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4,4′-Dinitrocarbanilide (DNC) concentrations in egg shells as a predictor of nicarbazin consumption and DNC dose in goose eggs†

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Abstract: Nicarbazin is being investigated as an infertility agent for the control of non-migratory Canada geese (*Branta canadensis* L) populations. Nicarbazin is presently registered for use as a coccidiostat for poultry. Geese fed sufficient quantities of nicarbazin will lay non-viable eggs. We established nicarbazin consumption by measuring the concentration of a component of the formulation, 4,4′-dinitrocarbanilide (DNC) in the egg contents (yolk, albumin) in non-viable eggs. To estimate the nicarbazin consumption of birds that laid viable eggs (eggs that hatched or contained an embryo), a high-performance liquid chromatography method was developed to measure the concentration of DNC in egg shells. A statistically significant correlation was established using linear regression between the mean concentrations of DNC in the egg shell and in the egg contents in non-viable eggs. Viable eggs were estimated to contain lower levels of DNC than non-viable eggs. DNC concentrations in both the egg contents and the egg shell increased with increases in nicarbazin dose in feed. Our method allows for the estimation of nicarbazin consumption and DNC dose in eggs under field conditions, which is important in developing an effective infertility agent for over-abundant non-migratory goose populations.
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Keywords: nicarbazin; egg shell; Canada goose; infertility

1 INTRODUCTION

Non-migratory Canada goose (*Branta canadensis* L) populations are increasing across the USA. Geese populations come into conflict with humans and pose increased health risks near bodies of water that they frequent. Acceptable population control strategies have often focused on relocation or hazing and these have met with limited success. Due to the proximity of many of these non-migratory populations to urban centers, hunting may not be a viable population control strategy. These issues have led to the investigation of infertility agents as population control strategies. Nicarbazin, an equimolar mixture of 4,4′-dinitrocarbanilide (DNC, Fig 1A) and 4,6-dimethyl-2-pyrimidinol (HDP, Fig 1B) has been investigated as a means of reducing the viability of eggs laid by geese at the United States Department of Agriculture/Animal Plant Health Inspection Service/Wildlife Services/National Wildlife Research Center. Nicarbazin is used as a coccidiostat in the broiler chicken industry, and has been observed to reduce hatchability in laying chickens.[1] Where hatchability is reduced, nicarbazin appears to break down the vitelline membrane (surrounding the yolk) allowing the yolk and the albumin to mix. Early work with ducks and geese indicated that nicarbazin decreased egg viability in a similar manner.[2,3]

To assess the nicarbazin dose required to prevent hatchability in the Canada goose, we performed a pen study with breeding pairs. We sought to correlate the feed dose, blood plasma levels and concentration of nicarbazin detected in unhatched eggs. Birds were fed extruded feed formulated with nicarbazin at 500, 250, 125 $\mu g\,g^{-1}$. Occasionally some, but not all, eggs laid in clutches at all three dose levels hatched. This raised the need to determine the threshold dose that was required to prevent hatchability and ascertain whether the egg shell could be assayed to predict the dose of nicarbazin in the egg contents (albumin and yolk). The objectives of the work reported here were: (1) to develop an analytical method to detect DNC as a measure of nicarbazin residues in egg shells, (2) to assess the relationship between shell concentration and the concentration in the egg contents, and (3) to use this relationship to predict the dose of DNC in viable eggs.

A high-performance liquid chromatography (HPLC) method was developed to assay nicarbazin levels in the egg shell by measuring DNC.[4] DNC is the active component of the mixture that comprises the nicarbazin formulation; HDP is thought to facilitate uptake of DNC in the gut.[5] For each treatment group,

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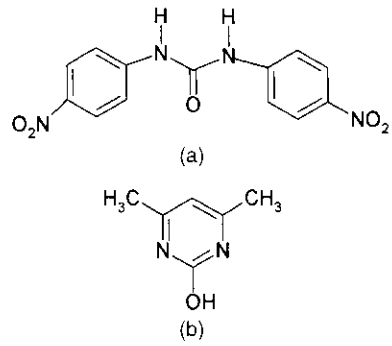

Figure 1. Chemical structures of the compounds that comprise nicarbazin; (A) 4,4′-dinitrocarbanilide (DNC) and (B) 4,6-dimethyl-2-pyrimidinol (HDP).

DNC concentrations in the shells were correlated with the concentration of DNC determined for the egg contents (yolk and albumin, combined).[6] The levels of DNC in the egg shells from eggs that were viable were then assayed.[4] The results of these analyses indicated a strong linear correlation between DNC found in the egg contents and in the shell of non-viable eggs. The linear regression of this relationship has been used to predict the concentration of DNC in goose eggs that were viable. The study demonstrated that in viable eggs the levels of DNC predicted were significantly lower than those observed in non-viable eggs.

2 MATERIALS AND METHODS

2.1 Canada goose feeding study

Eighty-eight mated pairs of Canada geese were maintained in outdoor pens at a farm in Fillmore County, MN. The pairs were randomly assigned to a nicarbazin feeding regimen. Both birds in a pen were fed, daily, a total of 200 g of extruded feed formulated to provide nicarbazin at levels of 500, 250, 125 or 0 $\mu g\,g^{-1}$ feed on a dry weight basis, ($n = 22$ pairs per treatment group). Feed was formulated at a local feed mill using a proprietary recipe developed by the owners of the geese. The addition of nicarbazin was the only modification to the recipe. Once the treated feed had been consumed, a maintenance diet of untreated feed was provided. The treatment was initiated approximately 7 days before the first egg was laid in a clutch. This diet was maintained until all eggs in a clutch were laid, for a total treatment period ranging from 15 to 18 days for 80% of the pairs.

2.2 Egg collection and handling

Eggs were labeled with the date on which they were laid. Un-hatched eggs and the egg shells from hatched eggs were collected 14–30 days after being laid. Eggs were stored at −12 °C until analyzed. The shells were separated from the egg contents, rinsed with de-ionized water and stored frozen until analyzed. Seven un-hatched eggs were randomly selected at each of the feeding treatment levels for analysis. Nineteen eggs were analyzed for DNC concentration in the egg contents as well as DNC concentration in the egg shell.

2.3 Analysis of egg shells[4]

Shells were cut into strips and 5-g samples were ground with a Brinkman Polytron (Brinkmann Instruments, Inc, Westbury, NY, USA) in 7 ml of acetonitrile. The suspension was then shaken on an Eberbach horizontal shaker (Eberbach Corp, Ann Arbor, MI, USA) for 10 min. The suspension was centrifuged for 2 min at 2500 *g* (Fisher Centrific centrifuge; Fisher Scientific, Pittsburgh, PA, USA). The supernatant was decanted and the extraction repeated twice more, both times in 5 ml of acetonitrile. The extraction volumes were combined and filtered through a 0.45-μm Teflon filter (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA). The solution was blown down under a gentle nitrogen stream at 60 °C using an N-Evap (Organomation, South Berlin, MA, USA). The solution was brought to a final volume of 1.00 ml. The solution was sonicated for 10 min using a Bransonic 20 ultrasonic bath (Branson Cleaning Equipment Co, Shelton, CT, USA) and an aliquot was filtered through a 0.45-μm Teflon filter into an LC vial and capped. Each shell was assayed twice and a mean shell concentration was calculated from the two values. Duplicate analysis were not rejected on the basis of a wide range in measured concentration, as two 5-g samples from a shell consumed the majority of the shell, precluding a repeat analysis. DNC concentrations were determined on an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA). A 30-μl sample was injected onto a Keystone ODS/H C-18 column, 5-μm particle size, 4.6 × 250 mm using a Keystone ODS/H 4.6 × 15 mm guard column (Keystone, Bellfonte, PA, USA). The separation was performed using a gradient elution in a mobile phase of acetonitrile + water, (40 + 60 by volume) increasing to 60 + 40 by volume over 15 min. The final mobile phase composition was maintained for 10 min. The flow rate was 1 $ml\,min^{-1}$ with a column temperature of 40 °C. DNC was measured with an Agilent UV multiple wavelength detector at $\lambda = 347$ nm. Standards (DNC, Phibro Animal Health., San Diego, CA) were prepared in acetonitrile. The limit of determination for DNC using chicken egg shells as a matrix was 0.0015 $\mu g\,g^{-1}$ with recoveries of 90.2% for shells fortified at a mean concentration of 0.047 $\mu g\,g^{-1}$ ($n = 7$) and 90.8% for shells fortified at a mean concentration of 0.93 $\mu g\,g^{-1}$ ($n = 7$).

2.4 Analysis of egg contents for DNC content[6]

An egg was cracked open, the contents were poured into a container, and homogenized with a hand blender. A 5.0-g portion was extracted three times in acetonitrile + *N*,*N*-dimethylformamide (1 + 1 by volume, 7.0 ml). The extraction solutions were combined and brought to volume in a 25-ml type A volumetric flask. At each extraction the sample was placed in an ultrasonic bath for 10 min and then centrifuged for 5 min. An aliquot of the solution was filtered with a 0.45-μm Teflon filter into an LC vial and capped. The samples were analyzed on an HP 1090 HPLC system with a diode-array detector (Agilent,

Palo Alto, CA, USA). A 60-µl sample was injected onto a Keystone ODS/H 250 × 4.6 mm column, 5-µm particle size, with a Keystone ODS/H 15 × 4.6 mm guard column (Keystone, Bellfonte, PA, USA) at 35 °C. The mobile phase was acetonitrile + water (60 + 40 by volume) and was an isocratic elution. DNC was measured at $\lambda = 347$ nm. Duplicate samples from every egg were analyzed with acceptance criteria that the replicates had to differ by ≤30%. The concentrations determined for each replicate were averaged. The method had a limit of detection of 0.075 µg g^{-1} for fortified chicken egg samples.

3 RESULTS

3.1 DNC concentration in goose egg shell

The mean DNC levels determined for the egg shell samples, which included the membrane for each of the treatments were: control—all less than the method limit of detection; 125 µg g^{-1} treatment = 0.080 (±0.073) µg g^{-1} (mean ± 1standard deviation); 250 µg g^{-1} treatment = 0.166 (±0.122) µg g^{-1}; and the 500 µg g^{-1} treatment = 0.341 (±0.241) µg g^{-1}. Mean egg shell DNC concentration was proportional to the nicarbazin concentration in the feed. This suggests that DNC egg shell concentration can be used as a predictor of nicarbazin consumption. The variability may be due in part to the non-uniform distribution of the membrane on the egg shell. In earlier work[4] the mass of membrane separated from the shell varied from 8 to 17% of the total mass of the 5-g shell sample. The DNC concentrations in the egg contents (albumin and yolk) averaged respectively for the same treatments: less than the method limit of detection, 2.28 (±1.17), 3.72 (±2.16) and 8.27 (±4.94) µg g^{-1}. The general increase in concentration in both the shells and the egg contents is consistent with earlier work where increases in tissue concentrations of DNC were observed with increased doses in chickens.[7,8] The mean DNC concentration in the egg shell are plotted against the mean DNC concentration in the egg contents for the same egg in Fig 2. The relationship between the mean DNC concentration in the shell versus the mean egg contents DNC concentration was (linear regression (SAS, version 8.2)):

$$\text{egg shell DNC concentration} = -0.000351 + 0.04346 \times \text{egg contents DNC concentration};\ r^2 = 0.838.$$

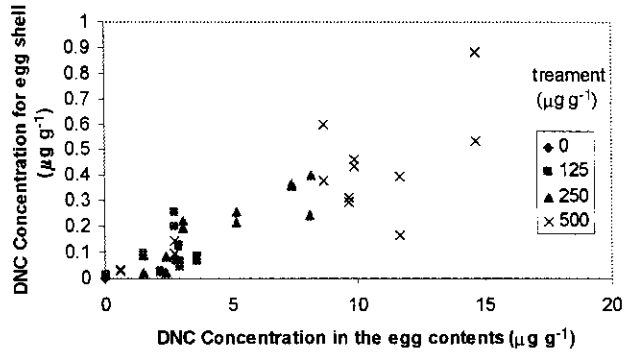

Figure 2. Concentration of DNC measured for two replicate samples of an egg shell plotted against the mean concentration of DNC measured in the egg contents for the same egg. Values are grouped by feed treatment dose level.

3.2 Comparison of DNC concentration in the egg shell from viable and non-viable goose eggs

The concentrations of DNC determined in the egg shell for both viable and non-viable eggs are presented in Table 1. The means were compared using Student's *t*-test for each treatment assuming unequal variances (Microsoft Excel, Office 2000). The *t*-test results at each treatment level are also presented in Table 1. For each of the treatment groups the viable eggs had significantly lower DNC concentrations in the shells than the non-viable eggs. The means for the DNC concentrations in the egg shell from the viable eggs were tested for significant differences using ANOVA (Microsoft Excel, Office 2000). The means across the three feeding treatments were found not be significantly different at $\alpha = 0.05$, $F_{\text{calculated}} = 1.60$, $F_{\text{critical}} = 3.39$. This contrasts with the non-viable eggs where the egg shell DNC concentration increased with increasing DNC concentration in the feed.

The uniformly low level of DNC in the viable eggs suggests to us that there is a threshold concentration of nicarbazin that the embryo must receive to prevent vitelline membrane formation. That viable eggs can be laid at any feed treatment level reflects the fact that both a hen and a gander were in a pen and the amount of treated feed consumed by the hen may not be adequate to prevent egg viability or that exposure must occur at a specific point in egg development prior to laying. We focused on the female as the male's fertility should not have been affected by consuming the nicarbazin-treated feed. Nicarbazin

Table 1. Comparison of DNC concentrations in the egg shells determined for viable and non-viable eggs

	DNC Concentration (µg g^{-1}) (±SD)		
Feeding treatment	125	250	500
Viable eggs	0.023 (±0.014)	0.031 (±0.013)	0.032 (±0.017)
Non-viable eggs	0.094 (±0.071)	0.19 (±0.13)	0.34 (±0.24)
Degrees of freedom	14	13	13
$T_{\text{calculated}}$	3.44	4.34	4.78
T_{critical}	1.76	1.77	1.77
$P(T_{\text{calculated}} \leq T_{\text{crit-cal}})$	0.002	0.0008	0.0004

consumption was determined to have no effect on the fertility of (chicken) cockerels at levels comparable to those administered in this study.[9] Consumption patterns are further complicated by the fact that nicarbazin may not be retained for long periods. Work done with chickens indicated that DNC could not be detected in the plasma or tissues of birds 48 h after a single dose.[7,10] The use of C^{14}-labeled DNC in nicarbazin fed to chickens indicated that only traces could be detected in tissues after four days following the discontinuation of the treatment.[11] In a comparative study, chickens, ducks and geese were all fed 8.4 mg kg^{-1} nicarbazin for eight days.[12] For the ducks and geese, the plasma concentrations of DNC fell below the MLOD within four days, and for the chickens, within six days after stopping treatment. The plasma levels of DNC in geese fed 8.4 mg kg^{-1} (treatment mass/body mass) nicarbazin never exceeded 1.5 μg ml^{-1} DNC.[12] Also, DNC is sparingly water soluble. Early work done analyzing the DNC in feces of ducks fed nicarbazin-formulated feed indicated that the majority of the dose in the feed passed through the GI tract unabsorbed.[13]

3.3 Prediction of DNC concentration in hatched eggs from egg shell concentration

From the viable eggs, 16 were randomly selected and the shells were extracted and analyzed for DNC concentration in the shell. These values were then used with the linear regression of DNC concentration in the shell versus DNC concentration in the egg contents for eggs that were not viable to calculate the concentration of DNC in the viable egg. The results for these 16 eggs are presented in Table 2. The values are presented by feeding treatment group. The means for the predicted DNC level for the viable eggs as well as the means of the egg DNC concentrations for the viable eggs are plotted in Fig 3 versus feeding treatment nicarbazin concentration, demonstrating the differences in viable and non-viable eggs by feed treatment group. At all three feeding treatment levels the predicted DNC concentration in the viable eggs was less than that observed in the non-viable eggs. The mean predicted DNC concentration

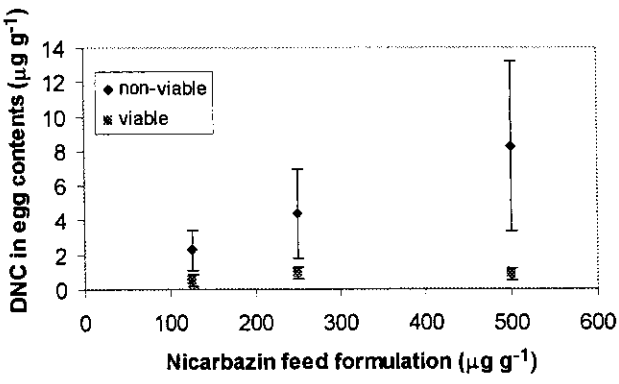

Figure 3. Predicted DNC concentration in the egg (viable eggs) and measured DNC concentration in the egg (non-viable) plotted against feed treatment dose level. Bar = SD.

Table 2. Analysis of hatched goose egg shell data using the regression equation of DNC concentration in the composite shell versus the DNC concentration in the egg contents

Sample	Treatment DNC conc (μg g^{-1})	Mean shell conc (μg g^{-1})	Calculated DNC conc in egg from shell conc (μg g^{-1})
1	500	0.053	1.30
2	500	0.024	0.63
3	500	0.019	0.52
4	500	0.027	0.69
5	500	0.041	1.02
6	250	0.030	0.77
7	250	0.020	0.54
8	250	0.043	1.07
9	250	0.057	1.39
10	250	0.020	0.55
11	250	0.047	1.17
12	125	0.016	0.45
13	125	0.0067	0.23
14	125	0.039	0.98
15	125	0.015	0.44
16	0	<MLOD	nc
	Mean	0.028	0.75
	SD	0.016	0.37

MLOD = method limit of detection.
nc = not calculated.

in the egg contents for the viable eggs calculated from the DNC concentration in the shell was 0.75 (±0.37) μg g^{-1}. Regardless of the concentration of nicarbazin in the feed, viable eggs appeared to receive approximately the same dose. The low DNC levels, across the feed treatment levels, predicted from the non-viable egg data suggest that the DNC concentration in the egg shell reflects the dose received by the embryo.

4 CONCLUSIONS

The ability to measure the DNC concentration in the egg shells of geese has been useful in making predictions about the effective dose required to cause the laying of non-viable eggs by geese. The need to control non-migratory geese populations in a socially acceptable manner is driving the need to develop these kinds of analytical tool. This method is particularly useful in assaying viable and non-viable eggs regardless of the stage of development. The method offers the opportunity to access the effectiveness of a feeding program in the field. For example, if two eggs in a clutch hatch, and the remainder do not, the shells can be analyzed to determine whether the eggs that did not hatch were a result of a nicarbazin feeding program or some other environmental factor. The results from this work are presently being incorporated in evaluating new feed formulations to improve the consumption of nicarbazin by geese in an effort to ensure that the eggs in a clutch receive a minimum effective dose.

REFERENCES

1 Sherwood DH, Milby TT and Higgins WA, The effect of nicarbazin on reproduction in White Rock breeder hens. *Poult Sci* **35**:1014 (1956).

2 VerCauteren KC, Pipas MJ and Tope KL, Evaluation of nicarbazin-treated pellets for reducing the laying and viability of Canada goose eggs, *Proceedings of the 9th Wildlife Management Conference* ed by Brittingham MC, Kays J and Mcpeake R, Pennsylvania State University, University Park, PA, pp 337–345 (2001).

3 Johnston JJ, Britton WM, McDonald A, Primus TM, Goodall MJ, Furcolow CA, Buttgenbach TL, Kohler DJ, Miller LA, Yoder CA and Fagerstone KA. Quantification of plasma and egg 4,4′-dinitrocarbanilide (DNC) residues for the efficient development of a nicarbazin based contraceptive. *Pest Manag Sci* **58**:197–202 (2002).

4 Stahl RS, VerCautern KC, Buettgenbach TL and Johnston JJ, Determination of 4,4′-dinitrocarbanilide (DNC), a component of Nicarbazin, in Canada goose (*Branta canadensis*) eggshells using high-performance liquid chromatography. *J Agric Food Chem* **51**:1130–1135 (2002).

5 Rogers EF, Brown RD, Brown JD, Kazazis DM, Leanz WJ, Nichols JR, Ostlind DA and Rodino TM, Nicarbazin complex yields dinitrocarbanilic acid as ultrafine crystals with improved anticoccidial activity. *Science (Washington)* **222**:630 (1983).

6 Primus TM, Kohler DJ, Goodall MA, Yoder C, Mathies T, Miller L, Johnston JJ and VerCauteren KC, Determination of 4,4′-dinitrocarbanilide (DNC), the active component of the antifertility agent nicarbazin in chicken, duck, goose, and snake eggs. *J AOAC International* submitted.

7 Cuckler AC, Malanga CM and Ott WH, The antiparasitic activity of nicarbazin. *Poult Sci* **35**:98 (1958).

8 Francois AC and Pihet AJ, Metabolism of nicarbazin in chickens. *Ann Inst Nat Rech Agron Sec D Ann Zoolech* **9**:345 (1960).

9 Chapman HD, A review of the biological activity of the anticoccidial drug nicarbazin and its application for the control of Coccidiosis in poultry. *Poultry Sci Review* **5**:231–243 (1994).

10 Porter CC and Gilfillan JL, The absorption and excretion of orally administered nicarbazin by chickens. *Poultry Sci* **34**:95 (1955).

11 Clark I, Geoffrey RF, Gilfillan JL and Porter CC, Adsorption and elimination of carbon 14 labeled Nicarbazin by chickens. *Proc Soc Exp Biol Med* **91**:4 (1956).

12 Primus TM, Kohler DJ, Goodall MJ, Yoder C, Griffin D, Miller L and Johnston JJ, Determination of 4,4′-dinitrocarbanilide (DNC), the active component of the antifertility agent nicarbazin, in chicken, duck, and goose plasma. *J Agric Food Chem* **49**:3589–3593 (2001).

13 Stahl RS and Johnston JJ, High-performance liquid chromatography-based determination of nicarbazin excretion in waterfowl. *J Chromatogr B* **775**:103–108 (2002).