of geese (Forbes, 1993; Stout et al., 1997).

Nicarbazin (**NCZ**) is a coccidiostat routinely used in the poultry industry since the 1950s to control protozoan cecal and intestinal infections by *Eimeria* species in broiler chickens. It is an equimolar complex consisting of 4,4'-dinitrocarbanilide (**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 coccidiostat (Cuckler et al., 1955; Rogers et al., 1983). When fed to laying hens, NCZ impacts reproduction by either reducing egg hatchability or reducing egg production (Jones et al., 1990a; Hughes et al., 1991; Chapman, 1994). Although these effects are undesirable in the poultry industry, NCZ can potentially be used as a waterfowl contraceptive.

Mallards treated with 500 ppm (34 mg/kg) NCZ had 26% hatchability compared to 55% hatchability in the control group (Chapter 3). Because the initial testing indicated 500 ppm (33.75 mg/kg BW) was the minimum dose needed to affect reproduction, this study used higher doses of NCZ. Because geese in the field would be given NCZ on treated bait rather than by gavage, we chose to feed NCZ coated onto cracked corn *ad libitum* for this study.

Many contraceptive studies rely on initial testing using gavage procedures to determine a dose level for further testing. Therefore, we wanted to determine how plasma DNC levels compared when NCZ is given either as a bolus dose or fed continuously throughout the day. We achieved this objective by monitoring plasma DNC levels for mallards given NCZ either by liquid gavage, capsule, or in the daily feed ration.

The objectives of the reproductive portion of the study were to determine plasma and egg DNC levels when NCZ is fed *ad libitum* coated onto cracked corn, and whether plasma DNC levels differ between male and female mallards. Further objectives included assessing the effect of NCZ on reproduction by monitoring egg production and hatchability. We also wanted to determine whether NCZ negatively impacts general health by monitoring bird weight, hematocrit, hemoglobin, oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), bicarbonate levels (HCO_3^-), saturated oxygen levels (sO_2), and blood pH.

METHODS

Comparison of Delivery 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 as follows: 1) NCZ in capsules, 2 females and 4 males; 2) NCZ suspended in corn oil, 2 females and 5 males; 3) NCZ suspended in water, 3 females and 4 males; 4) NCZ in feed, 3 females and 4 males. Mallards (Whistling Wings, Inc., Hanover, IL) were randomly assigned to treatment groups and cages, and were housed individually. Males were included to determine whether NCZ absorption differed between sexes. All birds were approximately 52 wk of age at the beginning of the study, and a 17L:7D light cycle was maintained throughout. Mallards 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.

All treatment groups received 34 mg NCZ /kg BW daily for 12 d. Capsules were loaded with 25% NCZ on wheat middlings (Phibro Animal Health, Inc., Fairfield, NJ), such that each capsule contained 34 mg pure NCZ. Corn oil and water suspensions were made by vortexing 25% NCZ on wheat middlings in the appropriate medium to achieve an even suspension, such that 2 mL of suspension contained 34 mg pure NCZ. Mallards in the free feeding group received a daily ration of 68 g pelleted feed treated at 500 mg NCZ/kg feed.

A total of 3 mL of blood was obtained from the brachial vein on days 2, 4, 6, 8, 10, and 12 of treatment. Blood was centrifuged and the plasma stored at -70°C until analysis of DNC concentration using HPLC (Primus et al., 2001).

Reproductive Trial

The experiment consisted of 4 female and 2 male treatment groups as follows: 1) 0 ppm NCZ (Phibro Animal Health, Inc., Fairfield, NJ), 12 females; 2) 0 ppm NCZ, 5 males; 3) 750 ppm NCZ, 12 females; 4) 1000 ppm NCZ, 13 females; 5) 1000 ppm NCZ, 5 males; 6) 1500 ppm NCZ, 13 females. Mallards (Whistling Wings, Inc., Hanover, IL) were randomly assigned to treatment groups and cages, and were housed individually. Because males would consume bait in the wild, they were included to determine the effect of NCZ on male health. All birds were 30 wk of age at the beginning of the study, and a 17L:7D light cycle was maintained throughout. Mallards 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. Untreated male mallards were placed with control and treated female mallards, and treated males were placed with untreated

females 2 to 3 times each week. Pairs were observed until copulation occurred, and then males were removed.

Doses were made by overcoating cracked corn with 0, 750, 1000, or 1500 ppm NCZ using 5% corn oil and 5% milk powder. Bait was formulated such that a mallard eating 65 g of feed daily would receive 50.4, 67.2, or 100.8 mg/kg BW in the 750, 1000, or 1500 ppm groups respectively. Each mallard was offered 75 g of treated feed daily for 14 consecutive days. Any feed remaining after 24 hr was collected, dried in an oven dryer at 200°F overnight, and weighed to determine actual dose received.

We randomly selected 7 females from each group prior to onset of treatment for blood sampling. The same 7 females from each group were used for blood sampling throughout the study, and blood samples were drawn from all males. A total of 3 mL of blood was obtained from the brachial vein once pretreatment, on days 4, 7, 10, and 14 of treatment, and on days 2 and 5 post-treatment. Two microhematocrit tubes per blood sample were filled and analyzed for hematocrit (Dein, 1986). Blood was placed on ice until blood gas analysis (approximately 30 min) of a 0.2 mL subsample using a Radiometer ABL5 (Radiometer America Inc., Westlake, OH). The remainder of the blood was centrifuged and the plasma stored at -70°C until analysis of DNC concentration using HPLC (Primus et al., 2001). All birds were weighed at the time of bleeding.

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 for DNC analysis were broken open and the degree of mottling assessed. Mottling was assessed using the following scale: 0 = no mottling, 1 = mild mottling, 2 = moderate

mottling, and 3 = severe mottling. The shells were removed and the remainder of the egg was homogenized using a Cuisinart handheld blender and analyzed for DNC content by HPLC (Johnston et al., 2002). Because egg production was severely reduced in all groups during treatment, no eggs were incubated.

Statistical Analyses

Delivery methods. Data were analyzed as a mixed effects model (PROC MIXED; SAS Institute Inc., 2003), and significance was defined as $P \leq 0.05$. Data were analyzed for sex, delivery method, treatment day, and all interaction effects. Means separations were carried out using PDMIX800 (Saxton, 1998).

Reproductive trial. We divided the study into 5 time periods among groups for all statistical analyses 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) post-treatment 1 (day 2 post-treatment); and 5) post-treatment 2 (day 5 post-treatment). All data were analyzed as a mixed effects model (PROC MIXED; SAS Institute Inc., 2003), and significance was defined as $P \leq 0.05$ for all analyses. Data were analyzed for sex, treatment, time period, and all interaction effects. Means separations were carried out using PDMIX800 (Saxton, 1998). Because we had no data for the treatment periods for egg mottling scores, mottling data were not analyzed. Correlations were determined between plasma and egg DNC levels, and also between plasma or egg DNC levels and numbers of eggs laid, egg weight, body weight, hemoglobin, hematocrit, blood pH, pCO₂, pO₂, sO₂, and HCO₃⁻.

RESULTS

Delivery methods

Plasma DNC levels did not differ between sexes ($P = 0.9283$), but differed among delivery methods ($P = 0.0073$), treatment days ($P < 0.0001$), and a significant method by day interaction existed ($P < 0.0001$; Figure 4.1). Peak DNC levels were 3.58 ± 0.28 , 3.17 ± 0.33 , and 2.85 ± 0.29 , 2.10 ± 0.29 $\mu\text{g/mL}$ in the corn oil suspension, capsule, water suspension, and free feeding groups, respectively. Males received an average dose of 25.8 ± 1.8 mg/kg BW compared to an average dose of 34.3 ± 2.1 mg/kg BW for females ($P = 0.0031$) in the free feeding group.

Reproductive trial

Plasma and egg DNC levels differed among treatment groups and time periods, and a significant treatment by period interaction existed (Table 4.1). There was no difference in plasma DNC level between male and female mallards ($P = 0.5854$). Peak plasma DNC levels were 2.69 ± 0.41 , 3.64 ± 0.32 , and 5.38 ± 0.43 $\mu\text{g/mL}$ in the 750, 1000, and 1500 ppm groups, respectively. Peak egg DNC levels were 10.60 ± 4.41 , 22.80 ± 4.42 , and 16.65 ± 2.25 $\mu\text{g/g}$ in the 750, 1000, and 1500 ppm groups, respectively. Mallards received an average dose of 30.8 ± 1.2 , 34.2 ± 1.3 , and 49.1 ± 2.4 mg/kg BW in the 750, 1000, and 1500 ppm groups, respectively.

There was no effect of NCZ on the number of eggs laid per female per day ($P = 0.8179$), but there was a significant period effect (Table 4.2), with fewer eggs laid during both treatment periods and post-treatment period 1. Egg weights differed among treatment groups and time periods, and a significant treatment by period interaction existed (Table 4.3).

Bird weights differed between sexes, with males weighing more than females (Table 4.4). Body weights differed among time periods (Table 4.5), but there was no effect of NCZ on body weight ($P = 0.0861$). Hemoglobin concentrations and hematocrit differed between sexes, with females having a lower hemoglobin concentration and hematocrit than males (Table 4.4). Hemoglobin concentrations and hematocrit differed among time periods (Table 4.5), but there were no differences among treatment groups ($P = 0.7028$ and $P = 0.8249$, respectively). Blood pH, $p\text{CO}_2$, and $s\text{O}_2$ differed between sexes (Table 4.4) and among time periods (Table 4.5), but there were no differences among treatment groups ($P = 0.8324$, $P = 0.5994$, and $P = 0.7400$, respectively). Males exhibited a higher blood pH and $s\text{O}_2$ than females, but lower $p\text{CO}_2$ than females. Partial pressure of oxygen ($p\text{O}_2$) and HCO_3^- differed among time periods (Table 4.5), but there were no differences between sexes or among treatment groups ($P = 0.6185$ and $P = 0.7097$, respectively).

Body weights and $p\text{CO}_2$ tended to decrease during the treatment periods and post-treatment period 1. Hemoglobin and hematocrit tended to decrease over time, with the lowest levels occurring during the post-treatment periods. Blood pH decreased from pretreatment levels, but there was no difference in blood pH between treatment and post-treatment periods. Saturated oxygen ($s\text{O}_2$) decreased during treatment period 1, then increased, but remained lower than pretreatment levels. The partial pressure of oxygen ($p\text{O}_2$) decreased during the treatment periods, but returned to pretreatment levels during the post-treatment period. The highest HCO_3^- levels occurred during treatment period 2, and the lowest level occurred during post-treatment period 2.

Overall, plasma DNC levels were negatively correlated with the number of eggs laid per female per day, body weight, pO₂, and sO₂ (Table 4.6). Plasma DNC levels were positively correlated with egg DNC levels during treatment period 1 and post-treatment period 2. Plasma DNC levels were negatively correlated with body weight during treatment period 2. Plasma DNC levels were negatively correlated with body weight in the 750 ppm group, and positively correlated with hemoglobin in the 750 and 1500 ppm groups. Plasma DNC levels were negatively correlated with pO₂ and sO₂ at all treatment levels. Egg weight and the number of eggs laid per female per day were negatively correlated with plasma DNC in the 1000 ppm group.

DISCUSSION

Delivery methods

Differences in plasma DNC levels obtained by utilizing different delivery methods can be explained by differences in the bioavailability of DNC with each method. The increased anticoccidial activity of NCZ is believed to be due to the formation of smaller DNC crystals in the gut (Rogers et al., 1983). The aqueous environment of the gut causes HDP to leach out of the NCZ complex. Although DNC crystals tend to aggregate in aqueous environments, HDP prevents DNC from aggregating in larger crystals by keeping the DNC crystals more separated than if DNC is fed alone (Rogers et al., 1983). The average diameter of wetted DNC crystals formed from DNC alone is 0.53 µm compared to an average diameter of 0.11 µm obtained from the wetted NCZ complex. (Rogers et al., 1983). Because of the smaller size, the DNC crystals have a larger surface area, resulting in greater absorption.

Nicarbazin suspended in corn oil is likely protected from “pre-wetting”, keeping crystal sizes small. In addition, NCZ is distributed throughout the suspension, making NCZ more available to the gut. The corn oil suspension produced the highest peak DNC levels of any of the delivery methods tested. A water based suspension would be expected to produce lower plasma DNC levels because the NCZ is already in an aqueous environment prior to ingestion, and the results of this study confirmed this. Nicarbazine delivered in a capsule produced plasma DNC levels higher than the water based suspension, but lower than the corn oil suspension. Likely, this is because NCZ is dry upon ingestion, but then is subsequently exposed to the aqueous environment in the gut. This may result in crystal sizes intermediate to those in the corn oil and water based suspension groups.

The lowest plasma DNC levels were obtained by feeding pelleted bait. Pellets have a lower exposed surface area than suspension or capsule based delivery methods. Because of this, less NCZ is immediately available for uptake in the gut, and lower plasma DNC levels result. Male mallards received only 26 mg/kg BW rather than the predicted 34 mg/kg BW dose, but females received a 34 mg/kg BW dose. Although the lower dose level in the males may have contributed to lower plasma DNC levels overall, no significant differences were found in plasma DNC levels between sexes.

During the reproductive trial, mallards fed 1000 ppm NCZ on cracked corn received an average dose of 34 mg/kg BW. Because mallards in the delivery method trials also received 34 mg/kg BW doses, and both trials used 25% NCZ on wheat middlings, plasma DNC levels can be compared. Mallards receiving cracked corn had higher peak plasma DNC levels than any of the other four delivery methods tested (Figure 4.1, Figure 4.2).

This may be due to two factors. First, coating NCZ onto cracked corn gives more surface area than a pellet, resulting in more NCZ being immediately available to the gut. Second, corn oil was used as a sticker for the NCZ on cracked corn, and this likely protected the NCZ some from pre-wetting, resulting in formation of smaller DNC crystals in the gut.

Reproductive trial

Peak plasma and egg DNC levels were in the range predicted to have a contraceptive effect in waterfowl. A minimum plasma level of 2.9 $\mu\text{g/mL}$ is needed to induce reproductive effects in waterfowl (Jones et al., 1990a; Yoder et al., 2005). Plasma DNC levels in this study ranged from 2.7 to 5.4 $\mu\text{g/mL}$. Despite decreased food consumption, mallards in the 1000 and 1500 ppm groups received enough NCZ to obtain adequate plasma DNC levels to induce contraceptive effects. Reduced egg hatchability in chickens was observed at a minimum egg DNC level of 6 $\mu\text{g/g}$ (Jones et al., 1990a), and similar levels are likely needed in waterfowl eggs to prevent hatchability. Egg DNC levels in this study ranged from 10.6 to 22.8 $\mu\text{g/g}$, indicating that even 750 ppm NCZ in feed could reduce egg hatchability.

The mallards utilized in this study were accustomed to a pelleted waterfowl diet, and did not accept a cracked corn diet, even in the control group. Egg production and BW decreased as a result of the decreased food intake in all treatment groups, but began to return to pretreatment levels during post-treatment period two. Thus, we were unable to evaluate the effects of nicarbazin on hatchability.

Treatment with NCZ resulted in a decrease in egg weight. This is consistent with the results of previous studies on chickens (Jones et al., 1990a,b; Hughes et al., 1991). Mean egg weights were 50.7 ± 1.1 , 47.8 ± 1.2 , 46.0 ± 1.1 , and 49.4 ± 1.3 g in the 0, 750,

1000, and 1500 ppm groups, respectively. During treatment period 1, egg weights were reduced by 10% in the 1500 ppm group. There were no eggs laid in the control group during treatment period 2 to compare with the treatment groups. Egg weights decreased by 10% and 19.7% in the 750 and 1000 ppm groups, respectively during post-treatment period 1. During post-treatment period 2, egg weights were reduced by 21.7%, 24.8%, and 17.9% in the 750, 1000, and 1500 ppm groups, respectively. In the 1000 ppm group, egg weight decreased as plasma DNC increased.

Male mallards in this study had a higher hematocrit and hemoglobin concentration than females. In geese, administration of estrogen causes a decrease in red blood cell counts (Hunsaker, 1968). While androgen has no effect on hematocrit in geese, it increases the red blood cell count in chickens and quail (Burton and Smith, 1972; Nirmalan and Robinson, 1972). Although there are differences among species, males tend to have a higher hematocrit than females (Sturkie, 1986). Because hemoglobin concentration is related to hematocrit (Campbell, 1995; Fudge, 2000), males also have a higher hemoglobin concentration than females (Sturkie, 1986).

Increased plasma DNC was associated with decreases in egg production and BW in this study. This is consistent with the finding that egg production in chickens decreased as NCZ dose in feed increased (Jones et al., 1990a, Hughes et al., 1991). At levels ≥ 100 ppm NCZ, feed efficiency and weight gain are decreased in chickens (Bartov, 1989a,b; Sorribas et al., 1993).

Increased plasma DNC was also correlated with decreased pO_2 and sO_2 . Because this effect occurred consistently in all treatment groups, it is likely a genuine effect. Saturated oxygen levels would be expected to follow the trend of pO_2 , and this was true

in this study. However, there was no treatment effect of NCZ on pO₂ or sO₂, and the correlations are weak ($r = -0.25$ and $r = -0.22$, respectively), therefore this likely is not a biologically significant effect.

Plasma and egg DNC levels high enough to induce contraceptive effects were obtained during this study at ≥ 1000 ppm NCZ in feed. Although cracked corn bait produced the highest plasma DNC levels, and would be more readily accepted by geese than pelleted bait, it is also associated with more nontarget hazards to songbirds. Because of this, it is recommended that NCZ be coated onto whole corn instead because the size of corn kernels will exclude most nontarget songbirds. Geese are accustomed to eating corn in fields during the winter months, therefore, they are more likely to accept corn bait than pelleted bait with which they have no prior experience. Geese in parks used to being fed by humans may be more likely to accept pelleted bait than geese not normally fed by humans. If pelleted bait is to be used in the field, a higher amount of NCZ must be incorporated into the pellet to allow for the decreased absorption of DNC. OvoControl G is a pelleted NCZ bread bait registered by the Environmental Protection Agency in 2005 for control of reproduction in resident Canada geese. It is treated with NCZ at a rate of 2500 ppm, requiring a goose to consume 50 g daily to achieve an approximate dose of 31.25 mg NCZ/kg BW (Bynum et al., 2006).

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Table 4.1. Plasma and egg 4,4'-dinitrocarbanilide (DNC) levels across sexes for mallards fed cracked corn treated with nicarbazin at 0 ppm, 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

Group	Period ¹	Plasma DNC (µg/mL)			Egg DNC (µg/g)		
		<i>n</i>	\bar{x} ²	SE	<i>n</i>	\bar{x} ²	SE
0 ppm	PRE	12	0.00 ^{a,b,c}	0.39	5	0.00 ^a	1.98
	TRT1	22	0.01 ^b	0.32	2	0.00 ^{a,b,c}	3.12
	TRT2	23	0.03 ^b	0.31	0		
	POST1	11	0.04 ^{a,b,d,e}	0.40	1	0.01 ^{a,b,c,d}	4.40
	POST2	11	0.00 ^{a,b,c}	0.40	3	0.08 ^{a,b,c}	2.55
750 ppm	PRE	7	0.00 ^{a,b,d,e}	0.51	3	0.00 ^{a,c}	2.56
	TRT1	13	2.69 ^{f,g}	0.41	2	6.88 ^{a,b,c,d}	3.13
	TRT2	14	2.35 ^{g,h}	0.40	0		
	POST1	7	0.76 ^{a,b,d,e}	0.51	1	10.60 ^{b,d,e}	4.41
	POST2	7	0.05 ^{a,b,d,e}	0.51	2	8.08 ^{b,c,d}	3.13
1000 ppm	PRE	12	0.00 ^{a,b,c}	0.39	5	0.00 ^a	1.98
	TRT1	21	3.64 ^f	0.32	0		
	TRT2	24	3.54 ^f	0.31	1	22.80 ^e	4.42
	POST1	12	1.11 ^{d,e}	0.39	2	18.69 ^e	3.12
	POST2	12	0.06 ^{a,b,c}	0.39	3	8.78 ^d	2.55
1500 ppm	PRE	7	0.00 ^{b,c,e}	0.51	3	0.08 ^{a,b,c}	2.55
	TRT1	12	5.38 ⁱ	0.43	0		
	TRT2	12	4.85 ⁱ	0.43	0		
	POST1	6	1.29 ^{a,d,h}	0.54	0		
	POST2	7	0.04 ^{a,b,d,e}	0.52	4	16.65 ^e	2.25

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = day 2 post-treatment, POST2 = day 5 post-treatment.

²Means within columns with different subscripts are significantly different. Means are from the LSMEANS option in PROC MIXED (SAS 9.1), and means separations were carried out using PDMIX800 (Saxton, 1998).

Table 4.2. Period effects across treatment groups for eggs laid per female per day for female mallards fed cracked corn treated with nicarbazin at 0 ppm, 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

Period ¹	<i>n</i>	\bar{x} ²	SE
PRE	655	0.54 ^a	0.03
TRT1	245	0.29 ^b	0.04
TRT2	191	0.01 ^c	0.04
POST1	141	0.18 ^d	0.04
POST2	518	0.44 ^e	0.03

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = day 2 post-treatment, POST2 = day 5 post-treatment.

²Means within columns with different subscripts are significantly different. Means are from the LSMEANS option in PROC MIXED (SAS 9.1), and means separations were carried out using PDMIX800 (Saxton, 1998).

Table 4.3. Egg weights (g) for female mallards fed cracked corn treated with nicarbazin at 0 ppm, 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

Group	Period ¹	<i>n</i>	\bar{x} ²	SE
0 ppm	PRE	55	50.7 ^{a,b}	1.2
	TRT1	3	41.4 ^{c,d,e,f}	2.8
	TRT2	0		
	POST1	3	51.7 ^{a,b,g,h}	2.8
	POST2	3	55.3 ^a	2.8
750 ppm	PRE	51	48.5 ^{b,g,h}	1.3
	TRT1	2	41.6 ^{c,d,e,f}	3.4
	TRT2	0		
	POST1	3	46.5 ^{b,c,d,g,h}	2.8
	POST2	4	43.3 ^{c,d,e}	2.5
1000 ppm	PRE	57	47.2 ^{c,g,h}	1.2
	TRT1	1	46.9 ^{a,b,c,d,e,g,h}	4.6
	TRT2	1	31.9 ^f	4.6
	POST1	4	41.5 ^{d,e,f}	2.4
	POST2	8	41.6 ^{d,e}	1.8
1500 ppm	PRE	49	50.4 ^{a,b,g}	1.3
	TRT1	2	37.3 ^{e,f}	3.4
	TRT2	1	50.4 ^{a,b,c,d,g,h}	4.7
	POST1	0		
	POST2	6	45.4 ^{c,d,h}	2.2

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = day 2 post-treatment, POST2 = day 5 post-treatment.

²Means within columns with different subscripts are significantly different. Means are from the LSMEANS option in PROC MIXED (SAS 9.1), and means separations were carried out using PDMIX800 (Saxton, 1998).

Table 4.4. Differences between sexes for body weight, hemoglobin, hematocrit, blood pH, partial pressure of carbon dioxide (pCO₂), and saturated oxygen (sO₂) for mallards fed cracked corn treated with nicarbazin at 0 ppm, 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

	Females			Males		
	<i>n</i>	\bar{x} ¹	SE	<i>n</i>	\bar{x} ¹	SE
Bird weight (g)	216	874.0 ^a	15.9	80	1063.8 ^b	26.2
Hemoglobin (g/dL)	179	15.1 ^a	0.3	70	19.1 ^b	0.4
Hematocrit (%)	188	40.7 ^a	0.5	70	48.8 ^b	0.9
pH	185	7.01 ^a	0.01	70	7.09 ^b	0.02
pCO ₂ (mm Hg)	185	87.4 ^a	1.4	70	71.2 ^b	2.4
sO ₂ (%)	185	67.0 ^a	1.4	70	72.8 ^b	2.3

¹Means within rows with different subscripts are significantly different. Means are from the LSMEANS option in PROC MIXED (SAS 9.1), and means separations were carried out using PDMIX800 (Saxton, 1998).

Table 4.5. Period effects across treatment groups and sexes for body weight, hemoglobin, hematocrit, blood pH, partial pressure of carbon dioxide (pCO₂), saturated oxygen (sO₂), partial pressure of oxygen (pO₂), and bicarbonate (HCO₃⁻) for mallards fed cracked corn treated with nicarbazin at 0 ppm, 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

	PRE ¹			TRT1			TRT2			POST1			POST2		
	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE	<i>n</i>	\bar{x}^2	SE
Bird weight (g)	76	1031.6 ^a	22.7	74	888.9 ^b	22.8	73	853.2 ^c	22.9	36	833.9 ^d	26.2	37	977.0 ^c	26.0
Hemoglobin (g/dL)	31	17.8 ^a	0.5	74	17.6 ^a	0.4	72	16.0 ^b	0.4	35	13.5 ^c	0.5	37	14.7 ^d	0.5
Hematocrit (%)	38	43.8 ^a	0.8	74	43.6 ^a	0.8	73	42.7 ^b	0.8	36	41.7 ^c	0.8	37	41.9 ^{b,c}	0.8
pH	35	7.09 ^a	0.02	74	7.00 ^b	0.01	73	7.04 ^c	0.01	36	7.03 ^{b,c}	0.02	37	7.00 ^b	0.02
pCO ₂ (mm Hg)	35	70.1 ^a	2.6	74	83.3 ^b	2.1	73	86.3 ^{b,c}	2.1	36	88.3 ^c	2.5	37	83.0 ^{b,c}	2.5
sO ₂ (%)	35	77.4 ^a	2.0	74	61.7 ^b	1.6	73	66.0 ^c	1.6	36	73.1 ^a	1.9	37	74.4 ^a	1.9
pO ₂ (mm Hg)	35	59.1 ^a	1.6	74	50.3 ^b	1.2	73	51.2 ^b	1.2	36	58.1 ^a	1.6	37	60.5 ^a	1.6
HCO ₃ ⁻ (mmol/L)	35	20.2 ^a	0.5	74	19.7 ^a	0.4	73	22.0 ^b	0.4	36	21.9 ^b	0.5	37	19.3 ^a	0.5

¹PRE = pretreatment days 1 to 14, TRT1 = treatment days 1 to 7, TRT2 = treatment days 8 to 14, POST1 = day 2 post-treatment, POST2 = day 5 post-treatment.

²Means within rows with different subscripts are significantly different. Means are from the LSMEANS option in PROC MIXED (SAS 9.1), and means separations were carried out using PDMIX800 (Saxton, 1998).

Table 4.6. Correlations between plasma and egg 4,4'-dinitrocarbanilide (DNC) levels and the number of eggs laid per female per day, egg weight, bird weight, partial pressure of oxygen (pO₂), and saturated oxygen (sO₂) for mallards fed cracked corn treated with nicarbazin at 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001

	Plasma DNC		Egg DNC	
	<i>n</i>	<i>r</i>	<i>n</i>	<i>r</i>
Eggs laid per female per day	185	-0.30165	37	0.28105 ¹
Egg weight	38	-0.23008 ¹	37	-0.38437
Bird weight	252	-0.19641	37	-0.33811
pO ₂	249	-0.24752	36	0.13937 ¹
sO ₂	249	-0.21678	36	0.06287 ¹

¹Correlations were not significant at $P < 0.05$. All other correlations were significant.

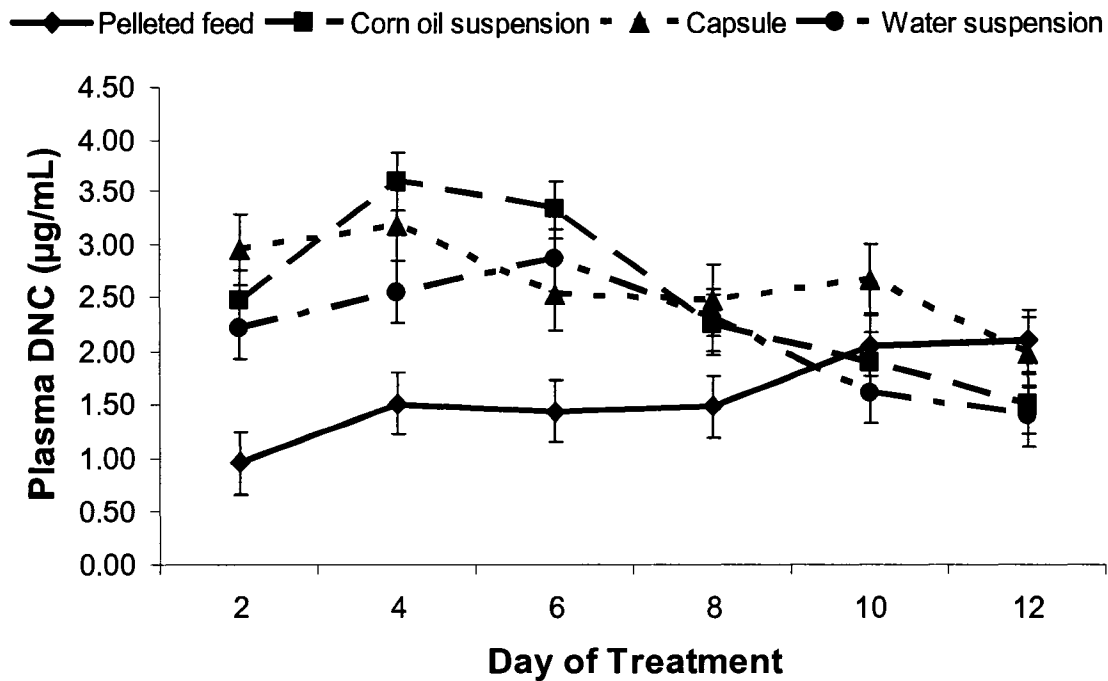


Figure 4.1. Plasma DNC levels of mallards given 500 ppm (34 mg/kg BW) nicarbazin either by gavage in a corn oil suspension, gavage in a water suspension, peroral administration of a capsule, or treated pellets fed *ad libitum* daily for 12 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, July 2001.

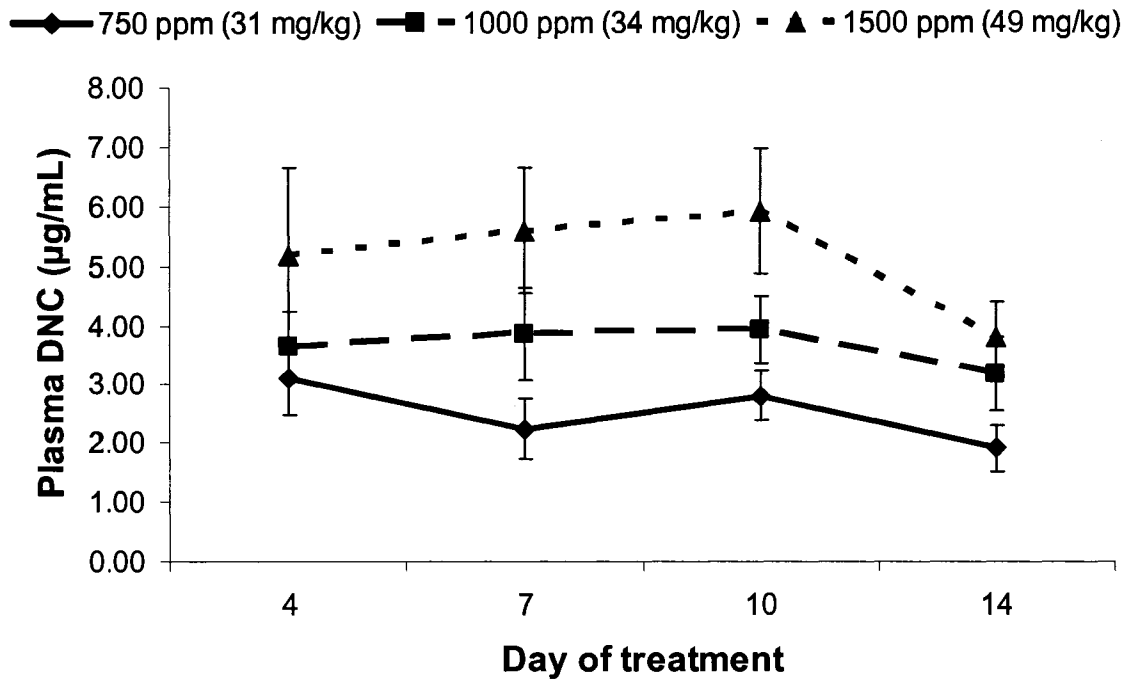


Figure 4.2. Plasma DNC levels of mallards fed cracked corn treated with nicarbazin at 750 ppm (30.8 mg/kg BW), 1000 ppm (34.2 mg/kg BW), or 1500 ppm (49.1 mg/kg BW) *ad libitum* daily for 14 d at the National Wildlife Research Center, Fort Collins, Colorado, USA, May to June, 2001.

CHAPTER 5

COLORADO FIELD STUDY

From 1966 to 2001, Canada goose populations in the United States experienced a nearly exponential rate of growth (Sauer et al. 2005). Nonmigratory populations are becoming more frequent in populated areas as development provides artificial sources of food and protection year round (Forbes 1993, Ankney 1996, Gosser and Conover 1999). Large numbers of resident geese concentrated in one area damage grass and can become aggressive (Conover and Chasko 1985, Forbes 1993). Ettl (1993) estimated geese defecate 5.2-18.8 times per hr, resulting in 1,248-4,512 g of feces for 40 geese over a 6 hr period. Large amounts of fecal material can contaminate drinking and recreational water supplies and cause over-fertilization of lakes (Manny et al. 1975, Conover and Chasko 1985, Fairaizl 1992).

Hunting is often not feasible in urban areas (Conover and Chasko 1985, Heusmann 1999), and use of special hunting seasons has had limited success in controlling resident goose populations (Heusmann 1999). Currently, there are few places that allow goose translocation, and annual roundups have met with public resistance in some areas. Additionally, translocation creates a “sink”, allowing other geese to quickly move into the area (Conover and Chasko 1985, Cooper 1987). Use of pyrotechnics and other scare devices often relocates the problem to another area without preventing new geese from moving into the hazed area (Aguilera et al. 1991). Contraception may provide an

acceptable alternative to help maintain bird populations at levels that minimize damage but allow for the existence of geese (Breault and McKelvey 1991, Forbes 1993).

Current reproductive control methods for Canada geese include egg oiling or addling (e.g. shaking the egg). These methods can be very effective in controlling goose populations and require intense effort for only a few days each season (Forbes 1993, Christens et al. 1995). However, the timing of egg oiling is critical to its success, as is the availability of trained personnel. Successful egg oiling programs often involve several applications to prevent egg hatching for late-nesting and renesting birds (Blackwell et al. 2000). Utilizing the public to incorporate egg oiling programs can be effective if people are trained in locating nests. However, adequate personnel may not always be available, and nests may be difficult to access. A contraceptive agent could be useful in these situations as it would not require personnel to access nests.

Nicarbazin (NCZ), is an anticoccidial drug routinely used in the poultry industry at 125 mg NCZ/kg feed to prevent coccidiosis in broiler chickens (*Gallus domesticus*). It is an equimolar complex consisting of 4,4'-dinitrocarbanilide (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 egg hatchability or rate of egg laying (Jones et al. 1990, Hughes et al. 1991, Chapman 1994). Mallards treated with 34 mg NCZ/kg body weight had 26% hatchability compared to 55% hatchability for control mallards.

The objective of this study was to determine the effect of NCZ on reproduction in free ranging populations of Canada geese in northern Colorado. We accomplished this

by determining egg hatchability, clutch size, egg fertility, the stage of embryonic development in unhatched eggs, and eggshell thickness. We wanted to determine whether egg DNC levels were correlated with egg hatchability, egg fertility, or stage of embryonic development in unhatched eggs. In addition, we wanted to determine if nesting success was correlated with distance from bait sites. We wanted to assess the effect of NCZ on incubation behavior by determining how long a female would continue to incubate a nest past its expected hatching date (EHD). We also wanted to determine the safety of NCZ by making observations of nontarget consumption of bait, nontarget nesting activity, and necropsy of birds found dead during the study.

STUDY AREA

The northernmost study site was located approximately 17 km north of Wellington, CO. It was a 296 ha utility facility site consisting of a single 171 ha lake used for water cooling that contained 2 permanent islands, and 1 intermittent island. The permanent islands were 1.2 and 1.7 ha in size, and the intermittent island was 1.1 ha. We placed nest boxes ($n = 31$) 62-76 m apart around the southwest perimeter of the lake.

The southernmost study site was located approximately 7 km southeast of Fort Collins, CO. It was a 41 ha reclaimed gravel pit consisting of 13 ponds and 10 islands. The ponds ranged from 0.1-5.4 ha, and the islands ranged from 0.002-0.08 ha. Nest boxes ($n = 32$) were placed ≥ 55.4 m apart around the edges of the ponds, and on 4 of the islands. This study area was 39 km from the utility facility study site.

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 study consisted of 3 yr of data collection from 2000-2002 at 2 study sites, with the first year as the control year. A third study site was planned as a control for the treatment years, but could not be used because the nesting area flooded during the last 2 yr of the study.

We made bait by coating cracked corn with 5% corn oil (w/w), 5% calf milk replacer (w/w), and 1,320 mg NCZ/kg corn. We drizzled the corn oil over the corn while the corn was being mixed in a Hobart feed mixer. We continued to mix the corn for 5 min to ensure even coating. Next, we slowly poured the calf milk replacer and NCZ over the corn as it was being mixed. We mixed the corn 5 min longer to ensure even coating. Areas observed to be common loafing areas were used as bait sites. The amount of bait placed at each bait site was based on the numbers of geese observed in the area each day and the amount of bait eaten each day. We placed bait on the bare ground at the gravel pit site from 16 March 2001 to 4 May 2001, and again from 25 March 2002 to 29 April 2002. We placed bait on the bare ground at the utility site from 31 March 2001 to 1 May 2001, and again from 25 March 2002 to 26 April 2002. Nontarget animals observed consuming bait were identified and recorded.

We applied 1,964.9 kg of treated bait to 38 sites at the gravel pit site in 2001, and 1,208.5 kg to 23 sites in 2002. We applied 547.5 kg of treated bait to 18 sites at the utility site in 2001, and 629.0 kg to 9 sites in 2002. We placed bait at stations at the gravel pit site for 42 d over a 50 d period in 2001, and for 33 d over a 36 d period in 2002. We placed bait at stations at the utility site on 23 d over a 32 d period in 2001, and on 31 d over a 33 d period in 2002.

We monitored nests at the gravel pit site for 7 weeks in 2000, from 27 March to 8 May. In 2001, we monitored nests at the gravel pit site for 8 weeks from 2 April to 24 May. During 2002, we monitored nests at the gravel pit site for 12 weeks from 28 March to 13 June. We monitored all nests 2-5 times per week until completion of the clutch. A clutch was determined to be complete when the same numbers of eggs were found in the nest for at least 5 d consecutively. We calculated an EHD for each nest by assuming a 28 d incubation period (Mowbray et al. 2002), and counting 28 d from the date the last egg was laid in the nest. If the date the last egg was laid was not known, it was estimated by assuming that a goose lays an egg every other day (Mowbray et al. 2002). We checked nests ≥ 5 times per week the last 2 weeks of the incubation period.

We monitored nests at the utility site for 5 weeks in 2000, from 10 April to 8 May. Due to logistical problems, we could only check nest boxes 2-3 times a week, and island nests twice during the entire 2000 nesting season. In 2001, we monitored nests 2-3 times per week at the utility site for 9 weeks from 4 April to 31 May. During 2002, we monitored nests at the utility site 2-6 times per week for 12 weeks from 30 April to 10 June. We calculated an EHD for each nest as described in the previous paragraph. We checked nests ≥ 3 times per week the last 2 weeks of incubation.

We collected unhatched eggs from nests that hatched or were abandoned past the EHD. Nests were considered to be abandoned if the eggs were cold and no geese were defending the nest, or it was ≥ 7 d past the EHD. We allowed females to incubate nests that did not hatch until they abandoned the nest to determine how long past the EHD they would remain on the nest.

We opened unhatched eggs and determined fertility of the egg. If no embryo or blood vessels could be distinguished, we considered the egg infertile. If an embryo was present, we estimated its age (Cooper and Batt 1972) and assigned a stage of embryonic development. We assigned stages of embryonic development as follows: 0 = infertile, 1 = 0-4 d, 2 = 5-8 d, 3 = 9-12 d, 4 = 13-16 d, 5 = 17 -20 d, 6 = 21-24 d, 7 = 25-28 d, 8 = hatched. We homogenized egg contents using a hand-held Cuisinart blender and analyzed the contents for DNC using high performance liquid chromatography (Johnston et al. 2002). Eggs with an embryo ≥ 7 d were not used for DNC analysis. We rinsed the eggshells with water, and measured shell thickness at 3 different locations using a calipers. We averaged the 3 thicknesses to obtain a shell thickness for each egg.

We marked all nest and bait sites using a hand-held GPS unit. We assigned the same number to bait sites that were common between years, and assigned a unique number to all other bait sites. We assigned nest boxes the same number throughout the study, but gave ground nests unique identifiers. We calculated distances from each nest to each bait site using ArcGIS (Environmental Systems Research Institute, Inc. 2005).

We searched for nontarget nests in all suitable habitats at both sites on 26 April 2001 and 4 May 2002. If possible, we removed a single egg from nests we found for DNC analysis. In addition, we recorded any nesting behavior we observed at the sites during the study. We collected intact bird carcasses we found throughout the course of the study and submitted them for necropsy to the Colorado Veterinary Diagnostic Laboratory in Fort Collins, CO.

Because of the lack of nesting data for the utility site in 2000, we only used the gravel pit site for analysis of the effect of NCZ on hatching success and clutch size.

However, we used the utility site data for correlation analysis of egg DNC levels and reproductive parameters. We only used nests of known fate for statistical analysis.

We calculated the percentage of eggs hatching in each nest at the gravel pit site by dividing the number of eggs that hatched by total number of eggs in the clutch and multiplying the result by 100. The total number of eggs in the clutch was assumed to be the number of eggs in the nest at completion of egg laying. Therefore, the number of unhatched eggs included eggs that rolled from the nest or were broken in the nest during incubation. We analyzed percent hatchability and clutch size as a mixed model, with nests as a random effect (PROC MIXED; SAS Institute, Inc. 2003). We performed means separations using PDMIX800 (Saxton 1998).

We calculated the percentage of fertile eggs in each nest at both sites by dividing the number of eggs that hatched or had an embryo by total eggs in the clutch and multiplying the result by 100. Eggs that disappeared during incubation that could not be located were assumed to be infertile. We assumed such eggs broke in the nest during incubation and were infertile because an embryo could not be located in the nest. We compared percent hatchability, clutch sizes, percent fertility, stage of embryonic development, eggshell thickness, and egg DNC levels between sites and treatment years using mixed model analysis (PROC MIXED; SAS Institute, Inc. 2003). We performed means separations using PDMIX800 (Saxton 1998). We determined the relationship between distance of a nest from bait sites and hatchability, clutch size, fertility, stage of embryonic development, shell thickness, and egg DNC level using a correlation analysis (PROC CORR; SAS Institute, Inc. 2003).

We used adult and gosling survival rates reported in the literature (Alisauskas and Lindberg 2002, Mowbray et al. 2002), and hatchability rates and clutch sizes determined during this study for control and nicarbazin treatment years to create a population model with stochastic survival rates, hatchability, and clutch sizes. Survival rates reported in the literature were used to create the model because we did not monitor survival in this study. Adult survival rates (S_A) ranged from 0.75-0.9, gosling survival rates (S_G) ranged from 0.49-0.61, control hatchability (H_C) ranged from 0.64-0.82, NCZ hatchability (H_N) ranged from 0.28-0.42, control clutch sizes (CS_C) ranged from 5.2-5.8 eggs/clutch, and NCZ clutch sizes (CS_N) ranged from 5.1-5.7 eggs/clutch. The starting population size was 140 geese. These models did not allow for immigration of new individuals into the population or compensatory mechanisms, and no carrying capacity was assumed. Additionally, the models assumed that all surviving adults returned to the natal area to breed the following year. The model did not allow for geese to breed until they were ≥ 2 yr old (Mowbray et al. 2002). The population size at $t + 1$, where $t =$ time, $NSA =$ surviving adults > 2 yr old, $NT =$ surviving 2-yr-olds, $HG =$ hatched goslings, and $NG =$ surviving goslings, was defined as $N_{t+1} = NSA_t + NT_t + NG_t$. The definitions of NSA_t , NT_t , and NG_t are as follows: $NSA_t = (NSA_{t-1} \times S_A) + (NT_{t-1} \times S_A)$; $NT_t = NG_{t-2} \times S_A \times S_A$; $NG_t = HG_t \times S_G$. The breeding population (NB) at t was defined as $NB_t = NSA_t + NT_t$. The number of nests at t was defined as $nests_t = NB_t/2$. The number of eggs at t for the control population was defined as $CE_t = nests_t \times CS_C$, and the number of eggs at t for the NCZ population was defined as $NE_t = nests_t \times CS_N$. The number of goslings hatching at t for the control population was defined as $HGC_t = CE_t \times H_C$, and the number of goslings hatching at t for the NCZ population was defined as $HGN_t = NE_t \times H_N$.

Population models that included periodic culling used a population reduction of 50%, therefore, N_{t+1} = post-cull population size at t . Models were created for using yearly contraception with culling occurring once every 3 yr, once every 4 yr, and once every 5 yr.

RESULTS

Hatchability was $73.7 \pm 5.9\%$ ($n = 21$), $32.2 \pm 4.6\%$ ($n = 35$), and $37.3 \pm 5.0\%$ ($n = 29$) in 2000, 2001, and 2002 at the gravel pit site, respectively ($P < 0.0001$). Nicarbazin decreased the percentage of eggs hatching by 56.3% in 2001 and by 49.4% in 2002. Clutch size did not vary among years ($P = 0.5421$).

Percent hatchability varied between sites (Table 5.1), but not between treatment years ($P = 0.9709$). Hatchability at the gravel pit site was 30.3% lower than at the utility site. During 2001, no eggs hatched in 28.6% of the nests at the gravel pit site compared to 22.2% of the nests at the utility site. During 2002, no eggs hatched in 27.6% of the nests at the gravel pit site compared to 20.0% of the nests at the utility site.

Clutch sizes varied between sites (Table 5.1), but not between treatment years ($P = 0.6147$). Percent fertility varied between sites, but not between treatment years ($P = 0.1118$). Fertility was 29.0% lower at the gravel pit site than the utility site. The stage of embryonic development varied between sites, but not between treatment years ($P = 0.2688$). Eggshell thickness did not vary between sites ($P = 0.9087$), but varied between treatment years ($P < 0.0001$). Mean eggshell thickness was 0.53 ± 0.02 mm ($n = 120$) during 2001, and 0.67 ± 0.02 mm ($n = 135$) during 2002. Egg DNC levels did not vary between sites ($P = 0.9393$) or treatment years ($P = 0.4310$). Mean egg DNC levels were

2.3 ± 0.8 µg/g (*n* = 190) at the gravel pit site, and 2.2 ± 2.0 µg/g (*n* = 35) at the utility site.

Egg DNC levels were negatively correlated with percent hatchability, clutch size, percent fertility, stage of embryonic development, and distance from the bait site (Table 5.2). Egg DNC levels and eggshell thickness were positively correlated. Clutch size was positively correlated with percent hatchability, percent fertility, and stage of embryonic development (Table 5.3). Eggshell thickness was negatively correlated with percent hatchability, clutch size, percent fertility, stage of embryonic development, and distance of the nest from a bait site (Table 5.4). Distance of the nest from a bait site was positively correlated with percent hatchability, percent fertility, and stage of embryonic development (Table 5.4). Distance of the nest from a bait site was negatively correlated with eggshell thickness.

In 2001, the average number of days spent incubating the nest past the EHD was 8 d at the gravel pit site and 6 d at the utility site. The range of days spent incubating the nest past the EHD in 2001 was 1-21 d at the gravel pit site, and 1-11 d at the utility site. In 2002, the average number of days spent incubating the nest past the EHD was 16.5 d at the gravel pit site and 9.3 d at the utility site. The range of days spent incubating the nest past the EHD in 2002 was 1-40 d at the gravel pit site and 7-13 d at the utility site.

We observed red-winged blackbirds (*Agelaius phoeniceus*) and mallards (*Anas platyrhynchos*) on bait sites during 2001 and 2002 at both sites. At the gravel pit site, we observed mourning doves (*Zenaida macroura*) on bait sites during 2001, and a single snow goose (*Chen caerulescens*) in 2002. We also observed cracked corn in raccoon (*Procyon lotor*) scat at the gravel pit site during both treatment years. At the utility site,

we observed ring-billed gulls (*Larus delawarensis*), California gulls (*Larus californicus*), American wigeons (*Anas americana*), green-winged teal (*Anas crecca*), American coots (*Fulica americana*), and horned larks (*Eremophila alpestris*) on bait sites during 2001 and 2002. During 2002, we also observed American crows (*Corvus brachyrhynchos*) on bait sites at the utility site.

We found 1 red-winged blackbird nest on 26 April 2001 at the gravel pit site. A single egg from the nest contained 0 µg/g DNC. On the same date, we observed an American robin (*Turdus migratorius*) building a nest at the gravel pit site. We located 1 mallard nest on an island at the gravel pit site on 30 April 2001. We did not find any nontarget nests at the utility site.

We located 1 mallard nest on an island at the gravel pit site on 18 April 2002. An unhatched egg from this nest contained 3.49 µg/g DNC, and 5 of the 9 eggs hatched. We found 4 common grackle (*Quiscalus quiscula*) nests, and observed an American robin gathering nesting materials during the nontarget nest search at the gravel pit site on 4 May 2002. We observed a red-winged blackbird gathering nesting materials on 14 May 2002, and located a mourning dove nest on 15 May 2002 at the gravel pit site. Both a grackle egg and a mourning dove egg from the gravel pit site contained 0 µg/g DNC. An American robin was observed sitting in a nest on 11 May 2002 at the utility site.

We submitted 3 Canada geese, 1 female and 2 males, for necropsy during 2001 from the gravel pit site. The female died from egg yolk peritonitis. Although the exact cause of death could not be determined for the males, 1 likely had an enteric bacterial infection leading to inflammation. The other male exhibited moderate myofiber degeneration, although no necrosis of the myofibers was observed. Death due to

coccidiostat intoxication could not be ruled out for this goose, although there was also evidence of a concurrent infectious or inflammatory disease.

We submitted 1 Canada goose, 1 mallard, and 1 Franklin's gull (*Larus pipixcan*) for necropsy during 2002 from the gravel pit site. The goose died as a result of pulmonary edema due to ingestion of lawn fertilizer. The mallard likely drowned during mating activity, and the Franklin's gull had a fractured bill which lead to starvation.

Population models showed the control population would continue to increase after 3 yr at an exponential rate, whereas the NCZ population would increase at a very slow rate (Figure 5.1). Culling once every 3 yr in the control population results in the population increasing in a relatively linear fashion over 20 yr. However, culling once every 3 years and using contraception yearly results in the population remaining relatively stable over 20 yr, with the population size fluctuating between 45-100 geese (Figure 5.2). Culling once every 4 yr in the control population results in the population expanding at a nearly exponential rate over 20 yr. Using contraception yearly and culling once every 4 yr results in a population that remains stable between 30-75 geese for 10 yr, then gradually increasing over the next 10 yr to 340 geese (Figure 5.2). Culling once every 5 yr in the control population results in an exponential growth rate over 20 yr. Using contraception yearly and culling once every 5 yr results in a population that remains stable between 45-280 geese for 10 yr, then gradually increasing over the next 10 yr to 820 geese (Figure 5.2).

DISCUSSION

Nicarbazin effectively reduced egg hatchability by 53% overall in this study. This correlates well with the results of a laboratory mallard study where 500 mg NCZ/kg feed

had 53% lower hatchability than control mallards (Chapter 3). However, this is greater than the 36% reduction in egg hatchability in a similar study in Oregon for Canada geese fed pelleted bait treated with NCZ at 2500 mg/kg feed (Bynum et al. 2006).

Nicarbazin increased the percentage of nests with $\leq 75\%$ hatchability. In 2000, all eggs hatched in 9.5% of nests at the gravel pit site, only 42.9% of nests had hatchability $\leq 75\%$, and there were no nests in which none of the eggs hatched. During 2001, there were no nests in which all eggs hatched, 94.3% of the nests had $\leq 75\%$ hatchability, and 28.6% of the nests had no eggs hatch. In 2002, all eggs hatched in 3.4% of the nests, 86.2% of the nests had $\leq 75\%$ hatchability, and 27.6% of the nests had no eggs hatch. The average rainfall in the area was 5.96 inches, 8.92 inches, and 5.91 inches in 2000, 2001, and 2002 respectively (Northern Colorado Water Conservancy District). Because there was no significant difference between the treatment years with respect to hatchability, rainfall likely had a minimal effect on hatching success.

Hatchability was negatively correlated with egg DNC levels. Prior studies with chickens, mallards, and Canada geese indicate a minimum egg DNC level of 6 $\mu\text{g/g}$ is needed to affect hatchability (Jones et al. 1990, Yoder et al. 2005). Mean egg DNC levels in this study were $2.3 \pm 0.8 \mu\text{g/g}$ and $2.2 \pm 2.0 \mu\text{g/g}$ at the gravel pit and utility sites, respectively. Levels ranged from 0.0-20.9 $\mu\text{g/g}$ throughout the study. With 1 exception, eggs with DNC levels $\geq 2.9 \mu\text{g/g}$ contained no embryos ($n = 33$). The 1 embryo that was found was approximately 2-3 d old. Therefore, it seems likely that a lower level of egg DNC is needed to reduce egg hatchability in geese than previously predicted.

At high doses, NCZ is associated with a reduction or cessation of egg production in chickens (Sherwood et al. 1956, Jones et al. 1990, Hughes et al. 1991). In 2000, 353 total eggs were laid at the gravel pit site compared to 242 and 201 eggs in 2001 and 2002, respectively. If the number of nests in 2000 is used to calculate the number of breeding geese, then there were 140 breeding geese based on the presence of 70 nests at the gravel pit site. Based on the mean clutch size of 5.5 eggs/clutch and mean hatchability of 73.7%, 284 new individuals could potentially be added to the population if no mortality occurred. Assuming a 90% adult survival rate and a 50% gosling survival rate (Mowbray et al. 2002), this would result in a population size of 268 geese in 2001.

Using the 90% survival rate for adults, and assuming all surviving breeding pairs returned to the site to breed the following year, there would be 242 geese returning at the start of the year. Because geese don't usually breed until their second year (Mowbray et al. 2002), there would be 126 breeding geese at the start of the year. During 2001, there were 48 nests with a mean clutch size of 5.5 eggs/clutch, but hatchability was only 32.2%. Based on these figures, 85 new geese could potentially be added to the population if no mortality occurred. Using 50% gosling survival rate, 43 goslings would be added to the population, resulting in 285 geese returning in 2002. There would be 113 breeding adults > 2 yr old. The surviving goslings from 2000 would breed in 2002, resulting in 115 2-year-old adults ($142 \times 0.9 \times 0.9$). The resultant breeding population in 2002 would be 228 geese. These models assume 100% fidelity to natal areas as breeding grounds, and that no compensatory mechanisms occur (e.g. decreased mortality rate, increased reproductive rate).

Calculating eggs/goose as an index, there were 2.5, 1.9, and 0.9 eggs/goose in 2000, 2001, and 2002, respectively. These figures indicate some geese may have consumed enough NCZ to severely reduce or cause complete cessation of egg production. In addition, clutch sizes in the study were negatively correlated with egg DNC level. In 1 instance, a goose failed to lay eggs, but incubated rocks she had rolled into the nest from nearby.

The population models show that although contraception can considerably slow the rate of population growth, the population will still continue to grow. Contraception combined with periodic culling can maintain the population within a given range while decreasing the amount of lethal control used. The population models show yearly use of NCZ and culling once every 3 yr is sufficient to maintain the population within 100 geese of the starting population for ≥ 20 yr. Longer periods between culling result in only being able to maintain the population within 100 geese of the starting population for ≤ 10 yr. Yearly contraception with periodic culling may be more publicly acceptable in urban areas where residents enjoy looking at geese, and thus would be more likely to oppose lethal control methods.

These results are in agreement with other studies modeling contraception in bird populations. A population model for Canada geese comparing an annual culling rate of 50% with an annual sterilization rate of 50% for 3 years showed that although sterilization did decrease the population at the end of 3 years, culling was more efficient for population reduction (Dolbeer 1998). A model comparing a 70% reduction in population with a 70% reduction in fertility in one generation of pigeons showed that the culled population recovered within 3 generations, and increased at a geometric rate

(Sturtevant 1970). The contracepted population decreased slowly over 3 generations, and did not recover for 9 generations. Our model shows the amount of culling necessary can be reduced if the population is first reduced to the desirable level with an initial cull, and then maintained through the use of contraception and periodic culling.

The stage of embryonic development was negatively correlated with egg DNC level. This agrees with the results of previous studies that suggest reduction in egg hatchability for eggs fed NCZ may be due to an increase in embryonic mortality. Lucas (1958) found an increase in embryonic mortality during the first week of incubation for eggs of hens fed feed treated with NCZ at 125 mg NCZ/kg feed. A similar study found a 40-100% embryonic mortality rate for eggs of hens fed feed treated with NCZ at 50-700 mg NCZ/kg feed (Sherwood et al. 1956).

Interestingly, shell thickness was positively correlated with egg DNC levels. This is in contrast to chickens where NCZ is known to cause thinning of eggshells (Jones et al. 1990). However, it is in agreement with what occurs in mallards treated with NCZ (Yoder, unpublished data). In addition, DNC has been measure in eggshells of geese treated with NCZ (Stahl et al. 2003). Hatchability and clutch size were negatively correlated with eggshell thickness. Thicker eggshells may not allow adequate gas exchange, resulting in embryonic mortality.

Mean eggshell thickness was 0.55 ± 0.02 mm in 2001 and 0.65 ± 0.01 mm in 2002 at the gravel pit site, and 0.51 ± 0.04 mm in 2001 and 0.68 ± 0.03 mm in 2002 at the utility site. Egg DNC levels were 1.3 ± 1.1 $\mu\text{g/g}$ in 2001 and 3.4 ± 1.0 $\mu\text{g/g}$ in 2002 at the gravel pit site, and 1.8 ± 3.0 $\mu\text{g/g}$ in 2001 and 2.6 ± 2.1 $\mu\text{g/g}$ in 2002 at the utility site. A mean eggshell thickness of 0.65 ± 0.01 mm was reported for Canada geese (*Branta*

canadensis moffitti) in the Pacific flyway (Blus et al. 1979). Canada geese (*Branta canadensis canadensis*) in northern Germany had eggs with a mean eggshell thickness of 0.53 ± 0.07 mm (Bönner et al. 2004).

Distance of nests from bait sites was an important factor in reproductive success. Some geese may have utilized the bait site closest to their nest during the egg laying period as their primary food source. Eggs from nests closer to bait sites had higher egg DNC levels than eggs from nests farther from bait sites. The farther a nest was from a bait site, the higher the hatchability and overall nest fertility. In addition, nests farther from bait sites had larger clutch sizes. This indicates there may be some minimum distance a bait site must be located from a nest for hatchability to be affected. Nests located ≤ 70 m from a bait site had $\leq 45\%$ hatchability.

Geese at the gravel pit site stayed on the nest past the EHD longer than geese at the utility site. This was likely due to location of food resources in relation to the nesting area. The gravel pit site provided abundant food resources within 45 m of nesting areas, whereas food resources at the utility site were located ≥ 1100 m from the nesting areas. Although some geese remained incubating the nest for longer periods, most geese abandoned the nest about 1 week past the EHD.

Nontarget consumption of NCZ bait was a factor during the first half of the baiting period each year due to presence of migratory birds at each site. In order to minimize this hazard, bait should be formulated using whole corn or a pelleted bait to eliminate consumption by smaller birds. OvoControlG® is a pelleted NCZ bait that was registered with the Environmental Protection Agency for control of resident Canada geese in November 2005. With the exception of mallards, none of the nontargets consuming bait

had started nesting until the goose nesting season was nearly complete. In 2001, nontarget nesting activity was not observed until ≤ 9 d before the end of the baiting period. In 2002, only mallards began nesting before the end of the baiting period. Other nontarget nesting activity in 2002 occurred ≥ 6 d after the end of the baiting period. Because NCZ clears within 3-4 d from the blood stream (Yoder et al. 2005), nontarget nests started ≥ 4 d after the end of baiting should be unaffected. With 1 potential exception, none of the mortality that occurred during the study could be attributed to NCZ ingestion.

MANAGEMENT IMPLICATIONS

Nicarbazin reduces egg hatchability, and could be effectively used in an integrated management plan for resident Canada geese. Because effectiveness of NCZ is dependent on daily consumption of bait, variable results occur among geese. Therefore, it is not recommended that NCZ contraception be used as a stand-alone tool to manage resident flocks already larger than publicly acceptable levels. Such populations must first be reduced through round-ups and hunting, then maintained with contraception. This plan would reduce the frequency of culling programs (Ettl 1993). It is recommended that contraception be used yearly, and culling programs be implemented once every 3 yr to maintain the population at a given size.

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Table 5.1. Average hatchability (%), clutch size (eggs/clutch), fertility (%), and stage of embryonic development of Canada geese treated with 1,320 mg nicarbazin/kg cracked corn at a gravel pit site and a utility facility site in northern Colorado during 2001 and 2002 from March to June.

Parameter	Gravel pit site			Utility site			<i>P</i>
	\bar{x}	SE	<i>n</i>	\bar{x}	SE	<i>n</i>	
Hatchability	34.7A ^a	3.8	64	49.7B	6.5	24	0.0493
Clutch size	5.4A	0.1	75	4.8B	0.2	34	0.0127
Fertility	52.2A	4.5	50	73.6B	7.8	20	0.0205
Development stage	3.6A	0.3	273	5.4B	0.6	94	0.0089

^aMeans within rows with different letters are significantly different.

Table 5.2. Correlations between egg 4,4'-dinitrocarbanilide (DNC) levels ($\mu\text{g/g}$) and hatchability (%), clutch size (eggs/clutch), fertility (%), stage of embryonic development, eggshell thickness (mm), and distance of the nest from the bait site (m) for Canada geese treated with 1,320 mg nicarbazin/kg cracked corn at a gravel pit site and a utility facility site in northern Colorado during 2001 and 2002 from March to June.

	Egg DNC level		
	<i>n</i>	<i>r</i>	<i>P</i>
Hatchability	1764	-0.08222	0.0005
Clutch size	1863	-0.14069	< 0.0001
Fertility	1604	-0.21866	< 0.0001
Stage of embryo development	1604	-0.12013	< 0.0001
Eggshell thickness	1960	0.06583	0.0035
Distance	2153	-0.09554	< 0.0001

Table 5.3. Correlations among clutch size (eggs/clutch) and hatchability (%), fertility (%), and stage of embryonic development for Canada geese treated with 1,320 mg nicarbazin/kg cracked corn at a gravel pit site and a utility facility site in northern Colorado during 2001 and 2002 from March to June.

	Clutch size		
	<i>n</i>	<i>r</i>	<i>P</i>
Hatchability	2332	0.18720	< 0.0001
Fertility	1768	0.11538	< 0.0001
Development stage	1759	0.15731	< 0.0001

Table 5.4. Correlations among shell thickness (mm) and distance of a nest from the bait site (m), and hatchability (%), clutch size (eggs/clutch), fertility (%), stage of embryonic development, and distance of a nest from the bait site (m) for Canada geese treated with 1,320 mg nicarbazin/kg cracked corn at a gravel pit site and a utility facility site in northern Colorado during 2001 and 2002 from March to June.

	Eggshell thickness			Distance from bait site		
	<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
Hatchability	1764	-0.05040	0.0343	2332	0.09841	< 0.0001
Clutch size	1886	-0.17893	< 0.0001	2771	0.01296	0.4951
Fertility	1604	-0.15065	< 0.0001	1768	0.11792	< 0.0001
Development stage	1604	-0.14853	< 0.0001	1759	0.15959	< 0.0001
Distance	2153	-0.06595	0.0022			

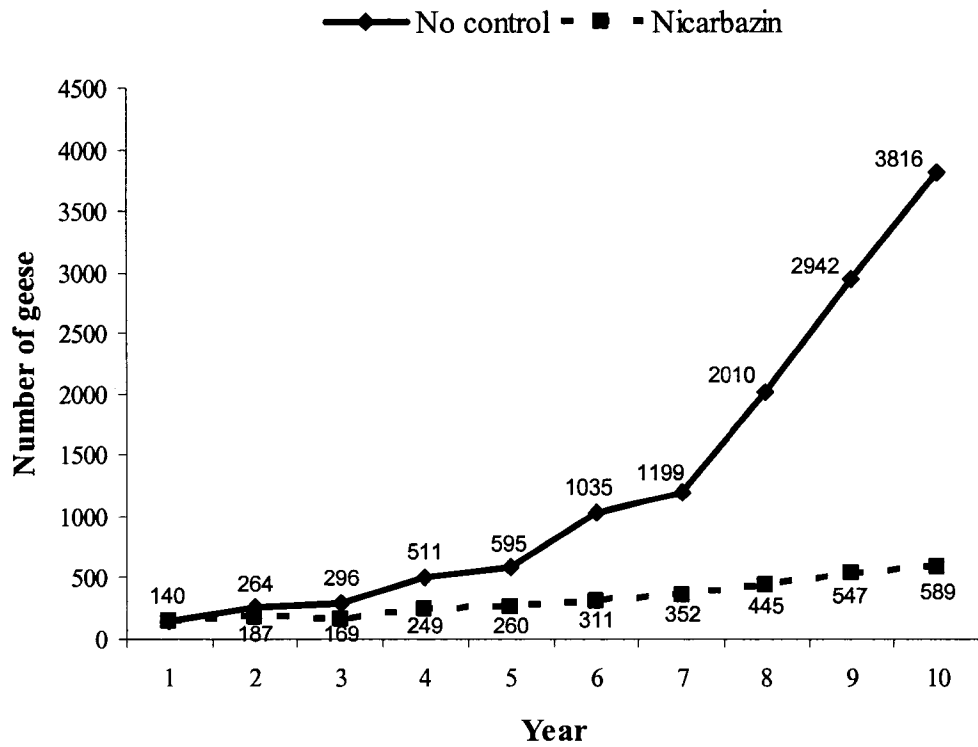
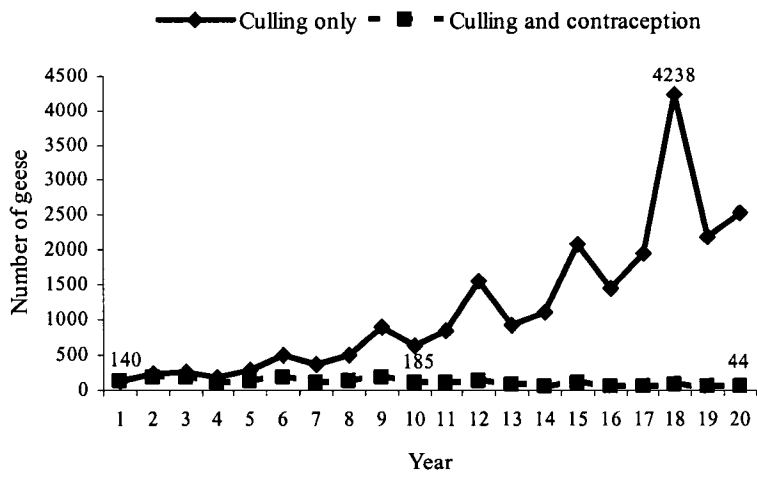


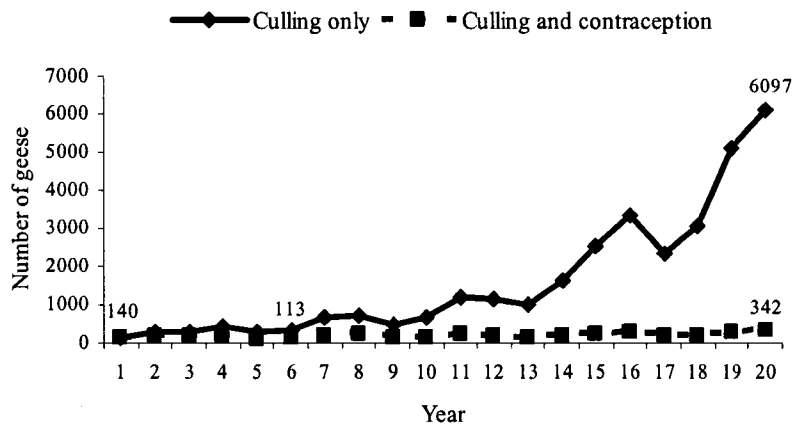
Figure 5.1. Model for Canada goose populations with no control or yearly treatment with nicarbazin contraception over 10 yr. Adult survival rates ranged from 0.75-0.9, gosling survival rates ranged from 0.49-0.61, control hatchability ranged from 0.64-.82, NCZ hatchability ranged from 0.28-0.42, control clutch sizes ranged from 5.2-5.8 eggs/clutch, and NCZ clutch sizes ranged from 5.1-5.7 eggs/clutch. The starting population size was 140 geese. These models did not allow for immigration of new individuals into the population, compensatory survival, and no carrying capacity was assumed. Additionally, the models assumed that all surviving adults returned to the natal area to breed the following year. The model did not allow for geese to breed until they were ≥ 2 yr old.

Figure 5.2. Models for Canada goose populations controlled with periodic culling, or populations controlled with yearly contraception and periodic culling. Adult survival rates ranged from 0.75-0.9, gosling survival rates ranged from 0.49-0.61, control hatchability ranged from 0.64-.82, NCZ hatchability ranged from 0.28-0.42, control clutch sizes ranged from 5.2-5.8 eggs/clutch, and NCZ clutch sizes ranged from 5.1-5.7 eggs/clutch. The starting population size was 140 geese. Culling involved removal of 50% of the population. These models did not allow for immigration of new individuals into the population, and no carrying capacity was assumed. Additionally, the models assumed that all surviving adults returned to the natal area to breed the following year. The model did not allow for geese to breed until they were ≥ 2 yr old. The maximum, and minimum population sizes for the contracepted population are noted on the graphs. Note: A) Culling once every 3 yr, B) Culling once every 4 yr, C) Culling once every 5 yr.

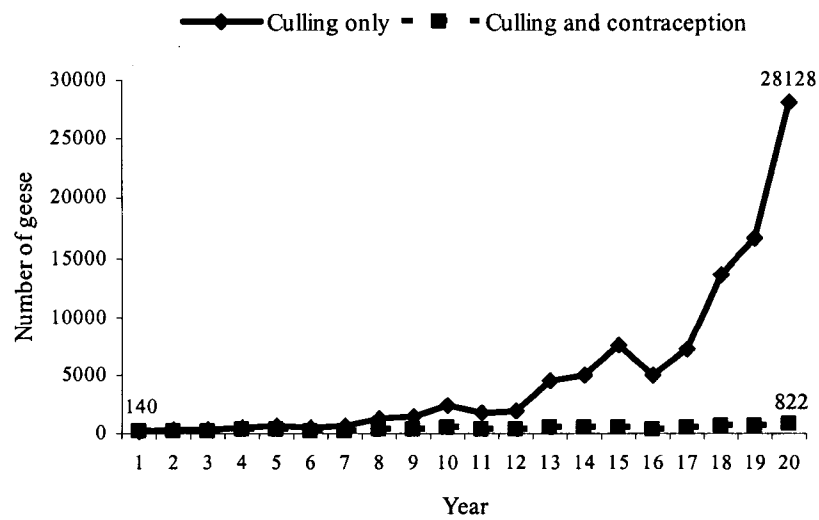
A)



B)



C)



CONCLUSIONS

The results of these studies can be used to formulate conclusions about the effect of nicarbazin on reproduction at the molecular level and on the level of the whole animal. At the molecular level, nicarbazin increases the activity of lipoprotein lipase, which causes premature degradation of VLDL in the blood stream, preventing deposition of lipids in the egg yolk. In addition, nicarbazin acts as a calcium ionophore, inserting itself into the vitelline membrane, and causing yolk mottling. Nicarbazin may also affect the outer vitelline membrane proteins VMOI and VMOII. The degradation of the vitelline membrane creates an inhospitable environment for the embryo, ultimately resulting in reduced egg hatchability.

At the level of the whole animal, mallards fed nicarbazin coated cracked corn achieved higher plasma DNC levels than mallards fed nicarbazin treated pellets. If pelleted bait is to be used in the field, it must be manufactured with a higher level of nicarbazin than cracked corn to achieve comparable plasma DNC levels. Measurements of fecal fluorescein were correlated with plasma DNC levels. Fecal fluorescein measurements provide a non-invasive technique to measure nicarbazin bait consumption in the field.

Finally, when Canada geese were baited with cracked corn coated with 1,320 mg nicarbazin/kg corn, egg hatchability was reduced by 53%. Nests located ≤ 70 m from bait sites had $\leq 45\%$ hatchability, whereas nests located farther from bait sites had higher hatchability. Egg DNC levels were negatively correlated with hatchability, fertility, clutch sizes, and stage of embryonic development, and were positively correlated with eggshell thickness. Baiting Canada geese with nicarbazin treated bait is effective in

reducing reproductive rates, but cannot reduce the population size overall at the level of contraception achieved in this study. Population modeling indicates that treating geese every year with nicarbazin bait in addition to culling 50% of the population every 3 years (e.g. through round up and removal) is sufficient to maintain a population within 100 geese of the starting population for ≥ 20 years.