POTENTIAL FOR AFLATOXICOSIS IN NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) EXPOSED TO CONTAMINATED GRAIN AT FEEDING STATIONS

By

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Bachelor of Science in Wildlife Ecology and Management

University of Arizona

Tucson, Arizona

2011

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2014

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ACKNOWLEDGMENTS

Funding for this research was provided by the Oklahoma Department of Wildlife Conservation through a graduate research assistantship at Oklahoma State University. For administrative and logistical support I thank the Department of Natural Resource Ecology and Management, the Oklahoma Cooperative Fish and Wildlife Research Unit, and the Oklahoma Agricultural Experiment Station. My special gratitude goes to Dr. Timothy O'Connell for the help, advice, and encouragement he provided during the last two years and for the careful revision of thesis drafts. I extend special thanks to the other members of my graduate committee, Drs. Dwayne Elmore and Jason Belden, for their respective expertise and guidance. Additionally, I wish to thank Fred Guthery for his preliminary work that led to the development of this study, Adam Gourley for assistance in field work and data collection, Mark Payton for his help with study design and statistical analysis, and Ryan Lerch of the Laboratory Services Division of the Oklahoma Department of Agriculture, Food & Forestry for analytic training and support. Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

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ABSTRACT

There is concern about the potential impacts that supplemental feeding may have on wildlife populations. Possible negative effects of wildlife feeding include altered fecundity, recruitment, survival, predation, pathogen transmission, and exposure to aflatoxins through contaminated grain. Aflatoxins are produced by toxigenic strains of *Aspergillus flavus* and *A. parasiticus* and are considered the most toxic of all naturally occurring mycotoxins. Wildlife may be exposed to aflatoxins in agricultural grains during supplemental feeding and baiting practices. Although most supplemental feeding stations are designed to benefit white-tailed deer (*Odocoileus virginianus*), non-target species also visit bait stations leading to potential exposure to aflatoxins in contaminated grain. This is a particular concern for Northern Bobwhite (*Colinus virginianus*), a species in decline rangewide that has been demonstrated to be highly susceptible to aflatoxicosis. We used infrared-triggered cameras to assess non-target species visitation and potential for contact with aflatoxin at bait stations on the Cross Timbers Experimental Range in Payne County, OK in September 2012 and January 2013. Six species of birds and 10 species of mammals were photographed during the September survey. Species richness was higher during the January survey, with 17 bird species and 9 mammal species. Visitation increased from 1 non-target capture per hour in the fall to 2 non-target captures per hour in the winter. Northern Bobwhite visitation accounted for 0.03% and 0.23% of non-target captures in fall and winter, respectively. Aflatoxin formation in supplemental feed was also assessed to identify contributing factors. Greenhouse trials were conducted in August, September, and December of 2013 in Payne County, OK, with average greenhouse temperatures of 27°C, 23°C, and 15°C, respectively. A split-plot design was used to compare aflatoxin concentrations for experimental units (n = 96) within each trial. Experimental units varied by grain type (milo vs. corn), feeding method (broadcast vs. piled), precipitation presence (dry vs. wet), and duration (1, 2, 3, and 4 weeks). Corn piled in wet conditions resulted in the highest individual concentration of 3230 ppb. Results suggest that aflatoxin formation in wildlife feed can be reduced by selecting milo instead of corn, broadcasting grain instead of distributing in piles, and limiting the length of time that grain persists before ingestion. Feeding should be avoided during wet conditions when daily temperatures exceed 18°C. Given the ease with which aflatoxin developed in the greenhouse trials, those involved in wildlife feeding/baiting are urged to weigh the possible benefits with the known risks that baiting and supplemental feeding may pose to wildlife species.

Chapter I

SEASONAL VARIATION IN NON-TARGET VISITATION TO WHITE-TAILED DEER BAIT STATIONS.

Introduction

Baiting and supplemental feeding of white-tailed deer (*Odocoileus virginianus*) are common practices among land owners, hunters, and wildlife managers, with an estimated 136 million kg of whole kernel corn distributed for wildlife feeding annually in Texas alone (Wilkins 1999). When baiting deer, grain is typically piled, scattered along trails, or contained in stationary feeders where it attracts a variety of wildlife species (Lambert and Demarais 2001, Campbell et al. 2013). There is concern regarding the impact that feeding may have on white-tailed deer (Brown and Cooper 2006) as well as trepidation about influences on other wildlife populations (Boutin 1990, O'Donoghue and Krebs 1992, Cooper and Ginnett 2000). In addition to possible effects on fecundity, recruitment, survival, and predation (Selva et al. 2014), direct or indirect pathogen transmission may occur as a result of increased density of individuals and intra- and interspecific interactions (Thompson et al. 2008, Sorensen et al. 2014).

Among the multiple effects of supplemental feeding, wildlife could be exposed to aflatoxins in contaminated grain. Aflatoxins are produced by toxigenic strains of *Apergillus flavus* and *A. parasiticus* and are considered the most toxic of all naturally occurring mycotoxins (Stoloff 1980). Wildlife may be exposed to aflatoxins in agricultural grains that have become

contaminated prior to harvest, during curing and storage, or while in use as feed (Woloshuk and Shim 2013). Grain used as wildlife feed has been repeatedly shown to contain aflatoxins (Oberheu and Dabbert 2001, Schweitzer et al. 2001). Henke et al. (2001) found that 17% of purchased wild bird seed contained aflatoxin exceeding 100 ppb. Fischer et al. (1995) reported that 41% of corn collected from bait piles and storage bins contained > 20 ppb and 10% contained aflatoxin exceeding 300 ppb.

Aflatoxins were first identified in 1961 when the fungal metabolite caused acute toxicity in commercial turkeys (*Meleagris gallopavo*). Since then, mass waterfowl die-offs have been attributed to acute aflatoxicosis (Couvillion 1991, Cornish and Nettles, 1999). The gregarious nature of waterfowl has facilitated the identification of aflatoxin as a cause of mortality (Robinson et al. 1982). Acute toxicity is characterized by hepatic injury, coagulopathy, hemorrhage, icterus, and death; chronic ingestion is associated with reduced weight gain, immune system suppression, and negative reproductive effects (Pier 1992). Species suffering from toxicity that are cryptic or less gregarious would likely go unnoticed, with the sick and dead likely consumed by predators and scavengers.

While there is considerable variation in species susceptibility, birds appear to be the most susceptible (Huff et al. 1986, Creekmore 1999). Although the majority of aflatoxin research has explored the effects of aflatoxin on domestic birds, the susceptibility of these species leads to concern regarding wildlife exposure. This concern is warranted, as researchers observed blood-clotting abnormalities and immune dysfunction in domesticated turkeys at levels as low as 100 ppb (Giambrone et al. 1985). The susceptibility of quail to aflatoxicosis is well documented (Wilson et al. 1978, Stewart 1985, Ruff et al. 1992), and recent findings suggest that wild

individuals may be substantially more susceptible (Moore et al. 2013). Aflatoxicosis may be responsible for wildlife deaths for which there is no documented cause.

Bait stations for white-tailed deer may serve as an exposure route by which wildlife ingest aflatoxin-contaminated grain. Warm, wet conditions are conducive to fungal growth and aflatoxin production (Bhatnagar 2006), therefore there is concern regarding when baiting occurs. To accurately assess the risk of aflatoxin exposure to wildlife, relative visitation to bait stations by wildlife must be known. The objectives of my study were to (1) determine which non-target species are attracted to bait stations for white-tailed deer during both fall and winter and (2) determine if highly susceptible bird species are attracted to bait when aflatoxin formation is likely to occur. We assume that visitation by non-target species will increase as temperatures decrease and food becomes limited. A decrease in temperature would be associated with a decrease in aflatoxin formation within bait piles (Choudhary and Sinha 1993). If non-target visitation does increase with decreasing temperatures, then we would expect to observe an increase in species composition and non-target occurrence from fall to winter.

Methods

The study was conducted on the 736-ha Cross Timbers Experimental Range (CTER) of the Oklahoma Agricultural Experiment Station in Payne County, OK. The CTER is located in the Cross Timbers forest that is defined by a mosaic of upland deciduous forest, savanna, and tallgrass prairie that typifies the broad region between the eastern deciduous forest and the grasslands of the southern Great Plains (Küchler 1964). Upland forest patches dominated by blackjack (*Quercus marilandica*) and post oak (*Q. stellata*) are interspersed with tallgrass prairie in the Cross Timbers. In a vegetation survey of the CTER, Ewing et al. (1984) found understory

woody species to be dominated by coralberry (*Symphoricarpos orbiculatus*), eastern redcedar (*Juniperus virginiana*), poison ivy (*Toxicodendron radicans*), roughleaf dogwood (*Cornus drummondii*), redbud (*Cercis canadensis*), and American elm (*Ulmus americana*). Dominant herbaceous vegetation included little bluestem (*Schizachrium scoparium*), Indiangrass (*Sorghastrum nutans*), western ragweed (*Ambrosia psilostachya*), and rosette panicgrass (*Panicum oligosanthes*). Soils are predominantly Stephenville–Darnell–Niotaze associations that are ustalfs of a fine, sandy loam texture (Soil Survey Staff 2008). Annual precipitation for the study area averages 930 mm with peak rainfall normally occurring in May. Average daily temperatures for the region vary seasonally from approximately 34° C (summer) to 0° C (winter) (Brock et al. 1995, McPherson et al. 2007). Relatively low annual rainfall of about 63–100 cm, together with sandy, low-fertility soil, accounts for a reduced diversity of trees in the cross timbers compared to elsewhere in the oak-hickory forest (Risser and Rice 1971).

We used infrared-triggered cameras (ITCs) to assess visitation by non-target wildlife to bait stations for white-tailed deer on the CTER. We established 20 bait stations throughout the study area, recording all visits 7–21 Sep. 2012 and 6–23 Jan. 2013. We piled 23 kg of whole kernel corn on the first day of monitoring and replenished every 3 days during the trials. We distributed bait stations as uniformly as possible, while still being accessible by vehicle, using the same locations for both surveys.

We deployed Moultrie I40 digital game cameras (Ebsco Industries, Birmingham, AL) equipped with data stamp (exposure date and time), frame advance, night vision, and digital memory cards for data storage. We mounted ITCs on metal posts at a height of 1m and 3m from bait piles. We set the ITCs to trigger after a pulse delay of 1 second when motion was detected

on or near the bait pile. We used manufacturer's instructions for date, time, sensitivity, and camera's activation interval.

Bait station visitation was assessed using total surveillance time, total number of photographs taken, non-target captures, and the duration of time spent by non-target species on or near bait piles (occurrence). Photographs including at least 1 non-target species were considered captures. A single photograph could contain multiple captures if multiple species were present at the same time. The number of individuals for each species was counted and recorded per capture. Photographs were taken every minute when motion was detected, allowing us to estimate the duration of time spent on or near bait piles for each species. For example, a single photograph containing 5 Northern Bobwhite and 3 Wild Turkey would be recorded as 2 captures with a combined occurrence of 5 minutes for Northern Bobwhite and 3 minutes for Wild Turkey. Occurrence was summed for each species at each bait station. This likely provided an underestimate of non-target visitation. Individuals attracted to bait stations could have vacated the area before a photograph was taken; individuals could also have been present near the bait station but outside of the frame of the photograph. We assumed an equal probability for underestimates in visitation by species and survey period. We identified individuals from photographs using field guides to birds (Sibley 2000) and mammals (Bowers et al. 2007). We used descriptive statistics to summarize data from the two surveys, and one-way ANOVA ($\alpha = 0.05$) to compare visitation by selected species and species groups.

Results

During the 14 day survey period in September 2012, we photographed a total of 16 non-target species of birds (6) and mammals (10) at the CTER bait stations (Table 1). The survey

included 6,496 hours of data collection, with a total of 7,346 non-target captures. Non-target visitation occurred at a rate of 1 capture per hour. Birds were captured in 7.7% of survey photographs, and constituted 60.4% of non-target captures. Mammals were captured in 5.1% of survey photographs, and constituted 39.6% of non-target captures (Figure 1).

During the 17 day survey period in December 2013, we photographed a total of 26 non-target species of birds (17) and mammals (9) at CTER bait stations (Table 1). The survey included 7,961 hours of data collection, with a total of 16,834 non-target captures. Non-target visitation occurred at a rate of 2 captures per hour. Birds were captured in 27.3% of survey photographs, and constituted 74.2% of non-target captures. Mammals were captured in 9.5% of survey photographs, and constituted 25.8% of non-target captures (Figure 1).

We used occurrence data to test for differences in non-target visitation. With the exception of grouped "all birds" and "all mammals", comparisons displayed homogeneity in variance by season. For all birds and all mammals, we analyzed $\log_{10}$ transformed data by season to stabilize the variances. Visitation by "all wildlife" and "all birds" was higher in winter than in fall ($F_{1, 39} = 19.59$, $p < 0.001$). Visitation by all mammals, mesocarnivores, and upland game birds did not differ by season (Table 2). Of the individual upland game bird species tested, visitation did not differ by season for Wild Turkey, Northern Bobwhite, or Mourning Dove (*Zenaida macroura*).

Discussion

Visitation to baited infrared-triggered cameras was observed for non-target birds and mammals. Visitation by non-target species occurred frequently within our study area, with an average of 1 capture per hour in the fall and 2 captures per hour occurring in the winter. The

increases in species richness (16 to 26) and non-target occurrence in January provide support for the hypothesis that visitation increases as temperatures decrease and food becomes limited.

Birds made up the majority of non-target captures in both surveys, comprising 60.4% and 74.2% in September and January, respectively. Although toxicity studies have not been conducted for the majority of bird species, aflatoxin is considered hazardous to all species (Patterson 1973). Although the degree of susceptibility is species specific and highly variable, the reported susceptibility of poultry (Dalvi 1986) raises concern regarding aflatoxin exposure in wild birds. The observed increase in non-target species composition in winter is the result of dietary shifts in residents and migrant Passeriformes that were not present within the study area in fall. Given that decreased temperatures correspond to a decrease in aflatoxin production (Schindler et al. 1967), we are less concerned with exposure risk for species that only visited bait piles in winter, e.g. Dark-eyed Junco (*Junco hyemalis*).

Upland game bird visitation was high in the fall, implying that these species are at risk of consuming contaminated grain in the fall, when relatively higher temperatures and humidity create optimal conditions for aflatoxin production. Wild Turkey captures occurred at 65% and 55% of the bait stations in fall and winter, respectively. Visitation occurred frequently, accounting for 9% of non-target captures in both fall and winter. Captures of Northern Bobwhite, a species in the midst of a precipitous, long-term, rangewide decline (Sauer et al. 2014) increased from fall (2) to winter (10). Northern Bobwhite spent more time on or near bait piles in winter (179 minutes) compared to fall (37 minutes), although this was not found to be a significant increase due to small sample size. Of the 20 bait stations established, Northern Bobwhite individuals were photographed by a single camera in September and two cameras in January. This may be a result of low attraction to corn by the species or small population numbers at

CTER during the survey periods (Adam Gourley, personal correspondence, October 2012 and February 2013). The susceptibility of Northern Bobwhites to aflatoxins is well documented (Stewart 1985, Wilson et al. 1978, Moore et al. 2013) and baiting for white-tailed deer may represent an exposure route for this and other wildlife species.

Although our data were not recorded to quantify wildlife interactions, Campbell et al. (2013) estimated 38.4 wildlife contacts per kg of corn used at feed stations. Within our study, individual photographs often included captures of multiple species on or near the bait pile. This occurred most frequently among birds, with multiple Passerines occupying a bait pile simultaneously. Mammalian interactions also occurred, with white-tailed deer feeding alongside Northern Bobwhite, North American raccoons, Eastern cottontails, and fox squirrels. These interactions may increase the risk of predation by other non-target species (Selva 2013) or create opportunities for direct or indirect pathogen transmission (Sorensen et al. 2014).

Given recent findings, there is sufficient information to conclude that providing food to wildlife through supplemental feeding or baiting has the potential to negatively impact individual health (Cooper and Ginnett 2000, Schweitzer et al. 2001, Brown and Cooper 2006) and represents a non-natural arena for disease transmission and preservation (Sorensen et al. 2014). Those involved in any form of wildlife feeding should be aware of the potential risks that these practices pose. The possible benefits of the practice should be weighed against known risks.

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Table 1. Non-target wildlife photographed at white-tailed deer bait stations in September 2012 and January 2013 in Payne County, OK.

Order	Scientific Name	Common Name
Accipitriformes	*Buteo lineatus*	Red-shouldered Hawk
Columbiformes	*Zenaida macroura*	Mourning Dove
Galliformes	*Meleagris gallopavo*	Wild Turkey
	Colinus virginianus	Northern Bobwhite
Passeriformes	*Corvus brachyrhynchos*	American Crow
	Cyanocitta cristata	Blue Jay
	Sayornis phoebe	Eastern Phoebe
	Cardinalis cardinalis	Northern Cardinal
	Baeolophus bicolor	Tufted Titmouse
	Turdus migratorius	American Robin
	Junco hyemalis	Dark-eyed Junco
	Passerella iliaca	Fox Sparrow
	Zonotrichia querula	Harris's Sparrow
	Melospiza lincolnii	Lincoln's Sparrow
	Melospiza melodia	Song Sparrow
	Pipilo maculatus	Spotted Towhee
Piciformes	*Melanerpes carolinus*	Red-bellied Woodpecker
Strigiformes	*Megascops asio*	Eastern Screech-Owl
Artiodactyla	*Sus scrofa*	feral hog
Carnivora	*Canis latrans*	coyote
	Urocyon cinereoargenteus	gray fox
	Lynx rufus	bobcat
	Mephitis mephitis	striped skunk
	Procyon lotor	North American raccoon
Cingulata	*Dasypus novemcinctus*	nine-banded armadillo
Didelphimorphia	*Didelphis virginiana*	Virginia opossum
Lagomorpha	*Sylvilagus floridanus*	Eastern cottontail
Rodentia	*Sciurus niger*	Eastern fox squirrel
	Erethizon dorsatum	North American porcupine

Table 2. Results of ANOVA tests for seasonal difference in non-target occurrence at white-tailed deer (*Odocoileus virginianus*) bait stations in September 2012 and January 2013

	Mean Occurrence (minutes)		ANOVA	
	September	January	$F_{1, 39}$	P
all wildlife	541.4	1283.8	19.59	< 0.001
mammals	163.8	257.3	2.52	0.121
mesocarnivores	111.6	108.2	0.01	0.920
all birds	322.3	1005.1	16.87	< 0.001
upland game birds	117.2	198.0	1.06	0.310
Wild Turkey	115.4	187.9	0.83	0.367
Northern Bobwhite	0.5	9.0	1.05	0.313
Mourning Dove	1.3	1.1	0.01	0.904

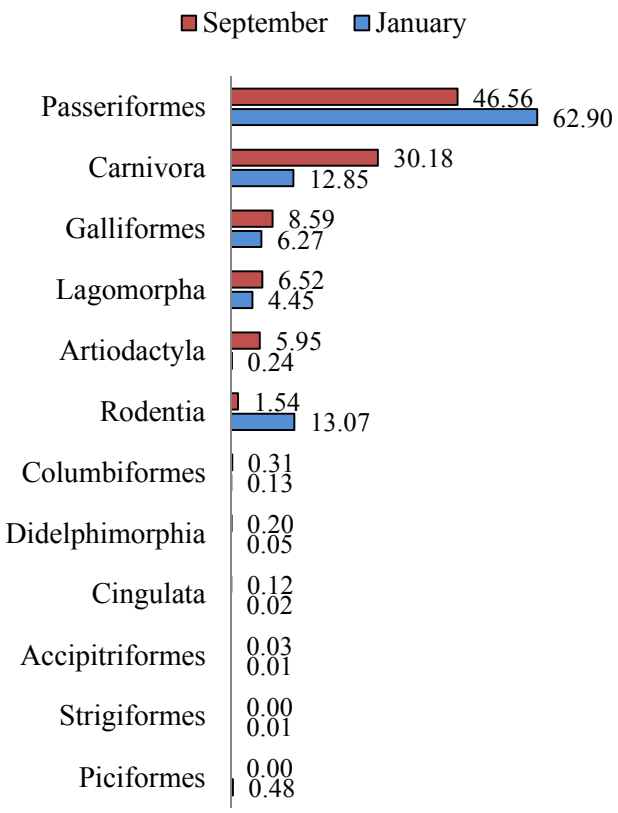

Figure 1. Seasonal variation of non-target wildlife attracted to bait stations for white-tailed deer. Values represent the percentage of 7,346 and 16,834 non-target captures in September 2012 and January 2013, respectively.

Chapter II

ENVIRONMENTAL INFLUENCES ON AFLATOXIN FORMATION IN WILDLIFE FEED

Introduction

Aflatoxins are carcinogenic, mutagenic, teratogenic, and immunosuppressive secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* (Stoloff 1980). Aflatoxins exert a range of acute and chronic pathological effects, and are considered the most toxic and carcinogenic of all naturally occurring mycotoxins. Aflatoxins were first identified in 1960, when approximately 100,000 domestic turkey poults (*Meleagris gallopova*) died from what was termed "Turkey X disease" (Blount 1961). While there is considerable variation in species susceptibility, birds appear to be the most susceptible (Huff et al. 1986, Creekmore 1999).

Mass die-offs of waterfowl have been attributed to acute aflatoxicosis, characterized by hepatic injury, coagulopathy, hemorrhage, icterus, and death (Robinson et al. 1982, Couvillion et al. 1991, Cornish and Nettles 1999). Two independent outbreaks occurred in Texas during the winter of 1977–78. The first outbreak resulted in the death of 500 Snow Geese (*Chen caerulescens*). Although the source was never identified, histopathologic lesions characteristic of aflatoxicosis were observed upon necropsy. The second outbreak was attributed to waste peanuts, and resulted in the death of 7,000 Mallards (*Anas platyrhynchos*). Researchers concluded that toxicity was related not only to the amount of toxin ingested, but also the period of time during which ingestion occurred (Robinson et al. 1982). Un-harvested corn in Louisiana resulted in the deaths of 10,500 Snow Geese along with hundreds of sick or dead White-fronted

Geese (*Anser albifrons*), Ross's Geese (*Chen rossii*), and Mallards between December 1998 and March 1999 (Cornish and Nettles, 1999). The gregarious nature of waterfowl has facilitated the identification of aflatoxin as a cause of mortality. Species suffering from acute toxicity that are cryptic or less gregarious would go unnoticed, with the sick and dead likely consumed by predators and scavengers.

Aflatoxicosis is manifested by a variety of clinical signs and disease states depending on animal species, dosage, and duration of exposure. As is common with hepatotoxins, the health, age, and sex of individuals may affect the degree of toxicity. Aflatoxins have been shown to cause liver damage, kidney disorders, gastrointestinal dysfunction, reduced productivity, decreased feed utilization and efficiency, decreased reproductive performance (including reduced hatchability, smaller eggs, and reduced eggshell quality), reduced milk or egg production, embryonic death, teratogenicity (birth defects), tumors, and suppressed immune system function, even when low levels are consumed (Iheshiulor et al. 2011). Researchers found that the ingestion of aflatoxin by turkeys altered protein synthesis by inhibiting nucleic acid transcription and interfering with RNA translation (Quist et al. 2000).

Quail are considered model organisms for a variety of clinical studies, and their susceptibility to aflatoxicosis well documented (Stewart 1985, Wilson et al. 1978). Researchers observed significantly decreased body weights, increased liver weights, and mortality in captive Northern Bobwhite fed a diet containing between 1250 and 5000 ppb aflatoxin (Ruff et al. 1992). Recent findings suggest that wild individuals may be substantially more susceptible to aflatoxicosis. Using wild bred Northern Bobwhites, researchers induced mortality in individuals after administering 100 µL of 500 ppb aflatoxin solution (Moore et al. 2013). The adverse effects of mycotoxins on reproduction have been reported in Japanese Quail (*Coturnix japonica*),

including reductions in fertility, hatchability, egg weight, egg quality, and increases in embryo death (Doerr and Ottinger 1980). Reproductive effects of aflatoxin on Northern Bobwhite has not been studied, although a halt in egg production was observed following with ingestion of 2000 ppb of aflatoxin (Stewart 1985). Reduced egg production in Japanese Quail has been observed following oral doses of 50 to 200 μg of aflatoxin per kg of body weight.

Chronic exposure to grain with low levels of aflatoxin is associated with reduced weight gain, suppression of the immune system, interference with reproductive function and neoplasia (Pier 1992). While far less research has been devoted to chronic ingestion of aflatoxin, and has been all but ignored for wildlife species, blood-clotting abnormalities and immune dysfunction has been observed in turkeys at levels as low as 100 ppb (Giambrone et al. 1985, Schweitzer et al. 2001). Additionally, chronic ingestion of aflatoxin has also been shown to cause hepatic carcinomas in ducklings after ingesting aflatoxin at levels of 30 ppb for 14 months (Carnaghan 1965). While chronic exposure to low levels of aflatoxin may not be a direct cause of mortality for individuals in some populations, deleterious effects would likely increase susceptibility to predation and disease (Giambrone et al. 1985). Aflatoxicosis may be responsible for many idiopathic wildlife deaths.

Among all the mycotoxins, aflatoxins result in the greatest grain losses and highest management costs due to their extremely high toxicity (Robens and Cardwell 2005). *Aspergillus* resides in soil and may colonize crops (Horn 2003). Pre-harvest aflatoxin contamination of corn is associated with drought and high temperatures during grain fill (Abbas and Shier 2009). While aflatoxin contamination is uncommon in Midwestern crops, severe drought conditions can favor both fungal growth and crop susceptibility, creating concerns for marketing and utilization. Aflatoxins may contaminate agricultural commodities prior to harvest (Abbas 2004), during

curing and storage (Thompson and Henke 2000), and while in use as wildlife feed (Fischer et al. 1995, Oberheu and Dabbert 2001*a*, Henke et al. 2001, Schweitzer et al. 2001). The fungal communities established during crop development greatly influence later aflatoxin contamination, with warm, moist conditions in the field or during transport and storage favoring increased aflatoxin concentrations (Cotty 1997, Cotty and Jaime-Garcia 2007). Due to its toxicity, the US Food and Drug Administration restricts the content of aflatoxin in human food and livestock and domestic animal feed supplies, with 20 ppb the maximum allowable for interstate shipment (USFDA 1979).

Wildlife may be exposed to aflatoxin through supplemental feeding and baiting practices. When baiting white-tailed deer, grain is typically piled, distributed in trails, or contained in stationary feeders, subsequently attracting a variety of wildlife species. Wild granivorous birds, such as Northern Bobwhite, consume a combination of native foods and agricultural grains, when available (Oberhue and Dabbert 2001*b*). Stationary and broadcast feeders have been used to supplement forage of Northern Bobwhite and reduce dispersal of coveys (Guthery et al. 2004). Managers have debated the benefits of supplemental feeding for Northern Bobwhite, with research suggesting conflicting effects. Sisson et al. (2000) suggested that supplemental feeding may reduce fall-spring covey home ranges, increase hunter success, and increase survival. Other researchers have noted negative or neutral effects on survival and reproductive performance (Townsend et al. 1999, Doerr and Silvy 2002, Guthery et al. 2004).

Aflatoxin concentrations in grain increase with length of storage, regardless of storage container (Thompson and Henke, 2000). Researchers found that increases in aflatoxin also occurred following placement in feeders, but that changes in concentrations were variable (Oberheu and Dabbert 2001*a*). Grain is inspected and tested for aflatoxin, but not assessed for

the presence of the fungal producers. Aflatoxin concentrations are an estimate of current contamination, providing no information on the aflatoxin producing potential of any undetected fungus. Grain that has been tested free of aflatoxins at harvest will remain susceptible to contamination until its destruction.

Grain that has been improperly handled may accumulate aflatoxin while in storage, substantially increasing the risk of deleterious effects to wildlife. The initial aflatoxin concentration of grain is an important consideration when undertaking wildlife feeding. Only grain that has been tested and approved for human consumption (aflatoxin < 20 ppb) should be purchased to feed wildlife, with proper handling practices employed to reduce aflatoxin formation in storage. In a move that could increase aflatoxin levels in wildlife feeds, the Food and Drug Administration (FDA) has relaxed standards for aflatoxin abatement. Due to widespread drought and resulting aflatoxin occurrence, state officials may request the ability to blend corn containing aflatoxin with "clean" corn (aflatoxin < 20 ppb). Under the waivers, grain handlers may blend corn containing aflatoxin levels up to 500 ppb, thus enabling producers to sell corn that would have otherwise been destroyed.

While previous research on aflatoxin has provided information regarding contamination of grains occurring prior to harvest and during curing and storage, it is not well understood how grain choice, feeding method, and environmental conditions may influence aflatoxin formation in wildlife feed. We require information on the degree to which grain becomes contaminated given variations in grain type, feeding method, temperature, precipitation, and the length of time that grain persists before ingestion. The primary objective of this study was to determine if grain choice (milo vs. corn), feeding method (broadcast vs. piled), environmental conditions (precipitation presence and temperature), and the length of time that grain persists (1, 2, 3, and 4

weeks) influence aflatoxin formation in wildlife feed. Identifying potential alterations to feeding practices may decrease the risk that aflatoxins pose to wildlife. We tested the general hypothesis that common conditions under which supplemental grains are offered to wildlife (i.e. fall feeding of piled corn) can lead to detrimental and, in some cases, lethal concentrations of aflatoxin.

Methods

Study Design

Greenhouse trials were conducted in August, September, and December of 2013 in Payne County, OK. Average temperature within the greenhouse varied between trials, representing the upper (36^{o}C), optimal (29^{o}C), and lower (20^{o}C) limits of aflatoxin production for *A. flavus* and *A. parasiticus*. Forty-eight nested split-plots were established within each trial. Plots consisted of 50 cm x 45 cm x 6 cm plastic growing trays with holes, filled with 9 kg of organic top soil (Hope Agri Products Inc.) and covered with landscape fabric (Greenscapes Home & Garden Inc.). The use of landscape fabric allowed for efficient collection of grain. Plots were partitioned to include a small (10 cm diameter) sub-plot nested within the main plot. Both areas contained 75 g of grain (hereafter referred to as experimental units), with the main plot representing low-density broadcast feeding, and the nested plot representing high-density pile feeding. Plots were randomly assigned one of 16 treatments, resulting in 3 replicates per treatment per trial (n = 98). Treatments included all possible combinations of grain type (corn, milo), precipitation presence (wet, dry), and grain persistence (1–4 weeks). Grain was dispensed at the start of each trial. Additionally, 2 75-g samples (hereafter referred to as control units) were collected from each bag of corn and milo to provide initial aflatoxin concentrations. Watered plots received approximately 10 liters of water once per week (days 1, 8, 15, and 22). The entire experimental

unit was collected according to its treatment. Control and experimental units were labeled and stored in a freezer immediately after collection (-18^{o}C) to halt any further production of aflatoxin (Schindler et al. 1967).

Sample Preparation, Extraction and Analysis

Following each trial, control and experimental units were sent to the Oklahoma Department of Agriculture, Food & Forestry (ODAFF) Laboratory Services Division (Oklahoma City, OK) for grinding. Upon return, all control and experimental units were analyzed by a single researcher. We quantified aflatoxin using monoclonal antibody-based affinity chromatography and fluorometric detection (AflaTest®, VICAM, Milford, MA). Our laboratory methods followed the standard operating procedures established by ODAFF for the analysis of aflatoxins in feeds. To extract the aflatoxins, 50 g of ground grain was blended with 5 g of salt (NaCl) and 100 mL of reagent grade methanol:water (80:20, VICAM) for 1 minute, and then filtered (Fisherbrand™ Qualitative Grade Plain Filter Paper Circles - P8 Grade, Waltham, MA). Ten mL of filtrate was diluted to 50 mL with distilled water, and then passed through a filter (Fisherbrand™ Glass Fiber Circles). We passed 2 mL aliquot under vacuum through an AflaTest®-P affinity column at a rate of 1–2 drops per second until air came through the column. The column was rinsed with 10 mL of distilled water at a rate of 1–2 drops per second. We eluted aflatoxins from the column by passing 1.0 mL reagent grade methanol (VICAM) through the column at a rate of 1–2 drops per second, and collected in a glass cuvette. One mL of AflaTest® Developer (VICAM) was added to the eluate, mixed well, and then placed in a calibrated fluorometer (VICAM, series 4EX). Aflatoxin concentration was read after 60 seconds.

Quality Control

Calibration methods were followed to assure accurate results in our analysis. We internally calibrated the fluorometer with a range of 0–300 ppb at the start of each day and calibration was repeated every 20 units. Samples outside of this range were diluted using a 16% methanol solution, and rerun. We conducted external calibration by analyzing blank and proficiency samples (external quality assurance samples purchased from Trilogy Labs, Washington, MO) every 20 units. A positive reading for blank samples signified contamination in the test procedure, resulting in an invalid run. Proficiency samples had to be within 3 standard deviations of the study average to pass; otherwise the run was determined to be invalid. In the event of an invalid run, corrective actions were taken to bring the analysis within acceptance parameters and the affected samples were rerun.

Statistical Analyses

All statistical analyses were performed using SAS software (version 9.3). Split-plot comparisons were carried out using a model that assumed a split-plot design with method as the split unit factor and grain, condition, and duration as the main unit factors. Each trial was analyzed separately using $\alpha < 0.05$. Replicate analysis was conducted on randomly selected experimental units and proficiency samples to calculate the relative percent difference (RPD). RPD was used to calculate the precision of analysis from duplicate measurements. It is a measure of reproducibility and is calculated using the following equation:

$$\text{RPD} = \frac{|\ \text{Result 1} - \text{Result 2}\ |}{\text{Average Result}} \times 100$$

Results

Although all samples tested negative for aflatoxin at the beginning of trials, 26% developed aflatoxin concentrations in excess of 20 ppb by the end of the 4-week sampling periods. Corn piled in wet conditions produced the highest individual concentrations in each trial. The highest individual concentrations for each trial resulted from wet piling of corn. The concentrations were 2640 ppb in August, 3230 ppb in September, and 150 ppb in December. Average greenhouse temperatures in August, September, and December were 27ºC, 23ºC, and 15ºC, respectively.

Comparisons of grain type, given the other factors, resulted in statistical differences in all three trials (Figure 1). In the August trial, milo resulted in lower aflatoxin concentrations than corn for wet broadcast (weeks 2, 3, and 4) and wet pile (all weeks). The September trial had similar results, with milo resulting in lower aflatoxin concentrations for wet broadcast (weeks 3 and 4) and wet pile (all weeks). Concentrations were markedly reduced in the December trial, with milo resulting in lower aflatoxin concentrations than corn for wet pile (weeks 1 and 2).

Comparisons of feeding method, given the other factors, resulted in statistical differences in all three trials (Figure 2). In the August trial, broadcasting resulted in lower aflatoxin concentrations than piling for wet corn (all weeks) and wet milo (weeks 2, 3, and 4). The September trial had similar results, with broadcasting resulting in lower aflatoxin concentrations for wet corn (weeks 1 and 2) and wet milo (weeks 2, 3, and 4). Concentrations were markedly reduced in the December trial, with broadcasting resulting in lower aflatoxin concentrations for wet corn (weeks 1 and 2).

Comparisons of precipitation presence, given the other factors, resulted in statistical differences in all three trials (Figure 3). In the August trial, dry conditions resulted in lower aflatoxin concentrations than wet conditions for broadcast corn (weeks 2, 3, and 4), piled corn (all weeks), and piled milo (weeks 2, 3, and 4). The September trial had similar results, with dry conditions resulting in lower aflatoxin concentrations for broadcast corn (weeks 3 and 4), piled corn (all weeks), and piled milo (weeks 2, 3, and 4). Concentrations were markedly reduced in the December trial, with dry conditions resulting in lower aflatoxin concentrations than wet conditions for piled corn (week 2).

Comparisons of grain persistence, given the other factors, resulted in significant differences in all three trials (Figures 4 and 5). In the August trial aflatoxin concentrations increased with duration for wet broadcast corn, wet piled corn, and wet piled milo. The September trial showed increases in aflatoxin concentrations with increased duration for wet broadcast corn and wet piled milo. Concentrations were markedly reduced in the December trial, with significant differences between weeks only observed for wet piled corn.

Calculations of RPD was conducted using 18 duplicate analyses. Two duplicates were removed, due to the inability to calculate RPD when aflatoxin concentrations are 0 ppb. Overall RPD for the remaining 16 duplicates was 9%. This value falls within the acceptance range established by ODAFF (< 20%).

Discussion

We determined that common environmental conditions and supplemental feeding practices can result in deleterious levels of aflatoxin formation in grain provided to wildlife, even when that grain did not have detectable aflatoxin when it was made available. We identified

feasible alterations to feeding practices that may decrease the risk that aflatoxins pose to wildlife. The conditions under which supplemental grains are currently offered to wildlife (i.e. fall feeding of piled corn) can lead to detrimental and, in some cases, lethal concentrations of aflatoxin. The highest individual concentrations within each trial resulted from the wet piling of corn. FDA guidelines recommend that grain fed to wildlife not exceed 20 ppb aflatoxin. Aflatoxin concentrations in August, September, and December greenhouse trials exceeded this limit in 39%, 38%, and 2% of experimental units, respectively. In August and September, concentrations exceeded 200 ppb in 26% and 24% of the experimental units, respectively (Table 1).

Aflatoxin concentrations in supplemental feed were compared between treatments that varied by grain type, feeding method, temperature, precipitation, and the length of time that grain persists before ingestion. These factors were selected due to the regulation of both fungal development and aflatoxin production by a range of environmental and development cues. Temperature, followed by pH, nitrogen source, and carbon source are most deterministic for gene transcription specific to the aflatoxin pathway (Price et al. 2005). Milo and corn are similar in their total dissolved nitrogen (USFDA 2007) and average pH (Hall et al. 2009). Unlike the production of most secondary metabolites that are repressed by simple sugars, aflatoxin synthesis is stimulated by glucose (Davis and Diener 1968). Therefore, we anticipated differences in aflatoxin concentrations for different grain types. *Aspergillus flavus* is capable of growing over a wide range of temperatures, with optimal growth occurring at 37ºC. This temperature also represents the upper limit for aflatoxin synthesis (Bhatnagar et al. 2006). Aflatoxins are produced optimally between 28 and 30ºC, with production decreasing linearly as temperature is increased to 37ºC or decreased to 18ºC (O'Brian et al. 2007).

Greenhouse trials were conducted when temperatures would most closely proximate upper, optimal, and lower temperature thresholds for aflatoxin production. Average greenhouse temperatures in August (27°C) and September (23°C) were conducive to aflatoxin production. As we anticipated, September temperatures provided optimal conditions for aflatoxin production, with a 10% increase in aflatoxin production compared to August. Reduced temperatures in the December greenhouse trial limited aflatoxin production, producing less than 2% of the aflatoxin analyzed in August or September.

Moisture is required both for the fungal growth of *Aspergillus* and the production of its toxic secondary metabolite (Bhatnager et al. 2006). While in use as supplemental feed or bait, *Aspergillus* may begin to produce aflatoxin without a significant precipitation event. Aflatoxin formation occurs rapidly when grain moisture content is 18% or greater (Moreno et al. 2011). During the August and September greenhouse trials, wet treatments (n = 96) produced aflatoxin in all but 7 experimental units. Additionally, dry treatments did not result in aflatoxin concentrations exceeding 200 ppb (n = 288). High humidity or accumulation of dew may provide sufficient moisture for aflatoxin formation in grains used as wildlife feed.

By selecting milo in place of corn for wildlife feeding, aflatoxin concentrations can be significantly reduced. Our results show that regardless of other factors, milo resulted in significantly lower aflatoxin concentrations than corn. Additionally, the method in which grain is dispensed is of importance. Grain density may influence grain moisture content, subsequently influencing aflatoxin formation. This may help explain the difference in aflatoxin concentrations observed between piled and broadcast grain. Aflatoxin concentrations were higher for piles, but significant differences were only observed for wet treatments. Grain density did not significantly influence aflatoxin concentrations when grain remained dry.

Reducing the length of time that grain persists before ingestion may help decrease the risk of wildlife exposure to aflatoxin. My data shows that if conditions are conducive to aflatoxin formation, concentrations increase linearly with time. Grain persistence can be reduced by limiting the amount of grain dispensed at a given time, and promptly removing uneaten grain.

Conservationists have become concerned that supplemental feeding and baiting practices could expose wildlife to toxic amounts of aflatoxin in contaminated grains (Fischer et al. 1995, Oberheu and Dabbert 2001*a*, Henke et al. 2001, Schweitzer et al. 2001). Our results support this, with observed aflatoxin concentrations high enough to produce deleterious, and in some cases, lethal effects in multiple wildlife species. Although consumption of aflatoxins is hazardous to all individuals, species vary in their susceptibility (Creekmore 1999). Poultry are among the most highly susceptible, exhibiting noticeable effects and mortality at lower doses than other food producing animals (Dalvi 1986). Among them, poults and goslings are the most sensitive, quail are intermediate, and domestic chicks the most resistant (Arafa et al. 1981). Lethal dose (LD_{50}) values range from 300 μg/kg of body weight for day-old ducklings to 6300 μg/kg for adult broilers (single dose of orally administered aflatoxin B1) (Edds et al. 1973).

Supplemental feeding and baiting practices represent a significant exposure route for aflatoxin in wildlife populations. Our results indicate that current practices, in particular the piling of corn, may pose a substantial risk. Aflatoxin concentrations in August and September exceeded the LC10 for captive-bred Northern Bobwhites (Ruff et al. 1992) in > 8% of experimental units (n = 192). Within the scope of our study, corn piled in warm, wet conditions resulted in the highest individual concentration of 3230 ppb. Although this concentration represents the upper range of our observed aflatoxin production, concentrations would likely have been increased even further if aflatoxin was initially present in the grain, or if additional

moisture would have been dispensed on the plots. Feeding should be avoided during wet conditions when daily temperatures exceed 18°C.

The frequency and duration of feeding events is likely to greatly affect the degree of toxicity experienced by those ingesting contaminated grains. While decreasing aflatoxin concentrations in wildlife feed may be possible, it will not eliminate the risk of aflatoxin exposure to wildlife. Aflatoxin is considered unavoidable and unpredictable, with safety control efforts focused on minimizing their presence to the greatest extent feasible (Park and Troxell 2002). Those involved in any form of wildlife feeding should be aware of the risks that aflatoxins pose. The possible benefits of the practice should be weighed against these risks.

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Table 1. Percentage of all experimental units (n = 96), wet piled corn (n =12), and wet piled milo (n = 12) with aflatoxin concentrations exceeding 20 ppb, 200 ppb, 500 ppb, and 1000 ppb for August, September, and December greenhouse trials.

Trial	Ave. Temp.	Treatment	> 20 ppb	> 200 ppb	> 500 ppb	>1000 ppb
August	27°C	*all*	39%	26%	15%	9%
		wet piled corn	100%	100%	75%	58%
		wet piled milo	83%	58%	33%	17%
September	23°C	*all*	38%	24%	20%	8%
		wet piled corn	100%	83%	83%	42%
		wet piled milo	83%	58%	25%	0%
December	15°C	*all*	2%	0%	0%	0%
		wet piled corn	17%	0%	0%	0%
		wet piled milo	0%	0%	0%	0%

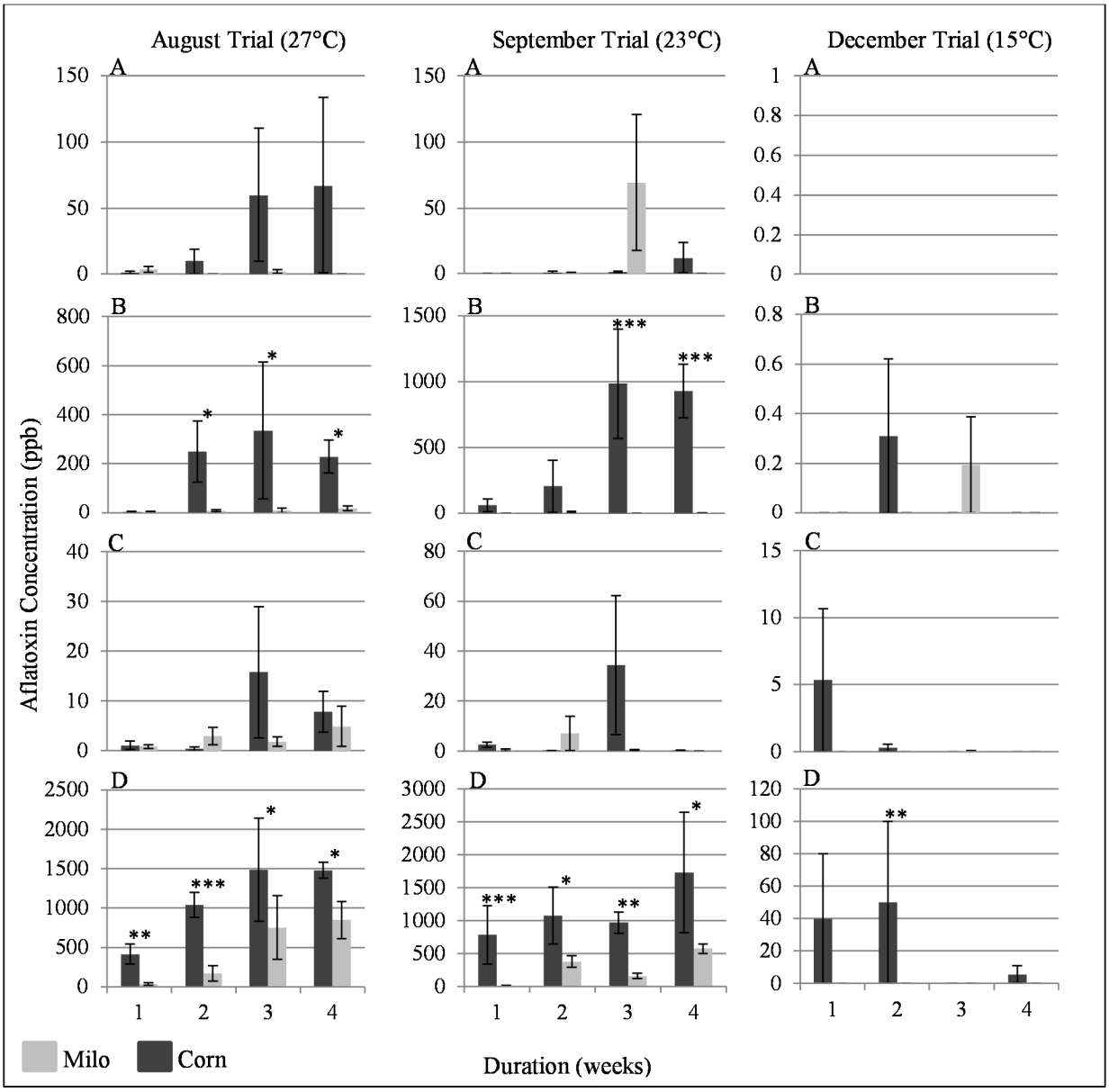

Figure 1. Aflatoxin concentrations of milo and corn. Mean aflatoxin concentrations (ppb) and standard errors are shown for (A) dry broadcast, (B) wet broadcast, (C) dry piled, and (D) wet piled. Split-plot comparisons of precipitation presence identified significant differences in aflatoxin concentrations between treatments (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

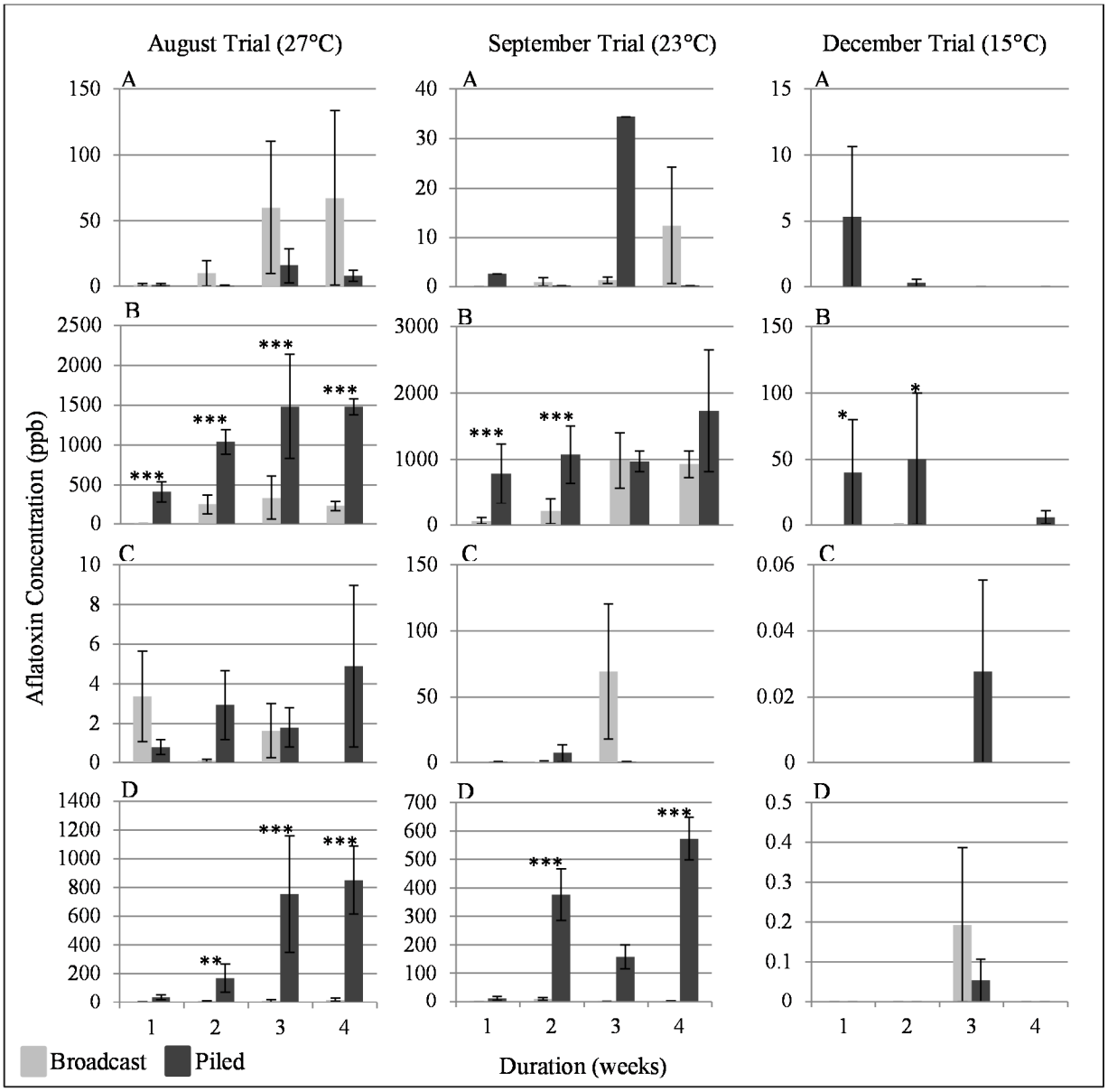

Figure 2. Aflatoxin concentrations of broadcast and piled grain. Mean aflatoxin concentrations (ppb) and standard errors are shown for (A) dry corn, (B) wet corn, (C) dry milo, and (D) wet milo. Split-plot comparisons of precipitation presence identified significant differences in aflatoxin concentrations between treatments (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

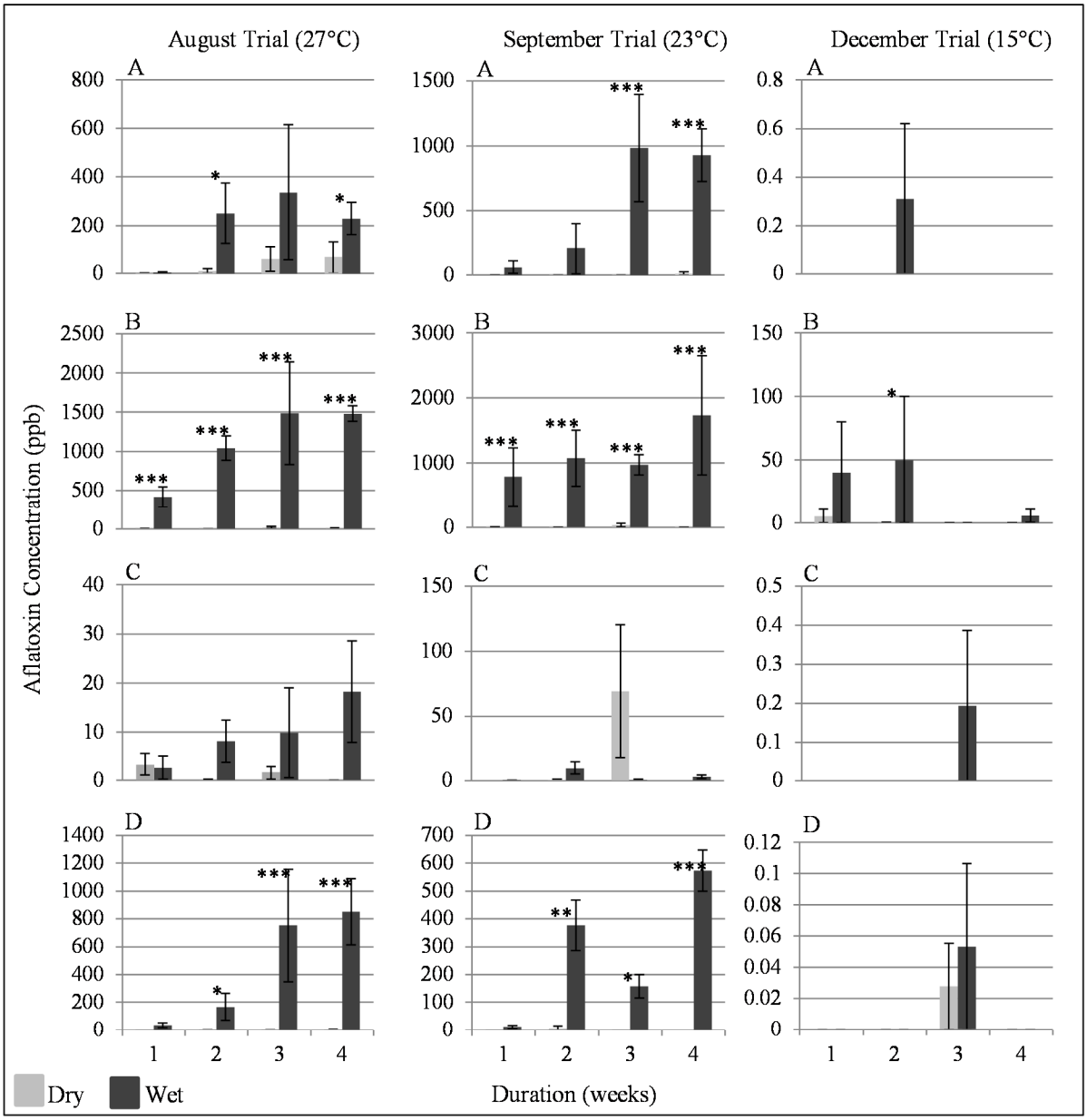

Figure 3. Aflatoxin concentrations of dry and wet grain. Mean aflatoxin concentrations (ppb) and standard errors are shown for (A) broadcast corn, (B) piled corn, (C) broadcast milo, and (D) piled milo. Split-plot comparisons of precipitation presence identified significant differences in aflatoxin concentrations between treatments (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

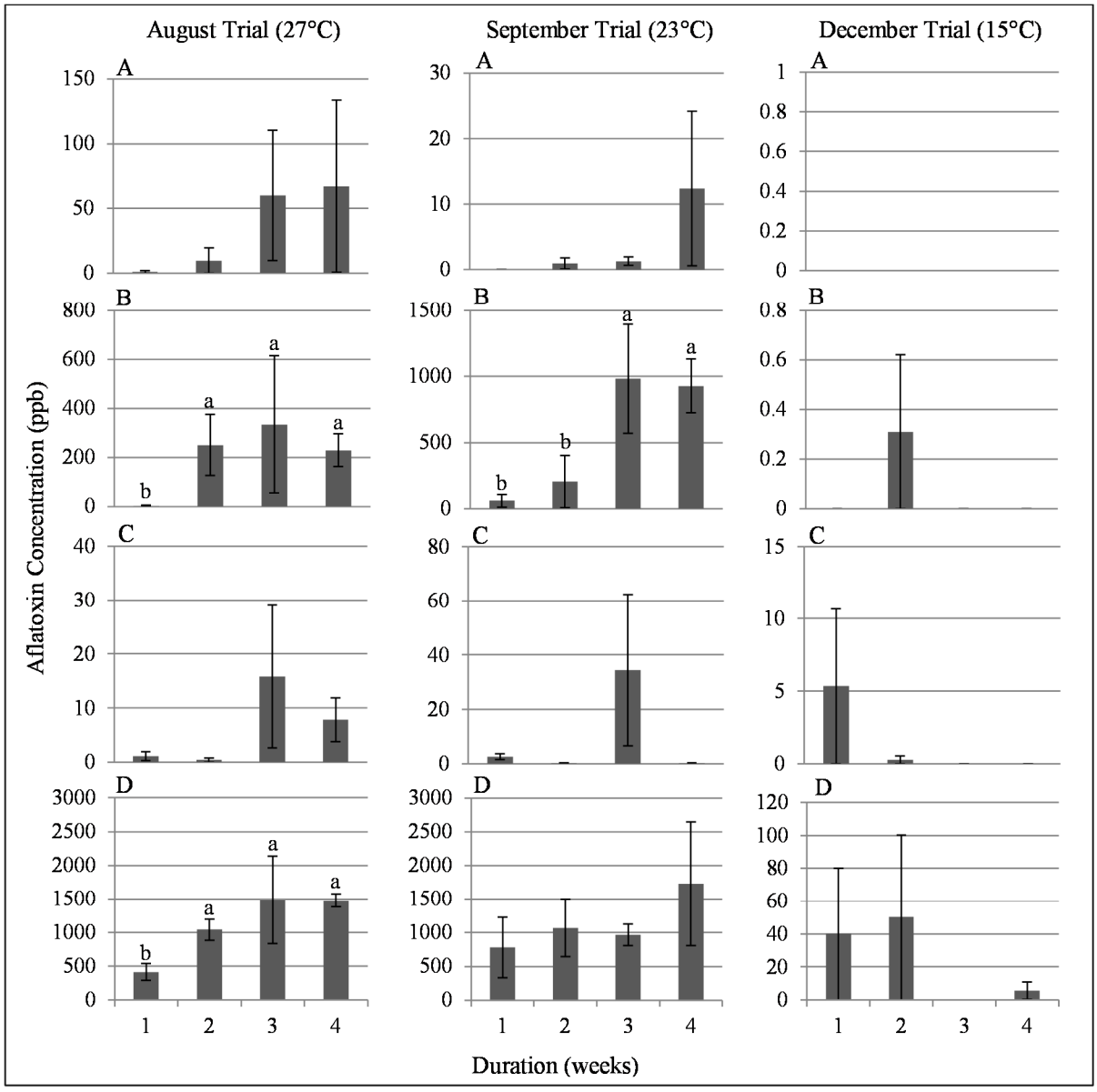

Figure 4. Aflatoxin concentrations of corn. Mean aflatoxin concentrations (ppb) and standard errors are shown for (A) dry broadcast, (B) wet broadcast, (C) dry piled, and (D) wet piled corn. Two means with a different letter are significantly different ($p < 0.05$).

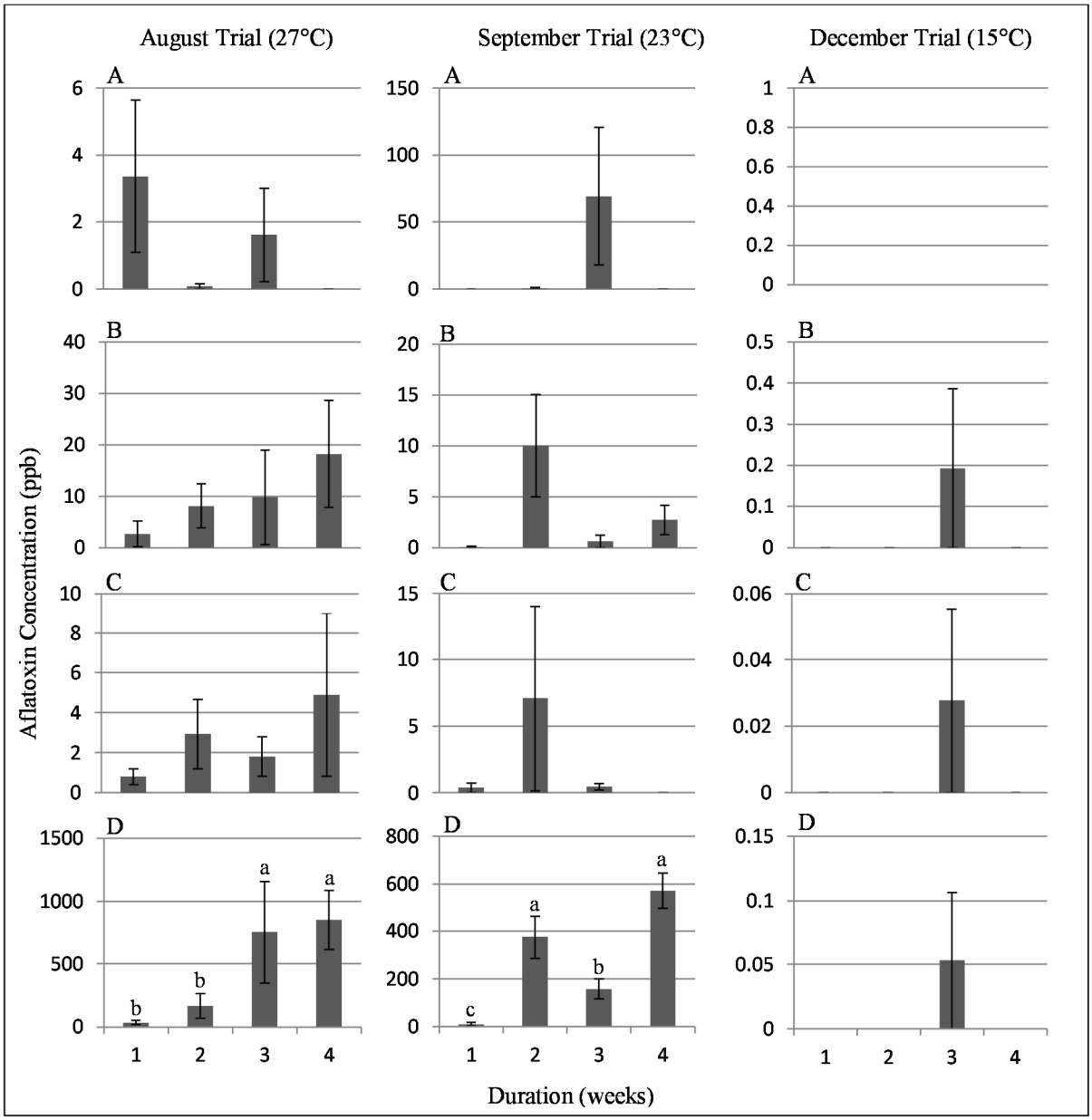

Figure 5. Aflatoxin concentrations of milo. Mean aflatoxin concentrations (ppb) and standard errors are shown for (A) dry broadcast, (B) wet broadcast, (C) dry piled, and (D) wet piled milo. Two means with a different letter are significantly different ($p < 0.05$).

VITA

Leah L. Dale

Candidate for the Degree of

Master of Science

Thesis: POTENTIAL FOR AFLATOXICOSIS IN NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) EXPOSED TO CONTAMINATED GRAIN AT FEEDING STATIONS

Major Field: Natural Resource Ecology and Management

Biographical:

Personal Data: Born in Aurora, Colorado, on August 12, 1980. Married to Joseph J. Dale.

Education: Completed the requirements for the Master of Science in Natural Resource Ecology and Management at Oklahoma State University, Oklahoma in July 2014; received Bachelor of Science degree in Wildlife Conservation and Management from University of Arizona, Tucson, Arizona in May, 2011; graduated from Tombstone High School, Tombstone, Arizona, 1998.

Experience: Worked as an intern for the Arizona Department of Game and Fish, Black-footed Ferret Reintroduction Program, in Seligman, Arizona in the summer of 2010. Volunteered with the University of Arizona, The Nature Conservancy, and the Bureau of Land Management conducting Aravaipa Canyon native fish surveys 2009–2011. Volunteered with the Arizona Game and Fish Department conducting endangered species surveys 2009–2011. Worked as an undergraduate lab assistant for Dr. Brian McGill of the School of Natural Resources at the University of Arizona in 2010. Volunteered with Fort Huachuca Game Management conducting deer and quail hunting check stations in 2009. Volunteered with Arizona Department of Game and Fish conducting pond restoration and non-native removal in 2009. Volunteered as a research assistant for Michele Lanan, a graduate student with the Ecology and Evolutionary Biology Department at the University of Arizona in the summer of 2009.

Professional Memberships: The Wildlife Society

Name: Leah L. Dale Date of Degree: July 2014

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: POTENTIAL FOR AFLATOXICOSIS IN NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) EXPOSED TO CONTAMINATED GRAIN AT FEEDING STATIONS

Pages in Study: 42 Candidate for the Degree of Master of Science

Major Field: Natural Resource Ecology and Management

Scope and Method of Study: The provisioning of wildlife with supplemental food can have unintended consequences on non-target species. One concern is the likelihood of exposure to toxins that develop in grain that has been dispensed to attract deer. Grains left exposed to the elements provide a medium for the colonization of *Aspergillus* spp. fungi, and these organisms can produce deadly aflatoxins. We set out to determine first what species of non-target wildlife visit bait stations for deer during fall and winter, using camera trap arrays at 20 locations in central Oklahoma. We were specifically interested in quantifying use by Northern Bobwhite (*Colinus virginianus*) due to that species' long-term, rangewide decline and demonstrated susceptibility to aflatoxicosis. Next, we examined the conditions that contribute to aflatoxin formation in otherwise uncolonized grains. In a series of greenhouse trials, we used a split-plot design to provide information on the contribution of grain type, dispensation, temperature, moisture, and duration exposed to the development of aflatoxin.

Findings and Conclusions: We photographed 18 species of birds, including Northern Bobwhite, and 12 species of mammals at bait piles of whole corn set for deer. Non-target visitation was twice as high during winter than during fall. We determined that aflatoxin development increased with duration of exposure, moisture, grain density, and temperature. Regardless of ambient conditions, corn developed aflatoxin more often and at higher levels than did milo. Piles of wetted corn produced alfatoxin concentrations high enough to be acutely toxic to Northern Bobwhite, with a peak sample of 3230 ppb (compared to the FDA limit for interstate trade of 20 ppb). We encourage those who choose to provide supplemental feed to consider the potential for negative consequences of their actions, and to take steps to reduce the amount of aflatoxin that can form in the grain they provide.

ADVISER'S APPROVAL: Dr. Timothy J. O'Connell