The Pennsylvania State University

The Graduate School

Graduate Program in Animal Science

CONTRACEPTIVE ACTION OF NICARBAZIN IN WHITE PEKIN DUCKS

A Thesis in

Animal Science

by

Valerie Reinoso

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2008

The thesis of Valerie Reinoso was reviewed and approved* by the following:

Guy F. Barbato
Associate Professor of Poultry Science
Thesis Advisor

Daniel Hagen
Professor of Animal Science

Troy Ott
Associate Professor of Reproductive Physiology

Ramesh Ramachandran
Assistant Professor of Molecular Endocrinology

Robert Elkin
Professor of Poultry Science
Head of the Department of Poultry Science

*Signatures are on file in the Graduate School

ABSTRACT

Nicarbazin is a potential contraceptive for controlling population growth in waterfowl; however, the mode of action for nicarbazin is unknown. In previous research, it was noted that the eggs of chickens with a low molecular weight variant of zona pellucida protein 3 (ZP3) laid eggs had a weak yolk membrane similar to eggs from nicarbazin-fed birds. Zona pellucida 3 is the putative sperm receptor and plays a structural role in the perivitelline membrane of birds. The objectives of this study were: 1) to assess the well-known nicarbazin-influenced decline in egg production and fertility in the White Pekin duck and 2) analyze the effect of dietary nicarbazin on ZP3 expression in the perivitelline membrane of the White Pekin duck egg.

Six doses of nicarbazin (0, 31, 63, 125, 250 and 500 ppm) were fed to female ducks for 14 days. Eggs were collected daily and incubated weekly. Ducks fed 0 ppm nicarbazin (control) had an average rate of lay (# eggs/duck/day) of 0.95 ± 0.15 with a mean viable fertility (#ducklings/egg set) of 87%. Ducks administered 250 or 500 parts per million (ppm) nicarbazin had significantly lower egg production within 2 days after the start of treatment. By the end of the treatment period, all nicarbazin groups had significantly lower egg production than the control group. Recovery of egg production after withdrawal of the nicarbazin treated diets occurred in reverse order of treatment dose, with complete recovery occurring within 4 days. All nicarbazin groups had significantly lower fertility than the control group within 3 days of the start of treatment. Seven days after the start of nicarbazin treatment, the 500 ppm group had no fertile eggs (hen/day egg production of 20%). The 125 and 250 ppm treatments eliminated fertility to

0 ducklings/egg set by 12 days post-treatment. Recovery of fertility occurred in inverse order of nicarbazin dose in the diet, preceding recovery of egg production by 2 to 3 days.

The relative abundance of the ZP3 glycoprotein in the perivitelline membrane of White Pekin ducks fed varying concentrations of nicarbazin was estimated by Western blot analysis. Western blot analysis of heat-solubilized perivitelline membranes from nicarbazin treated ducks exhibited a decrease in the abundance of ZP3 protein. Repeated measures ANOVA suggested a significant decline between internal control Week 1 and Week 3 of treatment for doses 63 ppm, 125 ppm, 250 ppm and 500 ppm of nicarbazin ($P<0.05$). Taken together, decreased fertility and diminished protein abundance of nicarbazin treated ducks demonstrates a negative effect of nicarbazin on ZP3, the putative sperm receptor, in the avian perivitelline membrane.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I wish to express my sincerest gratitude to Dr. Guy Barbato for his years of guidance as my thesis advisor. I would also like to thank him for his support and mentorship throughout my undergraduate career that cultivated my appreciation for research.

I want to thank Dr. Daniel Hagen, Dr. Troy Ott, and Dr. Ramesh Ramachandran for serving on my graduate committee and their advice and insight into improving the quality of my thesis. I greatly appreciate the time and patience they have given me.

My gratitude goes to my entire lab for their support and help. I could not have completed my thesis without the vital assistance of Robab Katani. Not only did her expertise as a laboratory technician help me, but also her constant encouragement and friendship. I would also like to thank the many undergraduates who spent their time cracking open hundreds of eggs with me, especially Kailash Lal and Daniel Woodburn.

I want to thank my fellow graduate students for the advice and camaraderie. I especially want to thank Olga Ocón-Grove and Susan Krzysik-Walker for answering my constant questions and sharing their experiences with me.

I thank the Department of Poultry Science and the Graduate School at the Pennsylvania State University for providing the support and opportunities that allowed me to pursue my graduate degree.

I would like to thank Dirk Wise, Gene Krout, and the employees at the Poultry Education and Research Center for taking care of the birds, collecting the eggs, and helping me handle the birds.

I would like to thank Alexander MacDonald and Innolytics Inc. for providing the financial support to make this research possible.

My love and gratitude goes to my family and friends for their overwhelming support. I am truly blessed to have my parents, Joyce and Anthony Reinoso, who give me strength and my many aunts, uncles and cousins that always think of me.

Chapter 1

Introduction

Approximately 80% of all urban goose flocks are increasing in size (Forbes 1993). Large non-migratory goose populations are considered pests and can pose a threat due to spread of disease, aggression, and present hazards for aircraft (Conover and Chasko 1985; Forbes 1993). Unfortunately, the majority of population control techniques are ineffective (Forbes 1993). A non-lethal method of pest control, in the form of contraception, is a potential solution for urban areas where hunting would be inappropriate and areas where public opinion does not favor pesticides. One potential avian contraceptive is the coccidiostat nicarbazin. Nicarbazin has been incorporated into OvoControl G® goose bait and registered with the Environmental Protection Agency (Bynum et al. 2007; Registration #80224-1). While nicarbazin is currently being marketed for contraceptive purposes, very few studies have been conducted to determine its mode of action.

The ability of nicarbazin to suppress reproduction is useful not only in the field of pest control, but also contributes a biochemical understanding to the structure and development of the perivitelline membrane. The formation of the perivitelline membrane is a necessary step for egg formation and oviposition. This study also offers the first step towards characterizing the genetic regulation of the sperm receptor and its relationship to fertility in poultry. This information may provide for future manipulation of the sperm

binding genes in breeding and laying hens, which could lead to increased fertility and hatchability.

The working hypothesis is that the reduction in egg production and viable fertility in birds exposed to nicarbazin is due to a decrease in the quantity of the zona pellucida 3 (ZP3) glycoprotein in the perivitelline membrane. The first experiment of this study addresses the effect of nicarbazin on egg production, fertility and hatchability. The zona pellucida family of proteins, particularly ZP3, are components of the putative sperm receptor in the avian species (Waclawek et al. 1998). In the second section of this study Western blotting was used to compare the amount of ZP3 in the perivitelline membranes of White Pekin ducks fed varying amounts of nicarbazin (Bellairs et al. 1963; Mori and Masuda 1993). The data collected also serves as confirmation that the White Pekin duck is an appropriate model for its wild relative, the Mallard duck, and for waterfowl in general (Tanabe et al. 1988; Scanes et al. 2004).

Chapter 2

Literature Review

Reproduction in the Female Bird

Egg Formation

In the avian species, an egg is formed within the female reproductive tract and oviposited approximately every 24 hours. Typically, only the left ovary is fully developed in an adult female (Scanes et al. 2004). At the time of hatching, there are an estimated 480,000 oocytes in the female, though only approximately 2,000 are visible in sexually immature chicken's ovary. Over the life of most domesticated species, approximately 250-500 oocytes will mature and ovulate (Sturkie 2000). Oocytes are contained within ovarian follicles, ranging in size according to their maturation stage. The ovum, including the yolk, is expelled from the follicle as the stigma ruptures at ovulation (Etches 1996; Scanes et al. 2004).

The oviduct is composed of three major sections: the infundibulum, magnum, and isthmus. The ovum is funneled into the entrance of the infundibulum, which is the site of fertilization, approximately 15 minutes after ovulation. The infundibulum secretes the first layer of albumen. Spermatozoa can be stored for 7-14 days in sperm-storage tubules located in both the uterovaginal region and infundibular-magnal region (Sturkie 2000). The ovum spends approximately 15 minutes within the infundibulum before it travels

through the magnum for approximately 3 hours. The secretory cells of the magnum produce the majority of the albumen and the majority of other egg white components that surrounds the ovum. The albumen-encased ovum moves to the isthmus, where it will spend approximately 1.5 hours. The inner and outer egg shell membranes are secreted in the isthmus. The next portion of the reproductive tract is the shell gland, where the ovum remains the majority of the time (18-22 hours) prior to oviposition. In the shell gland, the shell is formed by the deposition of calcium carbonate and other components of the shell, such as porphyrin pigments derived from hemoglobin metabolism, are added. The complete egg will move through the cervical-vaginal junction to be expelled through the cloaca (see review, Etches 1996).

Egg production and egg size vary widely among species. The White Leghorn reaches sexual maturity at 5-6 months of age and produces approximately 240 eggs/year. The meat-type Pekin duck reaches sexual maturity at 7-8 months of age and produces 110-175 eggs/year, whereas the layer-type ducks produce similar amounts to the White Leghorn chicken (Scanes et al. 2004). The egg of the Pekin duck (80 g) is also larger than the chicken's egg (58 g). The goose has a much larger egg at 215 g, however, she lays only 15-60 eggs/year (Scanes et al. 2004).

Ovarian Follicle Anatomy

The avian ovary contains a hierarchy of follicles that range in size, depending on their stage of growth. The largest follicle (F_1) releases the ovum during ovulation, the second largest follicle (F_2) will release the ovum during the next ovulation, continuing in

this pattern. There are usually 4-6 large (2-4 cm diameter) yolk-filled preovulatory follicles present in the ovary at one time. There are also many 6-12 mm prehierarchal follicles and a large number of small white follicles that are less than 6 mm at any given time (Sturkie 2000).

There are three stages in the growth of the follicle. In the first phase, follicles experience slow growth to 60-100 μm diameter, which will last months to years. The second phase consists of several months of faster growth where some yolk protein uptake occurs. The rapid growth phase is the third stage that occurs 6-11 days prior to ovulation. The majority of egg proteins and lipids are deposited during the rapid growth phase, where approximately 2 g of yolk per day are transported to the follicle, which results in growth from 8 mm to 37 mm in diameter (Sturkie 2000). Yolk consists of lipids or lipoproteins, mainly very low density lipoprotein (VLDL) or vitellogenin (VTG). The yolk proteins are synthesized in the liver and transported into the ovary via the blood. Yolk synthesis is regulated by gonadotropins and steroid hormones, which includes induction of VTG production in response to ovarian estrogen. The yolk is transported from the basal lamina to the oocyte's surface by traveling through the granulosa cells to be incorporated into the cell via adsorptive endocytosis as yolk spheres (Perry and Gilbert 1979).

The follicle contains several layers of different cell types (Figure **2-1**). These layers are the vitelline membrane (or oocyte plasma membrane), the perivitelline layer, the granulosa cells, the basal lamina (or basement membrane), and the theca interna and externa (Sturkie 2000). The inner perivitelline membrane is laid down in the follicle, whereas the outer perivitelline membrane (or extravitelline layer) is constructed as the

egg moves down the oviduct (Bakst and Howarth 1977b). The inner perivitelline membrane (PVM) is composed of four glycoproteins, and the outer PVM is composed of lysozyme, ovomucin, and vitelline membrane outer layer proteins I and II (Kido and Doi 1988; Kido et al. 1992). All together, the perivitelline membranes, continuous membrane located between the inner and outer PVM, and the vitelline membrane are often referred to as the yolk membrane (Bakst and Howarth 1977a).

In the prehierarchal follicles, the granulosa cells are tightly packed into layers and are cuboidal in shape. During the rapid growth phase, the granulosa cells are arranged in a single layer and change to a squamous form. At the time of follicular growth, the intercellular spaces widen between the granulosa cells to allow yolk transport. After ovulation, the granulosa and theca cells will remain behind to be reabsorbed. The post-ovulatory follicle is reabsorbed after 6-10 days in the chicken, but it will take several months for reabsorption in the mallard (see review, Sturkie 2000).

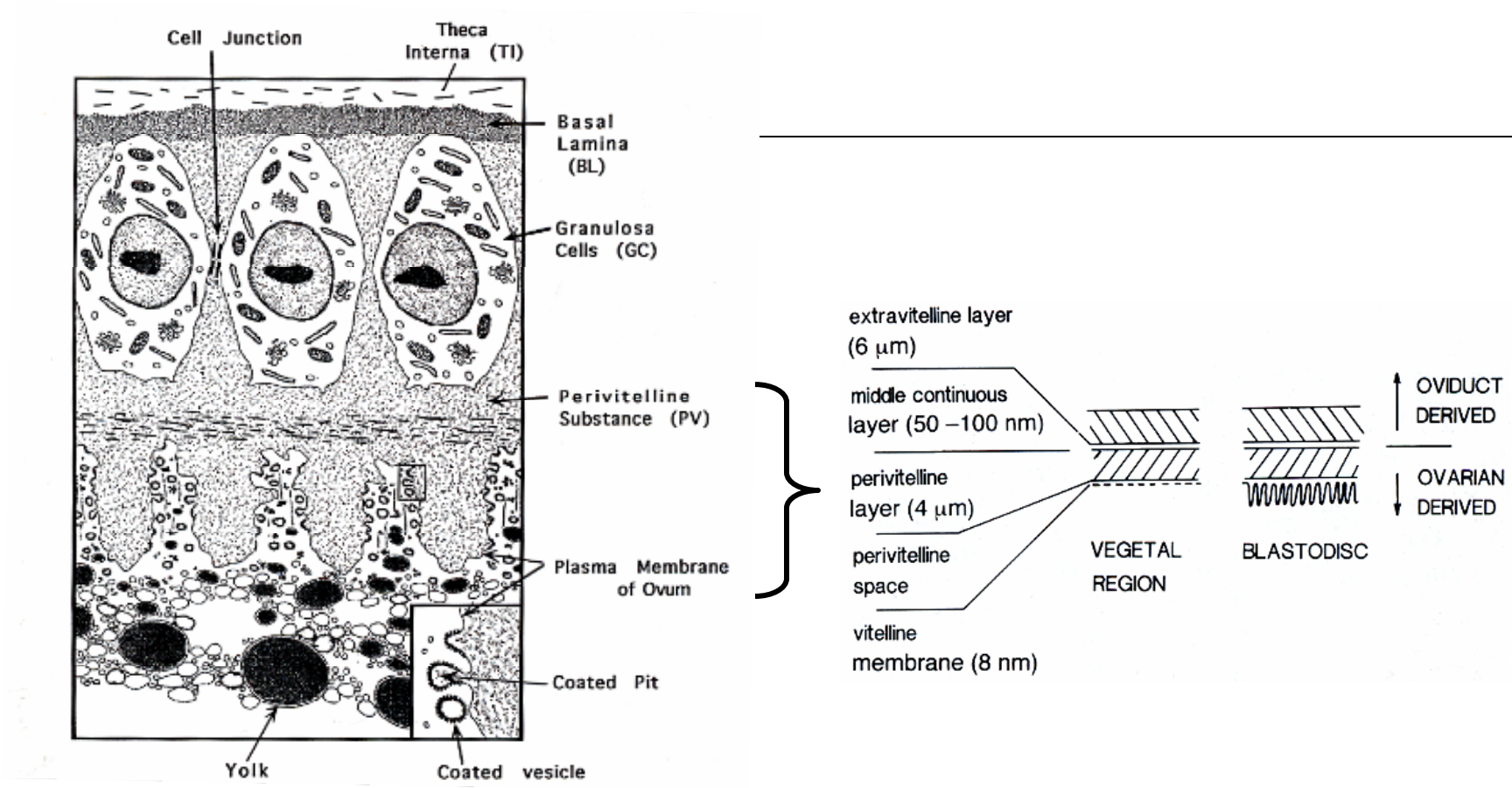

Figure **2-1**: a) The layers of the follicle (source: Sturkie, P. D. 2000. Avian Physiology. 5th ed. New York: Springer-Verlag) b) The perivitelline layers of the follicle (source: Etches, R. J. 1996. Reproduction in Poultry. CAB International: University Press, Cambridge, UK). The perivitelline layer is also known as the inner perivitelline membrane, while the extravitelline layer is known as the outer perivitelline membrane. The plasma membrane is also called the vitelline membrane.

Zona Pellucida Proteins

ZP Protein Family

The process of sperm binding to the egg, which leads to fertilization, is vital for reproduction. The site of sperm binding in mammals is a dense, fibrous extracellular membrane known as the zona pellucida (Wassarman et al. 1999). It is also known as the chorion in fish, the vitelline envelope in amphibians, and the perivitelline membrane in birds (Spargo and Hope 2003). This egg coat or envelope surrounds the oocyte. The zona pellucida matrix is laid down between the granulosa cells and oocyte during early follicular development (Lee et al. 1993). The proteins that are components of the zona pellucida and facilitate the sperm-egg interactions are known as zona pellucida (ZP) glycoproteins, regardless of the species.

The zona pellucida (ZP) serves several purposes: 1) it contains sperm receptors (Florman and Storey 1982; Berger et al. 1989), 2) it protects the gamete from physical damage (Sinowatz et al. 2001) and 3) after fertilization it functions as a block to polyspermy (Hoshi et al. 1988; Wassarman 1990). The mouse serves as the model for the majority of ZP information. In the mouse, there are three sulfated ZP glycoproteins, ZP1, ZP2 and ZP3 (Bleil and Wassarman 1980; Shimizu et al. 1983). In the murine model, it was suggested ZP glycoprotein presence was restricted to the oocytes (Epifano et al. 1995). However, ZP2- and ZP3-coding mRNA and proteins have been detected in the granulosa cells of cynomolgus monkeys and ZP3 in the granulosa cells of follicles in humans (Grootenhuis et al. 1996; Martinez et al. 1996).

The nomenclature for the zona pellucida (ZP) proteins and genes is inconsistent and thus the source of some confusion. The genes and the respective proteins they code for are: ZPA/ZP2, ZPB/ZP1, and ZPC/ZP3 (Sinowatz et al. 2001). The nomenclature for the zona pellucida proteins was initially determined by the order of their molecular weights in the mouse (Bleil and Wassarman 1980). It has also been suggested to refer to the zona pellucida proteins by the nomenclature used for their respective genes. The genes are named according to the size of their cDNA, starting with the largest cDNA being ZPA/ZP2 and the smallest ZPC/ZP3 (Kinloch et al. 1991). For clarity, the zona pellucida proteins will be referred to by their numeric protein names throughout this document.

Zona pellucida 3 protein (ZP3) is responsible for the binding of sperm in the mouse (O'Rand 1988; Rosiere and Wassarman 1992; Hardy and Garbers 1994; Wassarman and Litscher 2001). This is accomplished by the sperm recognizing and binding to specific serine/threonine-linked (O-linked) oligosaccharides located at the ZP3 combining site for sperm on the oocyte (Florman and Wassarman 1985; Liu et al. 1997). After binding to the sperm, ZP3 induces the acrosome reaction (Wassarman 1990; Liu et al. 1997). Once fertilization occurs, the ZP3 glycoproteins will undergo inactivation via proteolytic degradation (Bleil and Wassarman 1980; Wassarman 1990). The degradation of ZP3 causes it to no longer to be able to bind sperm and thus serve as a block to polyspermy (Noguchi et al. 1994). ZP3 also serves a structural role, which is evident in homozygous mutant female mice that have ZP3 inactivated. In these mice, ZP1 and ZP2 can be detected, but no zona pellucida matrix is present around the oocytes (Rankin et al. 1996).

Zona pellucida protein 2 (ZP2) is considered a secondary sperm receptor and it binds the inner acrosomal membrane of the acrosome-reacted sperm (Wassarman and Litscher 2001). ZP2 also undergoes proteolysis as a component to the block to polyspermy after fertilization (Moller and Wassarman 1989). In mice, ZP2 forms a heterodimer with ZP3, which is important for maintaining the connection between the acrosome-reacted sperm and the oocyte (Hinsch and Hinsch 1999). ZP2 serves as an anchor to sperm on the oocytes and allows the nuclear transfer to go to completion (Prasad et al. 2000).

In mice, zona pellucida protein 1 (ZP1) is primarily responsible for structural maintenance by connecting the ZP filaments to create a three dimensional matrix (Greve and Wassarman 1985). It is not required for sperm binding in mice, but is associated with sperm binding in rabbits and pigs (Rankin et al. 1999). However, early embryonic mortality and a loosely organized zona pellucida were observed in ZP1 knockout mice (Rankin et al. 1999; Sinowatz et al. 2001).

ZP Proteins in Birds

In the avian species, the egg envelope is referred to as the perivitelline membrane (PVM). With the chicken as a model, there are currently seven identified ZP genes or proteins within five ZP classes (reviewed in Hughes 2007). The classes are ZP1, ZP2, ZP3, ZPD and ZPAX (which continues to add confusion to the alphabetic/numeric nomenclature). ZP2 has been identified in birds and is assumed to contribute to the structure of the zona pellucida; although, its function is unknown (Smith et al. 2005).

ZPAX is described as a ZP2-like gene that has not yet been identified in mammals (Smith et al. 2005).

The major components of the avian egg envelope are considered to be ZP1, ZP3 and ZPD. ZP3 is the putative sperm receptor protein, but ZP1 also plays a role in sperm-egg interactions at the time of fertilization (Bausek et al. 2004). However, ZP1 begins to degrade once exposed to proteases from the sperm and ZP3 does not (Bausek et al. 2004). ZP1 is synthesized in the liver and travels to the perivitelline membrane via the bloodstream (Sasanami et al. 2003; Bausek et al. 2004). In addition to ZP1 and ZP3, the ZPD glycoprotein has been identified in the egg envelope and it has been suggested to play a role in sperm activation. In vitro, sperm incubated with ZPD displayed an increased incidence of having undergone the acrosome reaction (Okumura et al. 2004). ZPD displays variability between samples, and appears to be more readily released and separated from the egg envelope matrix, which makes it more difficult to detect (Okumura et al. 2004; Hughes 2007).

ZP3 has a molecular weight of 42 kDa, and is the major component of the perivitelline membrane (Takeuchi et al. 1999). The protein of ZPC (chZPC in chickens) has 41% homology to the mammalian ZPC gene class and ZP3 protein as indicated from GenBank database protein sequence comparisons and cDNA cloning (Waclawek et al. 1998; Takeuchi et al. 1999). ZP3 is synthesized in the granulosa cells, unlike mammalian ZP3, which is produced by both oocyte itself and the granulosa cells (Epifano et al. 1995; Waclawek et al. 1998; Takeuchi et al. 1999). In quail, ZP3 abundance has been shown to increase as the follicle develops (Pan et al. 2001). It has also been suggested that testosterone stimulates mRNA expression and ZP3 production (Pan et al. 2001).

Anti-chicken ZP3 antibodies can be used to identify ZP3 proteins via Western blotting across avian species due to highly conserved amino acid sequences. Antibody binding occurs in both inner and whole perivitelline membranes in the orders Galliformes, Anseriformes (family Anatidae), Estrildidae, Columbidae, Accipitridae and Spheniscidae (Stewart et al. 2004). The Khaki Campbell duck, which has shown ZP3 antibody cross-reactivity across avian species, was domesticated from the Mallard duck (Stewart et al. 2004). Similarly, the Pekin duck was also domesticated from the Mallard duck and thus has shown similar ZP3 antibody cross-reactivity (Tanabe et al. 1988; Scanes et al. 2004).

ZP-based Contraceptives

The zona pellucida glycoproteins, specifically the sperm-binding receptor ZP3, are important for successful fertilization and thus a subject of interest for studies involving improving fertility. Alternatively, the ZP proteins can be used for fertility control, via the development of immunocontraceptives or vaccines that target the sperm-binding receptor. Due to the variable conservation of the ZP proteins, the same vaccine may show cross-reactivity among several species (Naz et al. 2005). Immunization of female rabbits and monkeys with isolated porcine ZP resulted in a decline in fertility (Wood et al. 1981; Gulyas et al. 1983). The reduction in fertility from native porcine ZP was found to be caused by follicular atresia and abnormalities in the hormone presence. Contamination by other ovarian proteins may cause the abnormalities, thus purity of the

ZP as well as the agents used to increase efficacy are important factors to the success of such vaccines (Sacco et al. 1989; Bagavant et al. 1994).

Recombinant ZP vaccines are an alternative to the less abundant sources of native ZP (Naz et al. 2005). Marmosets immunized with recombinant human ZP3 displayed long-term infertility; however, it was associated with ovarian pathology as well (Paterson et al. 1998). In another study with recombinant human ZP proteins, it was suggested that ZP1 is an efficient anti-fertility agent, causing periods of infertility that ranged from 9-35 months (Martinez and Harris 2000).

Immunocontraceptives have the potential to be used for population control in wild and domestic animals as a humane alternative to killing as well as for use in zoos (Naz et al. 2005). ZP-based vaccines are being developed for species including feral horses (Turner et al. 2002), white-tailed deer (Miller et al. 1999; Naugle et al. 2002), African elephants (Fayrer-Hosken et al. 1999), dogs (Srivastava et al. 2002) and marsupials (McCartney et al. 2007). A study of feral female horses immunized against porcine ZP for four or five consecutive years indicated safe, reversible anti-fertility action. However, fertility did not return in the horses after seven years of treatment (Kirkpatrick and Turner 2002). Porcine ZP antigens do not cause infertility in feline species, illustrating that even considering sequence conservation; each species will have unique reactions to and requirements for a ZP vaccine (Naz et al. 2005). Though a human ZP-based vaccine has not yet been fully developed, there is great potential in using ZP proteins as a target for non-hormonal human contraception.

Nicarbazin

Chemical Overview

Nicarbazin (Figure **2-2**) is a common coccidiostat first introduced to the poultry industry in 1955 (Reid et al. 1984). Its primary use in poultry is to treat or prevent the transmission of an intestinal parasitic protozoa, coccidia (genus *Eimeria*), by inhibiting oocyst formation (Cuckler et al. 1956; Scanes et al. 2004). The mode of action for this inhibition is unknown, but it is believed to be associated with energy metabolism (Chapman 1997). Nicarbazin is often used in broiler chicken diets, since coccidia do not become resistant to it as with other drugs (Chapman 1997). Over $200 million annually has been lost in the US poultry industry due to coccidiosis and over $80 million per year has been spent on prevention (Scanes et al. 2004).

Nicarbazin is an equimolar complex of 4,4′-dinitrocarbinilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) (Ott et al. 1956). DNC is the active ingredient in nicarbazin, while HDP appreciably increases its solubility (Cuckler et al. 1955). The complex improves anticoccidial activity ten times that of DNC alone (Ott et al. 1956). HDP also enters and is excreted from the system more rapidly than DNC (Porter and Gilfillan 1955). DNC is nearly insoluble in water (2 μg/100 ml for DNC), and thus the nicarbazin complex must be dissolved in reagents such as dimethylsulphoxide (DMSO) (Rogers et al. 1983).

Nicarbazin has been established as a safe drug for use in the poultry industry. Nicarbazin has been approved by the US Food and Drug Administration (FDA) as a veterinary drug for use as an anticoccidial agent in poultry (Reid et al. 1984). The

recommended dose is 0.0125%, or 125 ppm, for treatment in poultry (Reid et al. 1984). A mallard would need to consume 3680 ppm nicarbazin to see lethal effects in 50% of the population, also known as the LD_{50} (EPA 2005). In periods of hot weather, chickens fed nicarbazin do show an increased tendency to exhibit heat stress (McDougald and McQuistion 1980). The FDA has also set a tolerance for nicarbazin residues in uncooked chicken tissue at 4 parts per million (EPA 2005). To ensure consumer safety, there is a 4 day withdrawal period for feeding nicarbazin to poultry prior to slaughter (Reid et al. 1984).

Research also indicates that nicarbazin poses little threat to mammalian health. Pigs can tolerate 50-100 times the approved range of nicarbazin recommended for chickens (Ott et al. 1956). In a two year study with rats chronically fed nicarbazin, a No Observed Effect Level (NOEL) was established at 400 mg/kg bw/day, and no toxic effects were observed at the highest dose (Roberts 1998). Studies in rats also indicate that there is no reproductive toxicity. Developmental toxicity in the form of depressed fetal growth and ossification was present in rats at high doses of 600 mg/kg bw/day (NOEL of 200 mg/kg bw/day). There is a NOEL of 240 mg/kg bw/day from a two year study in dogs due to slight bile-duct proliferation in one dog (Roberts 1998). It has also been determined that nicarbazin is not mutagenic or carcinogenic (EPA 2005). The US Environmental Protection Agency (2005) estimates that a child would have to consume 132 lbs of a 30% nicarbazin mixture to produce a lethal dose that would affect 50% of the population.

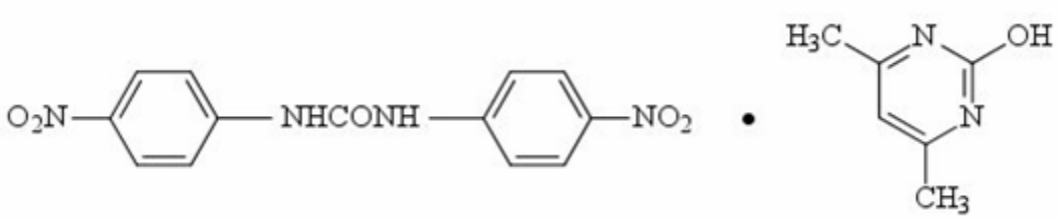

Figure **2-2**: Molecular structure of nicarbazin ($C_{19}H_{18}N_6O_6$). 1:1 complex with a molecular weight of 426.38 (Budavari 1989; photo from the National Wildlife Research Center)

Chemical Side Effects

While nicarbazin is an effective coccidiostat, there were several side effects noted in breeder and laying hens, which were cause for concern in the 1950's. There is a reversible reduction in hatchability by 60% with treatment levels of 50 ppm nicarbazin in the ration (Ott et al. 1956). There are similar reductions in brown egg layers fed 20 ppm nicarbazin and above, which have shown a drop to 0% hatchability after 8 days of 100 ppm nicarbazin treatment (Jones et al. 1990c). Broiler breeders also show a decrease in hatchability at these doses, including doses as low as 25 ppm (Jones et al. 1990a; Hughes et al.1991). These studies indicate an inverse linear relationship between nicarbazin level and hatchability.

When nicarbazin is added to the ration of hens, there is an observed decline in the rate of egg production. This reversible decrease in egg production was initially recorded at 400 ppm nicarbazin (Ott et al. 1956). However, egg production can decline at 125 ppm nicarbazin (Sherwood et al. 1956). At the level of 700 ppm nicarbazin in the ration, birds ceased oviposition (Sherwood 1956). Studies by Jones et al. (1990b,c) indicate that

White Leghorn layers show a reduction in egg production of 35% at 100 ppm nicarbazin and 92% at 125 ppm with similar results in Hisex brown egg layers. Jones et al. (1990a) did not see an effect on broiler breeders. A subsequent study by Hughes et al. (1991) observed that broiler breeders declined in egg production when fed nicarbazin ranging from 25-100 ppm for two days. These discrepancies are attributed to differences in genetic strain and age, emphasizing that the severity of the effects and metabolism of nicarbazin depends upon individual line and/or population species variation (Jones et al. 1990c; Hughes et al. 1991).

Eggs laid by hens fed nicarbazin show some alterations in their appearance. It is well-documented that nicarbazin causes the reversible depigmentation of brown and blue-green eggs. Doses as low as 25 ppm show a whitening of the shells of darker eggs after two days of exposure to nicarbazin in the feed of laying and broiler hens (Ott et al. 1956; Polin 1957; Jones et al. 1990a,c; Hughes et al. 1991). This is due to diminished levels of porphyrin in the uterus and egg shell (McClary 1955; Schwartz et al. 1975). The yolks of eggs from hens fed nicarbazin also exhibit a phenomenon called mottling. Mottling is the appearance of clear, white, or brown blemishes on a yolk. A mottled yolk appears as a combination of translucent and opaque areas that disturb the traditionally smooth and uniform surface (Baker et al. 1957). Egg yolks from hens display a 35% increase in mottling incidence after 3 days of 50 ppm nicarbazin in the diet and by 81% when fed 100 ppm nicarbazin (Polin 1957; Jones et al. 1990b,c). Mottled eggs also contain fewer solids and have higher water content. As with other effects of nicarbazin, mottling is reversible once nicarbazin is removed from the diet (Polin 1957; Silvestrini et al. 1965).

It is suggested that mottling occurs due to an increase in permeability of the vitelline membrane that allows the yolk and albumen to mix (Polin 1957; Cunningham 1977).

There are studies indicating several other changes to eggs produced by nicarbazin-fed poultry. There is some evidence that nicarbazin results in reduced egg weight and egg shell thickness. Reduced egg weight is observed in the eggs of hens fed 60-70 ppm nicarbazin (Baker et a. 1957; Polin 1957; Jones et al. 1990b; Hughes et al. 1991). It is possible egg shell thickness also decreases as a reduction can be seen in the ninth day after treatment with 125 ppm nicarbazin (Jones et al. 1990b). Several studies have noted no change in fertility in 100 ppm nicarbazin-fed hens (Jones et all. 1990ac; Hughes et al. 1991). Conversely, fertility appeared to be lower for nicarbazin-fed breeders, but the data were too inconsistent to conclude with certainty nicarbazin had a significant effect (Sherwood et al. 1956).

Contraceptive Use

While reduction in hatchability, egg production and other side effects were considered negative traits in the 1950's, these features of depressed reproduction may be considered assets in the field of wildlife population control. Populations of pest avian species are growing and new forms of management are needed, especially in urban areas where techniques, such as hunting, are not feasible. One group of target pest species are waterfowl, particularly resident Canada geese (*Branta canadensis*) that are known to frequent golf courses, airports and other urban locations (Forbes 1993; Ankney 1996). A survey of American households indicates that the public views lethal control techniques

as generally inhumane. In the survey, households rate the control of bird flocks near airports as a leading priority for federal involvement with wildlife, and they support research of non-lethal methods (Reiter et al. 1999). Since the ingestion of nicarbazin results in a reduction of reproductive efficiency, it has become a compound of interest to the National Wildlife Research Center (NWRC) as a potential contraceptive for overabundant waterfowl species.

The NWRC characterized and compared DNC presence in waterfowl, so the effectiveness of nicarbazin as a contraceptive could be evaluated. In 1998, nicarbazin was fed to Japanese quail to confirm that nicarbazin reduces hatchability in avian species other than the chicken. Hatchability in quail reached 0% after four weeks of treatment with 250 ppm nicarbazin in the diet (Bynum et al. 2005). The relationship between blood plasma DNC, egg DNC, and hatchability was determined using high-performance liquid chromatography (HPLC) techniques (Johnston et al. 2001). Egg and plasma DNC increased linearly as the dose of nicarbazin increased. Egg DNC also peaked after and decreased in the system slower than plasma DNC (Johnston et al. 2001). Over 97% of DNC plasma levels were able to be equally recovered via HPLC in chickens, Canada geese, and mallards (Primus et al. 2001).

After the collection of initial data, several comparative studies in waterfowl were performed. A comparison of nicarbazin absorption indicated that geese were less efficient in DNC absorption than mallards and mallards less than chickens (Yoder et al. 2005). Chickens reached peak plasma DNC levels (2.87±0.15 μg/ml) six days into treatment, while ducks (2.39±0.15 μg/ml) and geese (1.53±0.15 μg/ml) required eight days. Mallard plasma DNC levels were highly correlated with Canada goose plasma

DNC levels (Yoder et al. 2005). There is also no DNC plasma difference between genders in the mallard (Yoder et al. 2006b). Mallards and geese displayed similar characteristics for elimination of DNC from the plasma, indicating the mallard duck was an appropriate model for the Canada goose (Yoder et al. 2005). It was determined that greater than 125 ppm nicarbazin in the diet was necessary for waterfowl to reach similar plasma DNC levels to what is required in the chicken to reduce hatchability (Yoder et al. 2005). Further studies indicated that ideal plasma DNC levels for a decline in hatchability were reached at 500 ppm nicarbazin in treated feed (Yoder et al. 2006a).

The mode of action for nicarbazin's contraceptive activity is unknown. Micrographs of vitelline membranes from mallards fed 500 ppm nicarbazin displayed severe degenerative changes. Minor damage was observed at 125 ppm and 250 ppm dose groups in a dose-related manner (Yoder et al. 2006a). Preliminary in vitro studies indicated nicarbazin may be a calcium ionophore and increase intracellular calcium levels (Yoder et al. 2006c). It also increased lipoprotein lipase activity, which suggested the possibility nicarbazin causes early degradation of very low density lipoproteins (VLDL) that comprise the egg yolk (Yoder et al. 2006c). Overall, very few clues are available to describe nicarbazin's mode of action.

In 2005, the EPA approved the nicarbazin-based product OvoControl-G© from Innolytics, LLC to reduce egg hatch in resident Canada geese (EPA 2005). In a field trial with this product, there was a 62% reduction in nests having 100% hatchability and a 93% increase in nests having 0% hatchability (Bynum et al. 2007). When a flock of geese were fed a 2500 ppm OvoControl-G© bait, hatchability declined 36% overall from

control sites. Hatchability was inversely correlated with bait consumption (Bynum et al. 2007)

The use of nicarbazin as a contraceptive has also been applied to pigeon pest species. Initially, it was concluded that nicarbazin did not inhibit pigeon reproduction or did not have a strong enough effect for use in the field (Elder 1964; Giunchi et al. 2007). Further studies concluded the formulation of the treatment diet did not optimize absorption in pigeons and suggested much higher doses of 5,000 ppm nicarbazin were required (Avery et al. 2006). It was later determined that pigeons, in fact, required three times the amount of DNC in their plasma than geese and previous studies most likely did not use high enough doses (Avery et al. 2008). In captive pigeons fed 5,000 ppm nicarbazin, hatchability decreased by 59% and there was no reduction in egg production (Avery et al. 2008). In Italy, a nicarbazin-base contraceptive is currently in use to assist in control of urban pigeon populations (Giunchi et al. 2007).

Nicarbazin is a safe, established veterinary drug in the poultry industry. Previous trials have proven the low toxicity of nicarbazin in birds and mammals. While nicarbazin does result in negative side effects on reproduction in the poultry industry, the reduction in hatchability and egg production have become a benefit to pest species management. Nicarbazin has the potential for uses in contraception as seen with the pest avian control product OvoControl-G©. This still leaves the question of "how nicarbazin works". As an understanding of nicarbazin's mode of action is developed, the basic principles of the reproductive system can be better understood. This can lead to future contraceptive products for avian pest management.

Chapter 3

Nicarbazin Reduces Egg Production and Fertility in the White Pekin Duck

Introduction

Nicarbazin is a common coccidiostat introduced to the poultry industry in the 1950's. Nicarbazin is a complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), of which DNC is the active ingredient (Cuckler et al. 1955). While characterizing the biological features of this drug, Ott et al. (1956) noted a reversible reduction in New Hampshire chicken hatchability by 60% when a ration feed containing 0.005 % (50 ppm) nicarbazin. Egg production was significantly reduced with the addition of 0.04 % (400 ppm) nicarbazin. Sherwood et al. (1956) reported that egg production in White Rock breeder hens was lowered with nicarbazin doses as low as 0.0125 % (125 ppm). At the level of 0.07 % (700 ppm) nicarbazin in the ration, birds completely ceased egg production. In Sherwood's study, data were too variable to conclude nicarbazin had a significant effect on fertility; however, fertility appeared to be lower for nicarbazin-fed breeders than controls.

In 1990, Jones et al. suggested that intense selection for production traits among chicken populations may have increased or decreased susceptibility to nicarbazin's influence on reproduction. That study demonstrated a significant reduction in hatchability at 20 ppm nicarbazin and concluded that there was an inverse linear relationship between nicarbazin level and hatchability in broiler breeders (Jones et al. 1990a). Hughes et al. (1991) reported that hatchability decreased 54% after 4 days of

treatment with 25 ppm nicarbazin in broiler breeders, attributing the relatively large effect of the lower dose to both genotype and age (Jones et al. 1990a). Fertility was unaffected by nicarbazin up to a maximum dose of 100 ppm nicarbazin in brown egg layers and broiler breeders (Jones et al. 1990a,c; Hughes et al. 1991).

In recent years, nicarbazin has been studied as a potential contraceptive agent for pest bird species. The National Wildlife Research Center (NWRC) explored nicarbazin as a potential contraceptive for controlling overabundant waterfowl. At the NWRC, Yoder et. al. (2005) compared nicarbazin absorption in the plasma of chickens, mallards and Canada geese. From the comparison of nicarbazin absorptions, it was determined waterfowl require a higher nicarbazin dosage of 500 ppm than chickens (125 ppm) to achieve a significant reduction in egg production and/or fertility, though further dose-response studies are needed (Yoder 2006a).

Thus far, the mallard is the model used for determining the relationship between nicarbazin and egg production or fertility in waterfowl (EPA 1996). In the present study involving breeding and artificial insemination, the White Pekin duck provided a more suitable model for waterfowl. White Pekin ducks were domesticated from the mallard (*Anas platyrhynchos*) and thus share close homology (Scanes et al. 2004). Layer hybrid White Pekin ducks have high rates of egg production, averaging greater than 240 eggs per year (Scanes et al. 2004). Unlike pair-bonded mallards, Pekin ducks can be individually caged and artificially inseminated, allowing for the experimental dissection of the effect of any feed additive on males and/or females

The objectives of this study are to: 1) determine the dose-response relationship of dietary nicarbazin on egg production and fertility in White Pekin Ducks, 2) identify an

appropriate level of nicarbazin for contraceptive action with the Pekin duck as a model for waterfowl, and 3) compare the level of the active ingredient DNC in eggs and plasma of nicarbazin-fed ducks

Materials and Methods

Animals

Sixty female White Pekin ducks at 28 weeks of age were used under approved Institutional Animal Care and Use Committee (IACUC #21138) conditions for the following experiments. The ducks were raised in a small, mixed flock on raised wire floors with a 12 hour photoperiod. Ducks were individually caged at 16 weeks of age and lighting changed to a 16 hour light and 8 hour dark photoperiod. Water was available to the ducks *ad libitum*.

Nicarbazin Treatment

Sixty Pekin ducks were assigned to six groups of ten ducks/group. To adjust for pre-treatment differences, egg production and fertility data were collected two weeks prior to nicarbazin treatment. The ducks were then fed 300 g/day of a standard commercial layer diet (Appendix A) with either 0 ppm (control), 31 ppm, 63 ppm, 125 ppm, 250 ppm, or 500 ppm nicarbazin for a two-week period. Similar nicarbazin doses were used in previous experimentation involving waterfowl (Yoder 2005; Yoder 2006

a,b). After the two-week treatment period, egg production and fertility data were collected for a 16 day period post-treatment to assess recovery (Figure 3-1).

The period of blood and egg collection for DNC analysis consisted of three weeks, beginning with the second week of pretreatment and including both weeks of treatment. Blood (1 ml) was drawn from the brachial vein the morning of the seventh day of each week. Eggs were collected on the last three days of each week. Plasma and yolks of the collected eggs were pooled among individuals within dose treatments. The pooled samples of egg yolks and plasma from birds within each treatment group were assayed for DNC using HPLC with mass spectrometry by Exygen, Inc. (State College, PA).

Egg Production and Fertility Measurements

Eggs were collected daily, stored at 55°F and set in an incubator weekly to be hatched at 28 days of age. Incubator conditions were set at a temperature of 99.5°F and a relative humidity of 85%. During the 26-28th days of incubation, the temperature were adjusted to 98.5°F and 86%, respectively. Hen-day egg production (HDP) is defined as the average number of eggs laid per duck per day. Fertility was defined as the number of ducklings that hatched per egg set, since it is the presence of live offspring that dictates reproductive success (Barbato et al. 1998). Eggs were candled at 21 days after incubation to assess for early or late dead embryos and those that did not hatch were broken out and the contents evaluated.

Semen was collected from untreated drakes by abdominal massage. Semen was diluted to appropriate concentration with Minnesota A diluent, a semen extender

(Appendix B; Tajima et al. 1989). A sperm binding assay was also performed on 15 drakes to ensure they were not subfertile (Assay #3, Barbato et al. 1998; data not shown). Ducks were inseminated with 250 x 10^6 sperm/100 µl insemination dose from a pool of 10 untreated drakes having the highest sperm binding. Ducks were initially inseminated three separate times during the two week pretreatment phase of the experiment (Tuesday, Thursday, Tuesday) and weekly thereafter (on Tuesdays) to provide optimal fertility (Amann and Hammerstedt 2002).

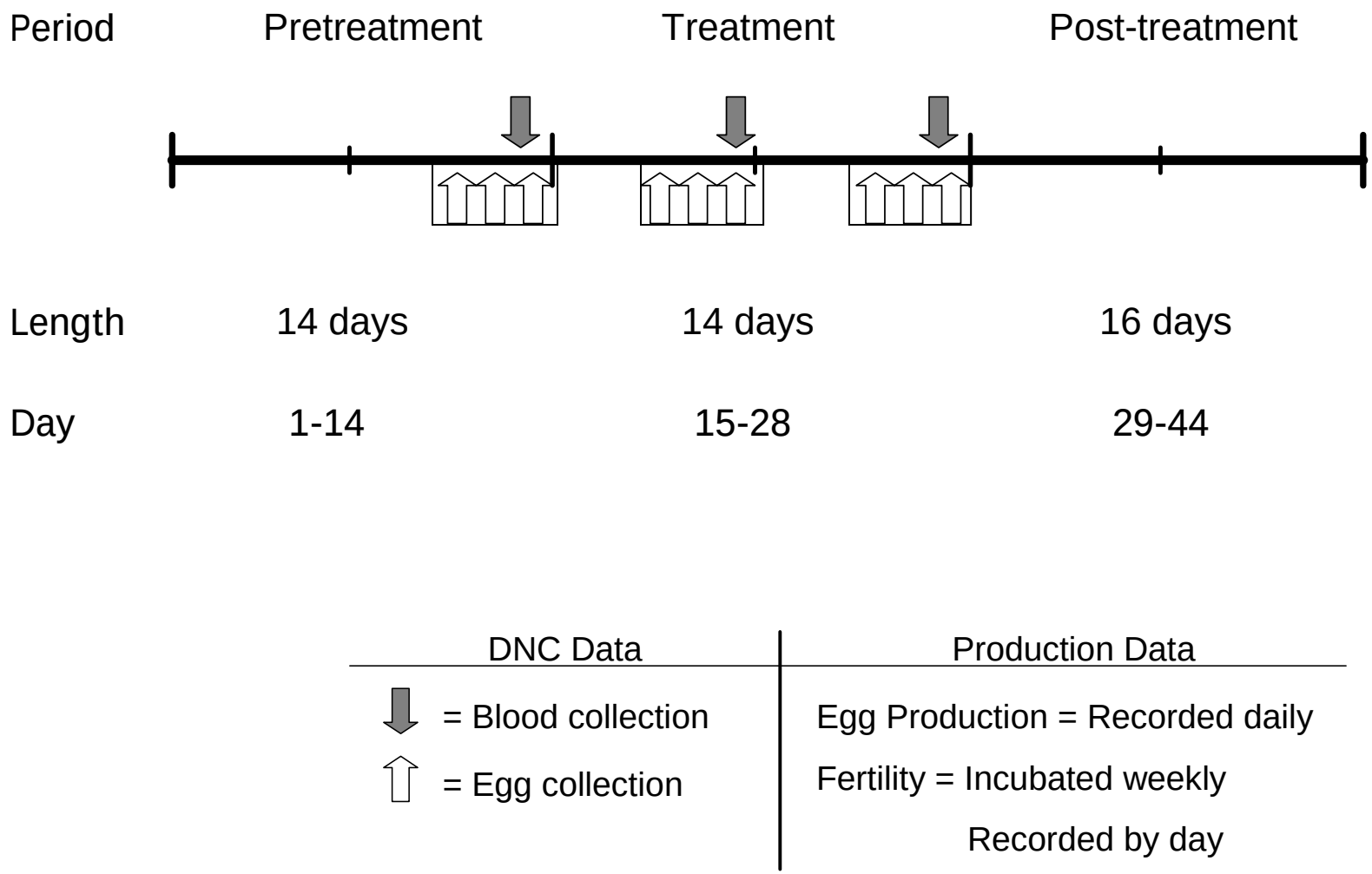

Figure **3-1**. Experimental design of data collection from nicarbazin-treated White Pekin ducks. Egg production was recorded from eggs that were collected daily. Eggs were set in incubator at the end of each week to determine fertility. One egg was collected at the end of each week and pooled for each dose group to use for determination of yolk DNC. This was the last egg laid by each duck during the period of three days indicated. Blood was collected on the last day of each week and pooled between dose group due to requirements for analysis of DNC.

Statistics

Within each treatment period (i.e., pre-treatment, treatment and recovery), egg production and viability (“*Y*”) were measured. All percentage data were transformed to arc sin square root percent, but presented as the nontransformed least square means for ease of reader interpretations. Linear regressions and all subsequent analyses were performed using JMP Statistical Software (SAS Institute Inc., Cary, NC). Due to the non-linearity of data across the three experimental periods, a repeated measures ANOVA was performed for each period using the following model.

$$Y_{ijk} = \mu + N_i + T_j + \beta(N*T)_{ij} + \varepsilon_{ijk}$$

where i = 0, 31, 63, 125, 250 or 500 ppm Nicarbazin (“*N*”) added to diet,
j = days (“*T*”) within each period, and
k = 10 ducks per subgroup

For ease of reader interpretation, all three periods were graphed with the 95% confidence interval represented by a shaded area within each graph. Any data points that are outside the shaded area are significantly different ($P<0.05$) from the control group.

Plasma and yolk DNC levels were determined via HPLC with mass spectrometry (Cannavan et al. 1999). Since the DNC assay is insufficiently sensitive to assess DNC in an individual blood sample or yolk – blood samples and yolks were pooled, and a single analytical determination made for each treatment and period subgroup. These data were analyzed via the following model:

$$Y_{ijk} = \mu + N_i + P_{ij}$$

where i = 0, 31, 63, 125, 250 or 500 ppm Nicarbazin added to diet,
j = last week of pretreatment and both the 1st and 2nd week of treatment.

In this model, Pij (period) becomes the error term to test for Nicarbazin effect (i.e., the period expected mean square was used in the F-test for Nicarbazin (*"N"*) main effect). Post-treatment (recovery) period was not analyzed for DNC since if recovery took place, DNC must have returned to pre-treated levels.

Results

In the two weeks prior to administering nicarbazin, the average hen-day egg production (HDP) for the control group was 0.95 ± 0.15 with a 95% confidence interval of 1.1 to 0.8 HDP (Figure **3-2**). The average egg production in the control group declined slightly to 0.75 ± 0.20 during the two-week nicarbazin treatment period due to an increase in environmental temperature from the mid 80's to mid 90's (°F). With the addition of nicarbazin to the diet, the egg production of all treatment groups decreased below the 95% confidence interval established from the control group. Egg production ($P<0.05$) was reduced in all treatment groups. Egg production dropped 72% from 0.9 HDP on the last day of pre-treatment (Day 14) to 0.25 HDP by the end of the first week of treatment (Day 21). The treatment groups from 31 ppm to 250 ppm did not reach their lowest production until the end of the two week treatment (Day 28), while the 500 ppm (50.88 mg/kg bw/day) had reached its lowest egg production by the end of the first week of nicarbazin treatment. The 125 ppm treatment group had a 70% decline in egg production, while the 250 ppm treatment group decreased by 67%. Egg production recovered in reverse order of treatment dose, beginning two days post-treatment. All dose groups recovered egg production with a 95% confidence interval of the control group between four and eleven days after withdrawal of nicarbazin from the feed.

Overall fertility of control duck eggs was 87% (49 eggs set), whereas at a dose of 500 ppm nicarbazin overall fertility was 20% (5 egg set) (Figure **3-3**). Fertility of the 500 ppm group reached 0% by the end of the first week of nicarbazin treatment (Day 21). Both the 250 ppm and 125 ppm nicarbazin treated ducks reached 0% fertility at Day 26 and 27, respectively. Eggs that did not hatch were determined to be infertile, as no embryos were detected. The control group had one early dead embryo, while the treatment groups had zero. Fertility recovered in reverse order of treatment dose, beginning the day after removal of nicarbazin from the feed. All treated groups recovered egg production with a 95% confidence interval of the control group between one and nine days after withdrawal of nicarbazin from the feed. The nicarbazin treated duck laid only infertile eggs with many fewer sperm.

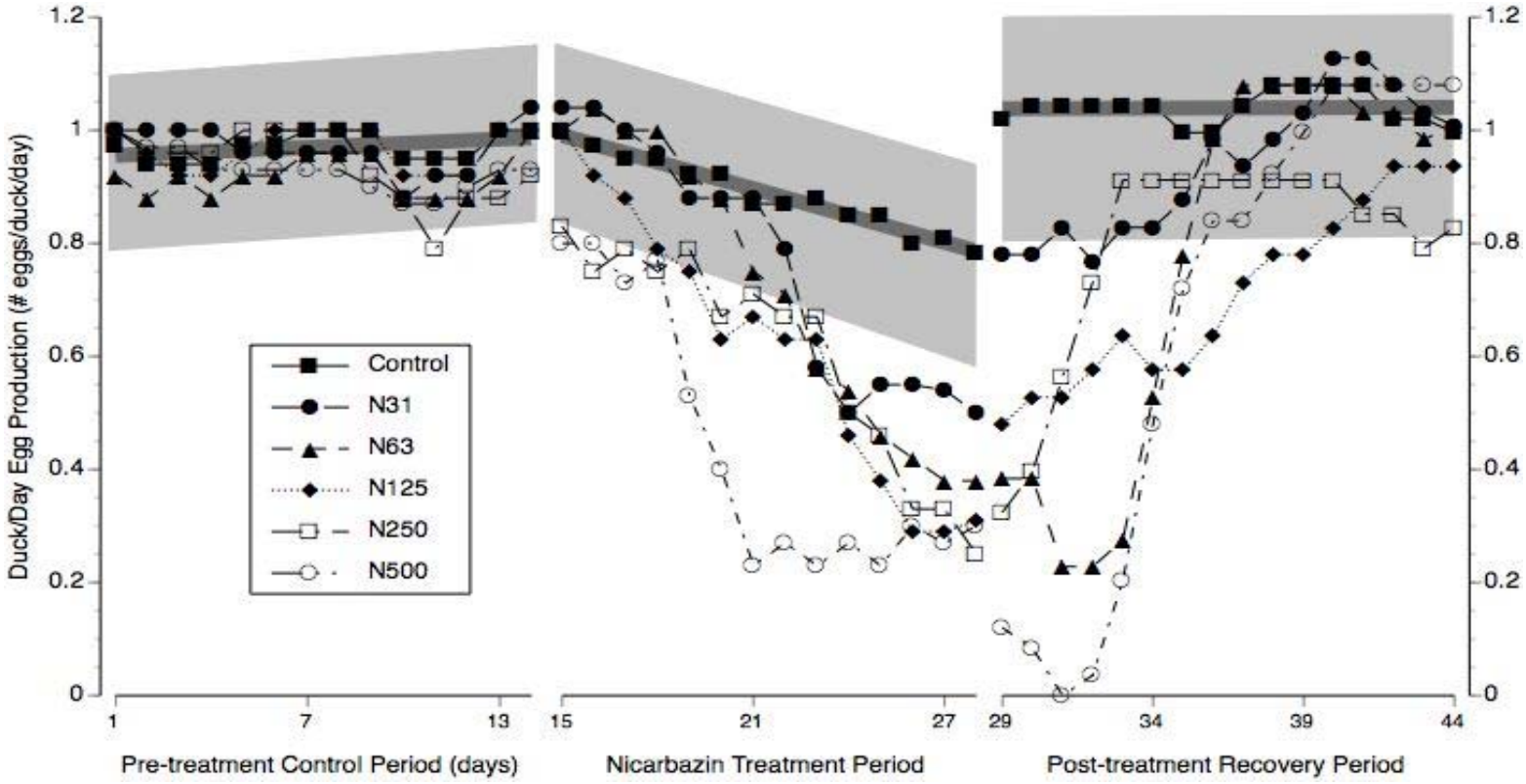

Figure **3-2**: Egg production of Pekin Ducks fed varying doses of nicarbazin. [The gray area represents the 95% confidence intervals of the regression of the control – any data points that fall outside these lines are significantly different from the controls.]

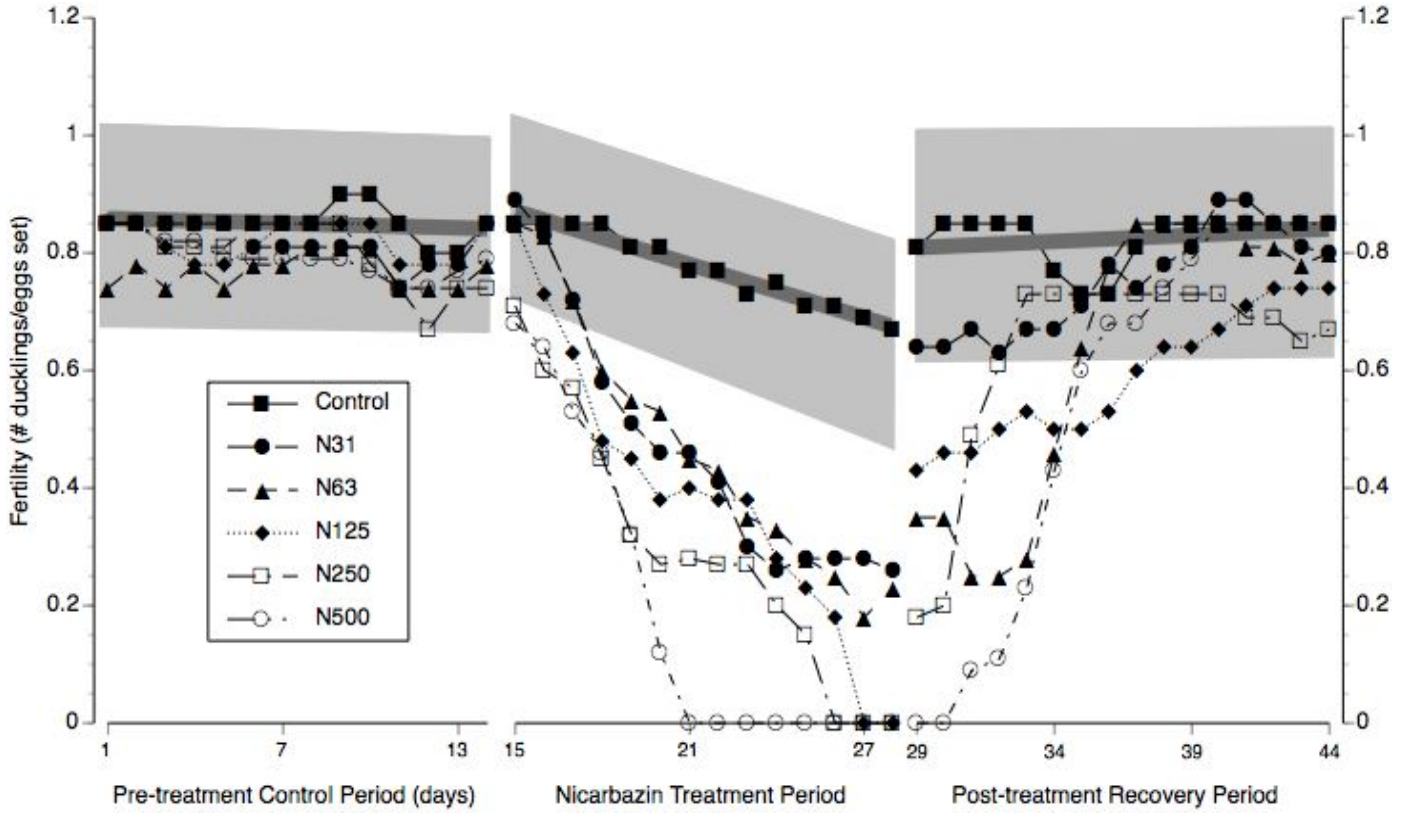

Figure **3-3**: Fertility of laid eggs obtained from Pekin Ducks fed varying doses of nicarbazin. [The gray area represents the 95% confidence intervals of the regression of the control– any data points that fall outside these lines are significantly different from the controls.]

33

Linear regression indicated that egg production significantly declined ($P<0.001$) with an increase in plasma DNC as indicated by the regression equation: Egg Production = 0.879 - 0.150 Plasma DNC. Individual nicarbazin dose regressions for plasma DNC and egg production do not have slopes significantly different from the control zero slope, except for the 125 ppm group (Table **3-1**). Overall regression of fertility indicated a decrease ($P<0.001$) in fertility per increase in µg/ml plasma DNC according to the equation: Fertility = 0.770 – 0.161 Plasma DNC. Individual dose groups 31 ppm, 63 ppm, and 500 ppm were different ($P<0.05$) from the control zero slope. Plasma DNC and egg DNC also share a significant positive relationship ($P<0.001$). This relationship can be characterized by the regression equation: Egg DNC = 4.16 + 0.00681 Plasma DNC. Individual dose groups other than 500 ppm were not statistically different ($P>0.05$) for the regression of plasma DNC and egg DNC.

Linear regression indicated that egg production decreased ($P<0.01$; $R2 = 66.9\%$) with an increase in plasma DNC as indicated by the regression equation: Egg Production = 0.879 - 0.000150 Plasma DNC. Individual nicarbazin dose regressions have slopes different than the control slope, except for the 250 ppm group. Plasma DNC and egg DNC also share a significant positive relationship ($P<0.01$; $R2 = 63.5\%$). This relationship can be characterized by the regression equation: Egg DNC = 4.16 + 0.00681 Plasma DNC.

Table **3-1**: Slope, R^2 and p-values for linear regressions with plasma DNC levels of nicarbazin-fed Pekin ducks as predictor

	Egg Production (# eggs/duck/day)			Fertility (# duckling/egg set)			Egg DNC (µg/g egg yolk)		
Dose (ppm)	Slope	R^2	P-Value	Slope	R^2	P-Value	Slope	R^2	P-Value
31	-0.21	0.989	0.07	-0.68	0.999	0.02*	7.47	0.373	0.58
63	-0.60	0.964	0.12	-0.65	0.999	0.02*	11.90	0.228	0.68
125	-0.26	0.998	0.03*	-0.48	0.988	0.07	15.50	0.883	0.22
250	-0.05	0.175	0.73	-0.09	0.249	0.67	7.45	0.585	0.45
500	-0.14	0.974	0.10	-0.16	0.996	0.04*	6.28	0.993	0.05*
Overall	-0.15	0.669	< 0.001*	-0.17	0.601	< 0.001*	6.81	0.617	< 0.001*

Egg production was measured on the day after the second week of treatment concluded, while fertility was measured the day before. This was due to the rapid increase in fertility after withdrawal of nicarbazin causing a skew in the regression line. Slopes represent the change in egg production/fertility/egg DNC for each unit (µg/ml) change in plasma DNC. N = 3 for individual dose rows and N = 15 for overall row.

* P-values are ≤ 0.05 and thus statistically significant from a slope of zero (control)

Discussion

Nicarbazin reduced egg production and fertility of White Pekin ducks with all dose ranges of 31 to 500 ppm within 8 days and 3 days of treatment, respectively. There was a striking decrease in the fertility of White Pekin ducks treated with nicarbazin. These results provide more consistent observations than those by Sherwood *et al.* (1956). Though diminished egg production was observed in all treatment groups, only doses of 125 ppm nicarbazin and above were statistically significant. The greatest and most rapid decrease in egg production was in the 500 ppm group. The 500 ppm level of nicarbazin was most effective in reducing egg production in Pekin ducks to near zero, while 125 ppm would be more appropriate for a partial reduction in reproductive success. The recommended value of over 125 ppm for full contraceptive action in waterfowl is in agreement with those presented by Yoder *et al.* (2006).

Egg production and fertility recovered to pretreatment values for all groups in inverse order of the nicarbazin dose. This displays the reversible nature of nicarbazin that has previously been seen in chickens (Ott et al. 1956; Sherwood et al. 1956). Sherwood et al. (1956) noted that White Rock breeder hens recovered egg production after 10 days of nicarbazin withdrawal, which is in agreement with the upper range of egg production recovery for White Pekin ducks. Due to its reversibility and lack of reproductive toxicity (EPA 2005), nicarbazin may be useful in reproductive studies as it may inhibit reproduction without permanent damage to the system.

The drop in egg production in the control group during the two weeks of treatment was due to an increase in ambient temperature during a 6-day period of hot weather (>90ºF). The temperature increase as well as the handling necessary to withdraw blood may have compounded the decline in egg production, however, it does not negate the fact that nicarbazin caused a significant decrease in fertility compared to controls. During the treatment period, the control group decline by approximately 15%, while the nicarbazin treatment groups declined by approximately 50-75%. The decline of the nicarbazin treated groups was much sharper and larger than that of the control group with a significant statistical difference for the higher doses of nicarbazin. Chickens fed nicarbazin exhibited an increased susceptibility to heat stress; this may have added to the overall decline in egg production of the treated ducks (McDougald and McQuinstion 1980).

In previous studies, hatchability was defined as the number of hatched chicks from fertilized eggs (Sherwood et al. 1956; Jones et al. 1990a). However, the number of eggs in the total set that are infertile due to nicarbazin treatment are then not taken into consideration. The term "fertility" is utilized in this study and refers to the number of viable ducklings that are produced from the entire egg set, rather than those that are only fertile. Yoder et al. (2006a) defined fertility as number of eggs hatched plus fertile unhatched eggs over total egg set. In the current study, only one egg in the control group showed early embryonic death and the rest that did not hatch were infertile, thus resulting in negligible differences between fertility calculations. The decline ($P<0.05$) in fertility observed in this study indicates that the number of infertile eggs increases with

nicarbazin treatment, which has not been clearly shown in previous studies (Ott et al. 1956; Sherwood et al. 1956; Jones et al. 1990a,c; Yoder 2006a).

As expected, egg production and fertility decreased as DNC, the active ingredient in nicarbazin, increased during the treatment period. Plasma DNC and egg DNC were positively correlated because more DNC would be deposited in eggs as it increased in a duck's system. Plasma DNC is more suitable to predict egg production and fertility because egg DNC did not show as strong of a correlation and thus it was not used. Individually the dose groups did not show significantly different slopes from the control for egg production and egg DNC. However, all p-values were 0.000 when dose groups were combined and the overall regressions are observed. As there are only three points for the dose group regressions, the lack of significance is most likely due to the small sample size, while the overall observations have much larger sample size.

The yolks of eggs from nicarbazin-fed ducks had observed changes in appearance and structure. Frequency of mottling increased to 81% in layer chickens that were fed 100 ppm nicarbazin (Jones et al. 1990b,c). In this study, mottling increased with nicarbazin dose and was very frequently noticeable in yolks from ducks fed 250 ppm and 500 ppm nicarbazin. There was also visual evidence of weakening of the yolk membrane during PVM collection. Membranes from ducks fed nicarbazin were fragile and easily broke while being handled, which increased in frequency for 250 and 500 ppm nicarbazin-treated ducks. Kerr (1998) and Block (2002) observed similar weak PVMs in a strain of chickens that were associated with low fertility. In ZP3 knockout mice, it was demonstrated that a lack of ZP3 results in infertility and a deficient zona pellucida matrix (Rankin et al. 1996). As the zona pellucida is equivalent to the perivitelline membrane in

birds, it is possible that the weakened yolk membranes were due to an alteration in the presence of ZP3 in the PVM.

Chapter 4

Nicarbazin Reduces ZP3 in the Perivitelline Membrane of White Pekin Ducks

Introduction

The coccidiostat, nicarbazin, has contraceptive activity that is useful in wildlife control. Nicarbazin is a complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), of which DNC is the active ingredient (Cuckler et al. 1955). The addition of nicarbazin to the feed of laying hens results in a reversible reduction of hatchability and egg production (Ott et al. 1956; Sherwood et al. 1956; Hughes et al. 1991). Due to its ability to reduce reproductive effectiveness, nicarbazin is being examined for its potential as a contraceptive for avian pest species (Fagerstone et al. 2006). However, the mechanism of action of nicarbazin as a contraceptive is unknown.

Yoder et al. (2006) investigated potential mechanisms for nicarbazin involving egg formation and embryogenesis; including lipoprotein lipase activity, vitellogenin phosphotase action, transglutaminase activity and calcium uptake. Overall, the studies were inconclusive, failing to identify a direct biochemical effect of nicarbazin; but suggesting that the drug had ionophore properties that would increase membrane permeability (Yoder et al. 2006).

Nicarbazin has shown to degrade the vitelline membrane that surrounds the yolk (Polin 1957). The inner perivitelline membrane is predominantly comprised of the zona

pellucida family of proteins (Bellairs et al. 1963; Mori and Masuda 1993). One member of that family is zona pellucida 3 (ZP3), which is involved in sperm binding in chickens (Waclawek et al. 1998). Mice that have ZP3 knocked out show a lack of zona pellucida matrix and suffer from infertility (Rankin et al. 1996). Hens known to have reduced or altered ZP3 have been observed laying eggs with similarly weak perivitelline membranes (PVM) to those of nicarbazin treated chickens (Kerr 1998; Block 2002).

Data from Chapter 3 demonstrated that nicarbazin reduced egg production, fertility and hatchability in the avian species. Zona pellucida 3 is the putative sperm binding receptor in birds (Waclawek 1998) and thus a likely candidate for nicarbazin's contraceptive mode of action. We hypothesize that nicarbazin inhibits fertility by altering ZP3 expression in the perivitelline membrane. The objective of this study is to analyze the presence of the ZP3 protein in the perivitelline membrane of nicarbazin treated ducks through western blotting.

Methods and Materials

Animals

Forty-nine female White Pekin ducks (Golden 300) were obtained from Metzer Farms (Gonzales, CA). Ducks were housed at the Pennsylvania State University Poultry Education and Research Center (University Park, PA). At 28 weeks of age, ducks had just passed peak egg production and were used under an approved Institutional Animal Care and Use Committee protocol (IACUC #21138). Ducks were individually caged at

16 weeks of age and lighting changed to a 16 hour light and 8 hour dark photoperiod. Water was available to the ducks *ad libitum*.

Nicarbazin Treatment

Forty-nine Pekin ducks were assigned to five groups of eight ducks/group and for the highest nicarbazin dose, a group of nine ducks/group. To adjust for pre-treatment differences, egg production data were collected two weeks prior to nicarbazin treatment. The ducks were fed 300 g/day of a standard commercial duck layer diet (Appendix A) with treatments of 0 ppm (control), 31 ppm, 63 ppm, 125 ppm, 250 ppm, or 500 ppm nicarbazin for a two-week period. Similar nicarbazin doses were used in previous reports involving waterfowl (Yoder 2005; Yoder 2006 a,b). After the two-week treatment period, ducks were monitored for a two-week period post-treatment to observe recovery of lay.

The period of blood and egg collection for DNC analysis consisted of three weeks, beginning with the second week of pretreatment and including both weeks of treatment. Blood (1 ml) was drawn from the brachial vein the morning of the seventh day of each week. Eggs were collected on the last three days of each week. Plasma and yolks of the collected eggs were pooled among individuals within dose treatments. The pooled samples of egg yolks and plasma from birds within each treatment group were assayed for DNC using HPLC with mass spectrometry by Exygen, Inc. (State College, PA).

Perivitelline Membrane Collection

Eggs were collected daily and used for perivitelline membrane (PVM) collection starting one week prior to treatment (Week 1) through the treatment period (Weeks 2 and 3). The eggs were broken and the yolk drained to leave the inner and outer perivitelline membrane, which was washed in 1X PBS (phosphate buffered saline). An average of three membranes were pooled by week for each duck. Membrane samples were placed in 0.75 ml/membrane of solubilizing solution containing 20mM tris(hydroxymethyl)-aminomethane (Tris), 2% sodium dodecyl sulfate (SDS), and 50mM dithiothreitol (DTT), then heat solubilized at 75°C to denature PVM proteins (Appendix C; Barbato et al. 1998). Membranes were frozen at -20°C and stored until needed.

BCA Protein Assay

The samples were thawed and vortexed before use. The samples were homogenized by aspiration through 21 gauge and 25 gauge needles to break down any protein aggregation. The samples were then briefly centrifuged at 10,000 rpm for 15 minutes to reduce the foaming of the detergents. Samples were used to make 1:25 and 1:50 dilutions with 1X PBS for protein assay. Dilutions (25 µl) of samples and standards were placed in duplicate on a 96-well plate and then 200 µl of BCA protein assay reagent (Pierce, Rockford, IL) was added. The plate was incubated at 37.5°C for 15 minutes. The assay was read at a wavelength of 562 nm in a Bio-Tek Microplate Autoreader (Model EL311; Bio-Tek Instruments, Winooski, VT). Results of the 1:25 and 1:50 dilutions were

averaged to estimate total protein. A standard curve ranging from 0 to 1500 µg/ml was used in each assay plate.

Western Blot Analysis

Duck PVM proteins (10 or 30 µg) were separated using a 12% SDS-PAGE (Laemmli 1970). Proteins were transferred from gels to 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) via semi-dry transfer for 2 hours at 45 mA per gel. Membranes were blocked for 45 minutes in a solution of PBS-T and nonfat dry milk (Appendix D). They were then probed with primary anti-chZP3 antibody serum at a 1:1500 dilution overnight. The antibody was made to the synthetic peptide TLINYDPSPASNPVIIR (TLINY) corresponding to animo acids 80-96 of native chicken ZP3 (chZP3) in male rabbits (Sigma-Genosys). The TLINY antibody has previously been verified to show specificity for ZP3 epitopes by comparing between pre-immune and immune blots (Block 2002). Immunoreactive ZP3 (irZP3) bands having molecular weights of 42 kD were further characterized by 2-dimensional gel electrophoresis, isolated and sequenced by HPLC mass spectrometry to confirm it matches the sequence of ZP3 (Hartmann and Barbato, unpublished data). After washing for 30 minutes in phosphate buffered saline–tween 20 (PBS-T), membranes were placed in secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase label (Vector, Burlingame, CA), diluted with PBS-T to 1:40,000. Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagents (GE Healthcare; Amersham, Buckinghamshire, UK) were used to visualize proteins on x-ray film.

Statistics

ImageJ Software (NIH, Bethesda, MD) was used to estimate band intensity ("*Y*") from Western blot films. Band intensity is the amount of light absorbed ('darkness') by the gel at the area of the target protein band (irZP3), measured in pixels. Week 1, 2, and 3 samples for each duck were run on the same film to reduce variation. All films were calibrated to the same optical density scale. Western blot data were analyzed via repeated measures ANOVA, using JMP Statistical Software (SAS Institute Inc., Cary, NC). Data points that were more than three standard deviations from the mean were removed. The mathematical model included main effects of nicarbazin dose ("*N*") and period in terms of week ("*T*"). The statistical model used was:

$$Y_{ijk} = \mu + N_i + T_j + \beta(N*T)_{ij} + \varepsilon_{ijk}$$

where i = 0, 31, 63, 125, 250 or 500 ppm Nicarbazin added to diet,
j = weeks 1, 2 or 3
k = 8 or 9 ducks per subgroup

Week 1 served as an internal control for comparisons between weeks. Results were confirmed by reanalysis of data using both Student's t-test and Scheffe's non-orthogonal linear contrasts. Tukey Student's t-tests on egg production in conjuction with the ANOVA model (similar to above) were performed using Minitab Statistical Software (State College, PA).

Results

Egg production showed a significant decrease with increasing doses of nicarbazin ($P<0.001$, R^2 of 0.77) (Figure **4-1**). The interaction between dose and week was not

significant (P=0.190); however week itself was significant (P<0.001). In Week 1, when no group was fed nicarbazin to serve as a control, the egg production for all dose groups was not different (P>0.97). For Week 2, the first week of nicarbazin treatment, the 500 ppm dose group had lower egg production (P=0.011) in comparison with the control group (0 ppm). In Week 3, which was the second week of nicarbazin treatment, the 125 ppm and 500 ppm dose groups displayed less egg production (P=0.048 and P=0.030) than the control group. When compared with the control Week 1 for individual dose groups, rather than the 0 ppm group, the 63 ppm, 125 ppm, 250 ppm, and 500 ppm treatments decreased egg production between Week 1 and Week 3 (P<0.05). The treatment groups 125 ppm and 500 ppm treatment groups also had lower egg production (P<0.05) between Week 1 and Week 2. Egg production pre-treatment and recovery data were similar to previous lab work (Chapter 3; not shown).

The blood plasma and egg yolks of the Pekin ducks within each treatment group were analyzed for DNC content. As expected, the control and the two week period of pre-treatment had no effect on DNC content. The 31 ppm group showed slightly higher plasma DNC levels than the 63 ppm group with 0.96 and 0.92 µg/ml plasma DNC, respectively, by the end of the second week of treatment. Plasma DNC levels reached 1.76 µg/ml and 4.27 µg/ml in ducks treated with 125 ppm and 500 ppm nicarbazin, respectively. However, plasma DNC levels for ducks treated with 250 ppm peaked in the first week at 3.98 µg/ml and declined to 2.84 µg/ml. The 125 ppm and 500 ppm groups had similar egg DNC levels at 25.8 and 25.7 µg/g yolk by the second week of treatment.

Immunoreactive ZP3 bands were identified at 45 kDa for all treatment groups on average (Figure **4-2**). A second band was detected around 117 kDa (Figure **4-3**), which

was determined by peptide mapping to be an aggregate of the ZP heterocomplex containing both ZP1 and ZP3 amino acid sequences (Hartmann and Barbato, unpublished data). The mean band intensity (±SE) of irZP3 for all untreated birds (n=49) during the first week (pretreatment) was 5702±502 pixels. Analysis of variance indicated that there were no differences ($P>0.20$) in irZP3 band intensity among untreated groups of ducks. Analysis of variance further indicated that dose of nicarbazin affected ZP3 abundance ($P<0.05$). Nicarbazin groups 63 ppm, 125 ppm, 250 ppm and 500 ppm had less immunoreactive ZP3 detected ($P<0.05$) between their respective internal control Week 1 and Week 3. There was no difference ($P>0.05$) between Week 2 and Week 3 for the 250 ppm dose group. Additionally, the 500 ppm group was the only one where Week 2 (first week of treatment) was also different ($P<0.05$) from Week 1 (control). Treatment groups 0 ppm and 31 ppm did not show a significant increase or decline in the amount of immunoreactive ZP3 ($P>0.05$) between their weeks of treatment. The comparisons of week within dose are shown in Table **4-1**.

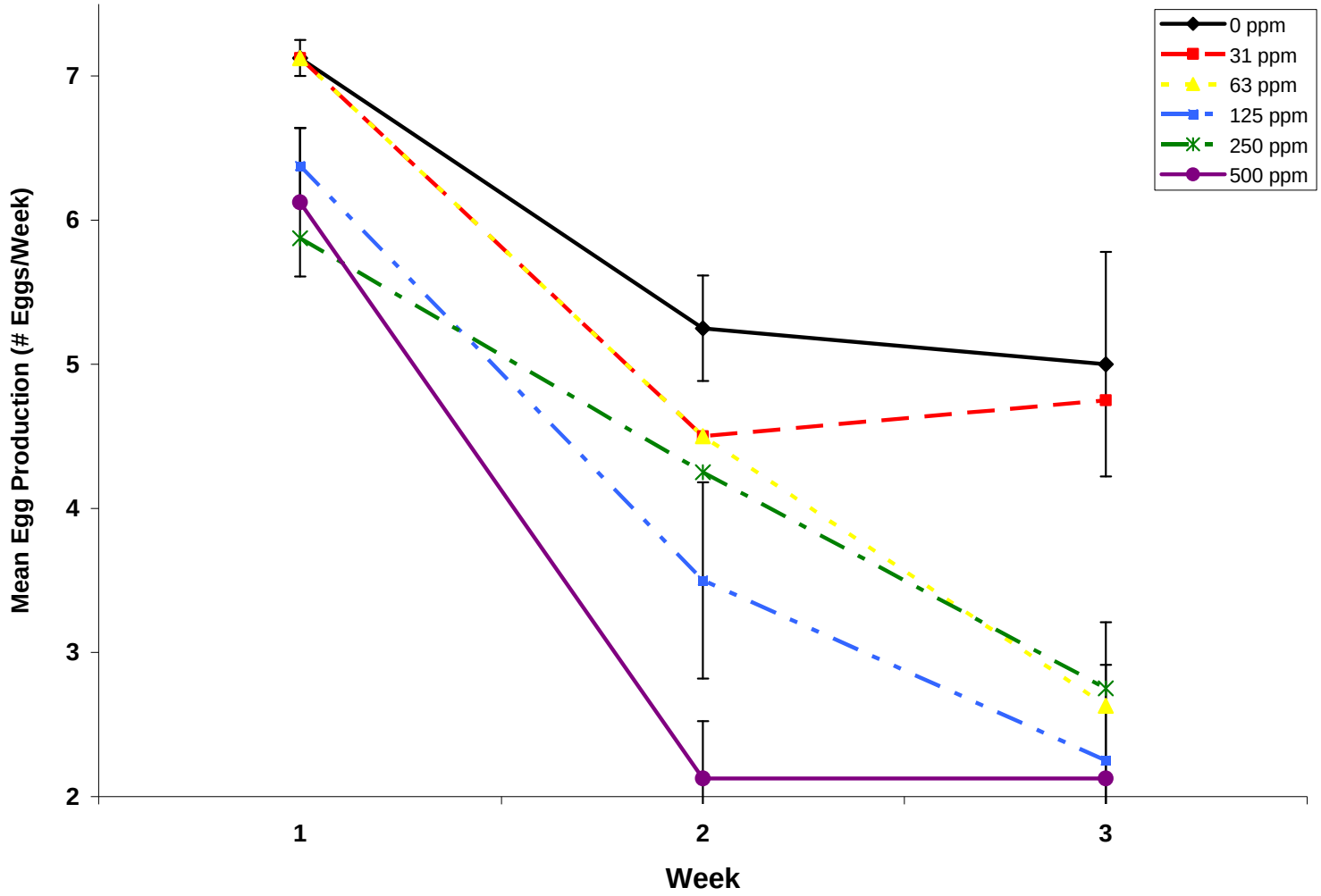

Figure **4-1**: Mean egg production (±SE) for dose of nicarbazin-fed White Pekin ducks (n=8 or 9/group) by week

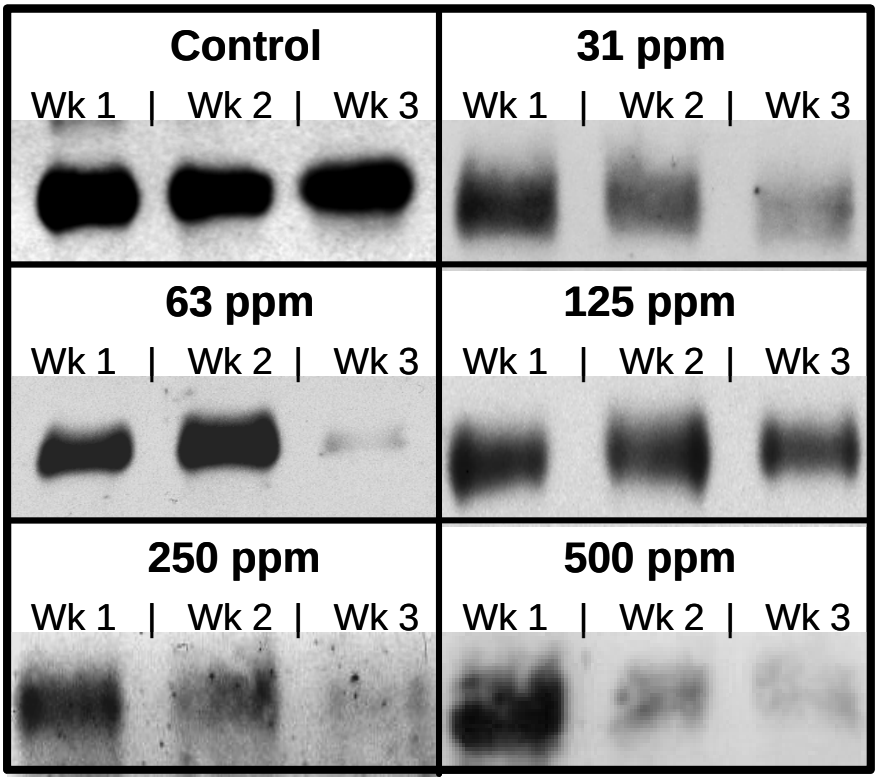

Figure **4-2**: Representative Western blots of irZP3 for each dose group, ranging from 0 – 500 ppm nicarbazin, arranged by week. Figure represents 3 samples for each duck (n=8 or 9) in each of 6 dose groups for a total of 147 samples analyzed through western blotting. Week 1 was a period of pretreatment, thus it serves as an internal control. A decrease in the abundance of ZP3 is visible in Western blots and confirmed in 63 ppm, 250 ppm, and 500 ppm dose groups via statistical analysis.

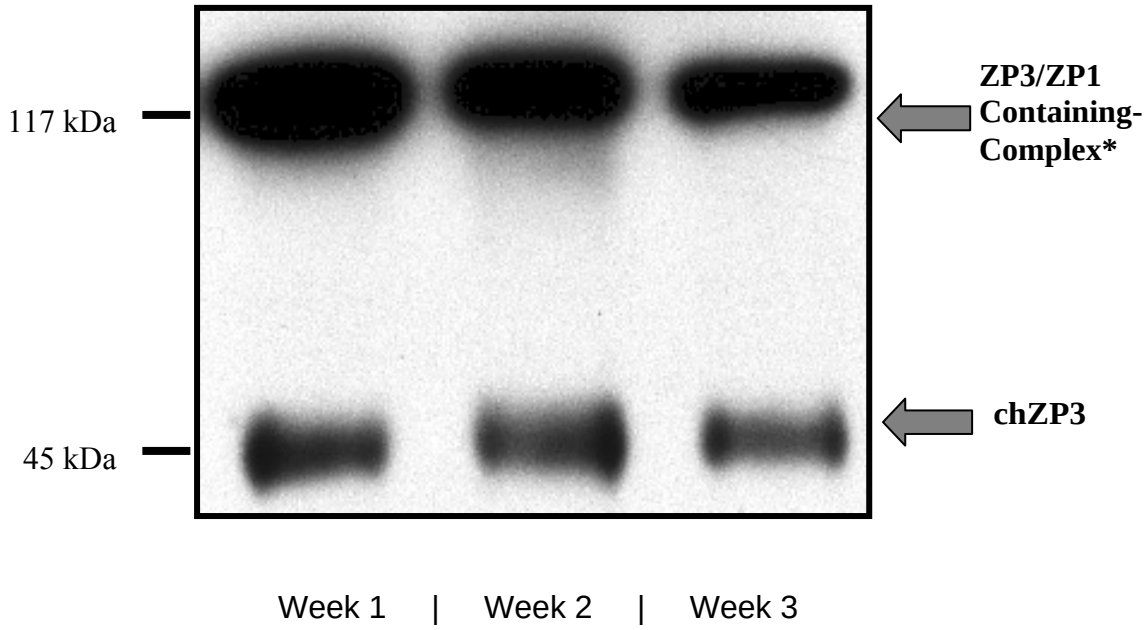

Figure **4-3**: 125 ppm dose sample, displaying identified bands. Bands were detected at 117 and 45 kDa on average for Western blots. Immunoreactive ZP3 is located at the 45 kDa band.

*Identified by Barbato and Hartmann (unpublished data) as containing both ZP1 and ZP3 by peptide mapping.

Table **4-1**: Least squares means (±SE) of area (in pixels) of immunoreactive ZP3 in White Pekin duck eggs from ducks (n=3 eggs/duck in 8 or 9 ducks/group) receiving different concentrations of nicarbazin in their feed. Band intensity (area of irZP3) was compared between weeks within dose, rather than between dose groups to control for variability.

	Band Intensity*		
Dose (ppm)	Week 1 (Pretreatment)	Week 2 (Treatment)	Week 3 (Treatment)
0	6094±627	5028±517	5569±523
31	6982±656	5284±473	6527±525
63	6726±662^{a}	6064±569^{a}	3738±267^{b}
125	4029±324^{a}	4080±401^{a}	2004±134^{b}
250	5453±366^{a}	5474±636^{ab}	2850±153^{b}
500	4930±353^{a}	980±92^{b}	150±7^{b}

* Means within a row having different superscripts are significantly different ($P<0.05$).

Discussion

As the previous experiment (Chapter 3) already displayed evidence of a decline in egg production due to the introduction of nicarbazin in the diet, egg production data were analyzed by week rather than day to coincide with the ZP3 data. As in the previous study, egg production was reduced in a dose-response fashion when nicarbazin was fed to the ducks. Significance was only seen in the higher dose groups when compared to the control group. This may be due to the fact that in the later weeks as the number of eggs produced decreases, so does the sample size and thus standard error increases. When compared within weeks for an individual dose group rather than between dose groups, all treatment groups except for the lowest (31 ppm) showed significant period effects. This indicated that there is a decrease in egg production for the dose groups. With a decline in egg production, there were enough functional effects to evaluate if ZP3 was declining as well.

In chickens, a minimum plasma level of 2.9 µg/ml plasma DNC is necessary for manifestation of reproductive effects (Jones et al. 1990b; Yoder et al. 2005). Mallard ducks treated with 125 ppm, 250 ppm, and 500 ppm nicarbazin peak at 1.36 µg/ml, 1.74 µg/ml, and 2.97 µg/ml plasma DNC, respectively (Yoder et al. 2006a). By the second week of treatment, Pekin ducks had plasma DNC levels that were well above these levels. Even with a decrease from 3.98 to 2.84 µg/ml plasma DNC, the 250 ppm nicarbazin treated group was still considerably high. Significant egg production treatment effects

may have been observed contrary to Yoder et al. (2006a) because the Pekin ducks absorbed the nicarbazin more efficiently.

Over time, the number of laid eggs decreased for the higher doses of nicarbazin, especially at 500 ppm. Therefore, fewer samples were available for collection and thus their mean value may not be atypical. For example, eggs from the 250 ppm group had higher levels of DNC, however, this may be due to the fact there were fewer eggs in the 500 ppm group rather than the 250 ppm group. In future experiments, though 500 ppm nicarbazin may have the most dramatic effect in terms of contraception potential, a lower dose of nicarbazin may be used to maintain sufficient egg production for collection.

The 45 kDa location of immunoreactive ZP3 closely aligns to the 42 kDa weight of chZP3 (Stewart et al. 2004; Waclawek et al. 1998). This may suggest that the ZP3 protein of the Pekin duck is slightly larger than that of the chicken. Heavy protein loads in the gels may also cause an apparent weight increase. Western blot indicated a decrease in the presence of irZP3 in four of the five dose groups, suggesting a direct and negative effect on ZP3. However, there was no significant decline if irZP3 seen in the 31 ppm dose group, most likely due to large standard errors. This could be addressed by increasing sample size, but this may be challenging in higher dose groups. An inherent difficulty with higher nicarbazin treatments is since it successfully reduces egg production; it also effectively diminishes the sample size. Of the nine ducks used in the 500 ppm dose group, one duck was removed due to an error in the sample collection and three additional ducks stopped producing eggs by the second week. In the other dose groups, only one duck completely stopped egg production in each.

There were several sources of possible variability in the Western blotting process. Variability in band intensity between films can lead to underestimating significant differences between dose groups. All films were calibrated on the same optical density scale to adjust for background inconsistencies between films. It is also difficult to compare high intensity values, since once a band is at the darkest intensity on film, it can not be given any higher of a value. Comparisons were made between weeks with a nested ANOVA rather than between doses. The Week 1 internal control was utilized to provide a standard for comparison. The combination of the sources of variation would most likely result in the underestimation of statistical differences.

Western blots were not normalized by probing the blots for a protein other than ZP3. The inner and outer PVM consist of glycoproteins, lysozyme, ovomucin, and vitelline membrane outer layer proteins I and II (Kido and Doi 1988; Kido et al. 1992). This leaves very few available targets for normalization. Since there are no cells in the PVM, probing for proteins such as beta-actin would not be useful. The effects of nicarbazin on ZP2 are unknown, so the best candidate to use for normalization would be ZP1, which does not decrease in the presence of nicarbazin. The protein assay was used to ensure that the same amount of protein was loaded in each well.

Fertility could not be measured with egg production in this study, because PVM samples must come from unfertilized eggs for western blot. Zona pellucida 3 undergoes proteolytic degradation upon fertilization and binding with sperm (Wassarman 1990; Kerr 1998). The degradation of ZP3 would make recovery of the protein extremely difficult and variable.

Zona pellucida protein 1 is also considered to be a component of the perivitelline membrane (Sasanami et al. 2003). In Western blots of PVM from 500 ppm nicarbazin treated ducks, immunoreactive ZP1 was detected using an antibody (anti-chZP1) specific to the amino terminus of chicken ZP1, and no pattern of decline in ZP1 protein was apparent (Barbato and Katani, unpublished data). Zona pellucida 1 is synthesized in the liver, rather than in the granulosa cells, and this may indicate that nicarbazin is acting on the granulosa cells that are responsible for ZP3 production (Bausek et al. 2000).

The significant decline in immunoreactive ZP3 in perivitelline membranes of laid eggs may suggest that less ZP3 is available to be incorporated into the structure of the PVM upon introduction of nicarbazin of 63 ppm doses or higher. Perivitelline membranes that were easily broken and appeared weak further displayed structural deficiencies in the PVM of Pekin ducks treated with nicarbazin, similar to observations from a previous study (Chapter 3). Sperm binding and structural support of the PVM both occur due to the presence of ZP3 (Rankin et al. 1996; Wassarman and Litscher 2001; Waclawek et al. 1998). It may be concluded from the combination of effects on the structure and function of the perivitelline membrane that nicarbazin has a direct and negative effect on ZP3.

Chapter 5

Summary

In previously cited literature, nicarbazin was shown to have negative effects on reproductive success in chickens, geese and mallards. While the Pekin duck is closely related to the mallard, the Pekin duck does not form pair bonds, has ten times greater egg production and can be artificially inseminated, making it an ideal model for studying compounds suspected of influencing reproduction. In the first study, nicarbazin significantly reduced egg production by 50-75% ($P<0.05$) in a dose-response fashion after two weeks. Fertility also declined by approximately 50-100% ($P<0.05$) with the addition of nicarbazin to the diet. After cessation of treatment, egg production and fertility recovered and were not different ($P>0.05$) from control values within two weeks of removing nicarbazin from the diet. The relationships between plasma DNC concentration and egg production and fertility are negatively correlated as expected.

As with many drugs, nicarbazin is absorbed differently in each species, thus it was necessary to characterize the specific dose-response relationship of nicarbazin in White Pekin ducks. These data also provide evidence that the White Pekin duck displays a similar response to nicarbazin as the mallard, reinforcing their known genetic and physiological similarities (Yoder 2005, 2006a,b). The current EPA standard for avian reproductive toxicology is the mallard; however, the White Pekin duck has several considerable advantages in a laboratory setting (EPA 2005). The White Pekin duck does not pair bond and also has high rates of egg production without being caged with a mate.

Not only does the first experiment prove egg production and fertility decline when nicarbazin is fed to the diet, but it also suggests that the White Pekin duck should be considered as a potential new model for EPA testing.

The second experiment addressed the possible connection between nicarbazin and the putative sperm binding receptor, ZP3. Previous research had noted that the perivitelline membrane of eggs from hens with a deficient ZP3 receptor were very similar to the eggs of birds fed nicarbazin (Kerr 1998; Block 2002). Western blotting revealed that the abundance of ZP3 declined ($P<0.05$) in the perivitelline membranes of White Pekin layer hybrid ducks fed nicarbazin. The present study is the first to suggest a correlation between nicarbazin dose and ZP3 in the perivitelline membrane.

There were challenges that came to light during the Western blotting process. As the perivitelline membrane is predominantly composed of ZP-related proteins, which are notoriously insoluble, there are few, if any, non-ZP-related proteins available to normalize the blots (Kido and Doi 1988; Barbato et al. 1998; Waclawek et al. 1998; Okumura et al. 2004). This may have an unintended consequence of increasing error due to increased variation both among and between blots. Since multiple films were used to develop the Western blots, there may be variation between films as well. However, the result would be the underestimation of differences, which further confirms the novel evidence put forth by this experiment.

While the ingestion of 63 ppm or more nicarbazin in the diet leads to a decline in ZP3, the mechanism of the decline is still unknown. Cell culture of granulosa cells should be performed in the future to determine if the granulosa cells that produce ZP3 are being damaged or inhibited, and if the effect is permanent for each individual cell. Real-

time PCR (RT-PCR) would also be useful for determining at what stage of production ZP3 declines in the perivitelline membrane. In conjunction with Western blotting, RT-PCR of cultured granulosa cells exposed to nicarbazin and/or DNC would provide evidence that an inhibitory mechanism of nicarbazin occurs at the level of translation of ZP3. The information from the current and future studies will greatly contribute to not only our understanding of nicarbazin's mode of action, but also how ZP3 functions.

Bibliography

Alexander, A. 1992. Effect of genotype on the fertility of chicken spermatozoa after dlution or cryopreservation. MS Diss. Pennsylvania State Univ., University Park.

Amann, R. P., and R. H. Hammerstedt. 2002. Detection of Differences in Fertility. J. Androl. 23:317-325.

Ankney, C. D. 1996. An embarrassment of riches: too many geese. J. Wildl. Manage. 60:217-223.

Avery, M. L., K. L. Keacher, and E. A. Tillman. 2006. Development of Nicarbazin Bait for Managing Rock Pigeon Populations. Proc. 22nd Vertebr. Pest Conf., 116-120, Univ. California, Davis.

Avery, M. L., K. L. Keacher, and E. A. Tillman. 2008. Nicarbazin bait reduces reproduction by pigeons (Columba livia). Wildl. Res. 35:80-85.

Bagavant, H., P. Thillai-Koothan, M. G. Sharma, G. P. Talwar, and S. K. Gupta. 1994. Antifertility effects of porcine zona pellucida-3 immunization using permissible adjuvants in female bonnet monkeys (Macaca radiata): reversibility, effect on follicular development and hormonal profiles. J. Reprod. Fertil. 102:17-25.

Baker, R. C., F. W. Hill, A. Van Tienhoven, and J. H. Bruckner. 1957. The effect of nicarbazin on egg production and egg quality. Poult. Sci. 36:718-726.

Bakst, M. R., and B. Howarth. 1977a. The Fine Structure of the Hen's Ovum at Ovulation. Biol. Reprod. 3:361-369.

Bakst, M. R., and B. Howarth. 1977b. Hydrolysis of the Hen's Perivitelline Layer by Cock Sperm in vitro. Biol. Reprod. 17:370-379.

Barbato, G. F., P. G. Cramer, and R. H. Hammerstedt. 1998. A practical in vitro sperm-egg binding assay that detects subfertile males. Biol. Reprod. 58:686-699.

Bausek, N., H. Ruckenbauer, S. Pfeifer, W. Schneider, and F. Wohlrab. 2004. Interaction of sperm with purified native chicken ZP1 and ZPC proteins. Biol. Reprod. 71:684-690.

Beebe, S. J., L. Leyton, D. Burks, M. Ishikawa, T. Fuerst, J. Dean, and P. Saling. 1992. Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. Dev. Biol. 151:48-54.

Bellairs, R., M. Harkness, and R. D. Harkness. 1963. The vitelline membrane of the hen's egg: a chemical and electron microscopical study. J. Ultrastruct. Res. 8:339-359.

Berger, T., K. O. Turner, S. Meizel, and J. L. Hedrick. 1989. Zona pellucida-induced acrosome reaction in boar sperm. Biol. Reprod. 40:525-530.

Bleil, J. D., J. M. Greve, and P. M. Wassarman. 1988. Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev. Biol. 128:376-385.

Bleil, J. D., and P. M. Wassarman. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev. Biol. 76:185-202.

Block, M. 2002. Proteomic and Genomic Analysis of Female Subfertility in Chickens and Turkeys. PhD Diss. Pennsylvania State Univ., University Park.

Budavari, S., 11th ed. 1989. Nicarbazin. The Merck Index.

Bynum, K., J. Eisemann, G. Weaver, C. Yoder, F. Fagerstone, and L. Miller 2007. Nicarbazin OvoControl G Bait Reduces Hatchability of Eggs Laid by Resident Canada Geese in Oregon. J. Wildl. Manage. 71:135-143.

Bynum, K. S., C. A. Yoder, J. D. Eisemann, J. J. Johnston, and L. A. Miller. 2005. Development of nicarbazin as a reproductive inhibitor for resident canada geeese. Proc. 11th Wildlife Damage Management Conference, 11:179-189.

Chapman, H. D. 1997. Biochemical, genetic and applied aspects of drug resistance in Eimeria parasites of the fowl. Avian Pathol. 26:221-244.

Conover, M. and G. Chasko. 1985. Nuisance Canada Goose Problems in the Eastern United States. Wildl. Soc. Bull. 13:228-233.

Cuckler, A. C., C. M. Malanga, A. J. Basso, and R. C. O'Neill. 1955. Antiparasitic activity of substituted carbanilide complexes. Science 122:244-245.

Cuckler, A. C., C. M. Malanga, and W. H. Ott. 1956. The antiparasitic activity of nicarbazin. Poult. Sci. 35:98-109.

Cunningham, F. E. 1977. Properties of albumen from eggs having mottled yolks. Poult. Sci. 56:1819-1821.

Elder, William H. 1964. Chemical Inhibitors of Ovulation in the Pigeon. J. Wildl. Manage. 28:556-575.

EPA. 1996. Ecological Effects Test Guidelines: Avian Reproduction Test. Environmental Protectional Agency.

EPA. 2005. EPA Pesticide Fact Sheet for Nicarbazin. United States Environmental Protection Agency.

Epifano, O., L. F. Liang, M. Familari, M. C. Moos, and J. Dean. 1995. Coordinate expression of the three zona pellucida genes during mouse oogenesis. Development 121:1947-1956.

Etches, R. J. 1996. Reproduction in Poultry. CAB International: University Press, Cambridge, UK.

Fagerstone, K., L. Miller, K. Bynum, J. Eisemann, and C. Yoder. 2006. When, Where and for What Wildlife Species Will Contraception Be a Useful Management Approach? Proc. 22nd Vertebr. Pest Conf., 45-54, Univ. California, Davis.

Fayrer-Hosken, R. A., H. J. Bertschinger, J. F. Kirkpatrick, D. Grobler, N. Lamberski, G. Honneyman, and T. Ulrich. 1999. Contraceptive potential of the porcine zona pellucida vaccine in the African elephant (Loxodonta africana). Theriogenology 52:835-846.

Florman, H. M., and B. T. Storey. 1982. Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization in vitro. Dev. Biol. 91:121-130.

Florman, H. M., and P. M. Wassarman. 1985. O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell 41:313-324.

Forbes, J. E. 1993. Survey of nuisance urban geese in the United States. Proc. 11th Great Plains Wildlife Damage, 92-101, Kansas State Univ., Manhattan.

Giunchi, D., N. E. Baldaccini, C. Sbragia, and C. Soldatin. 2007. On the use of pharmacological sterilisation to control feral pigeon populations. Wildl. Res. 34:306-318.

Greve, J. M., and P. M. Wassarman. 1985. Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J. Mol. Biol. 181:253-264.

Grootenhuis, A J, H L Philipsen, J T de Breet-Grijsbach, and M van Duin. 1996. Immunocytochemical localization of ZP3 in primordial follicles of rabbit, marmoset, rhesus monkey and human ovaries using antibodies against human ZP3. J. Reprod. Fertil. Suppl. 50:43-54.

Gulyas, B. J., L. C. Yuan, R. B. Gwatkin, and E. D. Schmell. 1983. Response of monkeys to porcine zona pellucida as detected by a solid-phase radioimmunoassay. J. Med. Primatol. 12:331-342.

Hardy, D. M., and D. L. Garbers. 1994. Species-specific binding of sperm proteins to the extracellular matrix (zona pellucida) of the egg. J. Biol. Chem. 269:19000-19004.

Hinsch, K. D., and E. Hinsch. 1999. The zona pellucida 'receptors' ZP1, ZP2 and ZP3. Andrologia 31:320-322.

Hoshi, K., A. Sato, and R. Yanagimachi. 1988. Effects of agents used for zona pellucida removal on hamster oocyte penetration by human spermatozoa. Fukushima J. Med. Sci. 34:1-9.

Hughes, B.L., J.E. Jones, and J.E. Toler. 1991. Effects of Exposing Broiler Breeders to Nicarbazin Contaminated Feed. Poult. Sci. 70:476-482.

Hughes, D. C. 2007. ZP genes in avian species illustrate the dynamic evolution of the vertebrate egg envelope. Cytogenet. Genome Res. 117:86-91.

Johnston, J. J., W. M. Britton, A. MacDonald, T. M. Primus, M. J. Goodal, C. A. Toder, L. A. Miller, K. A. Fagerstone. 2001. Quantification of plasma and egg 4-4'-dinitrocarbanilide (DNC) residues for the efficient development of a nicarbazin-based contraceptive for pest waterfowl. Pest Manag. Sci. 58:197-202.

Jones, J. E., B. L. Hughes, J. Solis, D. J. Castaldo, and J. E. Toler. 1990c. Effect of Nicarbazin on Brown-Egg Layer-Breeders. Appl. Agric. Res. 5:149-152.

Jones, J. E., J. Solis, B .L. Hughes, and D. J. Castaldo. 1990a. Reproduction Responses of Broiler-Breeders to Anticoccidial Agents. Poult. Sci. 69:27-36.

Jones, J. E., J. Solis, B. L. Hughes, D. J. Castaldo, and J. E. Toler. 1990b. Production and Egg-Quality Responses of White Leghorn Layers to Anticoccidial Agents. Poult. Sci. 69:378-387.

Kerr, C. 1998. Genetics of Female-Related Infertility in Chickens. PhD Diss. Pennsylvania State Univ., University Park.

Kido, S., and Y. Doi. 1988. Separation and properties of the inner and outer layers of the vitelline membrane of hen’s eggs. Poult. Sci. 67:476-486.

Kido, S., A. Morimoto, F. Kim, and Y. Doi. 1992. Isolation of a novel protein from the outer layer of the vitelline membrane. Biochem. J. 286:17-22.

Kinloch, R. A., B. Ruiz-Seiler, and P. M. Wassarman. 1991. Genomic organization and polypeptide primary structure of zona pellucida glycoprotein hzp3, the hamster sperm receptor. Dev. Biol. 145:203-204.

Kirkpatrick, J. F., and A. Turner. 2002. Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (Equus caballus). Reprod. Suppl. 60:197-202.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee, V. H., E. Schwoebel, S. Prasad, P. Cheung, T. M. Timmons, R. Cook and B. S. Dunbar 1993. Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. J. Biol. Chem. 268:12412-12417.

Liu, C., E. S. Litscher, and P. M. Wassarman. 1997. Zona pellucida glycoprotein mZP3 bioactivity is not dependent on the extent of glycosylation of its polypeptide or on sulfation and sialylation of its oligosaccharides. J. Cell Sci. 110:745-752.

Martinez, M. L., G. K. Fontenot, and J. D. Harris. 1996. The expression and localization of zona pellucida glycoproteins and mRNA in cynomolgus monkeys (Macaca fascicularis). J. Reprod. Fertil. Suppl. 50:35-41.

Martinez, M. L., and J. D. Harris. 2000. Effectiveness of zona pellucida protein ZPB as an immunocontraceptive antigen. J. Reprod. Fertil. 120:19-32.

McCartney, C. A., M. S. Harris, J. C. Rodger, and K. E. Mate. 2007. Towards a ZP-based contraceptive for marsupials: comparative analysis and developmental expression of marsupial ZP genes. Mol. Reprod. Dev. 74:1581-1589.

McClary, C. F. 1955. The restriction of ooporphyrin deposition on egg shells by drug feeding. Poult. Sci. 34:1164-1165.

McDougald, L. R., and T. E. McQuistion. 1980. Mortality from heat stress in broiler chickens influenced by anticoccidial drugs. Poult. Sci. 59:2421-2423.

Miller, L. A., B. E. Johns, and G. J. Killian. 1999. Long-term effects of PZP immunization on reproduction in white-tailed deer. Vaccine 18:568-574.

Moller, C. C., and P. M. Wassarman. 1989. Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev. Biol. 132:103-112.

Mori, M., and N. Masuda. 1993. Proteins of the vitelline membrane of quail (Coturnix coturnix japonica) eggs. Poult. Sci. 72:1566-1572.

Naugle, R. E., A. T. Rutberg, H. B. Underwood, J. W. Turner, and I. K. Liu. 2002. Field testing of immunocontraception on white-tailed deer (Odocoileus virginianus) on Fire Island National Seashore, New York, USA. Reprod. Suppl. 60:143-153.

Naz, R. K., S. K. Gupta, J. C. Gupta, H. K. Vyas, and G. P. Talwar. 2005. Recent advances in contraceptive vaccine development: a mini-review. Hum. Reprod. 20:3271-3283.

Noguchi, S., N. Yonezawa, T. Katsumata, K. Hashizume, M. Kuwayama, S. Homano, S. Watanabe, and M. Nakano. 1994. Characterization of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. Biochim. Biophys. Acta. 1201:7-14.

Okumura, H., Y. Kohno, Y. Iwata, H. Mori, N. Aoki, C. Sato, K. Kitajima, D. Nadano, and T. Matsuda. 2004. A newly identified zona pellucida glycoprotein, ZPD, and dimeric ZP1 of chicken egg envelope are involved in sperm activation on sperm-egg interaction. Biochem. J. 384:191-199.

O'Rand, M. G. 1988. Sperm-egg recognition and barriers to interspecies fertilization. Gamete Res. 19:315-328.

Ott, W. H., S. Kuna, C. C. Porter, and A. C. Cuckler. 1956. Biological Studies on Nicarbazin, a New Anticoccidial Agent. Poult. Sci. 35:1355-1367.

Pan, J., T. Sasanami, Y. Kono, T. Matsuda, and M. Mori. 2001. Effects of Testosterone on Production of Perivitelline Membrane Glycoprotein ZPC by Granulosa Cells of Japanese Quail (Coturnix japonica). Biol. Reprod. 64:310-316.

Paterson, M., M. R. Wilson, K. D. Morris, M. van Duin, and R. J. Aitken. 1998. Evaluation of the contraceptive potential of recombinant human ZP3 and human ZP3 peptides in a primate model: their safety and efficacy. Am. J. Reprod. Immunol. 40:198-209.

Perry, M. M., and A. B. Gilbert. 1979. Yolk transport in the ovarian follicle of the hen (Gallus domesticus): lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. J. Cell Sci. 39:257-272.

Polin, D. 1957. Biochemical and Weight Changes of Mottled Yolks in Eggs from Hens Fed Nicarbazin. Poult. Sci. 36:831-835.

Porter, C. C., and J. L. Gilfillan. 1955. The absorption and excretion of orally administered nicarbazin by chickens. Poult. Sci. 34:995-1001.

Prasad, S. V., S M. Skinner, C. Carino, N. Wang, J. Cartwright, B. S. Dunbar. 2000. Structure and function of the proteins of the mammalian Zona pellucida. Cells Tissues Organs 166:148-164.

Primus, T. M., D. J. Kohler, M. A. Goodall, C. Yoder, D. Griffin, L. Miller, J. J. Johnston. 2001. 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.

Rankin, T., M. Familari, E. Lee, A. Ginsberg, N. Dwyer, J. Blanchette-Mackie, J. Drago, H. Westphal, and J. Dean**.** 1996. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 122:2903-2910.

Rankin, T., P. Talbot, E. Lee, and J. Dean. 1999. Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development 126:3847-3855.

Reid, W. M., P. L. Long, and L. R. McDougald. 1984. Coccidiosis. Pages 705-708 in Diseases in Poultry. Ames, IA: Iowa State University Press.

Reiter, D. K., M. W. Brunson, and R.t H. Schmidt. 1999. Public Attitudes toward Wildlife Damage Management and Policy. Wildl. Soc. Bull. 27:746-758.

Roberts, G. 1998. Toxicological evaluation of certain veterinary residues in food. Geneva: World Health Organization.

Rogers, E. F., R. D. Brown, J. E. Brown, D. M. Kazazis, W. J. Leanza, J. R. Nichols, D. A. Ostlind, and T. M. Rodino. 1983. Nicarbazin complex yields dinitrocarbanilide as ultrafine crystals with improved anticoccidial activity. Science 222:630-632.

Rosiere, T. K., and P. M. Wassarman. 1992. Identification of a region of mouse zona pellucida glycoprotein mZP3 that possesses sperm receptor activity. Dev. Biol. 154:309-317.

Sacco, A. G., E. C. Yurewicz, and M. G. Subramanian. 1989. Effect of varying dosages and adjuvants on antibody response in squirrel monkeys (Saimiri sciureus) immunized with the porcine zona pellucida Mr = 55,000 glycoprotein (ZP3). Am. J. Reprod. Immunol. 21:1-8.

Sasanami, T, J. Pan, and M. Mori. 2003. Expression of perivitelline membrane glycoprotein ZP1 in the liver of Japanese quail (Coturnix japonica) after in vivo treatment with diethylstilbestrol. J. Steroid Biochem. Mol. Biol. 84:109-116.

Scanes, C., G. Brant, and M. E. Ensminger. 2004. Poultry Science. 4th ed. Upper Saddle River, New Jersey: Pearson Education, Inc.

Schwartz, S., B. D. Stephenson, D. H. Sarkar, and M. R. Bracho. 1975. Red, white, and blue eggs as models of porphyrin and heme metabolism. Ann. N. Y. Acad. Sci. 244:570-588.

Sherwood, D. H., T. T. Milby, and W. A. Higgins. 1956. The Effect of Nicarbazin on Reproduction in White Breeder Hens. Poult. Sci. 35:1014-1019.

Shimizu, S., M. Tsuji, and J. Dean. 1983. In vitro biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. J. Biol. Chem. 258:5858-5863.

Silvestrini, D. A., L. E. Dawson, R. J. Evans, and J. A. Davidson. 1965. Effects of nicarbazin in diet on mottled yolks. I. Incidence and degree of mottling, and certain yolk proteins. Poult. Sci. 44:467-473.

Sinowatz, F., S. Kölle, and E. Töpfer-Petersen. 2001. Biosynthesis and expression of zona pellucida glycoproteins in mammals. Cells Tissues Organs 168:24-35.

Smith, J., I. R. Paton, D. C. Hughes, and D. W. Burt. 2005. Isolation and mapping the chicken zona pellucida genes: An insight into the evolution of orthologous genes in different species. Mol. Reprod. Dev. 70:133-145.

Spargo, S., and R. Hope. 2003. Evolution and Nomenclature of the Zona Pellucida Gene Family. Biol. Reprod. 68:358-362.

Srivastava, N., R. Santhanam, P. Sheela, S. Mukund, S. S. Thakral, B. S. Malik, and S. K. Gupta. 2002. Evaluation of the immunocontraceptive potential of Escherichia coli-expressed recombinant dog ZP2 and ZP3 in a homologous animal model. Reproduction 123:847-857.

Stewart, S. G., N. Bausek, F. Wohlrab, W. Scheider, J. Horrocks, and G. Wishart. 2004. Species specificity in avian sperm:perivitelline interaction. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 137:657-663.

Sturkie, P. D. 2000. Avian Physiology. 5th ed. New York: Springer-Verlag.

Tajima, A, E. F. Graham, and D. M. Hawkins. 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J. Reprod. Fertil. 85:1-5.

Takeuchi, Y., K. Nishimura, N. Aoki, T. Adachi, C. Sato, K. Kitajima, and T. Matsuda. 1999. A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida . Its first identification, cDNA cloning and granulosa cell-specific expression. Eur. J. Biochem. 260:736-742.

Tanabe, Y., D. Hetzel, M. Kasai, T. Nakano, M. Mizutani, and B. Gunawan. 1988. Genetic Relationships among Asian Duck Breeds Studied by Biochemical Polymorphisms of Blood Proteins. Proc. Int. Symp. Waterfowl Prod. The Satellite Conference for the XVIII World's Poultry Congress, Beijing, China: International Academic Publishers.

Turner, J. W., I. K. Liu, D. R. Flanagan, K. S. Bynum, and A. T. Rutberg. 2002. Porcine zona pellucida (PZP) immunocontraception of wild horses (Equus caballus) in Nevada: a 10 year study. Reprod. Suppl. 60:177-186.

Waclawek, M., R. Foisner, J Nimpf, and W. J. Schneider. 1998. The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. Biol. Reprod. 59:1230-1239.

Wassarman, P. M. 1990. Profile of a mammalian sperm receptor. Development 108:1-17.

Wassarman, P. M., and E. S. Litscher. 2001. Towards the molecular basis of sperm and egg interaction during mammalian fertilization. Cells Tissues Organs 168:36-45.

Wassarman, P., J. Chen, N. Cohen, E. Litscher, C. Liu, H. Qi, and Z. Williams. 1999. Structure and function of the mammalian egg zona pellucida. J. Exper. Zoo. 285:251-258.

Wishart, G. J. 1987. Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. J. Reprod. Fertil. 80:493-498.

Wood, D. M., C. Liu, and B. S. Dunbar. 1981. Effect of alloimmunization and heteroimmunization with zonae pellucidae on fertility in rabbits. Biol. Reprod. 25:439-450.

Yoder, C. A., J. K. Graham, L. A. Miller, K. S. Bynum, J. J. Johnston, and M. J. Goodall**.** 2006b. Effect of method of delivering nicarbazin to mallards on plasma 4,4'-dinitrocarbanilide levels and reproduction. Poult. Sci. 85:1442-1448.

Yoder, C. A., J. K. Graham, L. A. Miller, K. S. Bynum, J. J. Johnston and M. J. Goodall, 2006a. Evaluation of Nicarbazin as a Potential Waterfowl Contraceptive Using Mallards as a Model. Poult. Sci. 85:1275-1284.

Yoder, C. A., J. K. Graham, and L. A. Miller. 2006c. Molecular effects of nicarbazin on avian reproduction. Poult. Sci. 85:1285-1293.

Yoder, C. A., L. A. Miller, and K. S. Bynum. 2005. Comparison of Nicarbazin Absorption in Chickens, Mallards, and Canada Geese. Poult. Sci. 84:1491-1494.

Appendix A

Diet Ingredient and Nutrient Compositions

Diet Ingredient Composition

Ingredient	Percent
Corn Chopped	49.240
Soybean	20.602
Wheat Middlings	6.001
Bakery Products	6.001
Distillers Grains	5.000
Limestone	4.700
Layer Concentrate	2.500
Calcium Chips	2.500
Fat, Blend	2.050
Dicalcium Phosphate, 18.5% Bulk	0.950
Liquimeth (Methionine)	0.295
Vitamin Mix, Breeder	0.050
Biotin 100MG	0.050
Liquid Mold Guard	0.050
Salt, Granular – Bulk	0.011

Diet Nutrient Composition

Nutrient Name		Amount
Protein		16.625 %
Fat		5.205 %
Fiber		2.454 %
Calcium		3.101 %
Phosphorus		0.559 %
Available Phosphorus		0.307 %
Salt		0.338 %
Sodium		0.133 %
Chloride		0.190 %
Poultry ME		1,300.30 KCAL
WPQ		59.775 %
Mixer Fat		5.146
Choline		663.06 MG
Linoleic Acid		1.773 %
Arginine, Dig. P.[1]		0.963 %
Lysine, Dig. P.		0.731 %
Methionine, Dig. P.		0.354 %
Methionine & Cystine, Dig. P.		0.603 %
Tryptophan, Dig. P.		0.175 %
Threonine, Dig. P.		0.529 %
Isoleucine, Dig. P.		0.607 %
Xanthophyll		2.907 MG
Bulk Density		41.036 LB/CF
Dust Control		2.223 %
Arginine		1.048 %
Lysine		0.827 %
Methionine		0.376 %
Methionine & Cystine		0.667 %
Tryptophan		0.200 %
Threonine		0.612 %
Isoleucine		0.668 %

[1] Digestible Protein

APPENDIX B

Minnesota Avian Semen Extender Composition[1,2]

Compound	**g/L**
BES[a]	3.0
Glucose	10.0
HEPES[b]	4.0
L-Glutamic Acid (monopotassium salt)	2.1
L-Glutamic Acid (monosodium salt)	6.0
Magnesium Sulfate	0.35
Potassium Acetate (anhydrous)	2.1
Potassium Citrate	0.5
Potassium Hydroxide	1.0
Potassium Phosphate (dibasic)	7.0
Potassium Phosphate (monobasic)	1.6
Sodium Acetate	2.5
Sodium Phosphate (dibasic)	0.8
Sorbitol	0.7
TES[c]	4.0
pH	7.1
Osmotic Pressure	370 (mOsm/Kg)

[1] Tajima, A, E. F. Graham, and D. M. Hawkins. 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J. Reprod. Fertil. 85:1-5.

[2] Alexander, A. 1992. Effect of genotype on the fertility of chicken spermatozoa after dlution or cryopreservation. MS Diss. Pennsylvania State Univ., University Park.

[a] NN-Bis(2-hydroxyethyl)-2-aminoethane) sulfonic acid
[b] N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
[c] N-tris(hydroxymethyl)methyl-2 aminoethane) sulfonic acid

APPENDIX C

Protocol for Egg Membrane Heat Solubilization

Solutions

10X PBS

240 g NaCl
6 g KCl
34.5 g $Na_2HPO_4:7H_20$[1]
6 g KH_2PO_4[2]
Bring to 3 L with double distilled water (DDH_2O)

20 mM Tris

2.42 g Trizma Base
Bring to 1 L with DDH_2O
pH = 8 with HCl

1X PBS

100 ml 10X PBS[3]
900 ml DDH_2O

Solubilizing Solution

0.7713 g DTT[4]
2 g SDS[5]
100 ml 20mM Tris
pH = 8 with HCl

Heat Solubilizing Samples

1) Set water bath to 75°C
2) Crack egg into palm or egg separator and dispose of egg white. Hold top of yolk with thumb and cut bottom to drain as much yolk as possible.
3) Place membrane in a sieve partially submerged in 1X PBS. Gently shake sieve until all yolk has dissolved from PVM.
4) Pool 2-5 PVM in a 15 ml conical centrifuge tube with 0.75 ml of solution (Either solubilizing solution or 20 mM Tris)
5) Homogenize the PVM samples with a tissumizer for 40 seconds
6) Place samples in 75°C water for 30 minutes
7) Cool centrifuge to 4°C
8) Centrifuge samples at 27,000 X G for 14 minutes at 4°C
9) Collect the supernatant
10) Store supernatant in -20°C until needed

---

[1] Sodium phosphate dibasic heptahydrate
[2] Monopotassium phosphate
[3] Phosphate buffered saline
[4] Dithiotheritol
[5] Sodium dodecyl sulfate

APPENDIX D

Western Blot Solutions

4X Reducing Sample Buffer
5 ml Upper Stock
4 ml Glycerol
0.8 g Sodium dodecyl sulfate (SDS)
0.31 g Dithiotheritol (DTT)
400 ml Beta-mercaptoethanol
Bromophenol Blue

Upper Stock
6.05 g 20mM Tris
pH = 6.8
0.4 g SDS
Bring to 100 ml with double distilled water (DDH_2O)

10X Laemmli Running Buffer
30.3 g Tris Base
144.2 g Glycine
10 g SDS
pH = 8.3
Bring to 1 L with DDH_2O

Transfer Buffer
5.8 g Tris Base
2.9 g Glycine
0.37 g SDS
pH = 8.3
200 ml Methanol after pH
Bring to 1 L with DDH_2O

PBS-T
300 ml 10X Phosphate buffered saline (PBS)
3 ml Tween-20
Bring to 1 L with DDH2O

Blocking Solution
80 ml PBS-T
4 g Nonfat Dry Milk

VITA

Valerie Reinoso

Valerie Peer Reinoso was born in Pittsburgh, Pennsylvania on the 2nd day of May 1983. She graduated from Montour High School in 2001. On a full Bunton-Waller scholarship, Valerie attended the Pennsylvania State University where she obtained her Bachelor's Degree in Animal Bioscience. Under the guidance of Dr. Guy Barbato, Valerie joined the M.S. program of the Graduate Program in Animal Science. She studied the characteristics of the zona pellucida 3 protein and the mode of action involved in the contraceptive action of nicarbazin. She is expected to graduate in August 2008 with a major in Animal Science. Valerie has been accepted into the Fall 2008 class at the Virginia-Maryland Regional College of Veterinary Medicine.