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Safety evaluation of lasalocid use in farm-reared pheasants

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Safety evaluation of lasalocid use in farm-reared pheasants

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TABLE OF CONTENTS

LIST OF TABLES iv

LIST OF FIGURES v

ABSTRACT vi

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW 1

Thesis Organization 1

Literature Review 1

Pheasant background 1

Coccidiosis 3

Classification of pheasants 5

Extra-label drug use (ELDU) 5

Ionophores 6

Lasalocid 7

Lasalocid toxicity 7

Current use of lasalocid 9

FDA drug approval process 10

Investigational new animal drug (INAD) exemption 11

New animal drug application (NADA) information 12

Conclusion 14

CHAPTER 2. SAFETY EVALUATION OF LASALOCID USE IN RING NECKED PHEASANTS (*PHASIANUS COLCHICUS)* 17

Abstract 17

Introduction 18

Materials and Methods 19

Results 24

CHAPTER 3. SAFETY EVALUATION OF LASALOCID USE IN RING NECKED PHEASANTS (*PHASIANUS COLCHICUS)* 31

Abstract 31

Materials and Methods 32

Results 34

CHAPTER 4. REFERENCE INTERVALS FOR CLINIAL PATHOLOGY PARAMETERS FOR RING-NECKED PHEASANTS (*PHASIANUS COLCHICUS)* AT 6 WEEKS OF AGE 38

Abstract 38

Introduction 38

Materials and Methods 39

Results 41

CHAPTER 5. DISCUSSION 45

CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE WORK 51

ACKNOWLEDGEMENTS 53

REFERENCES 55

LIST OF TABLES

CHAPTER 2

Table 1. Test group diet analyses reported by Alpharma Inc. 28

Table 2. Test group means and standard deviations (SD) for live weights, organ and weights overall feed consumption and feed conversion rates. 29

Table 3. Test group means and standard deviations (SD) from clinical pathology sample testing performed in controls and pheasants treated with lasalocid sodium. 30

CHAPTER 3

Table 1. Test group diet analyses reported by Alpharma Inc. 35

Table 2. Test group means and standard deviations (SD) for live weights, absolute organ and weights overall feed consumption and feed conversion rates. 36

Table 3. Test group means and standard deviations (SD) from clinical pathology sample testing performed in controls and pheasants treated with lasalocid sodium. 37

CHAPTER 4

Table 1: Hematology and serum biochemistry variables for Chinese ring-necked pheasants at 6 weeks of age 43

LIST OF FIGURES

Figure 1. Chemical structure of lasalocid sodium……. 16

ABSTRACT

Ring-necked pheasants are raised on farms under conditions similar to commercial production of broiler chickens and turkeys. They are routinely infected with coccidia that cause outbreaks of clinical disease and sometimes death. Amprolium (Corid®) is the only approved drug for use against coccidiosis in this species and resistance has been reported. Lasalocid (Avatec®) is approved for use in broiler chickens, growing turkeys, and chukar partridges for the prevention of coccidiosis caused by *Eimeria* species specific for each of these birds. It is used extra-label on pheasant farms and has been demonstrated in controlled trials to be effective against coccidia that cause disease on pheasant farms. In order to add pheasants to the Avatec® label, information regarding its efficacy and safety is required by the U.S Food and Drug Administration. The current work focused on target animal safety evaluation of lasalocid use in ring-necked pheasants. No treatment related effects were observed in physical, clinical pathologic and tissue evaluations when lasalocid was dosed orally at 1, 2 and 3 times the recommended high dose for treatment of coccidia in other avian species for 6 weeks, equivalent to 3 times the duration of treatment.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Thesis Organization

This thesis comprises six chapters. Chapter 1 is a literature review that familiarizes the reader with background information about pheasants and coccidiosis as well as reasons why the research presented was performed. Chapters 2 and 3 report the target animal safety of lasalocid (Avatec®) when used in ring-necked pheasants at 1, 2 and 3 times the label dose of other species of poultry for 6 weeks which is equivalent to three times the normal treatment length. Chapter 4 describes how reference intervals for clinical pathology parameters were determined. Chapter 5 discusses the results of the target animal safety evaluations. Chapter 6 concludes this thesis and briefly highlights further investigations required to provide data that that will be considered towards the FDA approval of Avatec® in ring-necked pheasants. Chapters 2, 3 and 4 were adapted from manuscripts that will be submitted to the *Journal of Avian Diseases.* References cited are listed at the end of the thesis.

Literature Review

Pheasant background

Ring-necked pheasants *(Phasianus colchicus)* are large gallinaceous game birds that occur worldwide but are native to central and eastern Asia (53). Those occurring in North America were first introduced in 1881 from China, achieving stable breeding populations in at least 35 of the current 50 states by 1940 (28, 64). They were noted for their palatability, attractive nature, and ability to adapt to any habitat as well as potential to reproduce and thrive in

captivity. They became even more desirable because they were relatively resistant to many parasites and other diseases common to domestically raised birds (63).

In the last century, ring-neck pheasants have become an important source of meat and wildlife activities in the United States. According to the U. S. Department of Agriculture (USDA), about 10 million pheasants are sold annually for human meat consumption (15). In comparison, 8 billion chickens, 220 million turkeys, 37 million quail, 4 million chukar partridges and 1 million mallard ducks are also sold locally for feeding humans and other animals (15, 44). While the contribution by pheasants is small when compared to other poultry, they still represent an important sector to U.S. poultry production and wildlife-associated recreation. In the most recent national survey by the U.S. Fish and Wildlife Service (USFWS), pheasants were the 5th most hunted animal species in 2006 (after deer, wild turkey, leporids and squirrels), attracting 1.6 million hunters in a cumulative 12 million days. They contributed significantly to approximately $2.4 billion worth of spending on small game hunting compared to $11.8 billion spent on big game hunting (70).

Changes in land use policies and agricultural practices in the last 100 years have led to the destruction of naturally occurring pheasant habitats and a consequent decline in their population (22). In order to increase their numbers, pheasants are now intensively raised on game farms under conditions similar to commercial poultry production (62). Under these systems, pheasants succumb to bacterial, viral and parasitic infections including salmonellosis, colibacillosis, coccidiosis, hexamitiasis, histomoniasis, syngamiasis, avian encephalomyelitis and adenovirus amongst others (4, 23, 51, 59, 62, 72).

Coccidiosis

Coccidiosis is a parasitic disease of the intestine of many animals caused by obligate intracellular protozoa (coccidia) belonging to the family *Eimeriidae* (32). Coccidia that cause illness in pheasants belong to the genus *Eimeria* (45, 59, 62). The most commonly encountered coccidia in outbreaks of clinical disease are *Eimeria colchici, E. phasiani and E. duodenalis* although *E. tetartooimia* has also been described (23, 42, 45). At least six other species of *Eimeria* have been described in the literature and are reported to cause mild to severe disease (45, 49). Coccidiosis is most prevalent in pheasants between 2 and 6 weeks of age although younger and older birds can be affected (39). Outbreaks of coccidiosis in confined pheasants has been associated with overcrowding, poor sanitation and concurrent disease because these conditions favor high parasite reproduction (23). Cross infection with other species and birds has also been described (45).

The life cycle of coccidia and pathogenesis of coccidiosis in pheasants is similar to that observed in other avian species (39). Briefly, oocysts are passed in feces of infected birds, sporulate and become infective under favorable conditions of temperature, oxygen and humidity. These sporulated oocysts are then ingested in feed, water, litter and/or soil. Once in the intestine, sporozoites invade the intestinal mucosa and develop into schizonts and merozoites. Merozoites invade adjacent cells until they develop into gametocytes and fertilize to produce oocysts that are passed out with feces and the cycle repeats itself (11).

Coccidia invasion of the intestine is responsible for the observation of diarrhea, the character of which will vary from watery to mucohemorrhagic. In chronic cases, pheasants begin to lose weight, become dehydrated and eventually die. Severity of infection depends on the

Eimeria species involved and on the number of ingested sporulated oocysts. Although multiple species of *Eimeria* are usually isolated from farms undergoing active disease outbreaks (23, 45), fatal disease has been demonstrated experimentally when a single species is involved (24). Intestinal lesions caused by *Eimeria* in pheasants are similar to those reported for other birds (3, 27, 73).

Treatment of coccidiosis is aimed at reducing the severity of clinical disease while allowing for the development of immunity (46). Amprolium, a thiamine derivative, is the only U.S Food and Drug Administration (FDA) -approved drug for use in pheasants against coccidiosis. It is marketed by Merial Limited as Corid® (NADA 012-350), a type A medicated article, containing 25% amprolium. It is added to feed at 175 ppm as a continuous dose up to 8 weeks of age. Patton *et al.,* (1984), demonstrated the efficacy and safety of amprolium against *E. colchici, E. duodenalis* and *E. phasiani* (50). They found that when they challenged 2 week old pheasant chicks with a high dose of infective oocysts, and subsequently fed medicated diets that contained 125 ppm, 175 ppm and 250 ppm amprolium, pheasant chicks receiving medicated diets did not die compared with those receiving a non-medicated diet. No information regarding subsequent oocyst shedding was reported. While amprolium has been used for several decades, there have been reports of reduced efficacy in the control of coccidiosis on intensively reared pheasant operations (47).

Other methods such as immunization of pheasants using sub-lethal oral doses of *E. colchici* oocysts (33) or treatment of *E. colchici* oocysts with ozone to inhibit sporulation (34) have been demonstrated to reduce the severity of clinical disease under battery trials but the efficacy of these techniques as well as the cost considerations are unknown at this time.

Classification of pheasants

The U.S. Department of Agriculture (USDA) allows pheasants reared in captivity, not hunted in the wild, to be sold for human consumption (15). According to the U.S. Food and Drug Administration (FDA), birds, other than chickens or turkeys, are considered minor species (67) . Farm-reared pheasants are, therefore, classified as minor food-producing animals and use of drugs in this species must follow the same federal guidelines as for other food-producing animals.

Extra-label drug use (ELDU)

Many of the diseases that occur on pheasant farms can be medically managed through the administration of medication in feed or water because this is practical and convenient. By law, the drug must be labeled for use in this species, and use of an unapproved animal drug in the feed of a food-producing animal, such as pheasants is prohibited. The number of FDA-approved drugs available for use in game birds in general remains extremely low, with only 5 for pheasants, 2 for chukar partridges, 3 for ducks and 8 for quail. In comparison at least 250 medications are labeled for chickens and turkeys (44).

Extra-label drug use (ELDU) in food-producing animals is acceptable under conditions described by the Animal Medicinal Drug Use Clarification Act (AMDUCA) (9, 14). ELDU in pheasants is acceptable under the same conditions as AMDUCA according to supplemental information described in the FDA's Compliance Policy Guidance (66). The exceptions state that ELDU in feed is permitted under veterinary guidance for up to 6 months and the FDA "will not ordinarily consider regulatory action against a veterinarian" provided specific regulations are followed. The roles of veterinarians who prescribe suitable extra-

label medications on pheasant farms are to create a valid veterinarian-client-patient relationship, determine the appropriate dose for the flock being treated, use a medication that is approved in at least one other major species, ensure appropriate labeling of feed, keep accurate records, confine the population and establish appropriate withdrawal period. In order for veterinarians to make treatment decisions, as well as remain within accepted legal practice, they require access to therapeutic drugs as well as scientific data that demonstrates effectiveness, target animal safety and potential drug residues remaining in edible animal tissues that could potentially enter the human food chain. The use of ionophores in pheasants is currently considered ELDU.

Ionophores

Ionophores are antibiotics produced by *Streptomyces* bacterial species. They were originally developed for poultry as coccidiostats but have been added to feed for many decades as growth promotants in ruminants (38). Ionophores possess a broad anticoccidial and predominantly gram positive antibacterial spectra. They are classified as either channel formers or ion carriers. Channel forming ionophores aggregate within a cell membrane and create a hydrophilic channel across the membrane with hydrophobic residues outside the cell. In this way, various ions from outside a cell environment can be transported into a cell. Ion carriers (neutral or carboxylic), complex with monovalent and/or divalent ions forming lipid complexes that are then transported across surface membranes via passive diffusion (18). Ionophores commonly used in livestock production are lasalocid, monensin and salinomycin (6, 58).

Lasalocid

Lasalocid is a carboxylic polyether ion carrier ionophore produced by the bacterium *Streptomyces lasaliensis*. It is used as a sodium salt (CAS No. 25999-20-6), and its structure as depicted by Ripoli *et al.*, (54) is shown in Figure 1. Lasalocid is unique in that it facilitates the transport of both monovalent (H^+, Na^+ and K^+) as well as divalent ($Ca^{2+}$and Mg^{2+}) cations across cell surface membranes (18). Other molecules such as norepinephrine and epinephrine can be complexed *in vitro* to lasalocid, allowing them to be transported across surface membranes. Because of this, lasalocid has been used by physiologists and biochemists to study transport mechanisms in various membrane systems (18). Lasalocid is marketed by Pfizer Animal Health as Avatec®, a type A medicated article containing 20% lasalocid sodium.

The effect of lasalocid on coccidia has been studied in chicken-specific *Eimeria* species (*E. tenella* and *E. acervulina*) using light and electron microscopy (8, 43). Lasalocid increases the osmolality in 1st and 2nd generation extracellular sporozoites and merozoites, causing them to absorb water and consequently rupture. Intestinal mucosa invasion and oocyst shedding are reduced (36). At 75, 90, 100 and 125 ppm, Bains (1980) showed that lasalocid was effective against major coccidia species in chickens (2). In pheasants, lasalocid has been shown to be more effective against major coccidial species at 75 ppm and 120 ppm compared to other ionophores such as monensin and salinomycin (29, 41).

Lasalocid toxicity

In general, lasalocid and other ionophores are safe in poultry and ruminants if administered at recommended doses. Numerous reports are available on the toxicity of lasalocid in various

species (17). Most toxicity issues are encountered following accidental ingestion, overdosing and/or feed mixing errors (20, 48). Once absorbed, lasalocid is distributed into muscle, liver, skin, fat, heart, thymus, lung and spleen. Peak plasma concentrations in chickens can be obtained within 2 hours after administration, while about 95% remains in the intestines and is excreted in feces. Signs of toxicity will vary but result from the disruption of normal physiologic ionic gradients. Cardiac and skeletal muscles appear to be the most sensitive organs.

Reduced weight gain, mortality, reduced fertility and hatchability were described by Perelman (1993) when broiler chickens were accidentally fed 115 and 150 ppm (52). Ionophore-induced neurotoxicity has also been reported where birds become ataxic and have leg weakness (12, 30). Death can either be due to heart failure as a result of myocardial necrosis or in chronic cases, low caloric intake associated with inappetence and anorexia (25). McDougald and McQuistion (1980) demonstrated that temporary compensatory growth can occur within a week of toxicity if anticoccidial drugs are removed from feed (40).

Information regarding the effect of lasalocid and other ionophores on clinical pathology parameters in poultry is lacking. In one study, 5-week old chickens that were treated with 300 or 400 ppm monensin for 16 days had elevated serum levels of aspartate aminotransferase, lactate dehydrogenase, malate dehydrogenase, creatine phosphokinase and malic enzyme detected in serum (26). The observed enzyme elevations were suggestive of cardiac muscle, skeletal muscle and liver dysfunction, but these were not accompanied by any observable clinical signs similar to observations in cattle and horses. No tissue gross or microscopic evaluations were reported. In another study, lasalocid had no effect on serum

glucose, triglycerides, cholesterol, total proteins and albumin, estradiol and progesterone levels when administered continuously through feed to turkey poults until they were 23 weeks old (55).

Current use of lasalocid

Lasalocid sodium (Avatec®) is approved for use in broiler or fryer chickens, growing turkeys, and Chukar partridges for the prevention of coccidiosis caused by a variety of *Eimeria* species specific for each of these animals (NADA 096-298). As a feed additive, it is given continuously to growing turkeys, broiler and fryer chickens at a dose of 68 ppm to 113 ppm and 113 ppm to chukar partridges from day 0 up to 8 weeks of age with no withdrawal period required. The use of Avatec® in ring-necked pheasants is ELDU. No scientific information is available regarding safety of this medication in this species or potential tissue residues that may inadvertently end up in the human food chain.

In ruminants lasalocid is marketed as Bovatec® (NADA 096-298). Its primary use is to improve weight gain and feed efficiency through altering ruminal microbial populations towards gram negative bacteria that increase propionic acid production and reduce acetate. In doing so, it 1) increases glucose that enters the blood stream; 2) conserves energy and amino acids. In addition to this, it improves magnesium, phosphorous, zinc, and selenium absorption, nutrients that are vital for energy and efficiency in ruminants (57). Bovatec® also has anticoccidial properties against *Eimeria bovis* and *E. zuernii* (61). It has been used in an extra-label manner in calves against *Cryptosporidium parvum* (5), although it is virtually ineffective against this organism.

FDA drug approval process

The Center for Veterinary Medicine (CVM) is the division of the FDA that governs the evaluation and approval of all animal pharmaceuticals, medicated feeds and animal devices. Prior to approval, the FDA/CVM requires that a new animal drug application (NADA) be submitted. The NADA must provide evidence that the drug is safe and effective for its intended use, and that the methods, facilities and controls used for manufacturing and packaging of the drug are adequate to preserve its identity, strength and quality.

Obtaining FDA approval for a drug is a lengthy and expensive process. The cost of obtaining information for FDA approval for a new animal drug is estimated between $10-$25 million and can take between 7 and 10 years depending on the nature of investigations required to obtain necessary data (68). Even after the studies are complete and the drug is approved, there are expenses associated with label changes, annual reports and adverse event monitoring. While most investigations aimed at providing data for a particular NADA can be performed by independent researchers, studies are typically sponsored by pharmaceutical companies that are heavily invested in the financial returns. In major species, the returns are high enough to be able to recover the costs incurred in investigating new animal drugs.

In a species such as pheasants, the market size is not large enough to guarantee sufficient economic return to a pharmaceutical sponsor. It may be less expensive to add pheasants to an existing label through providing supplemental information regarding safety and effectiveness of a product already on the market. Even with this provision, the cost of adding a new species to a label is between $2 and $8 million (average of $3.1 million) and research aimed at acquiring data for the label claim can take over 3 years (68).

To help facilitate the development of drugs for minor species, the Minor Use and Minor Species Animal Health Act was passed in 2004. Under this law, pharmaceutical companies were allowed to conditionally market a drug for use in minor species while providing additional time to collect efficacy and safety data. Federal funding was set aside for studies aimed at reducing the costs to complete investigations required by the FDA/CVM. The procedures investigators would need to follow towards adding a minor species to an existing label are regulated by the Office of the Minor Use/Minor Species (OMUMS) and the Office of New Animal Drug Evaluation (ONADE). Support for controlled research in minor food animal producing species is provided by National Research Support Projects (NRSPs) that have been facilitated by the USDA. According to the NRSP-7 2009 annual report, there are currently over 343 drug requests have been submitted to OMUMS for investigation, with about 40 of those drugs identified as urgently needed in minor species (68).

Studies aimed at providing data for safety evaluation of lasalocid in pheasants must be conducted under Good Laboratory Practices (GLP) for nonclinical laboratory studies. The requirements are codified under Title 21, Code of Federal Regulations Part 58 (21CFR Part 58) (69). The following is an overview of the drug approval process as it relates to lasalocid for its use in pheasants.

Investigational new animal drug (INAD) exemption

The INAD exemption requires that, prior to beginning research; investigators must receive FDA approval to administer the drug for experimental purposes in the intended species. In their application, they must propose the drug product, the label indications and the target animal in which the drug is to be used in. The investigator and the FDA/CVM then agree on

a product development plan that outlines requirements and conditions for the safety and efficacy trials.

New animal drug application (NADA) information

Once safety and efficacy information is acquired, it is then compiled and submitted as an original NADA or supplement to an existing NADA that seeks to add a species to a particular label.

Efficacy

The objective of an efficacy trial is to demonstrate that a drug is effective towards or against the condition for which its use is intended. This can be demonstrated *in vitro* or *in vivo* using laboratory animals or in the target species. Ideally, one would to simulate conditions that mimic natural infection and demonstrate a drug's potency against known isolates of the disease-causing agent within the host.

McQuistion was the first to report the efficacy of lasalocid against 4 *Eimeria* species, *E. phasiani, E. pacifica, E. duodenalis and E. tetartooimia* in pheasants (42). He administered coccidial suspensions to 3-week-old pheasant chicks, and then gave medicated feed containing 120 ppm lasalocid, salinomycin or monensin to each group of pheasants. While he observed a decreased mortality rate and significantly reduced oocyst shedding in all medicated birds, pheasants receiving lasalocid shed the lowest number of oocysts and had the highest weight gain per any of the groups. Fuller *et al.*, (16) also showed that lasalocid at 120 ppm decreased oocyst shedding and improved weight gain 5 days after pheasants were

inoculated with mixed coccidian isolates containing *E. phasiani, E. duodenalis,* and *E. colchici.*

Target animal safety (TAS)

The objective of a target animal safety evaluation is to investigate potential undesirable side effects in the target animal that could be attributed to the administration of a particular drug. FDA/CVM requires that the drug be tested at the claimed effective dose and progressively higher doses for an extended period of time beyond the duration a drug would ordinarily be used. The test subjects must be observed periodically for any harmful effects, including death, associated with these overdoses and/or increased duration of administration. Criteria for assessing safety should be indicated and should include but are not limited to clinical observations, clinical pathologic and gross and microscopic tissue evaluations.

Human food safety (HFS)

Human food safety is assessed through determining whether a drug, its metabolites or any other compounds formed as a result of use of that drug are detectable in edible tissues of that animal species. Ideally, levels must be non-detectable, below or within the allowable tolerances determined for other major avian species. Assessing tissue residues also requires knowledge of drug metabolism within the host animal. The goal is to establish a drug withdrawal period to ensure consumer safety. Methods used to analyze these tissue residues should be extremely accurate with a high level of sensitivity and specificity. High performance liquid chromatography (HPLC) has been used to determine lasalocid residues in chicken and turkey edible tissues. Tolerance levels established are; chicken skin and fat at 1.2 ppm, chicken liver at 0.4 ppm, turkey skin and fat at 0.4 ppm, and turkey liver at 0.4 ppm.

While the same analytical method can be applied to pheasant tissues, it must be bridged to pheasant tissues and validated by assessing specificity, linearity, recovery, accuracy, precision, limit of detection, limit of quantification and stability of extracts.

Pheasant edible tissues include muscle, liver, muscle fat, kidney and skin. Lasalocid is not known to break down into other compounds nor generate metabolites. Although lasalocid is known to be toxic to other animals when administered at high enough doses there are no reported cases of toxicity in humans, nor is it used in human medicine. Case reports are available for monensin and salinomycin in which patients died shortly after exposure (31, 60). Furthermore, it could still be absorbed into the circulatory system and exert cardiac and/or skeletal damage via similar mechanism as those in animals. Fortunately, levels in other species have remained sufficiently low that they have never been a threat to consumers (10)

Conclusion

Coccidiosis remains a significant threat to the welfare of farm- raised pheasants between the ages of 2 and 6 weeks. Most disease outbreaks are accompanied by diarrhea, dehydration, reduced feed intake, reduced weight gain, and sometimes death. Amprolium (Corid®) is currently the only drug on the market labeled for use for the prevention of coccidiosis caused by *E. colchici, E. phasiani and E. duodenalis* in growing ring-necked pheasants. Unfortunately resistant isolates have been reported on some farms. Lasalocid sodium (Avatec®) is effective against several field isolates of *Eimeria* species that infect ring-necked pheasants. It is approved for use in broiler chickens, fryer chickens, growing turkeys and chukar partridges for the prevention of coccidiosis specific to those species. Lasalocid is used

extra-label in pheasants and other game birds such as quail and mallard ducks that succumb to coccidiosis as well. Based on reports of its efficacy and its application in other poultry species, Avatec® may have a clinical application in the management of coccidiosis on pheasant farms. Before pheasants can be added to the Avatec® label, Avatec must meet the NADA requirements that demonstrate its safety in both the target species and in humans that may consume pheasant tissues. The target animal safety evaluation of Avatec® is presented in this thesis in partial fulfillment of NADA requirements for adding pheasants to the drug label.

FIGURES

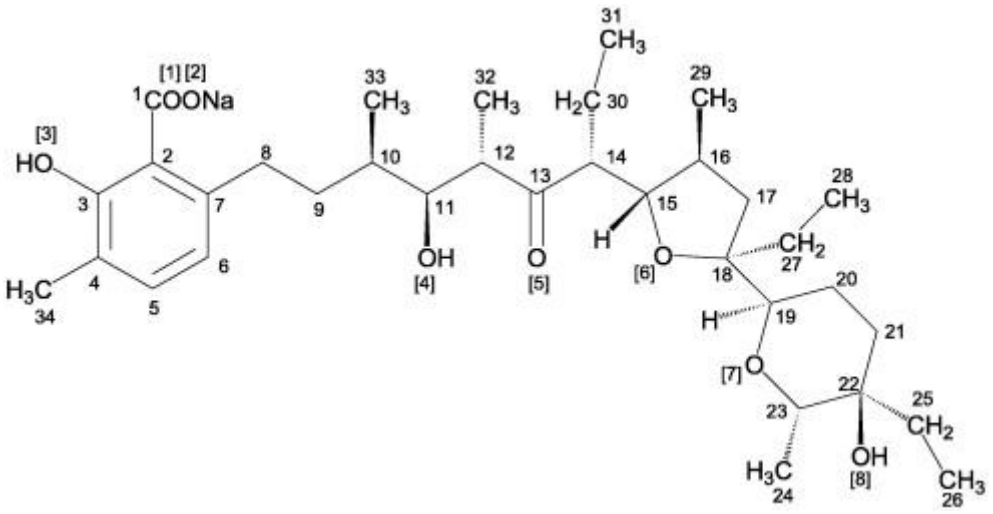

Figure 2. Chemical structure of lasalocid sodium (54).

CHAPTER 2. SAFETY EVALUATION OF LASALOCID USE IN RING NECKED PHEASANTS (*PHASIANUS COLCHICUS)*

Modified from a paper to be submitted to the *Journal for Avian Diseases*

Dzikamunhenga R.S., Wilberts B., Yaeger M., Bender H., Larson W., and Griffith R.W.

Abstract

The objective of the study was to gather data on the safety of lasalocid when fed to pheasants at levels of 0, 125, 250 and 375 ppm. These levels are equivalent to 0X, 1X, 2X and 3X the label dose for prevention of coccidiosis in broiler chickens, fryer chickens, chukar partridges and growing turkeys. One hundred and sixty pheasant chicks that were one day-old were randomly blocked by sex into 4 treatment groups. Group 1 pheasants (n=40) received a non-medicated basal diet and served as controls. Group 2 (n=40), Group 3 (n=40) and Group 4 (n=40) received a medicated diet that contained 125 ppm, 250 ppm and 375 ppm of lasalocid, respectively. Fresh feed was provided to all pheasants once daily for 6 weeks. Two pheasants (1.25%) were observed to be moribund and were humanely euthanized prior to study termination. Their illnesses were not related to lasalocid treatment. When the pheasants were 6 weeks old, they were humanely euthanized. Blood was collected for hematologic and serum biochemistry analyses. Necropsy and histopathologic evaluations were performed on the pheasant tissues. No adverse clinical signs related to the intake of lasalocid were observed during the six weeks of feeding. Liver weights were significantly higher for pheasants in the 2X and 3X groups when compared to controls. Red blood cell counts, packed cell volumes and alkaline phosphatase enzyme levels were significantly lower for

pheasants in the 3X group compared to controls. Female pheasants in the 3X group had significantly higher mean values for blood monocyte counts, total protein and calcium when compared to controls. Female pheasants in the 2X group had significantly higher serum calcium levels compared to controls. Collectively, these observations were of small magnitude, did not appear to be accompanied by other clinical or tissue reactions and/or were within established reference intervals such that they were considered incidental. No significant differences were observed in live weights; overall feed consumption; feed conversion rates; other clinical pathology variables; or gross and histopathologic tissue evaluations when treatment groups were compared to controls. The results of this study show that lasalocid fed at 1X, 2X and 3X the label dose for control of coccidia in other avian species is safe in ring-necked pheasants.

Introduction

Pheasants are raised on propagation farms in several states under conditions similar to the commercial production of poultry. They are routinely infected with coccidia such as *Eimeria colchici, E. duodenalis, E. phasiani* with outbreaks of clinical disease and sometimes high mortality being reported (23). Fatal infections are highest between 2 and 6 weeks of age and results in severe economic losses. Amprolium, a thiamine derivative, is currently the only drug approved for use in prevention of coccidiosis in growing pheasants. It is safe and effective at a dose of 175 ppm (50). Unfortunately, it has a limited species activity spectrum and resistant strains have been reported on some farms (46).

Lasalocid is a divalent carboxylic ionophore that facilitates the movement of monovalent and divalent ions across cell surface membranes (18). Lasalocid used in commercial operations is marketed as a sodium salt (Avatec®) and is labeled for use in broiler chickens, growing turkeys, and chukar partridges for the prevention of coccidiosis caused by a variety of *Eimeria* species specific for each of these birds. Lasalocid has been demonstrated to be effective against *Eimeria* species on pheasant farms at 120 ppm in feed (16, 42). Use of lasalocid on pheasant farms is extra-label and is acceptable under the Animal Medicinal Drug Use Clarification Act (AMDUCA) guidelines according to supplemental information described in the FDA's Compliance Policy Guidance (44). Before lasalocid can be approved by the U. S. Food and Drug Administration (FDA), information regarding its efficacy and safety must be documented in controlled trials. The objective of this study was to gather data on the safety of lasalocid when fed to pheasants from 0 to 6 weeks of age. It was hypothesized that no toxic effects would be associated with lasalocid administration.

Materials and Methods

Pheasants

One hundred and sixty Chinese ring-neck pheasant chicks (*Phasianus colchicus)* were purchased from Oakwood Game Farm (Princeton, MN). There were 80 males and 80 females. Pheasant chicks were one day old at study initiation and 6 weeks old at study termination.

Pheasant housing and maintenance

Pheasants were housed at a research facility at the Iowa State University Poultry Science Farm (Ames, IA). No acclimation period was used and pheasant chicks were placed in the

experiment on the day of arrival. Pheasants were raised in floor pens that measured 1.22 meters x 1.22 meters x 1.22 meters separated by wire partitions. Heat lamps suspended approximately 0.35 meters were used to provide artificial heat and light for the pheasant chicks up to 2-weeks of age. Once heat lamps were removed, room lighting was the only source of light for the pheasants. Pheasants were provided with approximately 16 hours of light and 8 hours of darkness per day. Wood shavings were used as bedding and were replenished as needed to maintain a sanitary and comfortable environment.

Treatment and control group layout

Pheasant chicks were blocked by sex and randomly assigned to four test groups of 40 pheasants each (20 males and 20 females). Eight pens (replicates) were used per test group and each pen housed 5 pheasants. There were a total of 32 pens of pheasants.

Test article and diet formulation

The basal diet used was a commercial starter preparation, Game Bird Startena^{TM} (Purina ®), formulated to meet the nutritional requirements of pheasants from 0 to 6 weeks old. Lasalocid (Avatec®) was obtained from Alpharma Inc., (Bridgewater, NJ) as a premix with 20% (199.54 g per kilogram) lasalocid sodium. Lasalocid was formulated for mixing with the basal diet using the following formula:

$$\frac{\text{Dose (mg/kg)x Weight of basal diet (kg)}}{\text{Concentration of lasalocid (g lasalocid/g premix)}} = \text{Test Article Dose (g) of premix per 45.4 kg bag of premix}$$

Approximately 0.5 kg of the medicated feed was collected from each prepared batch and submitted to Alpharma Inc., for assay at study initiation and at study termination.

Experimental design

There were 4 test groups in this investigation. Table 1 shows the formulation of the 4 test group diets. No lasalocid was added to the diet fed to the pheasants in test group 1 which were the controls. Pheasants in test groups 2, 3, and 4 were fed the basal diet with approximately 125 ppm, 250 ppm and 375 ppm lasalocid added, respectively. These doses were equivalent to 1X, 2X and 3X the approved dose of lasalocid in broiler or fryer chickens, growing turkeys, and Chukar partridges. Fresh feed was provided to the pheasants once daily for 6 weeks. Water was provided *ad libitum* via automatic drip tubes. Blinding was maintained by ensuring that persons that were involved at each level of clinical or pathologic evaluations had no knowledge of dosing. All phases of this study were conducted under Good Laboratory Practices (GLP) (21 CFR Part 58) guidelines for nonclinical laboratory studies, and were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University. Critical phases were monitored by quality assurance personnel appointed by the Minor Use Animal Drug Program (MUADP), National Research Support Porject-7 (NRSP-7).

Clinical observations

Study inclusion physical examinations were conducted by a veterinarian on each pheasant on day 0, and only normal and healthy pheasant chicks were included in the study. Pheasants were observed twice daily throughout the study to determine their general appearance as well as monitor consistency of fecal output, death and/or normal conditions. Pheasant general appearance for each pen was scored as 6=very excitable/agitated; 5=slightly agitated, excessive vocalization; 4=normal; 3=slight depression, feathers slightly ruffled, does not

appear to be gaining weight; 2=marked depression, ruffled feathers, obvious anorexia and 1=dead. Feces were described as one of the following: 5=severe watery diarrhea that may contain mucus and/or blood; 4=moderate diarrhea that may contain some mucus and/or blood; 3=slight diarrhea; 2=slight change from normal and 1=normal. A veterinarian confirmed any unusual observations. Moribund and/or dead pheasants were removed and necropsied. The chicks were weighed as a group at the time of placement into pens and individually at euthanasia at 6 weeks of age.

Feed consumption and feed efficiency

Fresh feed was weighed daily prior to being fed to pheasants. Feed remaining in feed containers or that may have inadvertently spilled during the day or night was noted in the morning, weighed and discarded. Food consumption and feed efficiency were determined and reported as average of the pen at the end of the study. The following formula was used to calculate adjusted feed conversion rate:

Total feed disappearance
[(total terminal pen bird weight + all dead and removed bird weights) – total bird weights in the pen at day 0]

Total feed disappearance (feed consumption) was defined as the sum of all feed additions to a pen *minus* the sum of all feed weighed back from that pen and the estimated or actual wastage.

Hematology and serum chemistry

At 6 weeks, pheasants were randomly sacrificed by direct intramuscular injection of pentobarbital solution into the breast muscle. As soon as the birds were unconscious and prior to cardiac arrest, approximately 2 ml of blood was collected via the vena cava. The

blood was divided between EDTA and heparinized vacutainer tubes and submitted to the Iowa State University, Veterinary Clinical Pathology Laboratory for hematology and serum biochemistry analyses. The following hematology variables were determined: red blood cell count; packed cell volume; mean corpuscular volume; white blood cell count; heterophils; eosinophils; lymphocytes; monocytes and thrombocyte counts. The following serum biochemistry values were determined: glucose, total protein; albumin, creatine kinase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, calcium, phosphorous, sodium, potassium, chloride, magnesium, amylase, uric acid, lactate dehydrogenase, alkaline phosphatase, total bilirubin, cholesterol and globulin.

Organ weights, necropsy and histopathology

Organ weights were obtained on heart, liver, spleen and thymus of the sacrificed birds. The following organs and tissues were examined grossly and histologically for any lesions or abnormalities: skin, eyes, liver, kidney, heart, lungs, trachea, adrenal gland, pancreas, esophagus, crop, spleen, proventriculus, ventriculus, intestines (upper, middle and cecum), bursa of fabricius, ovaries and oviducts, testes, bone, thyroid gland, thymus, parathyroid land, brain, spinal cord and pituitary body. If lesions were noted on gross necropsy, a full description was made and tissues collected for histologic examination. If no lesions were present, a representative sample of tissues was collected for histologic examination. The selected tissues were preserved in 10% neutral buffered formalin and prepared as paraffin-embedded sections on glass microscopic slides. Hematoxylin and eosin stained sections of the tissues were examined by light microscopy. Necropsy and histopathologic examinations were performed by a board-certified veterinary anatomic pathologist.

Data analyses

Primary variables for statistical analyses were morbidity, mortality, live weights (beginning and ending pen weights), organ weights, hematology and serum biochemistry parameters, food consumption and feed conversion rates. Statistical analyses were performed using SAS Enterprise Guide statistical software (version 4.3; SAS Institute, Inc., Cary, NC, USA). A mixed effect two way analysis of variance (ANOVA) was performed on the live weights, organ weights, organ/body weight ratios, feed consumption data, serum chemistry and hematology data. "Individual pens" was considered the random effect. There were no additional random effects. Test group and sex, and the interaction of test group and sex were considered fixed effects. There were no additional fixed effects. Least square means were used to compare the treated test groups to the control test groups. No adjustment of *p*-values was made. All statistical tests were conducted at 0.1 level of significance to meet the U.S Food and Drug Administration (FDA) protocol specifications evaluating effects of lasalocid administration. Feed conversion rates were calculated in Microsoft© Excel©. No statistical analyses were made for necropsy and histopathology.

Results

Diet analyses

Analyses performed on each dietary treatment group to confirm the target test article composition are shown in Table 1. Lasalocid was incorporated into three out of four test group diets. Beginning and ending trial composition for diets 1, 2 and 3 and the beginning trial composition for diet 4 were slightly higher than the targeted concentrations and

considered to be within ± 20% tolerance. The ending feed analysis of diet 4 was only slightly above the approved ±20% tolerance dose that had been targeted.

Pheasant observations, morbidity and mortality

One hundred and fifty eight out of one hundred and sixty pheasants received their intended doses for the entire six weeks. Two pheasants were observed to be moribund on days 17 (2X group) and 27 (3X group) respectively and these were humanely euthanized. Gross necropsy and histopathology of these pheasants revealed that the first bird had a generalized bacterial infection and the second bird had a dislocation of the spinal cord between cervical vertebrae C3 and C4. These deaths were not attributed to lasalocid administration. All other pheasants appeared to be in good health for the duration of the study and no other unusual or otherwise noteworthy findings were observed. The 1.25% mortality rate observed was within historical values of the facility. No statistical analyses were necessary for the variables morbidity and mortality.

Live weights

Mean test group pheasant weights at study initiation and termination are shown in Table 2. All pheasants gained weight over the course of the study. No significant difference in the mean ending body weights were seen when controls were compared to treatment groups *(p=0.2287).*

Feed consumption

The average amount of feed consumed per test group is shown in Table 2. There was no significant difference in mean food consumption *(p=0.9094)* were seen when controls were compared to treatment groups.

Feed conversion

Feed conversion data is used to assess the amount of feed required for weight gain when reported as grams of feed per gram weight gain. The mean feed conversion rate, corrected for body weight, by test group for the control and each treatment group is shown in the Table 2. No significant difference was found in mean feed conversion rates *(p=0.5563)* amongst the test groups.

Organ weights

Test group mean terminal organ weights are shown in Table 2. Mean liver weights were significantly higher for pheasants in the 2X (8.65 g, *p*) and 3X (8.61 g) groups compared to those in controls (7.88 g). No other significant differences were observed in weights of other organs or organ to body weight ratios in any lasalocid treatment groups.

Hematology and serum biochemistry

Hematology and serum biochemistry test group means are summarized in Table 3. Males had higher mean monocyte counts and higher total protein and calcium values when compared with female pheasants. Red blood cell counts and packed cell volumes (PCV) were lower for pheasants in the 1X, 2X and 3X groups compared to controls. Alkaline phosphatase enzyme levels were also significantly lower for pheasants in the 3X group when compared to controls.

Necropsy and histopathology

One pheasant, in the 3X group had moderate subacute myocardial hemorrhage, necrosis and mineralization of the myocardium that could have been associated with lasalocid toxicity. Microscopically, mild focal lymphocytic and heterophilic infiltrates were seen on various tissues including myocardium, kidneys, lungs and spleens. These lesions were spread out amongst all birds.

Table 1. Test group diet analyses reported by Alpharma Inc.

Diet	Target dose g/t (ppm)	Pre-trial lasalocid analysis g/t (ppm)	Post-trial lasalocid analysis g/t (ppm)
Control	0 (0)	<0.5 (0)	<0.5 (0)
1X	113 (125)	133.6 (147)	110.3 (122)
2X	204 (250)	231.8 (256)	244.7 (270)
3X	340 (375)	424.7 (468)	272 (300)

Table 2. Test group means and standard deviations (SD) for live weights, organ and weights overall feed consumption and feed conversion rates.

Variable	Control	1X Group	2X Group	3X Group
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Beginning body weight	90.38 (2.77)	90.13 (1.73)	93.50 (4.72)	88.63 (5.15)
Ending body weight	358.50 (43.95)	368.41 (46.80)	372.85 (47.44)	362.48 (51.30)
Feed consumption	3992.63 (254.05)	4024.25 (385.63)	4054.00 (456.73)	4114.38 (333.66)
Feed conversion rate	2.35 (0.08)	2.36 (0.09)	2.35 (0.07)	2.39 (0.07)
Liver weight	7.88 (1.07)	8.28 (1.04)	8.65 (1.18)*	8.61 (1.30)*
Heart weight	1.69 (0.29)	1.81 (0.28)	1.78 (0.27)	1.80 (0.27)
Spleen weight	0.25 (0.08)	0.33 (0.14)	0.32 (0.13)	0.34 (0.12)
Thymus weight	1.22 (0.40)	1.34 (0.39)	1.36 (0.30)	1.37 (0.44)

*indicates significant difference at $p<0.10$ observed between controls and lasalocid treated pheasants.

Table 3. Test group means and standard deviations (SD) from clinical pathology sample testing performed in controls and pheasants treated with lasalocid sodium.

Variable (units)	Control	1X	2X	3X
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
RBC ($x10^6$/µl)	3.05 (0.52)	2.87 (0.41)	2.77 (0.41)*	2.72 (0.31)*
PCV (%)	35.86 (2.44)	34.30 (2.17)*	34.34 (2.89)*	33.87 (2.45)*
MCV (fl)	120.27 (17.01)	121.85 (17.43)	126.23 (17.62)	125.60 (11.85)
WBC ($x10^3$/µl)	9.07 (4.96)	8.24 (4.45)	9.4 (4.33)	11.17 (9.38)
-Heterophils ($x10^3$/µl)	2.81 (3.27)	1.99 (2.43)	2.01 (2.23)	3.86 (6.80)
-Eosinophils ($x10^3$/µl)	0.05 (0.09)	0.07 (0.10)	0.07 (0.12)	0.08 (0.13)
-Basophils ($x10^3$/µl)	0.49 (0.42)	0.60 (0.32)	0.58 (0.35)	0.63 (0.39)
-Lymphocytes ($x10^3$/µl)	5.60 (3.22)	5.45 (3.29)	6.39 (2.72)	6.04 (3.29)
-Monocytes ($x10^3$/µl)	0.20 (0.21)	0.17 (0.16)	0.34 (0.48)	0.56 (1.24)
Glucose (mg/dl)	344.60 (26.32)	344.05 (36.76)	349.26 (30.48)	340.75 (25.77)
TP (mg/dl)	2.82 (0.24)	2.84 (0.23)	2.86 (0.26)	2.92 (0.46)
Albumin (mg/dl)	1.44 (0.15)	1.42 (0.10)	1.43 (0.13)	1.42 (0.13)
Creatine kinase (IU/L)	3376.40 (941.28)	3395.92 (1379.59)	3199.92 (926.82)	2896.08 (801.91)
AST (IU/L)	402.43 (93.11)	371.47 (57.27)	362.38 (42.79)	374.05 (48.93)
ALT (IU/L)	7.33 (6.12)	6.87 (3.24)	5.49 (1.90)	6.55 (4.21)
GGT (IU/L)	4.20 (1.70)	4.08 (1.94)	3.74 (1.73)	3.93 (1.56)
Calcium (mg/dl)	10.01 (0.57)	9.88 (0.94)	10.37 (0.58)	10.29 (0.87)
Phosphorous (mg/dl)	9.76 (1.91)	9.19 (1.98)	9.20 (1.28)	8.40 (0.92)
Sodium (mEq/L)	153.33 (3.00)	152.44 (2.16)	153.13 (2.96)	152.93 (2.53)
Potassium (mEq/L)	4.26 (1.54)	4.08 (1.50)	3.55 (0.92)	3.43 (0.74)
Chloride (mEq/L)	112.08 (2.15)	112.13 (2.32)	111.56 (3.00)	111.93 (2.68)
Magnesium (mg/dl)	2.53 (0.27)	2.42 (0.30)	2.52 (0.28)	2.40 (0.23)
Amylase (mg/dl)	2582.83 (856.96)	2607.03 (680.19)	2421.33 (657.68)	2438.48 (627.80)
Uric Acid (mg/dl)	20.41 (8.47)	15.12 (6.07)	18.43 (6.81)	16.06 (7.87)
LDH (IU/L)	776.25 (328.70)	683.92 (174.04)	660.42 (136.33)	649.60 (196.99)
ALP (IU/L)	1286.68 (269.31)	1205.21 (242.75)	1211.74 (221.95)	1044.75 (259.68)
Total bilirubin (mg/dl)	0.21 (0.10)	0.25 (0.15)	0.26 (0.17)	0.26 (0.19)
Cholesterol (mg/dl)	104.23 (14.94)	111.55 (13.58)	108.18 (15.88)	112.08 (16.71)
Globulin (mg/dl)	1.36 (0.20)	1.42 (0.18)	1.78 (2.05)	1.49 (0.44)

Variables: RBC, red blood cell count; PCV, packed cell volume; MCV, mean corpuscular volume; WBC, white blood cell count; heterophils; eosinophils; lymphocytes; monocytes; glucose, TP, total protein; albumin, CK, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; calcium; phosphorous; sodium, potassium; chloride; magnesium; amylase; uric acid; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; total bilirubin, cholesterol and globulin

*indicates significant difference at $p<0.10$ observed between treatment group and controls.

CHAPTER 3. SAFETY EVALUATION OF LASALOCID USE IN RING NECKED PHEASANTS (*PHASIANUS COLCHICUS)*

Modified from a manuscript to be submitted to the *Journal for Avian Diseases*

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Abstract

The objective of the study was to gather additional data on the safety of lasalocid (Avatec®) when fed to pheasants at levels of 0 and 375 ppm for 6 weeks. These levels are equivalent to 0X and 3X the label dose for prevention of coccidiosis in broiler chickens, fryer chickens, chukar partridges and growing turkeys. In the earlier study, feed samples were submitted to Alpharma Inc. following completion of the trial. The highest dose diet (3X) was found to be only marginally higher than the dose that had been targeted. It was decided to repeat the trial with only the 3X group and controls. Eighty pheasant chicks that were one day-old were randomly blocked by sex into 2 treatment groups. Group 5 pheasants (n=40) received a non-medicated basal diet and served as controls. Group 6 pheasants (n=40) received a medicated basal diet that contained 375 ppm of lasalocid. Fresh feed was provided to all pheasants once daily for 6 weeks. Two pheasants (2.50%) died prior to study termination. Their illnesses were not related to lasalocid treatment. When the pheasants were 6 weeks of age, 48 (3 per pen) pheasants were randomly selected and humanely euthanized. Blood was collected for hematologic and serum biochemistry analyses. Necropsy and histopathologic evaluations were performed on the pheasant tissues. No adverse clinical signs related to lasalocid intake were observed during the follow-up period. No significant differences were observed in live

weights; overall feed consumption; feed conversion rates; clinical pathology variables; or gross and histopathologic tissue evaluations when the treatment group was compared to controls. The results of this study show that lasalocid fed at 3 times label dose of lasalocid for the prevention of coccidiosis in broiler chickens, fryer chickens, chukar partridges and growing turkeys, is safe for ring-necked pheasants.

Materials and Methods

Forty male and forty female pheasant chicks (*Phasianus colchicus)* that were one day old were enrolled in this study. Pheasant chicks were blocked by sex and randomly assigned to two test groups of 40 pheasants each (20 males and 20 females). Control pheasants were fed a commercial non-medicated basal diet whereas pheasants in 3X group were fed the same commercial diet containing approximately 375 ppm of lasalocid sodium. Table 1 shows beginning and ending lasalocid analyses for the diets. Fresh feed in measured amounts was provided to the pheasants once daily for 6 weeks. Water was provided *ad libitum* via automatic drip tubes. Pheasants were observed twice daily throughout the study to determine their general appearance as well as monitor consistency of fecal output, death and/or abnormal conditions. The chicks were weighed as a pen at the time of placement into pens and individually at 6 weeks of age.

At 6 weeks, pheasants were randomly sacrificed by direct intramuscular injection of pentobarbital solution into the breast muscle. When pheasants were unconscious and before cardiac arrest, blood was collected and submitted for red blood cell count, hematocrit or packed cell volume, mean corpuscular volume, hemoglobin, white blood cell count, heterophils, band heterophils, eosinophils, lymphocytes, monocytes and thrombocyte counts.

The following serum biochemistry variables were determined: sodium, potassium, chloride, calcium, phosphate, magnesium, total protein, albumin, glucose, amylase, blood urea nitrogen, uric acid, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, alkaline phosphatase, creatine kinase, total bilirubin, cholesterol and globulin. The following pheasant tissues were evaluated for gross and histopathologic changes that could be related to lasalocid administration: skin, eyes, liver, kidney, heart, lungs, trachea, adrenal gland, pancreas, esophagus, crop, spleen, proventriculus, ventriculus, intestines (upper, middle and cecum), bursa of fabricius, ovaries and oviducts, testes, bone, thyroid gland, thymus, parathyroid land, brain, spinal cord, pituitary body**.**

Primary variables for statistical analyses were morbidity, mortality, live weights (beginning and ending pen weights), organ weights, hematology and serum biochemistry parameters, feed consumption and feed conversion rates. Statistical analyses were performed using SAS Enterprise Guide statistical software (version 4.3; SAS Institute, Inc., Cary, NC, USA). A mixed effect two-way analysis of variance (ANOVA) was performed on ending body weights, organ weights, hematology and serum chemistry data. "Individual pens" was considered the random effect. There were no additional random effects. Test group and sex, and the interaction of test group and sex were considered fixed effects. There were no additional fixed effects. Two-way ANOVA was used for feed consumption and feed conversion data. Least square means were used to compare the treated test groups to the control test groups. No adjustment of *p*-values was made. All statistical tests were conducted at 0.1 level of significance to meet the U.S Food and Drug Administration (FDA) protocol

specifications evaluating effects of lasalocid administration. Feed conversion rates were calculated in Microsoft© Excel©. No statistical analyses were made for necropsy and histopathology.

Blinding was maintained such that no person having knowledge of the dosing was involved in the clinical evaluations, necropsies or histopathologic examinations. All phases of the study were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University, conducted under Good Laboratory Practices (GLP) (21 CFR Part 58) guidelines and critical phases were monitored by quality assurance personnel from the Minor Use Animal Drug Program (MAUDP), National Research Project-7 (NRSP-7).

Results

All groups of pheasants received their intended diets for the entire 6 weeks. Two out of 80 pheasants (2.50%) died prior to study termination. Their deaths were not related to lasalocid administration. All other pheasants appeared to be in good health for the duration of the study. No significant differences were observed in live weights; overall feed consumption; feed conversion rates; clinical pathology parameters; or gross and histopathologic tissue evaluations when the treatment group was compared to controls.

Table 4. Test group diet analyses reported by Alpharma Inc.

Diet	Target dose g/t (ppm)	Pre-trial lasalocid analysis g/t (ppm)	Post-trial lasalocid analysis g/t (ppm)
Control	0	<0.5	<0.5
3X	340 (375)	302.4 (333)	378.0 (417)

Table 5. Test group means and standard deviations (SD) for live weights, absolute organ and weights overall feed consumption and feed conversion rates.

Variable	Control Mean (SD)	3X Group Mean (SD)
Beginning body weight	81.25 (3.49)	80.88 (4.45)
Ending body weight	369.51 (46.00)	362.79 (45.24)
Feed consumption	3600.50 (328.10)	3546.50 (278.58)
Feed conversion rate	2.10 (0.11)	2.11 (0.10)
Liver weight	7.60 (0.91)	7.61 (1.16)
Heart weight	2.10 (0.51)	2.06 (0.53)
Spleen weight	0.35 (0.10)	0.31 (0.09)
Thymus weight	1.20 (0.47)	1.15 (0.53)

Table 6. Test group means and standard deviations (SD) from clinical pathology sample testing performed in controls and pheasants treated with lasalocid sodium.

Parameter (units)	Control Mean (SD)	3X Group Mean (SD)
RBC ($x10^6$/µl)	2.64 (0.32)	2.46 (2.46)
PCV (%)	33.33 (2.71)	33.13 (3.11)
MCV (fl)	127.88 (16.03)	137.00 (18.39)
WBC ($x10^3$/µl)	9.99 (4.14)	8.90 (4.28)
-Heterophils ($x10^3$/µl)	1.79 (1.69)	1.35 (1.01)
-Eosinophils ($x10^3$/µl)	0.11 (0.11)	0.05 (0.11)
-Basophils ($x10^3$/µl)	0.37 (0.30)	0.36 (0.20)
-Lymphocytes ($x10^3$/µl)	7.36 (3.08)	6.78 (3.35)
-Monocytes ($x10^3$/µl)	0.30 (0.36)	0.37 (0.43)
Glucose (mg/dl)	281.21 (21.23)	291.96 (22.13)
TP (mg/dl)	2.87 (0.27)	2.90 (0.26)
Albumin (mg/dl)	1.10 (0.12)	1.13 (0.12)
Creatine kinase (IU/L)	5332.71 (2881.15)	6120.92 (2400.43)
AST (IU/L)	397.13 (50.65)	422.21 (51.19)
ALT (IU/L)	7.42 (3.87)	7.00 (1.84)
GGT (IU/L)	10.04 (0.20)	10.04 (0.20)
Calcium (mg/dl)	9.73 (0.48)	9.25 (1.78)
Phosphorus (mg/dl)	7.56 (0.92)	7.25 (5.7-10.3)
Sodium (mEq/L)	143.54 (2.21)	143.63 (1.76)
Potassium (mEq/L)	3.69 (0.36)	3.46 (0.38)
Chloride (mEq/L)	111.33 (1.74)	112.29 (2.07)
Magnesium (mg/dl)	2.04 (0.22)	2.05 (0.16)
Amylase (mg/dl)	447.00 (101.51)	420.39 (97.70)
Uric Acid (mg/dl)	7.01 (4.59)	8.28 (3.59)
LDH (IU/L)	3350.83 (644.82)	3783.38 (967.70)
ALP (IU/L)	440.08 (65.41)	407.04 (101.06)
Total Bilirubin (mg/dl)	0.10 (0.00)	0.10 (0.00)
Cholesterol (mg/dl)	97.63 (13.11)	100.88 (17.05)
Globulin (mg/dl)	2.03 (0.37)	2.03 (0.35)

Variables: RBC, red blood cell; PCV, packed cell volume; MCV, mean corpuscular volume; WBC, white blood cell; heterophils; eosinophils; lymphocytes; monocytes; glucose, T, total protein; albumin, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; calcium; phosphorous; sodium, potassium; chloride; magnesium; amylase; uric acid; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; total bilirubin, cholesterol and globulin

CHAPTER 4. REFERENCE INTERVALS FOR CLINIAL PATHOLOGY PARAMETERS FOR RING-NECKED PHEASANTS (*PHASIANUS COLCHICUS)* AT 6 WEEKS OF AGE

Modified from a manuscript be submitted to the *Journal for Avian Diseases*

Dzikamunhenga R.S., and Griffith R.W.

Abstract

The objective of this study was to determine and report reference intervals (RIs) for hematologic and serum biochemical parameters for ring-necked pheasants at 6 weeks of age. Data from one hundred and nineteen heparin and EDTA blood samples collected from clinically healthy Chinese ring-necked pheasants were available for statistical analyses. Reference intervals were generated in Microsoft® Excel® using Reference Value Advisor freeware. Ninety-five percent RIs were calculated using nonparametric methods following Clinical and Laboratory Standards Institute (CLSI) guidelines. These RIs will be useful for the monitoring of health and diagnosis of disease in confined pheasant populations.

Introduction

Hematology and serum biochemistry variables can be useful indicators of normal internal physiology or disease in animals and people. Reference intervals (RIs) are useful when clinical evaluation is based on analysis of multiple parameters. RIs for many species have been established and these continue to be updated according to changing population dynamics. Information regarding RIs for ring-necked pheasants is lacking with only selected clinical pathology parameters ever having been reported in literature. The objective of this study was to determine reference intervals for clinical pathology parameters in clinically

healthy pheasants at 6 weeks of age. These RIs were required so that values of clinical pathology parameters determined in 2 separate battery trials evaluating the safety of lasalocid and fenbendazole respectively in ring-necked pheasants could be compared to normal ranges.

Materials and Methods

Pheasants

A posteriori sampling as described by CLSI C28-A3 was followed where inclusion and exclusion criteria were set after blood sampling (1, 7). One hundred and nineteen blood samples from clinically healthy ring-necked pheasants were available for analysis. Pheasants had been one-day old when they were acquired and approximately 6 weeks old, weighing between 281g and 492g when they were euthanized. They had been fed a non-medicated commercial basal diet, Game Startena™ (Purina ®), for the entire duration. They had been raised five birds to a pen in floor pens measuring approximately 1.2 meters x 1.2 meters x 1.2 meters at an Iowa State University poultry research facility. Each pheasant chick had been physically examined on day 0 and at 6 weeks of age. Pheasants were observed twice daily throughout the study to determine their general appearance as well as monitor consistency of fecal output, death and/or normal conditions. Pheasant data was included if they had been determined to be clinically healthy at the time of euthanasia and excluded if they had shown obvious signs of disease prior to euthanasia.

Blood collection and analytical methods

Pheasants had been humanely euthanized via intramuscular injection of phenobarbital directly into the breast muscle. Approximately 1ml of blood was collected via the vena cava prior to cardiac arrest and placed in EDTA and heparinized vacutainer tubes for clinical

pathology analyses. Once collected, blood samples were placed on ice and submitted to Iowa State University, Veterinary Clinical Pathology Laboratory within one hour. Complete blood counts were performed by standard manual techniques for avian species using a hemacytometer and unopette (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and included red blood cell count, white blood cell count, packed cell volume, white blood cell count differential (based on 100 cells) including heterophils, eosinophils, lymphocytes and monocytes. Lipemic or hemolytic samples were not included. Air-dried Wright-stained blood smears were evaluated for cellular morphology and thrombocyte estimates according to the standard operating procedure in the laboratory. Serum biochemical variables were analyzed using Hitachi 912 chemistry analyzer (Roche Diagnostics Corp, Indianapolis, IN, USA) or Ortho Vitros® 5.1 FS (Ortho-Clinical Diagnostics, Inc. Rochester, NY, USA) and included glucose, total protein; albumin, creatine kinase; aspartate aminotransferase; alanine aminotransferase, gamma-glutamyl transferase, calcium, phosphorous, sodium, potassium, chloride, magnesium, amylase, uric acid, lactate dehydrogenase, alkaline phosphatase, total bilirubin, cholesterol and globulin.

Statistical Analyses

Data were initially examined visually for obvious outliers, with a focus on retaining the values rather than deleting them, if not known to be aberrant observations, as recommended by CLSI for reference intervals. Pheasant RIs were generated in Microsoft© Excel© using reference value advisor freeware as described by Geffré *et al*. (21). Pheasant 95% reference intervals were calculated by nonparametric methods following CLSI guidelines, and 90%

confidence intervals (CI) were determined for lower and upper limits. Means, medians, standard deviations and 95% RIs were determined for all parameters.

Results

Data from 119 blood samples were used for statistical analyses. Hematology parameters could not be determined from 12 heparin tubes because these clotted prior to analyses. RBC and MCV were only reported for 84 samples. Outliers were identified in 5 biochemical samples (lactate dehydrogenase, 11,582 IU/L; 51,41I U/L; creatine kinase 14,163 IU/L and 16,000 IU/L; aspartate aminotransferase 7,500 IU/L) and only these values were removed. Means, ranges, 95% RIs and 90% confidence intervals (CI) were determined for biochemical and hematologic variables for the pheasants are shown in Table 1.

Discussion

Using CLSI guidelines modified by the American Society of Clinical Veterinary Pathology (ASCVP), we were able to determine reference intervals for clinical pathology parameters in clinically healthy Chinese ring-neck pheasants at 6 weeks of age (1). Other reports available in the literature, describe selected clinical pathology parameters in terms of means and standard deviations (35, 56, 65) but do not report RIs that are more useful. We did not analyze males and females separately because at 6 weeks of age, pheasants are still juvenile and hormonal effects are likely to be minimal. Pheasants were considered clinically healthy if prior to euthanasia they had a normal appearance, were alert and obvious signs of disease including diarrhea or respiratory distress were absent. No medications were added to feed or water during the six weeks the pheasants were monitored that would otherwise affect clinical pathology parameters. Reports on gross and microscopic evaluations of individual birds were

available and analyzed in addition to the clinical pathology parameters. Pheasant tissues did not show any pathological changes that would otherwise influence determination of RIs.

The values reported here are useful for making clinically relevant decisions in confined pheasant populations. However, several factors may account for variability reported by different clinical laboratories such as diet, husbandry, pre-analytical and analytical methods as well as other factors not evaluated here such as age, sex or season. Not only this but excitement and the physiologic response to handling may affect certain parameters such as leukocytes, creatine kinase and aspartate aminotransferase (13, 37). We recommend caution when using these RIs for clinical decision-making or for transference and validation of RIs adopted from other sources.

Table 7: Hematology and serum biochemistry variables for Chinese ring-necked pheasants at 6 weeks of age.

	n	Mean	Median	SD	Range (Min – Max)	95% RI	90%CI for Lower Limit	90% CI for Upper Limit
Hematology parameter (units)								
RBC ($x10^6$/µl)	84	2.83	2.81	0.45	1.9-5.2	1.99-3.60	1.93-2.17	3.39-5.18
PCV (%)	107	34.5	35	3.2	23-41	28-40	23-29	39.3-41
MCV (fl)	84	124.74	122.85	15.66	71.4-181.4	90.58-164.29	71.4-105.33	150.65-181.4
WBC ($x10^3$/µl)	107	11.67	11.1	5.71	2.2 – 29.9	2.84-26.44	2.19-4	23.05-29.92
-Heterophils ($x10^3$/µl)	107	2.31	1.71	2.24	0.2-13.9	0.24-9.16	0.17-0.37	6.74-13.88
-Eosinophils ($x10^3$/µl)	107	0.09	0	0.12	0.0-0.6	0-0.45	0-0	0.36-0.57
-Basophils ($x10^3$/µl)	107	0.61	0.48	0.54	0-3.2	0-2.24	0-0.09	1.71-3.24
-Lymphocytes ($x10^3$/µl)	107	8.4	7.73	4.65	1.3-24.8	2.02-21.39	1.26-2.72	17.24-24.83
-Monocytes ($x10^3$/µl)	107	0.3	0.21	0.33	0-1.6	0-1.3	0-0	0.99-1.62
Serum biochemistry parameter (units)								
Glucose (mg/dl)	119	312	311	42.6	191-396	208-386	191-241	376-396
Total protein (gm/dl)	119	2.92	2.9	0.26	2.2-3.8	2.4-3.4	2.2-2.5	3.3-3.8
Albumin (gm/dl)	119	1.33	1.4	0.21	1-1.8	1-1.7	1-1	1.6-1.8
Creatine kinase (IU/L)	116	3903.4	3935	1601	256-9001	978-7352.5	256-1464.6	6506.8-9001
AST (IU/L)	118	400.7	381.5	94.4	240-922	285-689.3	240-304.8	622.2-922
ALT (IU/L)	119	8.2	6	6.6	4-42	4-31	4-4	23-42

Table 8. (continued)

	n	Mean	Median	SD	Range (Min – Max)	95% RI	90%CI for Lower Limit	90% CI for Upper Limit
GGT (IU/L)	119	6.4	6	3.3	0-12	1-10	0-1	10.12
Calcium (mg/dl)	119	9.96	10	0.6	7.6-11.7	8.4-11.1	7.6-9.1	10.9-11.7
Phosphorus (mg/dl)	119	8.84	8.6	1.66	6.3	6.4-13.3	6.3-6.5	12-15.7
Sodium (mEq/L)	119	150.2	151	5.7	138-165	142-161	138-142	159-165
Potassium (mEq/L)	119	4.07	3.7	1.75	2-15.5	2.3-10.7	2-2.6	6.5-15.5
Chloride (mEq/L)	119	112.3	112	2.5	106-120	108-118	106-109	116-120
Magnesium (mg/dl)	119	2.4	2.37	0.35	1.8-3.9	1.82-3	1.78-1.91	2.97-3.94
Amylase (IU/L)	118	1702.6	1863	1244	24.3-5141	221.63-4168.2	24.27-295.7	3781.3-5141
Uric acid (mg/dl)	119	13.22	12	8.07	3.5-45	3.9-34.7	3.5-4.1	29.8-45
LDH (IU/L)	118	2019	983	1492	505-5413	534-5340	505-597	4595-5413
ALP (IU/L)	119	949.4	1039	502.3	106-2006	317-1815	106-332	1752-2006
Total bilirubin (mg/dl)	119	0.21	0.15	0.16	0-0.9	0.07-0.69	0.04-0.1	0.54-0.88
Cholesterol (mg/dl)	119	109.6	109	17.3	69-150	81-140	69-83	135-150
Globulin (mg/dl)	119	1.66	1.6	0.36	1-2.8	1-2.6	1-1.2	2.4.2.8

Variables: RBC, red blood cell; PCV, packed cell volume; MCV, mean corpuscular volume; WBC, white blood cell; heterophils; eosinophils; lymphocytes; monocytes; glucose, TP, total protein; albumin, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; calcium; phosphorous; sodium, potassium; chloride; magnesium; amylase; uric acid; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; total bilirubin, cholesterol and globulin

1. All 90% CIs exceeded those recommended by the IFCC in CLSI C28-A3.
2. CI indicates confidence interval.

CHAPTER 5. DISCUSSION

Coccidiosis remains a major concern for the welfare of farm raised ring-necked pheasants and their producers. The intestinal damage impairs essential nutrient and water absorption leading to diarrhea, poor weight gain, dehydration and frequently death. The anaerobic environment created favors the proliferation of *Clostridium perfringens* Type A, that causes necrotic enteritis. Clostridial diarrhea may be poorly responsive to treatment because pheasants are too ill to drink medicated water; and mortality is usually 100%. The impaired feed conversion means pheasants consume more feed per pound of weight gain compared to uninfected birds. They take longer to attain market weight causing pheasant farmers to suffer huge economic losses since the cost of feed can be about 75% of the operation.

Several risk factors predispose farmed ring-necked pheasants to coccidiosis. Wet litter, poor ventilation, and contaminated drinkers and feeders favor oocyst sporulation and ingestion. Growing pheasants are often reared in net-covered pens and the soil in those pens can become heavily contaminated with coccidia. Improper mixing of coccidiostat with feed and inadequate medicated feed consumption will favor high parasite multiplication within the host and increase oocyst shedding. Stressors such as concurrent diseases or overcrowding may further exacerbate disease because the immune system is impaired. Multiple species of *Eimeria* are usually isolated from clinical outbreaks of disease (23). Because species are site specific, this may account for the varying degrees of diarrhea and intestinal lesions seen in clinically affected birds. While it is generally believed that the number of oocysts ingested is directly proportional to the intensity of the disease, it has been shown experimentally that a high number of oocysts of less pathogenic species, such as *E. duodenalis*, may actually need

to be ingested before clinical diarrhea occurs (24). It has never been determined whether, the acidic pH, enzymatic activity and high ingesta transit in the proximal intestine may be the reason why long term parasite survival is not favorable in the upper gastrointestinal tract compared to the lower intestine where the more virulent species, *E. colchici* and *E. phasiani,* attach.

Eradication of coccidia on pheasant farms is difficult because of the long term oocyst survival in the environment. Since pheasants are gallinaceous birds, similar to other birds in anatomy, physiology and some disease aspects, it is reasonable to assume that medications such as lasalocid, used in chickens and turkeys can be applied clinically, in ring-necked pheasants. The mechanism of action of lasalocid against coccidia has already been described (19). It achieves serum levels rapidly in chickens although about 95% remains in the intestines and is excreted in feces. It is active against extracellular stages of coccidia, reducing the severity of diarrhea, dehydration and oocyst shedding. Clinical efficacy against *Eimeria* species causing disease in ring-necked pheasants has been established at 120 ppm (16, 42). In comparison, clinical efficacy was established in other poultry species between 68-125 ppm. No reports are available that demonstrate that lasalocid would be efficacious for pheasant coccidia at lower doses. If approved, users would be encouraged to adhere to the recommendation of 125 ppm unless scientific data is provided that lasalocid can be effective at reduced doses. This is because, treating at lower doses would be extra-label, likely non-therapeutic and could result in emergence of resistant strains and subsequent failure of lasalocid therapy.

The objective of the work here was to assess the safety of lasalocid when given to ring-necked pheasants at 1, 2 and 3 times the recommended high dose of lasalocid in other species of poultry for six weeks which is equivalent to three times the normal treatment length. This was data required by the FDA/CVM in partial fulfillment of the NADA requirement for adding ring-pheasants to the Avatec® drug label. Most trials reported in literature that involve lasalocid toxicity in avian species examine feed intake, feed conversion, clinical behavior and oocyst shedding. In our study, we evaluated additional parameters including clinical pathology parameters, as well as gross and microscopic tissue changes.

Hematology and serum biochemistry changes following lasalocid administration in ring-necked pheasants have never been reported. Because Na^+, K^+, H^+, Ca^{2+} and Mg^{2+} are preferentially transported across cell membranes, it is reasonable to assume that these parameters may be decreased in serum (18). Since cardiac muscle, skeletal muscle and the liver have been reported to be sensitive to lasalocid in other avian species, serum enzyme elevations may be observed for creatine kinase, aspartate aminotransferase and lactate dehydrogenase. Even if these changes are observed, they would still need to be compared against established reference ranges. Unfortunately, reference intervals for clinical pathology parameters are lacking for ring-necked pheasants. Furthermore, pheasants like other game birds, are highly excitable and the stress and physiologic effects of handling can falsely affect clinical pathology data. Minimal muscle damage such as that occurring upon intramuscular injection of the phenobarbital prior to blood collection, can elevate serum levels of aspartate aminotransferase and lactate dehydrogenase enzymes (37). We therefore interpreted the hemograms and clinical chemistry panels cautiously.

We established reference intervals for pheasant clinical pathology parameters at six weeks of age using statistically acceptable methods (Chapter 4). We observed several values that would be considered elevated in mammalian and other avian species, for white blood cells counts, creatine kinase, alkaline phosphatase and lactate dehydrogenase. These were not associated with any obvious clinical observations or tissue changes. The observations made in the first trial where males had significantly higher monocyte counts, serum total protein and calcium levels and significantly lower serum alkaline phosphatase enzyme levels and red blood cell counts were considered incidental. This is because they did not appear to be dose related, were not repeatable in the second trial, were within reference ranges and were of such small magnitude that they were considered negligible.

Lasalocid-associated tissue changes following lasalocid toxicity have been reported for chickens, turkeys and ruminants (12, 20, 23) but not for pheasants. Cardiac and skeletal muscles are the most consistently affected although liver enlargement and ascites have been reported. The significance of the single pheasant heart that had changes suggestive of lasalocid toxicity could not be determined at this time. The pheasant weighed 316 g (range for the group was 197-467 g), was clinically normal, its clinical pathology variables were within the reference ranges and no other gross tissue or microscopic lesions were observed. The possibility that this could be an early case of toxicity cannot be ruled out but, because no other pheasants were observed with similar lesions, the lesions in this bird could have been incidental.

Lasalocid has been used on pheasant farms in an extra-label manner for the last 2 decades and is effective against pheasant specific *Eimeria* (16, 42). When its efficacy was first

demonstrated in 1987, lasalocid was not routinely used on pheasant farms. *Eimeria* isolates used in the study were likely naïve to lasalocid although isolates tested were only collected from a single farm. The isolates also did not include *E. colchici*, which is believed to be the most pathogenic of all the species. When its efficacy was evaluated by Fuller et al, 2008, isolates not only came from 2 geographically separate areas, but included *E. colchici*. While Fuller *et al.*, did not observe the same efficacy as McQuistion, against *E. phasiani*, it is possible that the concurrent infection with *E. colchici* could have masked the true efficacy of lasalocid. Although it is reasonable to assume that resistance to lasalocid may be emerging because of its extra-label use on farms over the last 20 years, the true effect of ELDU of lasalocid would need to be investigated using molecular techniques such as PCR to identify the presence of lasalocid resistance genes in coccidia. However, using data from this study, and information published on efficacy, lasalocid can be used judiciously at 120 ppm and delay the occurrence of resistant strains.

Although, ELDU of Avatec has become routine for controlling coccidiosis on pheasant farms, it should not replace good husbandry and biosecurity measures for all personnel involved in the care of pheasants or the facilities in which they are housed. Because oocysts can survive in the environment and on shoes, boots, vehicle wheels and clothing, good sanitation, proper drainage, decreasing stocking density and all in- all out practices may disrupt the life cycle of pheasant coccidia and lessen the occurrence of disease. Other therapies, reported in literature may be useful against coccidiosis in pheasants. Diclazuril at 2 ppm and 4 ppm has been described to be effective against *E. colchici, E. phasiani and E. duodenalis* (71). Treatment of *E. colchici* with ozone has been shown to inhibit sporulation

and reduce infectivity but it is likely to be impractical and expensive to implement (34). The possibility of vaccination against *Eimeria* species has been explored but its efficacy is unknown at this time.

In conclusion, physical, clinical pathologic and tissue evaluations were similar between control and lasalocid treated pheasants. The no-observed-effect-level (NOEL) for lasalocid sodium 20% when administered orally for six weeks was determined to be 375 ppm.

CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE WORK

The work presented here was a target animal safety evaluation required for FDA/CVM review towards adding ring-necked pheasants to the Avatec® label. We were able to show that Avatec fed at up to three times the recommended high dose in other avian species, at three times the normal treatment duration, resulted in no changes in feed consumption, feed conversion, and weight gain attributable to lasalocid treatment. We also evaluated additional parameters, i.e. clinical pathology variables and gross necropsy and histopathology tissue sections. No adverse effects associated with lasalocid treatment were observed in any of these additional parameters. This target animal safety data has been submitted and is under review by FDA/CVM. Data involving lasalocid efficacy has already been established and reported. Additional studies to evaluate consumer and environmental safety remain.

High performance liquid chromatography (HPLC) is the method used to detect lasalocid residues in chicken and turkey tissues. Before it can be applied to pheasants, FDA/CVM requires this technique be bridged for use in pheasant tissues. The goal of determining human food safety would be to determine adequate Avatec withdrawal periods; otherwise lasalocid could end up in human food chain. Tissue residues for lasalocid have been described for chicken and turkey tissues. Lasalocid is approved for a zero-day withholding period for other avian species primarily because the vast majority of the drug remains in the intestine and is not found in the edible tissues. The FDA/CVM would require that lasalocid in pheasant edible tissues should not exceed the levels described for chickens and turkeys.

Lastly information regarding environmental and user safety of lasalocid would need to be reviewed by FDA/CVM. Because, no changes in manufacturing, storage or distribution are being advocated, controlled trials are not warranted to demonstrate environmental or user safety. Material submitted to FDA/CVM will be extrapolated from assessments done in chickens and turkeys since conditions of use are likely to be similar.

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