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DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
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UNIVERSITY OF ARKANSAS
DALE BUMPERS COLLEGE
OF AGRICULTURAL, FOOD
& LIFE SCIENCES

DISCOVERY

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Undergraduate Research Articles

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Cover: Ashley Rodman works with her faculty mentor, Kristofor Brye, on research for her project, Growth and mortality of Ozark bass (*Ambloplites constellatus*) in streams of the Ozark Highlands. Photo by Fred Miller.

DISCOVERY

The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Vol. 15 **Fall 2014**

Undergraduate Research Articles *continued*

Letter from the Dean

Michael Vayda

DISCOVERY Expanded To Feature More Student Research

The Bumpers College of Agricultural, Food and Life Sciences is devoted to preparing our students for professional careers in the businesses associated with foods, agriculture and human quality of life. Our programs offer students value-added opportunities promoting their abilities as leaders, innovators, policy makers and entrepreneurs to make them first-choice candidates of prospective employers.

We are proud of our students and their accomplishments. The *DISCOVERY* undergraduate journal highlight just a few of those value-added learning opportunities by featuring results of research projects completed in partnership with faculty mentors.

The journal encourages students to engage beyond the classroom, offers an outlet to share results and findings in a citable publication, and provides a service to the college, the university, our friends and supporters, and society as a whole.

We encourage student research by awarding undergraduate research grants. Our students have been competitive for research and travel grants awarded by the Honors College and the Arkansas Department of Higher Education. Many projects are designed to meet the requirements for an honors thesis in the Bumpers College Honors Program and some have been funded by our Undergraduate Creative Projects/Research Grants Program.

We are pleased to present the exceptional work of our students. This year's *DISCOVERY* is the largest in the history of the publication, which is a testament to the quality of our students, our faculty mentors and their research. Congratulations to the student authors, and thank you to the faculty mentors and editors of this year's journal.

Michael Vayda, Dean and
Associate Vice President–Academic Programs

The influences of poultry litter biochar and water source on radish growth and nutrition

Julia M. Allen[*]*, David E. Longer*[†]*, Edward E. Gbur*[§]*, and Lichen Hao*[‡]

ABSTRACT

Many row-crop fields today have declined in soil fertility due to poor management practices and overuse of pesticides. Under these conditions, plant nutrient uptake can be sub-optimal. There are several soil amendments that can be used to improve soil quality and plant growth. This study focused on the addition of biochar to the soil and the use of structured water to enhance plant growth. Biochar is produced by pyrolysis of organic feedstocks. Previous studies which focused on biochar have shown an increase in plant yield, nutrient availability in the soil, and soil water holding capacity. Structured water is the liquid crystalline state of water which has unique characteristics due to the ordering of the hydrogen bonds in the water molecules. There have been numerous claims in the natural and organic health literature about the benefits of structured water in human and animal health, but little has been reported in the scientific literature concerning plant growth response. This study was conducted to evaluate the effect of biochar and structured water on the growth and nutrient content of radishes (*Raphahus sativa* L.). Data showed that the water type used had the most significant response. Biochar and tap water had a significant and positive interaction. Tap water and biochar used together resulted in higher yield, leaf area, plant fresh weight, and nutrient contents as the rate of biochar increased. Radish growth showed a negative response to structured water in almost every circumstance.

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[‡] Lichen Hao is a graduate student studying Statistics, and working toward an M.S. in Statistics.

MEET THE STUDENT-AUTHOR

Julia Allen

I was born in Lubbock, Texas, but grew up in Fayetteville, Ark. In high school I was in band and was an accomplished flutist and progressed to drum major. I chose the University of Arkansas to pursue a degree in Environmental, Soil, and Water Science with minors in Wildlife Habitat Management and Crop Science. I plan to pursue a M.S. degree in Crop and Soil Science after graduation. I have served as an officer in our departmental club for the past three years. To broaden my knowledge, I studied conservation in Scotland during the summer of 2013. While pursuing my studies, I have been apprenticing with a jeweler for the past 5 years and have learned to handcraft jewelry. I enjoy marksmanship, music, and art.

I would like to thank Dr. Longer for all his guidance on this project and Dr. Gbur and Lichen Hao for their help with the statistical analysis and the creation of the graphics.

INTRODUCTION

In most agricultural settings, soil fertility declines due to improper management (Laird et al., 2010). In order to support the projected population growth, more agriculture output will be required (Schult and Glaser, 2012). However, currently, soil degradation is becoming a more common problem worldwide due to climate change and over use from a rapidly expanding population which demands an increase in food supply. Plant uptake, leaching, runoff, and volatilization cause nutrient depletion over time. Yearly or periodic fertilizer applications are used to compensate for the loss, though this is usually a temporary and often costly solution (Laird et al., 2010). The application of biochar (BC) to agricultural fields may contribute to a long-term solution for increasing and maintaining minerals in the soil (Laird et al., 2010).

Biochar is carbonized biomass created from organic feedstocks which have undergone pyrolysis. Pyrolysis is defined as heating at extremely high temperatures in the absence of oxygen (Chan et al., 2007). Studies have indicated that BC is composed primarily of polyaromatic carbon and can resist decomposition for hundreds or more years (Doydora et al., 2011). This implies that BC may factor into a long-term solution for increasing and sustaining soil fertility due to the potential for this carbonaceous material to persist in the soil and provide benefits for extended periods of time.

Biochar can be made from essentially any type of organic matter. Two common sources for BC production are plant waste and animal waste (Chan et al., 2007). Biochar from plant material has high carbon content while other macro and micronutrients occur in smaller quantities. Biochar derived from wood has higher carbon concentration, around 70%, and has been associated with increased leaf area, leaf dry weight, and fruit yield (Lehmann et al., 2003). Poultry litter BC does not contain as high of carbon concentration, anywhere between 27% and 42%, but does contain larger concentrations of nitrogen (N), phosphorus (P), and potassium (K) compared to those made from plant matter (Revell et al., 2012a and 2012b).

Poultry litter based BC is becoming a favorable soil amendment option due to the vast amounts of feedstock being produced and the growing need for environmentally friendly ways of disposal. Poultry litter is high in phosphorus. One common use of poultry litter is land application to increase plant available nutrients. In areas with numerous poultry operations, excessive land applications of litter has led to a build-up of soil-test P and is a major contributor to surface water eutrophication (Moore and Miller, 1994). Converting poultry litter into BC is a viable option that can reduce costs and lead to sustainable agriculture (Revell et al., 2012a). Since BC often increases soil pH (Laird et al., 2010), traditional liming costs may also be reduced.

Most BC research has identified numerous benefits from its use as a soil amendment. Soil cation exchange capacity (CEC), pH, water holding capacity, and nutrient availability have all been shown to increase with application of BC (Adams et al., 2013). Biochar is alkaline by nature. The liming value of BC is roughly 30% $CaCO_3$ depending on the product origin (van Zwieten et al., 2010).

Numerous studies have shown BC can increase crop productivity, crop yield, and soil microbial biomass (Noguera et al., 2012). Positive yield results have been found when BC and N fertilizer are used on crops. Chan et al. (2007) conducted research to evaluate the effects of BC and N fertilizer interactions in the growth of radishes. Two rates of BC were used in the study: 50 t/ha and 100 t/ha. The results of this study showed a 320% increase in radish dry matter when 50 t/ha of BC and N fertilizer were combined.

Another relatively new area with the potential for study is the effect of "structured water" on plant growth. Structured water (SW) is the liquid crystalline form of water that has several unique characteristics when compared to water that has not undergone "structuring" (Pangman, 2011). In order to structure water, tap water (TW) is run through a vortex. Many units are sold that attach onto a faucet or can be installed into pipe work to create the vortex. Vortex structuring units alter the molecular structure of the water which removes the suspended solids and contaminants and keeps the beneficial minerals (Betterton, 2012). Differences have been detected in molecular stability, a negative electrical charge, greater viscosity, molecular alignment, and an improved ability to absorb a certain spectra of light (Pangman, 2011).

Structured water has to do with how water interfaces within cells. According to Pangman (2011), the water molecules line up and become ordered. In most water, hydrogen bonds are random. In SW, the hydrogen bonds gain some molecular stability while in motion. This is what happens when water molecules lose their randomness and become ordered. These ordered water molecules can create a few million molecular layers when a hydrophilic interface is present. Most constituents within cells are hydrophilic interfaces. The water molecules also contain a charge. Each molecule in the lineup has the opposite charge of the molecule beside it. This chain of charges acts like a battery and gives the SW energy (Mercola and Pollack, 2011).

Structured water is not a new concept but it is not well known. Among those in the scientific community who know about SW, according to Mercola and Pollack (2011), there is much controversy surrounding the concept and it has even been described as a hoax due to lack of scientific evidence. It was included as a factor in this research because it has grown in popularity with organic farmers and has provided yield and nutritional increases in numerous undocumented testimonies. One user of SW, Calvin Bey—retired from the USDA Forestry Service and now an organic farmer—uses SW in his personal gardens and has seen impressive growth and production without using fertilizer additives. The average tomato plant in his garden produced approximately 100 pounds of fruit in a growing season (C. Bey, pers. comm., 11 October 2013). The objectives of this study were to assess the effects of different rates of BC and SW on radish growth and plant nutrient development. Our hypothesis was that radish leaf area, total fresh weight, and root fresh weight would be greatest for radishes grown with 5000 kg/ha application rate of BC and watered with SW and that they would also have the highest plant nutrient content.

MATERIALS AND METHODS

The soil used in this study was obtained from a landowner in western Washington County, from a small field adjacent to his commercial organic vegetable garden. This soil had experienced 10 years of chemical-free operation prior to sampling. The soil was classified as a Captina silt loam (fine-silty, siliceous, mesic, Typic Fragiudult) and described as prime farmland by the Natural Resource Conservation Service (USDA, 2013). The two main factors under study in this project were BC and SW. The BC used in this study was derived from pyrolysed poultry litter as described previously in the Introduction. The BC used in this study was obtained from BioEnergy Systems, LLC based in Fayetteville, Ark. The nutrient content analysis is depicted in Table 1. The SW was donated by Calvin Bey for use in this project.

Radishes, *Raphahus sativa*, members of the Brassica family and grown worldwide, were used for this study due to their quick maturation time and being well suited for greenhouse culture. Radishes were grown from seed. Seeds were planted directly into the treatments.

This experiment was conducted in the Rosen Alternative Pest Control Center Greenhouse located on the University of Arkansas campus, in Fayetteville, Ark. The experiment was initiated on 30 October 2013 and ended on 5 December 2013. The study was established as a completely randomized design. There were 6 treatments: three rates of biochar and two water types. Each treatment was replicated 12 times.

Seventy-two 1-L plastic non-reactive pots were used. They were washed and sterilized prior to planting. A single coffee filter was placed in the bottom of each pot to prevent soil leaking from the base. Each pot was filled with approximately 1 L of the growing media which was a blend of 45% soil, 45% perlite, and 10% compost. The compost was uniform in appearance and texture and was

Table 1. Compositional analysis of BioEnergy Systems, LLC (BES) biochar.

Measured Property (unit)	Value
pH (pH units)[a]	10.2
Electrical Conductivity (µmhos cm^{-1})[a]	16680
P (mg kg^{-1})[a]	7076
K (mg kg^{-1})[a]	26412
Ca (mg kg^{-1})[a]	3217
Mg (mg kg^{-1})[a]	3071
S (mg kg^{-1})[a]	3525
Na (mg kg^{-1})[a]	6880
Fe (mg kg^{-1})[a]	32
Mn (mg kg^{-1})[a]	190
P (mg kg^{-1})[b]	46915
K (mg kg^{-1})[b]	72298
Ca (mg kg^{-1})[b]	67904
Mg (mg kg^{-1})[b]	15298
S (mg kg^{-1})[b]	10486
Na (mg kg^{-1})[b]	19919
Fe (mg kg^{-1})[b]	2453
Mn (mg kg^{-1})[b]	1397
Zn (mg kg^{-1})[b]	1261
Cu (mg kg^{-1})[b]	801
%Total N[c]	3.00
%Total C[c]	32.03

[a] pH (1:2 soil ratio), Mehlich-3 extractable (1:10 ratio) Analysis by SPECTRO ARCOS inductively coupled plasma spectrometer.
[b] Total Recoverable Metals, EPA method 3050, measured on Spectro Arcos inductively coupled plasma spectrometer.
[c] Total N and C by combustion, Elementar Variomax.

produced from lawn and plant waste. Biochar was then ground to a fine powder and weighed to the appropriate values and added to their respective pot. The BC was then incorporated into the top few centimeters of growing media in each pot. These rates were equivalent to BC applications to each pot at the following rates: 0 kg/ha, 5000 kg/ha, and 10,000 kg/ha.

The filled pots were transported from the preparation lab to the greenhouse and flushed with either SW or TW and allowed to drain overnight. When the soil settled, 3 radish seeds were planted per pot. The radishes were watered daily with 50 mL of their respective water type. Half of the radishes were watered with TW and the other half were watered with SW. Upon germination, each pot was thinned to one uniformly sized radish per pot. The radish pots were randomized and rotated weekly from one end of the bench to the other to avoid any biases from sunlight and air flow differences.

After the radishes completed their growth cycle, they were harvested and analyzed for total fresh weight, root fresh weight, leaf area, and root mineral content. Total plant and root fresh weights were determined by weighing at harvest on a Mettler analytical balance at the 0.00 g level of precision. Leaf area was analyzed using a LI-COR leaf area meter, LI-3100C Area Meter, (LI-COR Environmental and Biotechnology Research Systems, Lincoln, Neb.). Radish mineral content was determined with a Digital Hand-Held "Pocket" Refractometer PAL (ATAGO U.S.A., Inc.) which measures the amount of light refracted through a liquid, which can be used to detect sugar and mineral content of a liquid or slurry. The roots of the radish were pressed until liquid emerged. This liquid was used for the analysis. This instrument is often referred to as a BRIX meter. BRIX measures the amount of dissolved solids. The higher the value, the better the quality and flavor.

The data were analyzed using SAS PROC MIXED (SAS Institute Inc., Cary, N.C.). A completely randomized analysis of variance was performed to find the main effects and the interaction effect on the measurements. Estimates were calculated for each variable within each treatment. Significant differences in total fresh weight, root fresh weight, and leaf area were based on $P = 0.05$. Significant differences in BRIX measurements were based on $P = 0.10$.

RESULTS AND DISCUSSION

Radishes were used in this study due to their quick maturation time of approximately 30 days. Radishes are a vigorous, easy-to-grow, cool-season vegetable with potential for multiple crops per year. They are valued because of their ease of planting, their low management, and they can be eaten directly from the garden.

Fresh weight was significantly higher for radishes watered with TW than the radishes watered with SW ($P < 0.05$) (Fig. 1). The mean fresh weight for plants watered with TW was 15.34 g. Biochar application rates did not have a statistically significant influence on radish fresh weight (data not shown).

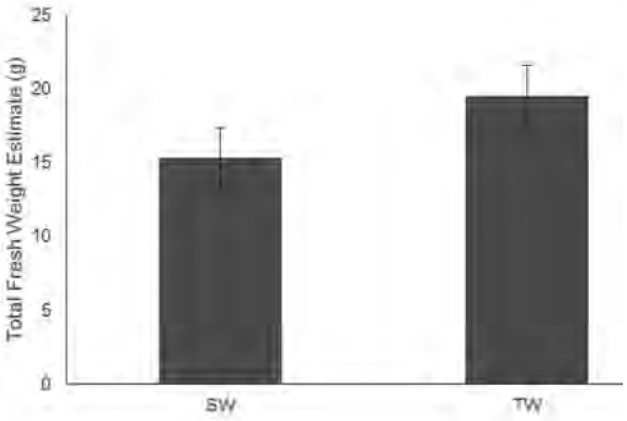

Fig. 1. Estimated total radish plant fresh weight for structured water (SW) and tap water (TW) treatments. Error bars indicate standard error.

The main effects of water type and BC were evaluated for their independent influences on root fresh weight. Water type alone had a statistically significant response ($P < 0.05$). Plants watered with TW had a larger mean root fresh weight (7.61 g) than those watered with SW (4.82 g) (Fig. 2). However, BC alone had a significant negative affect on the radish root fresh weight ($P < 0.05$) (Fig. 3). Mean root fresh weight decreased significantly when 1.00 g of BC was applied compared to the 0.00 g control.

Leaf area was affected by water type ($P < 0.05$) (Fig. 4) but not by BC application rate (data not shown). The average leaf area for radishes watered with TW was 300.80 cm^2; whereas plants watered with SW only averaged 257.83 cm^2 (Fig. 4).

The BRIX measurements showed a statistically significant interaction between water type and BC application ($P < 0.10$) (Fig. 5). Tap water alone gave a mean BRIX reading of 3%. The BRIX measurement increased to 3.79% for radishes treated with 10,000 kg/ha of BC and watered with TW. However, the treatments combining BC and SW had a very different reaction. When plants were only influenced by SW, the mean BRIX reading was 3.4%. When 5,000 kg/ha of BC was added, BRIX measurements dropped to 2.5%. With 10,000 kg/ha of BC added and watered with SW, the BRIX reading went back up slightly to 3.08%, which is still less than the plants with no BC added.

When examining the effect of water type on radish growth, TW outperformed SW in total fresh weight, root weight, and leaf area compared to SW. There was no interaction between the water type and the biochar rate except in the BRIX measurements.

Similar to the results found in the study by Chan et al. (2007), total weight and root weight were not affected by BC alone. Chan et al. (2007) saw a positive effect when BC was combined with N fertilizer. Van Zwieten et al.

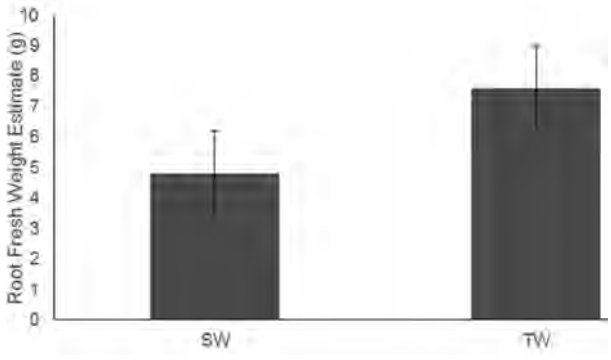

Fig. 2. Estimated root fresh weight for structured water (SW) and tap water (TW). Error bars indicate standard error.

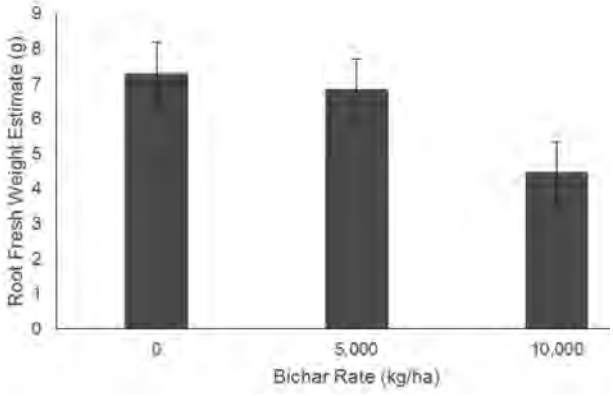

Fig. 3. Estimated root fresh weight for each biochar application rate. Error bars indicate standard error.

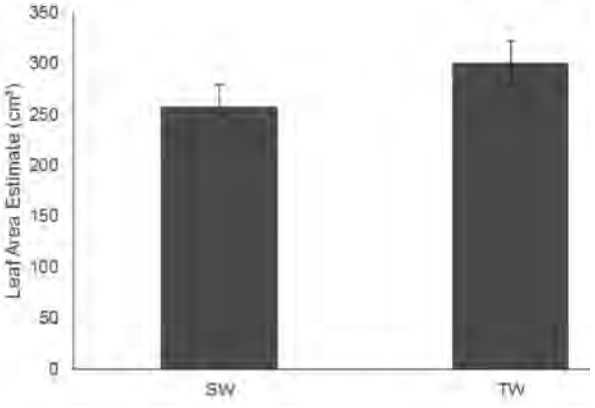

Fig. 4. Estimated leaf area for each structured water (SW) and tap water (TW) treatment. Error bars indicate standard error.

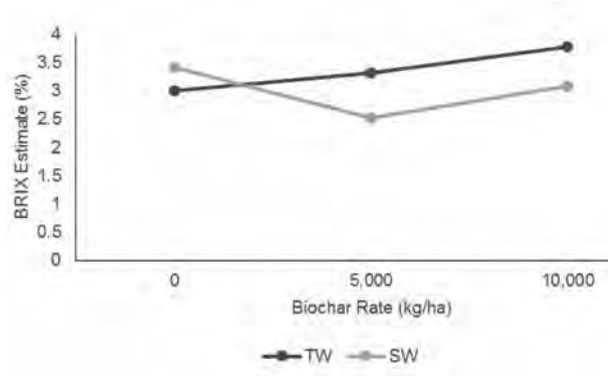

Fig. 5. Changes in BRIX estimate for each biochar treatment with structured water (SW) and tap water (TW) treatments. Error bars indicate standard error.

(2010) also concluded that biochar and N fertilizer had a significant interaction. As the rate of BC and N fertilizer increased, there was a significant increase in radish total weight compared to the control and to BC without N fertilizer. The results from our study indicate that BC did not significantly affect plant growth by itself but did have a positive effect on radish nutrient content when BC was combined with TW and a negative effect when in combination with SW as shown in Fig. 5. This interaction was significant ($P < 0.10$). When the interaction between TW and BC is examined for nutrient content, as the BC rate increases, BRIX measurement estimate increased.

Unlike the testimonies for the benefits of SW, our study showed that SW had a statistically significant ($P = 0.05$) negative impact on the growth parameters we examined in the radishes. Mercola and Pollack (2011) claims that drinking SW can be beneficial to cellular health in humans with the inference that all cellular health might benefit. In our study we saw that SW seemed to have the opposite effect of what we expected and hypothesized. We predicted that plants watered with SW would grow more vigorously and be larger, healthier plants than the radishes watered with TW. Total fresh weight, root weight, and leaf area were all significantly smaller for radishes watered with SW.

In summary, the data analyses indicated that the type of water used for irrigation had the most pronounced influence on radish growth and development. Analysis of variance values are displayed for the main effects and interaction effects in Table 2. Plants watered with TW had higher total fresh weights, root weights, and larger leaf areas. There was also an interaction between the water type and BC. When TW and BC were combined, the nutrient content increased as the BC rate increased. When SW and BC were combined, nutrient content was lower for plants that had BC added to the soil. Based on the results of this study, further trials to examine the effects of SW and the interaction between BC and water type would be appropriate.

Table 2. Analysis of variance table for main effects and interaction effects.

Source	DF	BRIX	RFW[a]	FW[b]	AREA[c]
BC	2	0.1793	0.0413	0.6572	0.1279
WATER	1	0.1190	0.0049	0.0001	0.0092
*BC*WATER*	2	0.0595	0.6948	0.5336	0.3698

[a] Root fresh weight.
[b] Fresh weight (total).
[c] Leaf area.

ACKNOWLEDGEMENTS

We would like to thank BioEnergy Systems LLC for producing the biochar used in this experiment. Thank you to Calvin Bey for supplying the structured water, soil, and compost as well as much guidance.

LITERATURE CITED

Adams, M.M., T.J. Benjamin, N.C. Emery, S.J. Brouder, and K.D. Gibson. 2013. The effect of biochar on native and invasive prairie plant species. Invasive Plant Sci. Manag. 6:197-207.

Betterton, C. 2012. Technical Data. <http://www.ultimatedestinyuniversity.org/>. Accessed 22 June 2014.

Chan, K.Y., L. Van Zwieten, I. Meszaros, A. Downie, and S. Joseph. 2007. Agronomic values of greenwaste biochar as a soil amendment. Aust. J. Soil Res. 45:629-634.

Doydora, S.A., M.L. Cabrera, K.C. Das, J.W. Gaskin, L.S. Sonon, and W.P. Miller. 2011. Release of nitrogen and phosphorous from poultry litter amended with acidified biochar. Int. J. Environ. Res. Public Health. 8:1491-1502.

Laird, D., P. Feming, B. Wang, R. Horton, and D. Karlen. 2010. Biochar impact on nutrient leaching from a Midwestern agricultural soil. Geoderma. 158, 436-442.

Lehmann, J., J. Pereira da Silva, C. Steiner, T. Nehls, W. Zech, and B. Glazier. 2003. Nutrient availability and leaching in an archeological anthrosol and a ferrosol of the Central Amazon basin: fertilizer, manure and charcoal amendments. Plant Soil. 249:343-357.

Mercola, J. (Interviewer) and G. Pollack (Interviewee). 2011. Water: the single most important element for your health [Interview transcript]. <http://articles.mercola.com>. Accessed 6 March 2014.

Moore, P.A. and D.M. Miller. 1994. Decreasing phosphorus solubility in poultry litter with aluminum, calcium, and iron amendments. J. Environ. Qual. 23:325-330.

Noguera, D., S. Barot, K.R. Laossi, J. Cardoso, P. Lavelle, and M.H. Cruz de Carvalho. 2012. Biochar but not earthworms enhance rice growth through increased protein turnover. Soil Biol. Biochem. 52:13-20.

Pangman, M.J. (2011). What is structured water? <http://www.dancingwithwater.com>. Accessed 18 April 2014.

Revell, K.T., R.O. Maguire, and F.A. Agblevor. 2012a. Field trials with poultry litter biochar and its effect on forages, green peppers, and soil properties. Soil Science. 177(10):573-579.

Revell, K.T., R.O. Maguire, and F.A Agbelvor. 2012b.Influence of poultry litter biochar on soil properties and plant growth. Soil Science. 177(6):402-408.

Schult, H., and B. Glaser. 2012. Effects of biochar compared to organic and inorganic fertilizers on soil qual-

ity and plant growth in a greenhouse experiment. J. Plant Nutr. Soil Sc. 175:410-422.

USDA. United States Department of Agriculture. 2013. Web soil survey. <http://websoilsurvey.sc.egov.usda.gov> Accessed 20 March 2014

Van Zwieten, L., S. Kimber, S. Morris, K.Y. Chan, A. Dowie, J. Rust, S. Joseph, and A. Cowie. (2010). Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant Soil. 327:235-246.

Isoflupredone acetate as ancillary therapy for bovine respiratory disease in high-risk stocker calves

Claire Crews[*], *Jeremy Powell*[†], *Elizabeth Kegley*[§], *Jana Reynolds*[‡], *and Pete Hornsby*[¶]

ABSTRACT

Bovine respiratory disease (BRD) is the most prevalent and devastating disease in U.S. feedlot cattle. This study evaluated the use of isoflupredone acetate in the treatment of BRD. Crossbred male beef calves (n = 192; body weight = 221 ± 3.9 kg) were acquired in two blocks from regional auction markets and transported to the University of Arkansas Stocker and Receiving Cattle Unit. Calves were observed daily for signs of respiratory illness. Antibiotic treatment was administered if calves displayed signs of respiratory illness and rectal temperature was ≥40 °C. Calves (n = 72) requiring antibiotic treatment were assigned randomly to either treatment 1 (florfenicol) or treatment 2 (florfenicol plus isoflupredone acetate). Treatment efficacy was determined by rechecking the rectal temperature of treated cattle 48 hours post treatment. Blood was collected (at treatment and recheck) via jugular venipuncture to evaluate complete blood count. Weights were recorded on days 0, 14, 28, 45, and 46. No difference existed for medical cost ($P = 0.54$) or temperature at recheck ($P = 0.43$). Upon recheck, neutrophils were higher and lymphocytes were lower in calves that received isoflupredone acetate ($P \leq 0.04$). No difference existed in overall white blood cell count at recheck ($P = 0.67$). Calves that received isoflupredone acetate tended to exhibit greater ($P = 0.09$) average daily gain (ADG) between days 14 and 28 of the study. Results indicate that using isoflupredone acetate as ancillary therapy in the treatment of BRD did not have a positive effect on overall ADG or medical costs.

* Claire Crews is a 2014 Honors graduate with a major in Animal Science.
† Jeremy Powell, the faculty mentor, is a professor in the Department of Animal Science.
§ Elizabeth Kegley is a professor in the Department of Animal Science.
‡ Jana Reynolds is a technical assistant in the Department of Animal Science.
¶ Pete Hornsby is a technical assistant in the Department of Animal Science.

MEET THE STUDENT-AUTHOR

Claire Crews

I am from Jacksonville, Arkansas, and graduated from North Pulaski High School in 2010. After spending four incredible years at the University of Arkansas, I graduated with a Bachelor of Science degree in Animal Science in 2014. This fall I will begin pursing a Doctor of Veterinary Medicine degree at the University of Missouri.

During my undergraduate career, I was a member of the Pre-Veterinary Club, Animal Science REPS (Representing, Educating, and Promoting Scholars), and Gamma Beta Phi Society. I also played clarinet in the Razorback Marching Band and the University of Arkansas Wind Ensemble. In the winter of 2012, I participated in a study abroad course in Belize in which I had the opportunity to provide free veterinary services to animals owned by low-income families. This experience helped me appreciate the ingenuity of cultures different from my own.

My honors research project was inspired by my desire to improve animal health. I would like to thank Jeremy Powell for helping me develop the topic for my project and for providing me with advice and encouragement throughout my research endeavors. I would also like to thank Elizabeth Kegley, Jana Reynolds, and Pete Hornsby for providing much assistance with the technical procedures of this project.

INTRODUCTION

Bovine respiratory disease (BRD) is the most prevalent and devastating disease in U.S. feedlot cattle. Every year, U.S. feedlot operations experience substantial economic loss due to the pervasiveness of the disease. It is estimated that the U.S. cattle industry experiences an annual economic loss of approximately $1 billion (Griffin, 1997). Preventative and treatment costs are over $3 billion annually.

There are many factors that contribute to the onset of bovine respiratory disease. The disease is often initiated when an animal is exposed to one or multiple stress contributors, such as dust, transportation, overcrowding, commingling with infected animals, weaning, castration, or poor nutrition (Powell, 2010). These stressors cause the animal's immune system to be suppressed, allowing viral and bacterial agents to enter and infect the body. The infection process usually begins with one or more viral agents, such as bovine viral diarrhea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza type-3 (PI3), or bovine respiratory syncytial virus (BRSV), entering the host in response to a weakened immune system due to various stressors. The viral infection hinders the immune system's ability to fight off infectious bacteria, such as *Mannheimia haemolytica*, *Haemophilus somnus*, or *Pasteurella multiocida*. Bacterial agents cause an even greater infection in the already-impaired respiratory tract, and the accumulation of these agents in the lungs can cause pneumonia (Faber et al., 1999).

Bovine respiratory disease is characterized by depression, isolation from the herd, decreased appetite, increased respiratory rate, fever, coughing, and nasal and/or ocular discharge (Powell, 2010). A clinical illness score (Table 1) should be assigned to any animal identified as sick. A rectal temperature of ≥40 °C (normal temperature = 38.6 °C) is also commonly associated with the disease.

Although antibiotics have been shown to be effective in treating BRD, many consumers are concerned that the excessive use of antibiotics may lead to the development of antibiotic-resistant bacteria in food animals (Hellwig et al., 2000). Non-steroidal anti-inflammatory drugs (NSAIDs) have proven to be beneficial in treating BRD when used in combination with antibiotics (Lockwood et al., 2003). These drugs do not impair the immune system and they help reduce pain and fever (Kaashoek et al., 1996; Lees, 2003). Steroidal anti-inflammatory drugs (SAIDs) have also been used as ancillary therapy, but studies have yielded conflicting results (Christie et al., 1977; Sustronck et al., 1997). These drugs have anti-inflammatory properties, but they are also immunosuppressive (Smith, 1996). Isoflupredone acetate is a SAID that has recently shown favorable results when used as combination therapy. However, only one publication has scientifically evaluated the drug's efficacy in the treatment of BRD. In that study, isoflupredone acetate prevented the

reduction in feed intake and average daily gain (ADG) in the first week after cattle were induced with the disease (Hewson et al., 2011). Faster clinical improvement was also seen in the cattle treated with isoflupredone acetate. Because this drug has not been extensively tested in cattle as ancillary therapy, there is a need for further data. This study was designed to provide supplementary data that can be used to evaluate the use of isoflupredone acetate in the treatment of BRD.

The objectives of this study were to determine the efficacy of isoflupredone acetate as ancillary therapy for bovine respiratory disease and to determine the cost-effectiveness of treatment using isoflupredone acetate as an adjunct to antibiotic treatment versus treatment using solely antibiotics.

MATERIALS AND METHODS

All methods used in this experiment followed the standard protocols that are used at the Stocker and Receiving Unit at the University of Arkansas System Division of Agriculture Experiment Station in Savoy. Additionally, the methods used were reviewed and approved by the University of Arkansas Animal Care and Use Committee.

The study utilized 192 commingled crossbred male beef calves (body weight = 221 ± 3.9 kg) acquired in two blocks (block 1 = 27 Sep 2012, block 2 = 11 Oct 2013). Calves were purchased from regional auction markets and transported to the University of Arkansas Stocker and Receiving Unit. Upon arrival (day -1), calves were individually weighed, identified with a uniquely numbered ear tag, and tested for a persistent infection of bovine viral diarrhea virus (PI-BVDV) using the antigen capture ELISA ear notch test (CattleStats LLC, Oklahoma City, Okla.). Calves were kept commingled overnight and were given ad libitum access to bermudagrass hay and water. The following day (day 0), calves were reweighed, castrated by banding (if applicable), and inoculated with a 5-way modified-live virus vaccine (Pyramid 5, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Mo.) and an 8-way clostridial vaccine (Covexin 8®, Merck Animal Health, Summit, N.J.). Calves were also treated for internal parasites (Cydectin, Boehringer Ingelheim Vetmedica, Inc.). They were then stratified by body weight and randomly assigned to 1 of 8 pens (0.42 ha) so that the average weights of the pens were similar. All calves were fed a grain supplement with a rate adjusted to a maximum of 1.9 kg per day per calf, which met or exceeded all nutrient requirements (NRC, 1996). The pre-determined quantity of feed was hand fed each morning (~8:30 a.m.). In addition, calves were given ad libitum access to bermudagrass hay and water.

Over the course of the 46-day study, all calves were observed daily (~8:00 a.m.) for signs of BRD. If 2 or more signs existed (i.e. depression, decreased appetite, coughing, nasal or ocular discharge), calves were pulled from the group and rectal temperature was recorded via digital thermometer (GLA Agricultural Products, San Luis Obispo, Calif.). If rectal temperature was ≥40 °C, calves were treated with 1 of 2 treatment methods (based on a pre-assigned treatment randomization sheet). Calves assigned to treatment 1 (control) received an injection of florfenicol (6 mL/45.4 kg) (Nuflor, Intervet Schering-Plough Animal Health, Summit, N.J.). Calves assigned to treatment 2 (ancillary therapy) received an injection of florfenicol (6 mL/45.4 kg) (Nuflor, Intervet Schering-Plough Animal Health) plus an injection of isoflupredone acetate (5 mL/45.4 kg) (Predef 2X, Pfizer Animal Health, Kalamazoo, Mich.). All treated cattle were bled via jugular venipuncture (7 mL) into evacuated tubes (Vacutainer, BD Inc, Franklin Lakes, N.J.) upon initial treatment and 48 hours post-treatment to evaluate overall white blood cell (WBC) count. Calves were reevaluated 48 hours post-treatment to determine if further antibiotic therapy was necessary. Usually cattle respond favorably

Table 1. Clinical illness scores (CIS) for calves.

Score	Description	Appearance
1	Slightly ill	Mild depression, gaunt, +/- ocular/nasal discharge
2	Moderately ill	Ocular/nasal discharge, lags behind other animals in the group, coughing, labored breathing, moderate depression, +/- rough hair coat, weight loss
3	Severely ill	Severe depression, labored breathing, purulent ocular/nasal discharge, not responsive to human approach
4	Moribund	Near death

Powell, 2010.

(80-85%) to the first line of treatment and no additional treatment is necessary (Edwards, 2010). Subsequent antibiotic therapy was administered if rectal temperature was still ≥40 °C or if the clinical illness score was greater than the initial score. Therapy 2, consisting of enrofloxacin (5.7 mL/45.4 kg) (Baytril, Bayer Animal Health, Shawnee Mission, Kan.), was given if calves failed to respond to the initial antibiotic therapy. Enrofloxacin was also administered if calves responded to therapy 1 but relapsed less than 21 days after receiving therapy 1. Therapy 3, consisting of ceftiofur hydrochloride (2 mL/45.4 kg) (Excenel, Pfizer Animal Health), was administered if calves did not respond to therapy 2 after 48 hours. Calves that did not respond to therapy 3 were considered "chronic" and were given no further antibiotic treatment. Therapy 3 was also used for calves that responded to therapy 2 but relapsed less than 21 days after receiving it.

Throughout the study, data were recorded for treatment groups 1 and 2 on the basis of morbidity (fever reduction, repull rate, rate of clinical improvement, failed treatments, chronic illness), performance (ADG and total weight gained) and economics (cost of treatments). Blood samples were analyzed using a Cell-Dyn 1700 Hematology Analyzer (Abbott Laboratory, Abbott Park, Ill.). Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc, Cary, N.C.) to compare the effectiveness of the two treatment methods.

RESULTS AND DISCUSSION

Seventy-two out of 192 calves received treatment for respiratory illness. Thirty-eight calves received the control and 34 calves received the ancillary therapy (Table 2). Antibiotic treatments occurred between day 2 and day 14 of the study. Body weights were recorded on day 0, 14, 28, 45, and 46. Average daily gain over the entire 46-day study was not different ($P = 0.88$) between treatment groups (Table 3). Calves that received isoflupredone acetate tended to exhibit greater ($P = 0.09$) ADG between day 14 and day 28 of the study compared to calves that received only antibiotic therapy, 1.06 kg and 0.77 kg, respectively (Table 3). This result contrasts a study conducted by Hewson et al. (2011), in which there were no differences in ADG throughout the study between calves that received isoflupredone acetate and calves that received only antibiotic therapy. No difference was evident between treatment groups for medical cost ($P = 0.54$) or repull rate ($P = 0.53$) (Table 2). Body temperature at recheck ($P = 0.43$) was also not different between treatment groups (Table 2). In Hewson's study (2011), body temperature was normalized sooner in the group that received isoflupredone acetate than in the group that received antibiotic therapy alone.

Upon recheck, neutrophils were higher and lymphocytes were lower in calves that received isoflupredone

Table 2. Effects of isoflupredone acetate as ancillary therapy for bovine respiratory disease on morbidity.

	Antibiotic treatment	Antibiotic treatment with isoflupredone acetate	*P*-value
Number of calves	38	34	--
Time to second pull, days	9	13	0.37
Calves treated two times	9	6	0.53
Repull rate, %	24	18	0.53
Calves treated three times	4	3	0.81
Second relapse, %	44	50	0.83
Medical cost, $	21.73	23.40	0.54
Temperature at treatment, °C	40.4	40.6	0.31
Temperature at recheck, °C	39.5	39.4	0.43

Table 3. Effects of isoflupredone acetate as ancillary therapy for bovine respiratory disease on growth performance.

Average daily gain (ADG) period	Antibiotic treatment	Antibiotic treatment with isoflupredone acetate	*P*-value
Day 0 to day 14, kg	1.23	1.03	0.27
Day 14 to day 28, kg	0.77	1.06	0.09
Day 28 to day 46, kg	0.87	0.76	0.49
Total, kg	0.95	0.94	0.88

acetate ($P \leq 0.04$) compared to calves that received only antibiotic therapy (Table 4). Consequently, the neutrophil to lymphocyte ratio was greater ($P < 0.01$) in calves that received isoflupredone acetate (Table 4). A higher neutrophil to lymphocyte ratio is an indication of stress which, in the case of this study, resulted from the administration of a drug that acts much like the natural stress hormone cortisol. It has been suggested that stress and viral infections may inhibit the recruitment of neutrophils to the lungs leaving a higher number in the peripheral blood (Caswell, 2014). No difference existed in overall WBC count at recheck ($P = 0.67$) (Table 4). This contrasts a study conducted by Sustronck et al. (1997) in which overall WBC count was significantly lower at recheck in calves that received a SAID (flumethasone) in addition to antibiotic therapy in comparison to calves that received only antibiotic therapy.

Results indicate that treatment of bovine respiratory disease with isoflupredone acetate as ancillary therapy to an antibiotic regimen did not have a positive effect on overall ADG or medical costs. The reason that a SAID rather than a NSAID was chosen as ancillary therapy in this study, despite conflicting results, is because SAIDs block molecules higher in the inflammatory cascade (Tsurufuji et al., 1981). It was thought that cattle treated with a SAID in addition to antibiotics would recover faster from BRD because they would not experience the negative effects that occur as a result of the inflammatory response. Isoflupredone acetate, in particular, was chosen in this study because it showed favorable results in the treatment of BRD during a study conducted by Hewson et al. (2011). The reason for the contrasting results between the current study and the study conducted by Hewson and colleagues is unknown. Perhaps replicating the current study using a larger sample group would yield results similar to those of Hewson and colleagues. Further research of isoflupredone acetate is needed in order to better evaluate the drug's effects on body weight gain performance and treatment expense. If this drug shows promising results in future studies, it could help prevent economic loss in the cattle industry due to poor performance, reduced carcass value, or death.

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LITERATURE CITED

Caswell, J.L. 2014. Failure of respiratory defenses in the pathogenesis of bacterial pneumonia of cattle. Vet. Pathol. 51:393-409.

Christie, B.M., R.E. Pierson, P.M. Braddy, D.E. Flack, D.P. Horton, R. Jensen, E.A. Lee, E.E. Remmenga, and K.G. Rutt. 1977. Efficacy of corticosteroids as supportive therapy for bronchial pneumonia in yearling feedlot cattle. Bovine Pract. 12:115-117.

Edwards, T.A. 2010. Control methods for bovine respiratory disease for feedlot cattle. Vet. Clin. Food Anim. 26:273-284.

Faber, R., N. Hartwig, D. Busby, and R. BreDahl. 1999. The costs and predictive factors of bovine respiratory disease in standardized steer tests. Beef Research Report. Iowa State University. A.S. Leaflet R1648.

Table 4. Effects of isoflupredone acetate as ancillary therapy for bovine respiratory disease on blood count analysis.

	Antibiotic treatment	Antibiotic treatment with isoflupredone acetate	*P*-value
At treatment			
White blood cells, n x 10^3/μL	10.7	10.8	0.96
Neutrophils, n x 10^3/μL	4.0	4.2	0.74
Lymphocytes, n x 10^3/μL	4.4	4.3	0.85
Neutrophil:Lymphocyte	1.0	1.1	0.73
Monocytes, n x 10^3/μL	0.7	0.7	0.44
Platelets, n x 10^3/μL	381.1	400.5	0.54
48 hours post treatment			
White blood cells, n x 10^3/μL	10.2	10.4	0.67
Neutrophils, n x 10^3/μL	3.4	4.3	0.01
Lymphocytes, n x 10^3/μL	4.9	4.1	0.04
Neutrophil:Lymphocyte	0.8	1.1	< 0.01
Monocytes, n x 10^3/μL	0.8	0.7	0.25
Platelets, n x 10^3/μL	405.0	438.0	0.25

Griffin, D. 1997. Economic impact associated with respiratory disease in beef cattle. Vet. Clin. N. Am-Food A. 13:367-377.

Hellwig, D.H., E.B. Kegley, Z. Johnson, and B. Hunsaker. 2000. Flunixin meglumine as adjunct therapy for bovine respiratory disease in stocker cattle. Arkansas Animal Science Report. AAES Research Series 478.

Hewson, J., L. Viel, J.L. Caswell, P.E. Shewen, and J.G. Buchanan-Smith. 2011. Impact of isoflupredone acetate treatment on clinical signs and weight gain in weanling heifers with experimentally induced *Mannheimia haemolytica* bronchopneumonia. Am. J. Vet. Res. 72:1613-1621.

Kaashoek, M.J., F.A.M. Rijsewijk, and J.T. Van Oirschot. 1996. Persistence of antibodies against bovine herpesvirus 1 and virus reactivation two to three years after infection. Vet. Microbiol. 53:103–110.

Lees, P. 2003. Pharmacology of drugs used to treat osteoarthritis in veterinary practice. Inflammopharmacology. 11:385–399.

Lockwood, P.W., J.C. Johnson, and T.L. Katz. 2003. Clinical efficacy of flunixin, carprofen, and ketoprofen as adjuncts to the antibacterial treatment of bovine respiratory disease. Vet. Rec. 152:392-394.

NRC. 1996. Nutrient Requirements of Beef Cattle, 7th Ed. National Academy Press, Washington, D.C.

Powell, J. 2010. Bovine respiratory disease (Livestock Health Series). University of Arkansas Cooperative Extension Service, Little Rock, Arkansas. FSA 3082.

Smith, R.A. 1996. Therapeutic management of the bovine respiratory disease complex. *In*: Bovine Respiratory Disease, Schering-Plough Animal Health, pp. 49-56.

Sustronck, B., P. Deprez, G. Van Loon, J. Coghe, and E. Muylle. 1997. Efficacy of the combination sodium ceftiofur-flumethasone in the treatment of experimental *Pasteurella haemolytica* bronchopneumonia in calves. J. Vet. Med. 44:179-187.

Tsurufuji, S., K. Sugio, H. Sato, K. Ohuchi. 1981. A review of mechanism of action of steroid and non-steroid anti-inflammatory drugs. Inflammation: Mechanisms and Treatment. 4:63-78.

College students' perceptions regarding sensory aspects of conventionally produced and unconventionally produced foods: implications for marketing to the Millennial generation

Christina Crowder[*], *Catherine W. Shoulders*[†], *and K. Jill Rucker*[§]

ABSTRACT

Consumers vote every day on which products line the shelves of grocery stores, co-ops, and niche markets. Public unrest with regard to the environmental, animal welfare, food purity, and human health impacts of agricultural production practices have led to the rise of unconventionally produced (UP) food products. While the sales of UP foods is increasing, studies regarding the qualities of such products that impact consumer purchases have yielded inconsistent results. This study examined students' perceptions of sensory aspects of conventionally produced (CP) and UP foods to better understand how sensory aspects impact decisions to purchase. Students reported consistent perceptions regarding the favorability of each sensory aspect of chicken and apples; the UP versions of the products yielded higher mean scores on every sensory aspect. However, students' perceptions of the sensory qualities of chocolate, milk, and beef were not consistent; for example, they reported more favorable perceptions of the appearance and smell of CP milk, but perceived a more favorable texture and flavor from the UP milk. The results of this study imply that when making purchasing decisions, consumers may value specific sensory attributes over others. One approach to marketing UP products is to focus on valued extrinsic aspects designed to attract consumers to purchase products even though they may have less favorable perceptions of certain sensory qualities.

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MEET THE STUDENT-AUTHOR

Christina Crowder

I am from Tulsa, Okla., majoring in Food, Human Nutrition, and Hospitality with a concentration in Dietetics. Currently, I am working on my Honors Thesis with Dr. Jamie Baum, studying the role of dietary protein on body composition, energy metabolism and metabolic health in young women. In support of my major, I was offered and accepted internships in the summer of 2013 with Chartwells Marketing at the University of Arkansas-Fayetteville, and with St. Vincent Sports Performance in Indianapolis, Indiana, under their sports dietitian. I served as Director of Dining Services for the Associated Student Government from 2012-2013 and as Campus Life Director during the fall semester of 2013. I also serve on the Bumpers Honors Student Board as the Outstanding Project/Thesis Competition Director and newly elected Vice Chair. My goals are to pursue my Registered Dietician licensing, and then continue in research or practice in nutrition as a medical professional. I would like to thank Dr. Catherine Shoulders for mentoring and contributing much to this project as a co-author. My personal growth in the realm of research was exponential because of this project and her devotion to me as a student. This project was also made possible by Dr. K. Jill Rucker, Ozark Natural Foods, the Associated Student Government, Lynne Williams, and Morgan Stout of Chartwells.

INTRODUCTION

Consumers vote every day on which products line the shelves of grocery stores. As agricultural technologies enable more people to work in areas outside of agricultural production, public concern regarding production practices has increased (Dimitri et al., 2005). Public unrest with regard to the environmental, animal welfare, food purity, and health impacts of agricultural practices has led to the rise of niche food products which boast the use of unconventional production practices on the label (Laux, 2012; GRACE Communications Foundation, 2013). These unconventionally produced (UP) products are labeled with messages such as organic (USDA certified), grass fed, locally grown, antibiotic free, hormone free, pasture raised, free range, and cage free (GRACE Communications Foundation, 2013), but are delivered to the consumer in retail products that are comparable to conventionally produced (CP) products; for example, consumers can purchase both CP and UP whole apples, chicken breasts, cartons of milk, and bars of chocolate.

In spite of the growth within the UP food industry, marketers lack a solid plan for advertising UP foods to potential consumers, partially because individuals' interpretation of the terms associated with UP foods varies (Hughner et al., 2007; Yiridoe et al., 2005). Through a review of research, Hughner et al. (2007) found that consumers could not distinguish organic from conventional food and recommended that marketers work to "better convey relevant information to consumers." With consumers making purchasing decisions based on their subjective experiences and perceptions of specific UP and CP foods, a better understanding of how consumers perceive these foods can help marketers advertise products accordingly (Hughner et al., 2007).

The purpose of this study was to evaluate Millennial generation members' (as accessed through a university setting) perceptions regarding the sensory characteristics of selected CP and UP foods. For the purposes of this study, "conventionally produced" was operationally defined as any product not indicating specific production methods on its label. "Unconventionally produced" was operationally defined as any product indicating a specific value-adding (as indicated by product cost) production method. To achieve this purpose, the following objectives were developed:

1. To describe students' preferences regarding CP and UP foods.
2. To describe students' perceptions regarding specific qualities of CP and UP foods.
3. To determine whether significant differences exist in how those that prefer a CP product perceive qualities of that product versus its UP alternative.

4. To determine whether significant differences exist in how those that prefer a UP product perceive qualities of that product versus its CP alternative.

MATERIALS AND METHODS

This study utilized a nonexperimental comparative design. A convenience sample of undergraduate students at [University] (N = 20,350) was recruited to participate via face-to-face methods at a central location on the campus from 5:00 pm to 7:00 pm during a publicized "food tasting" event. Sample size was calculated according to Israel (1992), and was determined to be 100 for a 10% precision level and confidence level of 95%. Students were offered samples of conventional and nonconventional foods, as was indicated on the food labels (Table 1).

Upon completion of the food sampling, participants were offered a paper-based, researcher developed, institutional review board (IRB) approved questionnaire. The questionnaire—which included 23 Likert-type items that ranged from 1 to 5, 1 being strongly disagree and 5 being strongly agree—asked participants to indicate their level of agreement with statements that expressed favorability with regard to food appearance, smell, texture, and flavor, which are selected intrinsic and extrinsic qualities as described in the Total Food Quality Model (Grunert, et al, 1996). Participants were then asked to select whether they preferred the CP or UP produced variety of each food. The survey was reviewed by a panel of experts in survey construction for face and content validity. Because responses were dependent upon the food tasted, the calculation of test-retest reliability was not deemed appropriate.

Data were analyzed using IBM SPSS Version 20 (IBM Corp., Armonk, N.Y.). Frequency, means, and standard deviations were reported for the first and second objective, which are descriptive in nature. The third and fourth objectives were carried out through the use of dependent samples *t*-tests. Cohen's effect sizes were used to describe differences in preference of individual sensory aspects for those who preferred a particular CP or UP food.

RESULTS AND DISCUSSION

Objective 1 was to describe students' preferences regarding specific CP and UP milk, chocolate, beef, chicken,

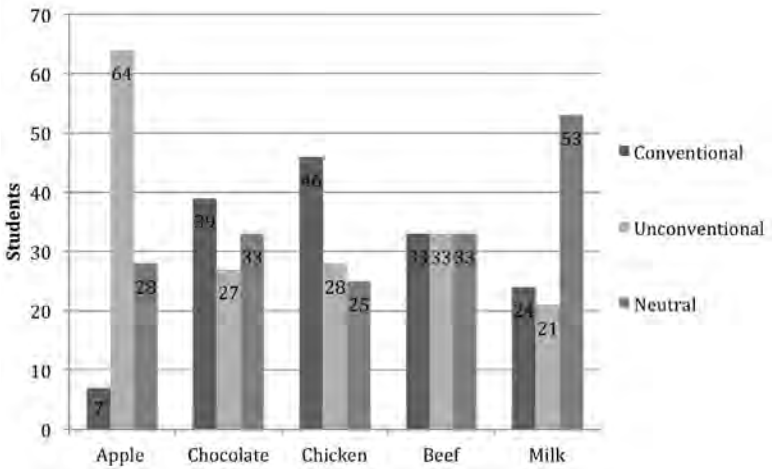

Fig. 1. Respondents' preferences with regard to conventionally and unconventionally produced milk, chocolate, beef, chicken, and apples.

Table 1. Food tasting panel: unconventionally produced and conventionally produced foods offered.

Item	Conventionally Produced	Unconventionally Produced
Milk	2%	USDA Organic 2%
Chocolate	Milk	USDA Organic Milk
Beef	Conventionally-raised	Grass-fed
Chicken	Conventionally-raised	GMO-free, Pasture Raised
Apple	Pink Lady	USDA Organic Pink Lady

and apples. Results are displayed in Fig. 1. Results showed that more students preferred CP chicken and chocolate, while more students preferred UP apples. Students displayed no preference with regard to milk, and were equally split in their beef preferences. These findings are partially supported by previous positions that the Millennial generation values UP products (Hughner et al., 2007), but suggests that Millenials may have specific preferences with regard to certain foods.

Objective 2 was to describe students' perceptions regarding specific qualities of CP and UP foods (Table 2; see Table 1 for specific labeling on CP and UP foods). Students reported consistent perceptions regarding the favorability of each sensory aspect of chicken and apples; the UP versions of the products yielded numerically higher mean scores on every sensory aspect. These findings support those found by Reganold et al. (2001), who reported that panelists described organic apples as sweeter and less tart. However, students' perceptions of the sensory qualities of chocolate, milk, and chicken were not consistent for each product; they reported more favorable perceptions of the appearance and smell of CP milk, but perceived a more favorable texture and flavor from the UP milk. Students' perceptions of CP chocolate were more favorable with regard to texture, but less favorable than the UP chocolate with regard to smell and flavor. Students' perceptions of the chocolates' appearance were equal. Conventionally produced beef yielded greater mean perception scores regarding appearance, smell, and flavor, but the texture of UP beef was perceived as more favorable. These findings are confirmed by the inconsistency found in previous research regarding sensory aspects of CP and UP foods (Bourn and Prescott, 2002), and suggest that while sensory-based intrinsic cues may influence a consumer's intentions regarding future purchases (Grunert et al., 1996), they may create mixed feelings about a product. The conflicting perceptions regarding the sensory aspects of a product imply that when making purchasing decisions, consumers may value specific sensory attributes over others, which contributes to the various subjective experiences in which consumers engage with their foods (Hughner et al., 2007).

Table 2. Mean perceptions scores regarding specific qualities of conventionally produced and unconventionally produced foods.

		Conventionally Produced		Unconventionally Produced	
Item		**M**	**SD**	**M**	**SD**
Milk					
	Appearance	3.90	0.83	3.77	1.01
	Smell	3.73	0.79	3.70	0.93
	Texture	3.79	0.81	4.01	0.85
	Flavor	3.68	0.87	3.90	0.87
Chocolate					
	Appearance	4.33	0.84	4.33	0.84
	Smell	4.15	0.88	4.31	0.84
	Texture	4.32	0.86	4.30	0.86
	Flavor	4.04	0.97	4.19	0.98
Beef					
	Appearance	3.48	1.05	3.04	1.13
	Smell	3.49	0.97	3.39	1.05
	Texture	3.31	1.04	3.58	1.02
	Flavor	3.38	0.98	3.35	1.12
Chicken					
	Appearance	3.76	0.96	4.18	0.93
	Smell	3.83	0.97	4.13	1.00
	Texture	3.63	1.08	4.24	0.93
	Flavor	3.79	1.13	4.35	0.92
Apple					
	Appearance	3.29	1.20	4.27	0.74
	Smell	3.65	0.99	4.27	0.71
	Texture	3.67	1.04	4.37	0.68
	Flavor	3.88	1.05	4.46	0.73

Objective 3 sought to determine whether significant differences exist in how those that prefer a CP product perceive qualities of that product versus its UP alternative, while Objective 4 sought to determine whether significant differences exist in how those that prefer an UP product perceive qualities of that product versus its CP alternative. Students who preferred CP milk (n = 24) reported higher mean scores on CP milk's appearance than UP milk's appearance (Table 3). The effect size was found to be medium (Cohen, 1988). Students who preferred UP milk (n = 21) reported a higher mean score on UP milk's smell, texture, and flavor. Effect sizes for those three sensory attributes were found to be medium and large (Cohen, 1988). Results showing that those preferring CP milk and those preferring UP milk perceived significant differences in sensory aspects of the milk samples imply that while their perceptions of the sensory aspects of the two products differ, those which in turn impacted their preferences may differ as well.

Students who preferred CP chocolate (n = 27) reported higher mean scores on CP chocolate's flavor (Table 4). The effect size was found to be medium to large (Cohen, 1988). Students who preferred UP chocolate (n = 39) reported higher mean scores on all four of the UP chocolate's qualities. Effect sizes were found to be medium for appearance, smell, and texture, and large for flavor (Cohen, 1988). Students who preferred CP chocolate scored it as significantly more favorable than the UP chocolate in flavor. However, those that preferred UP chocolate reported significantly higher scores on its appearance,

Table 3. Perceptions of qualities of conventionally produced (CP) and unconventionally produced (UP) milk among students who prefer CP milk or UP milk.

	Conventionally Produced		Unconventionally Produced			
Item	**M**	**SD**	**M**	**SD**	***t***	***P***
CP Milk						
Appearance	3.9	0.83	3.77	1.01	2.22	0.036
Smell	3.73	0.79	3.70	0.93	2.00	0.057
Texture	3.79	0.81	4.01	0.85	0.189	0.852
Flavor	3.68	0.87	3.9	0.87	1.86	0.076
UP Milk						
Appearance	3.82	0.80	4.18	0.85	1.63	0.119
Smell	3.45	0.86	4.09	0.92	3.31	0.003
Texture	3.59	0.91	4.23	0.69	3.52	0.002
Flavor	3.32	1.00	4.27	0.77	4.48	0.00

M = mean; SD = standard deviation; t = t-statistic; P = < 0.05; d = Cohen's effect size.

Table 4. Perceptions of qualities of conventionally produced (CP) and unconventionally produced (UP) chocolate among students who prefer CP or UP chocolate.

	Conventionally Produced		Unconventionally Produced				
Item	***M***	***SD***	***M***	***SD***	***t***	***P***	***d***
CP Chocolate							
Appearance	4.41	0.84	4.15	0.91	1.27	0.215	0.00
Smell	4.37	0.93	4.19	1.00	0.795	0.434	0.00
Texture	4.48	0.70	3.67	1.70	1.99	0.057	0.00
Flavor	4.52	0.75	3.67	1.07	3.79	0.001	0.73
UP Chocolate							
Appearance	4.26	0.86	4.71	0.52	3.33	0.002	0.54
Smell	4.18	0.83	4.61	0.64	2.66	0.012	0.43
Texture	4.18	0.83	4.63	0.68	2.9	0.006	0.47
Flavor	3.89	0.86	4.71	0.65	4.74	0.00	0.77

M = Mean; SD = Standard Deviation; t = t-statistic; P = <0.05; d = Cohen's effect size.

smell, texture, and flavor when compared to scores on CP chocolate. As was observed with student preferences regarding milk, the sensory aspects valued by those that preferred CP and UP chocolate differed. Findings suggest that while flavor was a factor in determining a preference for CP chocolate, those that preferred UP chocolate valued all four aspects.

Students who preferred CP beef (n = 33) reported higher mean scores on CP beef's appearance, and flavor (Table 5). Effect sizes were found to be medium (Cohen, 1988). Students who preferred UP beef (n = 33) reported higher mean scores on all four of the UP beef's qualities. Effect sizes were found to be small to medium for appearance, medium for smell, and large for texture and flavor. These results are similar to those obtained for chocolate in that fewer attributes (appearance and flavor) were scored higher for those students who preferred CP beef, while those who preferred UP beef displayed significantly higher scores on all four aspects.

Students who preferred CP chicken (n = 46) did not report higher mean scores on CP chicken's appearance, smell, texture, and flavor (Table 6). In contrast, students who preferred UP chicken (n = 28) reported higher mean scores on all four of the UP chicken's qualities. Effect sizes were found to be large for all qualities with the exception of smell, which was found to have a medium effect size (Cohen, 1988).

While no statistically significant differences were found among the perceptions of CP and UP chicken among students who preferred CP chicken, the students sampled

Table 5. Perceptions of qualities of conventionally produced (CP) and unconventionally produced (UP) beef among students who prefer CP or UP beef.

	Conventionally Produced		Unconventionally Produced				
Item	***M***	***SD***	***M***	***SD***	***t***	***P***	***d***
CP Beef							
Appearance	3.67	0.92	2.73	1.13	3.60	0.001	0.63
Smell	3.64	0.93	3.24	1.17	1.58	0.125	0.00
Texture	3.67	0.96	3.30	1.13	1.53	0.136	0.00
Flavor	3.76	0.75	3.00	1.09	3.23	0.003	0.56
UP Beef							
Appearance	2.97	1.13	3.55	1.06	2.2	0.035	0.38
Smell	3.00	1.00	3.70	0.98	3.11	0.004	0.54
Texture	2.73	1.04	3.85	0.91	4.65	0.00	0.81
Flavor	2.82	0.95	4.09	1.04	6.20	0.00	1.08

M = mean; SD = standard deviation; T = t-statistic; P = <0.05; d = Cohen's effect size.

Table 6. Perceptions of qualities of conventionally produced (CP) and unconventionally produced (UP) chicken among students who prefer CP chicken.

	Conventionally Produced		Unconventionally Produced				
Item	***M***	***SD***	***M***	***SD***	***t***	***P***	***d***
CP Chicken							
Appearance	4.14	0.93	4.21	1.07	0.26	0.795	0.00
Smell	4.07	1.02	3.82	1.12	1.02	0.316	0.00
Texture	4.32	0.82	4.11	0.99	0.86	0.396	0.00
Flavor	4.32	0.98	4.00	0.98	1.20	0.24	0.00
UP Chicken							
Appearance	3.69	0.095	4.53	0.73	5.55	0.00	0.83
Smell	3.71	0.99	4.40	0.94	3.73	0.00	0.56
Texture	3.38	1.05	4.44	0.87	5.56	0.00	0.83
Flavor	3.49	1.20	4.60	0.78	5.73	0.00	0.85

M = Mean; SD = standard deviation; t = t-statistic; P = <0.05; d = Cohen's effect size.

indicated that the UP chicken had a more favorable appearance. This conflicts findings in previous studies which reported that consumers were persuaded not to buy organic versions of food based on appearance (Hack, 1993; Jolly and Norris, 1991; Roddy et al., 1994). Results suggest that students preferring UP chicken value the sensory aspects of smell, texture, and flavor of the chicken products differently than those who preferred the CP chicken, as those students reported significantly higher scores on those aspects of the UP chicken, in addition to appearance.

Students who preferred CP apples (n = 7; Fig. 1) did not report higher mean scores on the CP apple's four sensory attributes tested in this study (Table 7). Similar to the results with chicken, students who preferred the UP apple (n = 64) reported a higher mean score for it on all four of the apples' aspects. Effect sizes were found to be medium to large for smell and flavor and large for appearance and texture.

No significant differences were found between scores of sensory aspects among students who preferred CP apples. These findings are in conflict with those of Hack (1993), Jolly and Norris (1991), and Roddy et al. (1994), who each reported that the appearance of organic foods was negatively perceived. Those that preferred the UP apple reported significantly higher scores on all four of the UP apple's sensory aspects.

The results of this study yield recommendations for both future research and those marketing CP and UP products. Those marketing CP and UP products should focus on the Millennial generation as an audience from which increased concern in food production practices will be seen. Agricultural communicators should focus on enhancing consumer awareness of the sensory aspects valued by those that prefer that product. For example, when marketing UP apples, communicators should highlight the appearance of the product in order to attract consumers typically purchasing CP apples, as this group reported higher scores regarding UP apples over their preferred CP apples. An alternative approach when marketing UP products is to focus on valued extrinsic aspects, such as environmental improvement, in communications designed to attract consumers to purchase products in spite of their perceptions of sensory aspects, which may be valued less than extrinsic aspects (Jolly et al., 1989).

This study was conducted at one institution, and should be replicated within and outside of the postsecondary educational environment. A main limitation of the study is the lack of a blind sensory panel, which was not feasible within the event in which the panel took place; participants were aware of the production method of each food as they were assessing sensory aspects, which could have impacted their perceptions and therefore presented a threat to the internal validity of the study. The researchers recommend that future research be conducted using a blind sensory panel to enhance validity.

As supported, consumers are not always consistent with their perceptions of a product's intrinsic and extrinsic qualities, and thus communicators should identify the aspects a consumer aligns quality with a product. Replication within and outside the postsecondary education environment is necessary to collect a broader sample. Although the limitation of this study included the lack of a blind sensory panel, these results are considered valid

Table 7. Perceptions of qualities of conventionally produced (CP) and unconventionally produced (UP) apples among students who prefer CP apples.

	Conventionally Produced		Unconventionally Produced				
Item	***M***	***SD***	***M***	***SD***	***t***	***P***	***d***
CP Apple							
Appearance	3.71	1.13	4.14	0.69	0.891	0.407	
Smell	3.71	1.13	4.14	0.69	1.16	0.289	
Texture	4.57	0.79	3.86	1.07	1.51	0.182	
Flavor	4.71	0.49	3.86	0.9	2.21	0.078	
UP Apple							
Appearance	3.25	1.23	4.35	0.79	6.43	0	0.8
Smell	3.52	1.08	4.4	0.71	5.72	0	0.72
Texture	3.43	1.08	4.49	0.62	7.18	0	0.9
Flavor	3.65	1.14	4.6	0.64	6.05	0	0.76

M= Mean; SD = standard deviation; t = t-statistic; P = <0.05; d = Cohen's effect size.

due to the nature of stores in which consumers would purchase these products. A consumer is aware of what "version", CP or UP, a product is when making the decision to purchase. Qualitative and quantitative methods could be used to more fully understand how individuals value different sensory aspects, and how those values influence consumer decisions, including instruments to measure price, acquisition, and future intentions to purchase. The Millennial generation is an audience from which increased concern in food production practices will be seen, and communicators should enhance customer awareness of sensory aspects valued by those that prefer a specific product.

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LITERATURE CITED

Bourn, D. and J. Prescott. 2002. A comparison of the nutritional value, sensory qualities, and food safety of organically and conventionally produced foods. Critical Rev. Food Sci. Nutr., 42, 1-334.

Cohen, J. 1988. Statistical power analysis for the behavioral sciences (2nd ed.). Hillsdale, NJ: Lawrence Erlbaum Associates, Inc.

Dimitri, C., A. Effland, and N. Conklin. 2005. The 20th century transformation of U.S. agriculture and farm policy. No. Bulletin 3. Economic Research Service/USDAGRACE Communications Foundation 2013. These labels are so confusing! Retrieved from http://www.sustainabletable.org/944/these-labels-are-so-confusing.

Grunert, K.G., H.H. Larsen, T.K. Madsen, and A. Baadsgaard. 1996. Market orientation in food and agriculture. Boston, MA: Kluwer Academic Press.

Hack, M.D. 1993. Organically grown products: Perception, preferences and motives of Dutch consumers. Acta Horticulturae.340:247-253.

Hughner, R.S., P. McDonagh, A. Prothero, C.J. Shultz II, and J. Stanton. 2007. J. Cons. Behav. 6:94-100. doi: 10.1002/cb.210.

Israel, G.D. 1992. Determining sample size (Fact Sheet PEOD-6). Retrieved from Florida Cooperative Extension Service: http://www.soc.uoc.gr/socmedia/papageo/metaptyxiakoi/sample_size/samplesize1.pdf

Jolly, D.A. and K. Norris, K. 1991. Marketing prospects for organic and pesticide-free produce. Amer. J. Altern. Agric. 6(4):174-179.

Jolly, D.S., H.G. Schutz, K.V. Diaz-Knauf, and J. Hohal. 1989. Organic foods: Consumer attitudes and use. Food Technology.43:60-66.

Laux, M. 2012. Pastured poultry profile. Retrieved from Agricultural Marketing Resource Center website:http://www.agmrc.org/commodities products/livestock/poultry/pastured-poultry-profile/.

Reganold, J.P., J.D. Glover, P.K. Andrews, and H.R. Hinman. 2001. Sustainability of three apple production systems. Nature. 410:926-930.

Roddy, G., C. Cowan, and G. Hutchinson. 1994. Organic food: A description of the Irish market. British Food J., 96(4), 3-10.

Yiridoe, E.K., S.S. Bonti-Ankomah, and R.C. Martin. 2005. Comparison of consumer perceptions and preference toward organic versus conventionally produced foods: a review and update of the literature. Renewable Agric. Food Sys. 20(4), 193-205.

Palatability of teff grass by horses

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ABSTRACT

Most forages commonly used to feed horses have potential detriments including blister beetles or excessive fiber concentrations. Teff grass (T), a warm-season annual forage, has the potential to be a good alternative for horses because of its lack of observed disorders. Our objective was to compare preference by horses for T harvested under different conditions with that of bermudagrass (B) harvested at two maturities. Six different forages were evaluated: T harvested at the late vegetative stage (TLV), at late bloom but that incurred 33 mm of rainfall between mowing and baling (TLBR), with caryopsis visible (TES), or at soft dough (TSD), and B harvested at late vegetative (BLV) and mid-bloom (BMB) growth stages. Five mature horses were used in a balanced incomplete block design where each horse received a different combination of 4 forages each day for 6 d. The 4 different forages were suspended in hay nets in each corner of each stall, and each hay was offered at 50% of the average daily hay consumption measured during a 12-d adaptation period. Forage preference as measured by individual forage dry matter (DM) consumption (kg and % of total DM consumed across the 4 forages) was greatest ($P < 0.05$) from TLV followed by BLV. Preference (kg and % of total DM consumed) of BMB was greater ($P < 0.05$) than that of TMBR, TES, and TSD, which did not differ from each other ($P \geq 0.63$). Therefore, within a specific growth stage, horses apparently preferred teff grass, but effects of maturity and rainfall had a more dramatic effect on preference by horses than forage species.

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MEET THE STUDENT-AUTHOR

Rachel Cummins

I grew up in Coppell, Texas where I graduated from Coppell High School in 2010. I came to the University of Arkansas and majored in animal science with an emphasis on pre-veterinary medicine and a minor in equine science. I am involved with many organizations on campus, including the Pre-Vet Club and the D.E. King Equine Program. I was also a member of the Razorback Marching Band Colorguard for 3 years. I have always hoped to pursue a career in veterinary medicine, and I have been accepted to University of Missouri's College of Veterinary Medicine. I will begin classes there in August of 2014. In my spare time I enjoy reading, hiking and playing video games.

INTRODUCTION

Teff grass is warm-season annual forage recently introduced in the United States from Ethiopia and Eritrea. Teff grass has already gained popularity in the western United States as a horse forage, especially as a forage for horses with metabolic disorders and obesity (Anonymous, 2012). Teff grass has the potential to be a viable alternative to other popular horse forages because of its lack of potential disorders. Alfalfa hay is a popular horse forage, but it is commonly contaminated with blister beetles which emit a chemical that can be fatal if consumed by horses (Echevarria and Hooser, 2006). Bermudagrass is another widely-utilized horse forage; however, it often has problems with low digestibility due to rapid maturity (Coleman et al., 2003). Teff grass does not mature as rapidly as bermudagrass (Miller, 2010), and does not have any observed insect problems. Teff grass is lower in nonstructural carbohydrates compared with cool-season forages (Staniar et al., 2010), thereby giving it potential as an alternative forage for horses. To be a contender as a replacement of bermudagrass and alfalfa hay, teff grass must first be established as a forage that horses will willingly consume. The purpose of this study is to determine the palatability of teff grass relative to that of bermudagrass at different maturities.

MATERIALS AND METHODS

All procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol no. 13055). Teff grass (T) was planted at the University of Arkansas System Division of Agriculture Watershed Research and Education Center (WREC) according to recommended practices on 29 May 2013. A comparable field of bermudagrass (B), a perennial warm-season grass, was also chosen to provide B hays for comparison with T. The field of B was harvested 15 June and baled for hay to initiate the regrowth process in an attempt to have both forages reaching comparable maturities under similar growing conditions. Both B and T were harvested beginning in late June. The forages included in the study were: T harvested at the late vegetative stage (TLV), T harvested at late bloom but that incurred 33 mm of rainfall between mowing and baling (TLBR), T harvested when the caryopsis was visible (early seed stage; TES), T harvested at soft dough (TSD), B harvested at the late vegetative stage (BLV) and B harvested at the mid-bloom (BMB) growth stage. All forages were allowed to dry in the field to a maximum of 20% moisture and packaged in small-rectangular bales. All bales were stored inside a metal enclosed shed until subsequent feeding.

Five mature horses [511 ± 17.4 kg body weight (BW)], 2 to 10 yr of age, were housed individually in stalls (3.7 × 3.7 m) at the Dorothy E. King (DEK) Equine facility for a 12-d adaptation period followed by a 6-d forage preference evaluation. During the adaption period, the horses were offered bermudagrass and teff grass hays that were harvested at the mid-bloom growth stage the previous year. This was done to acclimate the horses to each forage. Initially horses were offered 1% of their body weight of each forage divided equally into 2 hay bags. This resulted in a total of 2% of body weight from each forage offered in 4 different hay bags. One hay bag was placed at random in each corner of each stall, and the amount offered increased daily based on consumption. Triangular tarps were suspended beneath each hay bag to catch forage that was pulled from the bags but not consumed. The average daily dry matter (DM) consumption (ADC) for each horse was determined during the last 5 d of the adaptation period.

The preference portion of the experiment immediately followed the adaptation period and utilized a balanced incomplete block design (Plan 11.6 from Cochran and Cox, 1957) that was repeated twice. The original design was for 3 d, with each horse offered a total of 4 of the 6 forages each day. By repeating the design twice, we were able to offer each forage in combination with each other forage at least twice, and each forage was offered to each horse a total of 4 times during the 6-d period. Each horse had a different combination of 4 forages from each other horse, and the combinations were changed daily based on the experimental design (Fig. 1). In order to account for any idiosyncrasies, a number of factors were considered and randomized. First, horses were allocated to a different stall each day based on plans for 5 × 5 Latin Squares with one extra period. This resulted in each horse being housed in each stall at least one day during the study and in only 1 stall a second time. Secondly, the specific corner in which a particular forage was offered was randomized such that the particular forage was offered in all 4 corners of a stall for each individual horse. Each forage was offered at a rate of one-half of the total average daily consumption during the last 5 d of the adaptation period. This is done to ensure that the horses selected from and established a preference ranking for at least two of the forages each day. For example, if the total consumption of both B and T by horse "X" was 10 kg during the last 5 d of the adaptation period, then horse "X" was offered 5 kg of each of the 4 experimental forages.

Horses were given 2-h exercise periods twice daily in the morning at 6:30 AM and in the evening at 7:30 PM. During the morning exercise period, orts were removed and weighed and new forages were placed in the stalls. Each stall door also had a fan to ensure horses were not overheated. Stalls were bedded in sand and cleaned twice daily. No grain was offered during the adaptation period or trial period. Horses had unlimited access to water, even during the exercise periods.

Samples of each hay were taken daily at the time the hay bags were filled and were dried to a constant weight at 50 °C. Unconsumed hay was collected daily, weighed, and a representative sample was dried to a constant weight at 50 °C. Hay samples from each forage were maintained separately for each day and were ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, Pa.) and analyzed for neutral-detergent fiber, acid-detergent fiber, and acid-detergent lignin (Vogel et al, 1999).

Consumption data were analyzed using PROC GLM of SAS (SAS Institute Inc., Cary, N.C.). The model included the effects of horse, forage, day, stall, and corner. The effect of stall was included to ensure that location in the barn was not having an effect. The effect of corner was included to determine if horses preferred to consume forages out of a favorite corner. Stall affected ($P < 0.05$) each of the consumption measurements, but corner and day of study did not ($P \geq 0.56$) affect any of the consumption measurements. Therefore the final consumption model included effects of forage, stall, and horse. Means are reported as least-squares means. Pearson correlation coefficients were also determined among consumption measurements and fiber components using PROC CORR of SAS. The fiber components from each forage on each individual day were matched with consumption of that particular forage on a given day for correlation analyses.

RESULTS AND DISCUSSION

Weather data affecting the forages in the present study are presented in Table 1. When compared with the 30-yr averages, May of 2013 was relatively wet. This delayed the planting of the teff grass. June of 2013 was unusually dry, which allowed the late vegetative forages to be baled

Table 1. Weather data during the growing period for teff grass and bermudagrass in 2013.

Item	May	June	July	August
2013				
Avg. Temp. Min., °C	13.7	19.1	19.6	19.3
Avg. Temp. Max., °C	22.7	29.5	30.8	29.8
Rainfall, cm	26.7	3.6	8.7	15.5
30-year avg.				
Avg. Temp. Min., °C	13.3	18.3	20.6	20.0
Avg. Temp. Max., °C	24.4	28.9	31.7	31.7
Rainfall, cm	13.2	12.1	8.2	7.7

Fig. 1. Stall and corner layout for a study to evaluate the palatability of teff grass and bermudagrass harvested at different maturities. Forages were A – teff grass with the caryopsis visible; B – teff grass harvested at soft dough; C – teff grass harvested at late bloom that received 33 mm of rainfall; D – bermudagrass harvested at mid-bloom; E – bermudagrass harvested at the late vegetative stage; F – teff grass harvested at the late vegetative stage. Each horse's name is in the center cell of each block.

Table 2. Harvest dates and fiber components of forages offered to horses in a palatability study.[†]

	Forages[‡]						
Item[§]	**BLV**	**TLV**	**TLBR**	**BMB**	**TES**	**TSD**	**SEM[¶]**
Date baled	**1-July**	**28-June**	**18-Aug.**	**2-Aug.**	**24-Aug.**	**24-Aug.**	
NDF, %	67.6[b]	64.7[c]	73.5[a]	68.2[b]	73.6[a]	72.5[a]	0.86
ADF, %	28.4[c]	29.7[c]	35.2[b]	28.6[c]	37.7[a]	37.4[a]	0.60
Hemicellulose, %	39.2[a]	35.1[b]	38.3[a]	39.6[a]	35.9[b]	35.1[b]	0.68
Lignin, %	2.6[c]	2.7[c]	3.8[ab]	3.2[bc]	4.4[a]	3.9[ab]	0.33

[†]Means within a row without a common superscript letter differ ($P < 0.05$).
[‡]BLV = bermudagrass late vegetative; TLV = teff grass late vegetative, TLBR = teff grass late bloom with rain damage, BMB = bermudagrass mid-bloom, TES = teff grass with caryopsis visible, TSD = teff grass soft dough stage.
[§]NDF = neutral detergent fiber; ADF = acid detergent fiber.
[¶]SEM = standard error of mean.

under ideal conditions. However, the dry June also led to issues with growing the later maturities of the forages. Our original intention was to have 3 different maturities each of B and T. However, due to the slow growth rate, only 2 maturities of B were available because of field size limitations. August of 2013 had a greater rainfall compared with the 30-yr average, which delayed the baling of TLBR, TES and TSD. The TLBR also incurred 33 mm of rain damage between mowing and baling.

Forage fiber components are presented in Table 2. The NDF concentration of TES, TSD and TLBR were not different ($P \geq 0.40$) from each other, but were greater ($P < 0.05$) than the NDF concentrations of the other forages.The NDF concentrations of BMB and BLV were greater ($P < 0.05$) than those of TLV. The greater NDF concentration of TLBR suggests that the rain damage removed soluble components, resulting in NDF concentrations similar to that of a more mature forage. The TES and TSD forages also had the greatest ($P < 0.05$) ADF concentrations. These are followed by TLBR ($P < 0.05$). The two maturities of bermudagrass and TLV were not different from each other ($P \geq 0.14$), and had the lowest ($P < 0.05$) ADF concentrations. Lignin concentrations of TES, TSD and TLBR were greater ($P < 0.05$) than those from BLV and TLV. Lignin concentrations of TSD and TLBR are also not different ($P \geq 0.18$) from the lignin concentrations of TES or BMB. A previous study reported that rain damage increased all fiber components excluding hemicellulose in B and orchardgrass (Scarbrough et al., 2005).

Preference of the different hays by horses was expressed in three ways: kg of dry matter consumed per day (kg/d; Fig. 2), the amount of each forage consumed as a percentage of the amount of that particular forage offered (% offered daily; Fig. 3), and the amount of each forage

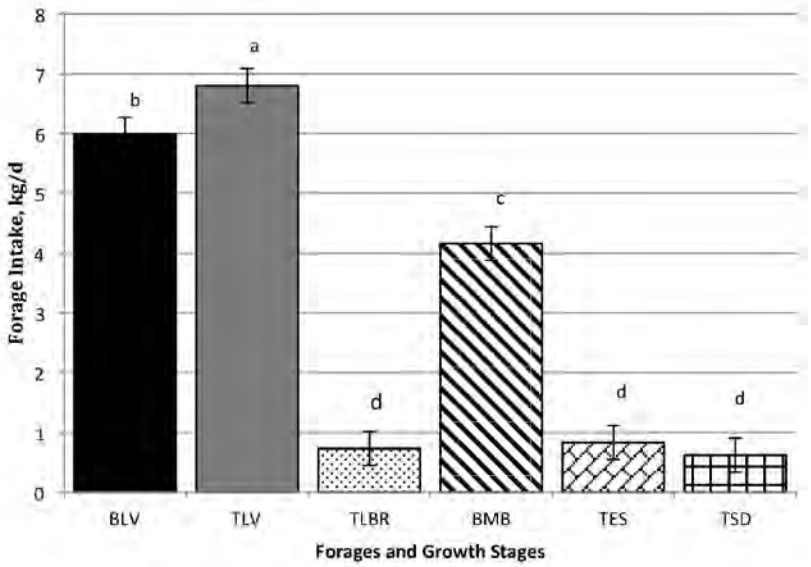

Fig. 2. Intake (kg/d) of teff grass and bermudagrass harvested under different conditions and offered to horses in combinations of 4 different forages each day for 6 days. Forages offered were bermudagrass late vegetative (BLV), teff grass late vegetative (TLV), teff grass late bloom with rain damage (TLBR), bermudagrass mid-bloom (BMB), teff grass with caryopsis visible (TES), and teff grass soft dough stage (TSD). Bars without a common superscript are different ($P < 0.05$).

consumed as a percentage of the total DM intake by each horse (% of DM intake; Fig. 4). Preference (kg/d) was greatest ($P < 0.05$) for TLV followed by BLV ($P < 0.05$). The least preferred ($P < 0.05$) forages were TLBR, TES and TSD. The low preference for TLBR, and the fact that the preference for TLBR was not different ($P \geq 0.63$) from that of TES and TSD suggests that the rainfall was just as damaging to preference as the increased maturity of TES and TSD. A study in cattle reported a 10% reduction of intake in response to rain damage on forages (Coblentz,

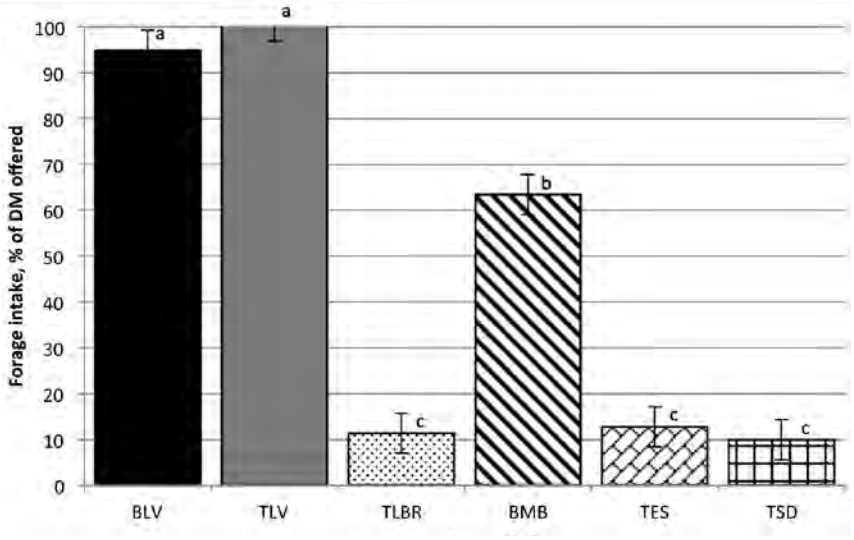

Fig. 3. Intake of teff grass and bermudagrass harvested under different conditions and offered to horses in combinations of 4 different forages each day for 6 days. Intake is expressed as a percentage of a particular forage offered. Forages offered were bermudagrass late vegetative (BLV), teff grass late vegetative (TLV), teff grass late bloom with rain damage (TLBR), bermudagrass mid-bloom (BMB), teff grass with caryopsis visible (TES), and teff grass soft dough stage (TSD). Bars without a common superscript are different ($P < 0.05$).

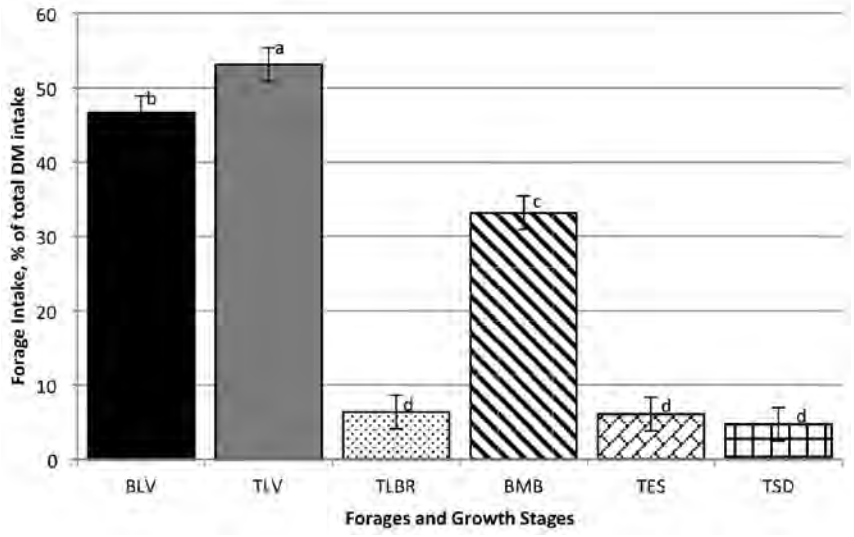

Fig. 4. Intake of teff grass and bermudagrass harvested under different conditions and offered to horses in combinations of 4 different forages each day for 6 days. Intake is expressed as a percentage of the total daily dry matter (DM) intake. Forages offered were bermudagrass late vegetative (BLV), teff grass late vegetative (TLV), teff grass late bloom with rain damage (TLBR), bermudagrass mid-bloom (BMB), teff grass with caryopsis visible (TES), and teff grass soft dough stage (TSD). Bars without a common superscript are different ($P < 0.05$).

2006). Preference expressed as a percentage of the total amount offered daily was greatest ($P < 0.05$) for BLV and TLV. The later maturities of T including TLBR were the least preferred forages ($P < 0.05$). This again suggests that the rainfall on TLBR was just as damaging to preference as increasing maturity. Preference expressed as a percentage of the total DM intake was greatest ($P < 0.05$) for TLV. Consumption of TLV was slightly above 50% of the DM intake for horses, which suggests that horses consumed all of the TLV offered, since each forage was offered at half of the estimated ADC. Preference was least ($P < 0.05$) for TLBR, TES and TSD, once again suggesting that rain damage and advanced maturity are equally detrimental to preference by horses.

Forage concentrations of NDF and ADF were both highly and negatively correlated with preference ($P < 0.05$; Table 3). Lignin content was also highly and negatively correlated with preference ($P < 0.05$), but not as highly correlated as NDF and ADF. Hemicellulose content was not correlated with preference ($P \geq 0.11$). In a previous study (Staniar et al., 2010), voluntary intake of T was less from late-heading maturity than from early-heading and boot stage maturities. In that study, concentrations of NDF and ADF were greatest from the late-heading T, lowest from the boot stage T, and intermediate from the early-heading T, which was not different from the late-heading T or the boot stage T in NDF concentrations (Staniar et al., 2010). These results are consistent with the results of our study, which demonstrate that an increase in maturity is detrimental to palatability, and that preference appears to follow closely with NDF and ADF concentrations.

CONCLUSIONS

When given a choice of different forages, horses preferred late-vegetative teff grass. However, forage maturity had a larger effect on preference than forage species when forages were compared across different maturities. This conclusion is drawn based on the relatively small difference in preference between bermudagrass and teff grass harvested at a comparable maturity, but a very large negative effect of maturity on preference of both forages. It is also apparent that rain damage can be just as detrimental to palatability as increasing maturity as preference for teff grass harvested at the late bloom stage was never different from preference for the later maturities of teff grass. Strong negative correlations among preference and NDF and ADF support the use of these measures to estimate preference by horses. Therefore, teff grass is palatable to horses, but forage maturity and rain damage are more important factors affecting palatability than forage species.

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Table 3. Pearson correlation coefficients relating forage quality measurements to palatability by horses across different forages.

Item[†]	DM consumption per forage, kg/d[‡]	DM consumption % of offer	DM consumption % of total DMI
NDF, %	-0.73	-0.74	-0.72
p-value	<0.01	<0.01	<0.01
ADF, %	-0.75	-0.76	-0.74
P-value	<0.01	<0.01	<0.01
Hemicellulose,%	0.13	0.15	0.13
P -value	0.14	0.11	0.14
Lignin, %	-0.55	-0.57	-0.55
P -value	<0.01	<0.01	<0.01

[†] NDF = neutral detergent fiber; ADF = acid detergent fiber.
[‡] DM = dry matter; DMI = dry matter intake.

LITERATURE CITED

Anonymous. 2012. Teff as an Irrigated Alternative Forage. Hay & Forage Grower, 27(3), 18.

Cochran, W. G. and G. M. Cox. 1957. Experimental Designs (2nd Ed.). pp. 472. John Wiley & Sons. New York, NY.

Coblentz, W. K. 2006. Rain Damage and Spontaneous Heating in Southern Forages Harvested as Hay. *In*: Proceedings of the 60th Southern Pasture and Forage Crop Improvement Conference, April 11-13, 2006, Auburn, Alabama. 2006 CD ROM.

Coleman, S. W., S. P. Hart, and T. Sahlu. 2003. Relationships among forage chemistry, rumination and retention time with intake and digestibility of hay by goats. Small Rumin. Res. 50: 129-140.

Echevarria, C. and S. Hooser. 2006. Blister beetle poisoning: cantharidin toxicosis in equines. https://www.addl.purdue.edu/newsletters/2006/Fall/EquineCT.htm. Accessed 18 April 2013.

Miller, D. 2010. Teff grass: crop overview and forage production guide. Producers Choice Seed. http://www.producerschoiceseed.com/documents/TeffGrassManagementGuide.pdf. Accessed 15 April 2013.

Scarbrough, D. A., W. K. Coblentz, J. B. Humphry, K. P. Coffey, T. C. Daniel, T. J. Sauer, J. A. Jennings, J. E. Turner, and D. W. Kellogg. 2005. Evaluation of dry matter loss, nutritive value, and in situ dry matter disappearance for wilting orchardgrass and bermudagrass forages damaged by simulated rainfall. Agron. J. 97:601-614.

Staniar, W. B., J. R. Bussard, N. M. Repard, M. H. Hall, and A. O. Burk. 2010. Voluntary intake and digestibility of teff hay fed to horses. J. Anim. Sci. 88:3296-3303.

Vogel, K. P., J. F. Pedersen, S. D. Masterson, and J. J. Troy. 1999. Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. Crop Sci. 39:276-279.

Nutrition knowledge of high school senior students in Northwest Arkansas

Grace Heymsfield and Cynthia K. Moore†*

ABSTRACT

Though there are many complex factors influencing diet, nutrition knowledge correlates with healthier food choices in older adolescents and can play a pivotal role in health. Nutrition curriculum was addressed in the state of Arkansas through Arkansas Act 1220 of 2003. Numerous changes have been seen in the school environment regarding nutrition, but there is no means of testing nutrition curriculum effectiveness in terms of nutrition knowledge of students. It is the purpose of this descriptive study to improve understanding of the nutrition knowledge of high school seniors. High school senior students (n = 25; males = 12, females = 13) successfully completed a validated *Survey to Assess the Knowledge of Conventional and Unconventional Dietary Methods of Weight Control* based on the *Dietary Guidelines for Americans 2010*. The survey also included demographic questions and items regarding sources of nutrition information. The mean nutrition knowledge score (out of 24) was 8.7 ± 2.8 or 36% (min. score = 4, max. score = 14). There was no significant difference in nutrition knowledge scores based on ethnicity, those on specialized diets, frequency of eating out, physical activity, gender, source of nutrition information, thoughts about food, or for any criteria based categorizing scores by High/Low. Though the study indicates better education is needed, this pilot test should be followed up with a larger sample size to confirm these results.

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MEET THE STUDENT-AUTHOR

Grace Heymsfield

I am from Elkins, Ark. and will be graduating from the University of Arkansas in December 2014 with a Bachelor of Science in Human Environmental Sciences with a major in Food, Human Nutrition, and Hospitality and a concentration in dietetics. I am proud to have represented the Razorbacks as a member of the cross country and track and field teams during my time at the University of Arkansas. Being a student-athlete allowed me to pursue my athletic dreams while also teaching me the value of perseverance and teamwork. Coupled with the opportunities afforded to me by the Honors College Fellowship such as the Honors Student Board and Honors menteeship, I believe I truly maximized my undergraduate experience.

After graduation, I will pursue a graduate degree in nutrition and a dietetic internship. In conjunction with my graduate degree, I hope to achieve my Registered Dietitian Nutritionist (RDN) credential.

Thank you to the outstanding dietetic faculty for all of their support and encouragement. I would particularly like to thank Cynthia Moore for her extensive guidance during the completion of this project, and committee members Kate Shoulders and Mechelle Bailey for their help with the completion of my Honors thesis. Thank you to Curt Rom for his outstanding leadership of the Bumpers Honors program, as well as for his personal support of my athletic and academic careers.

INTRODUCTION

Despite the increasing accessibility of health information in our country, adolescents continue to struggle with unhealthy weight. National rates of overweight or obese children ages 10-17 years reached 31.3% in 2011 (Ogden et al., 2012). Children who are overweight or obese are more likely to be overweight or obese in adulthood, and they are more likely to develop cardiovascular disease as well as Type II Diabetes Mellitus during childhood (CDC, 2014). Arkansas has historically had rates of overweight and obese adolescents that exceed the national average. Rates of overweight and obese Arkansas adolescents were 37.5% and 33.9% in 2007 and 2011, respectively (NSCH, n.d.). As of 2011, 15% of high school students in Arkansas were obese (CDC, n.d.).

As adolescents become more autonomous, the behavior patterns they acquire during this part of the life cycle are likely to affect long-term behaviors (Kelder et al., 1994). As they become more capable of controlling their own diet and food choices, increased nutrition knowledge during the adolescent stage is essential (Sichert-Heller et al., 2011).

Though diet is incredibly complex, nutrition knowledge has been shown to correlate with healthier food choices (Crockett and Sims, 1995; NSCH, 2012; Pirouznia, 2001; Rabiei et al., 2013; Worsley, 2002). A major study of over 1,000 adults ages 18-75 years identified that participants in the highest quintile of nutrition knowledge were nearly 25% more likely to meet current fruit, vegetable, and fat intake recommendations than those in the lowest quintile (Wardle et al., 2000). The HELENA Study was the first to examine the nutrition knowledge of a large sample of European adolescents (Sichert-Hellert et al., 2011). It was found that while body weight did not correlate with nutrition knowledge as much as parental education level, formal knowledge of dietary principles is required to make an informed food choice (Sichert-Hellert et al., 2011).

Factors influencing the health of children in Arkansas and a plan to decrease high obesity rates were both addressed in Arkansas Act 1220 of 2003. The Act outlined a set of initiatives to battle unhealthy body mass index (BMI) measures and required integration of nutrition education in school-wide curriculum (State of Arkansas, 2003). Each district's school nutrition and physical activity committee is responsible for integrating nutrition education into the overall curriculum (State of Arkansas, 2003). A means of testing the effectiveness of the nutrition curriculum in each district is lacking. High school students

are tested statewide in the areas of algebra, biology, geometry, and literacy per the Arkansas Department of Education (Arkansas Department of Education, 2014). No survey or test has been administered in Arkansas to test nutrition knowledge specifically.

The purpose of this study was to determine the level of nutrition knowledge of senior high school students in a Northwest Arkansas school district. Relationships between nutrition knowledge, sources of nutrition information, and demographic characteristics were also investigated. It was hypothesized that high school senior students have insufficient nutrition knowledge.

MATERIALS AND METHODS

The target population for this study was high school senior students from a Northwest Arkansas school district. The specific district was chosen for the study due to convenience and permission from the district. This district has only one high school. The sample selection for this study was taken from the senior graduating class of 2014. Only students who gave consent, or returned parental consent forms if less than 18 years old, were included in the study. The survey instrument was 32 items—24 of the questions were nutrition knowledge based, and there were eight added demographic questions. The 24 nutrition knowledge based questions were adapted for the purpose of this study from Cynthia Moore's *Survey to Assess the Knowledge of Conventional and Unconventional Dietary Methods of Weight Control* based on the *Dietary Guidelines for Americans 2010* (Moore, 2006).

Data collection for this study was done on-site. Paper assent forms and parental consent forms were distributed, and the researcher explained the study during a forty-minute college preparation class required of all senior students at the school district. All students were given parental consent forms regardless of age, though only students under age 18 were required to return one for participation in

Table 1. Demographic information and mean knowledge score.

Demographic[a]		Frequency[b]	Percentage	Mean[c]	Range	*P*-Value
Gender	Male	12	48%	8.5 ± 3		0.884[d]
	Female	13	52%	8.9 ± 2.8		
Ethnicity	White	19	76%			0.585[d]
	Other	6	24%	8.8 ± 2.6		
Frequency of eating out	None	7	28%	9.1 ± 2.4		0.659[e]
	1-6 times weekly	16	64%	8.8 ± 3.2		
	Daily	2	8%	7 ± 1.4		
Special Diet	Yes	1	4%	9		0.922[d]
	No	24	96%	8.7 ± 2.9		
Sources of Nutrition Info.[f]	Teacher	1	4%	14		0.055[d]
	Doctor	4	16%	9.5 ± 1.7		0.132[d]
Physical Activity	Light	11	44%	8.2 ± 2.8		0.29[e]
	30-60 mins/day	8	32%	8.3 ± 2.9		
	Hour+	6	24%	10.3 ± 2.7		
Nutrition Knowledge Score[g]				8.7 ± 2.8	4-14	

[a] Population = Senior class of high school.

[b] N = 25.

[c] Knowledge Score.

[d] t-test.

[e] ANOVA.

[f] Compared to other indicated sources of nutrition information.

[g] For entire sample.

the study. One month later, printed surveys were distributed to students with signed consent forms and parental assent forms, if under 18 years old. The guidance counselor at the school assisted in verifying birth dates according to school records. The students completed the survey with pencil under the researcher's supervision to ensure no collaboration and/or use of electronic devices to aid completion. No compensation or incentive was provided to the school district.

The software SPSS Statistics 21 (IBM Corporation, Armonk, N.Y.) was used to analyze descriptive statistics and to conduct a series of t-tests, as well as analysis of variance tests (ANOVA). The researcher investigated differences in knowledge score by gender, ethnicity, thoughts about eating, medical condition, reported sources of nutrition information, frequency of eating out, and physical activity level. Knowledge score was re-coded into "low" score and "high" score. "High score" was defined as greater than or equal to 50% (12 out of 24 correct answers or higher). The same series of t-tests and ANOVAs were conducted. Institutional Review Board approval was granted at the University of Arkansas.

RESULTS AND DISCUSSION

The survey was distributed to seventy students in a college preparatory class that is required of all students in the senior class at the school district. Forty-four students indicated a desire to participate in the study on the assent form. Nineteen students with signed assent forms were unable to complete the survey due to lack of parental consent or absence. Twenty-five students completed the survey, resulting in a response rate of 36%.

Of the twenty-five students who completed the survey, 12 (48%) were males and 13 (52%) were females (Table 1). Nineteen students (76%) identified themselves as "White (not Hispanic)" when asked about their ethnicity. The mean nutrition knowledge score (out of 24) was 8.7 ± 2.8, or 36% (Table 1). The minimum score recorded was 4, and the maximum score was 14. The mean score for females (8.9 ± 2.8) was not statistically different than the mean score for males (8.5 ± 3) ($P = 0.884$). There was no significant difference in nutrition knowledge scores based on ethnicity ($P = 0.585$), following a special diet for a medical condition ($P = 0.922$), or frequency of eating out ($P = 0.659$). No significant differences were found for any criteria based on High/Low scores.

Two listed sources of information nearly significantly correlated with nutrition knowledge scores: sources of information from a teacher ($P = 0.055$; n = 1) and sources of information from a doctor ($P = 0.132$; n = 4). It is worth noting that the survey participant who identified a teacher as a source of nutrition information also scored highest on the survey (14 questions answered correctly). A positive though not significant trend was noted between knowledge and physical activity ($P = 0.290$).

Five questions on the survey addressed fruit and vegetable consumption. Knowledge scores calculated with these five items were 40%. Sources of nutrition information varied among respondents (Table 2). Family was the most common source of nutrition information. The second most common source of nutrition information was a three-way tie between television, doctor, and Internet. When asked about daily thoughts about food, the majority of students tried to think about health when making food decisions but did not let it determine everything they ate. The second most common thought regarding food was eating whatever "sounds good at the time."

Though the findings of this study regarding nutrition knowledge were statistically insignificant, certain trends in the results were consistent with the literature. First and foremost, the results of this study supported the concerns raised by various sources regarding poor nutrition knowledge of adolescents. Adolescents in this study were found to have low nutrition knowledge scores as assessed by this instrument regardless of age, gender, and ethnicity.

A particularly troublesome area of adolescent nutrition is low fruit and vegetable intake. Five items on the survey instrument addressed fruit and vegetable recommendations. Nutrition knowledge calculated with these five items alone was 40%. This is higher than the overall score recorded on the survey (36%), but not substantially.

Table 2. Sources of nutrition information.

Response	Total (N)[a]	Percentage
Family	11	44%
Friends	3	12%
Television	4	16%
Dietitian	0	0%
Doctor	4	16%
Nurse	1	4%
Internet	4	16%
Magazines	1	4%
Books	0	0%
Texbooks	0	0%
Food Advertisements	2	8%
Other: _____		
Teacher	1	4%
Coach	1	4%
Do Not Receive Information	1	4%
School	1	4%

[a]Multiple responses allowed.

Students' responses regarding daily thoughts about eating were also consistent with the literature. Increasingly autonomous adolescents may not see the need to concern themselves with sound nutrition during their current stage of life; 52% did not let health concerns determine everything they ate, 20% ate according to convenience, and 32% ate whatever sounded good at the time. Thus, nutrition education and intervention efforts may need to address the importance of health during adolescence in terms of future consequences, particularly because obesity in adolescence is a strong risk factor for obesity in adulthood (CDC, 2014).

The trend identified in this study support the hypothesis that adolescents across the state and nation are lacking in nutrition knowledge. Research on this topic should not stop here. Assessment of nutrition knowledge of adolescents should be made region-wide (Northwest Arkansas) and statewide (Arkansas) if improvements in statewide nutrition curriculum are to be suggested. Replication of this pilot study with the revisions addressed earlier could aid in understanding how to best improve nutrition education for high school students.

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LITERATURE CITED

Arkansas Department of Education. 2014. Testing Calendar. <http://www.arkansased.org/events/event_categories/test-dates> Accessed 16 February 2014.

(CDC) Center for Disease Control and Prevention. 2014. Adolescent and school health: Childhood obesity facts. <http://www.cdc.gov/healthyyouth/obesity/facts.htm> Accessed 26 February 2013.

(CDC) n.d. Centers for Disease Control and Prevention. The obesity epidemic and Arkansas students. <http://www.cdc.gov/healthyyouth/yrbs/pdf/obesity/ar_obesity_combo.pdf> Accessed 5 March 2013.

Crockett S.J. and L.S. Sims. 1995. Environmental influences on children's eating. J Nutr Educ. 27(5):235.

Kelder S.H., Perry C.L., Klepp K., and L.L. Lytle. 1994. Longitudinal tracking of adolescent smoking, physical activity, and food choice behaviors. Am J Public Health. (07); 84(7):1121-1126.

Moore, C.K. 2006. Development and validation of an instrument to assess the knowledge of adults regarding conventional and unconventional dietary methods of weight control. University of Alabama.

(NSCH) n.d. National Survey of Children's Health. Arkansas State Fact Sheet. Data analysis provided by the Child and Adolescent Health Measurement Initiative, Data Resource Center. <http://childhealthdata.org/docs/nsch-docs/arkansas-pdf.pdf.> Accessed 5 March 2013.

(NSCH) National Survey of Children's Health. NSCH 2011/12. Data query from the Child and Adolescent Health Measurement Initiative, Data Resource Center for Child and Adolescent Health website.<www.childhealthdata.org.> Accessed 5 March 2013.

Ogden C.L., Carroll M.D., Kit B.K., and K.M. Flegal. 2012. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. J Am. Med. Assoc. 307(5):483-490.

Pirouznia, M. 2001. The association between nutrition knowledge and eating behavior in male and female adolescents in the US. Int. J. Food Sci. Nutr. 52(2):127-32.

Rabiei L., Sharifirad G., Azadbakht L., and A. Hassanzadeh. 2013. Understanding the relationship between nutritional knowledge, self-efficacy, and self-concept of high-school students suffering from overweight. J. Edu. Health Promot. (07);2:39.

Sichert-Hellert W., Beghin L., De Henauw S., Grammatikaki E., et al. 2011. Nutritional knowledge in European adolescents: results from the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) study. Public Health Nutr. (08);14(12):2083-2091.

State of Arkansas. 84th General Assembly. House of Representatives. 2003. Act 1220: An Act to Create a Child Health Advisory Committee; To Coordinate Statewide Efforts to Combat Childhood Obesity and Related Illnesses; To Improve the Health of the Next Generation of Arkansans; and for Other Purposes. 84th General Assembly, Regular Session, April 10, 2003.

Wardle J., Parmenter K., and J. Waller. 2000. Nutrition knowledge and food intake. Appetite. (06);34(3):269-275.

Worsley, A. 2002. Nutrition knowledge and food consumption: Can nutrition knowledge change food behaviour? Asia Pacific J Clin Nutr. 11(Suppl): 579-585.

Presence of antibiotic resistance genes from wastewater treatment plant effluent in Northwest Arkansas

Ryan S. MacLeod and Mary C. Savin†*

ABSTRACT

Antibiotic resistance genes (ARGs) among bacterial populations are causing increasing concern with medical and agricultural implications. While the effluent of wastewater treatment plants (WWTPs) is treated with a variety of antimicrobial methods, bacteria and the genetic material that is able to pass on antibiotic resistance to environmental populations are not completely destroyed. Ampicillin (amp), tetracycline (tet), and sulfonamide (sul) antibiotics have been detected in Northwest Arkansas (NWA) streams, and IncP plasmids—which are especially notorious for containing antibiotic resistance genes and have been detected after disinfection in NWA WWTPs—are known to carry ARGs for those antibiotics. The objective of this inquiry was to determine whether ARGs of commonly used antibiotics (*ampC* and *oxa2* for ampicillin, *tetA* for tetracycline, and *sul1* for sulfonamide resistance) were present in effluent following disinfection that demonstrated variable reduction in IncP plasmid numbers. DNA was extracted from water collected from a WWTP that uses ultraviolet light and a WWTP that uses chlorination and was participating in a pilot-scale ozonation test. Three of the four ARGs were detected using polymerase chain reaction (PCR) both before and after all three disinfection methods. The ampicillin resistance gene *ampC* was the only gene that was detected in less than two-thirds replications either before or after disinfection. Given the PCR results and previous quantitative PCR analysis of IncP plasmid concentrations, it appears there is little reduction of ARGs after disinfection. These data are important in understanding the role of WWTPs in contributing to the spread of antibiotic resistance in the environment.

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† Mary Savin is the faculty mentor and is a professor in the Department of Crop, Soil, and Environmental Sciences.

MEET THE STUDENT-AUTHOR

Ryan MacLeod

I am a proud Arkansan, and was born and raised in Little Rock. I graduated from Little Rock Central High School in 2010. The following fall semester, I began studying Biology at the University of Arkansas with a minor study in Environmental, Soil, and Water Science. I approached Dr. Mary Savin about research opportunities in her lab during my sophomore year, and she graciously allowed me to conduct my research study in her lab and provided me with mentoring and advising throughout the project. I received funding from the State Undergraduate Research Fellowship and the Honors College to facilitate my research endeavors.

I plan to attend medical school at the University of Arkansas for Medical Sciences where I am enrolling in a dual MD/PhD program in order to practice medicine and continue fulfilling my research goals. Ultimately, I hope to manage my own research programs in an academic hospital.

INTRODUCTION

Since the discovery of antibiotics and their medical, agricultural, and industrial uses, widespread use of antibiotics has contributed to antibiotic resistance in clinical and natural settings (Karlowsky et al., 2003, Wenzel et al., 2003). As antibiotic resistance spreads throughout bacterial species, many pathogenic bacterial infections are becoming increasingly difficult to treat in humans and agricultural animals (Aminov and Mackie, 2007). Within the last ten years, there has been worldwide recognition of the increase in rates of antimicrobial resistance, and an increase in the frequency of species with multidrug-resistance in a clinical setting has been demonstrated (Cantón, 2009).

One area of particular concern is the dissemination of broad-host range (BHR) plasmids containing antibiotic resistance genes (ARGs) throughout bacterial populations in the environment. Some examples of BHR plasmids are the IncP, IncN, and IncQ families of plasmids. They carry a wide range of resistances to compounds including antibiotics (β-lactams, tetracylines, sulfonamides, erythromycins, and others) and heavy metals (Schlüter et al., 2007). Broad-host range plasmids are able to transfer to and replicate within many different species of bacteria. Even when BHR plasmids themselves do not contain ARGs, they may still mobilize plasmids that do encode resistance yet have no mechanism to transfer themselves by facilitating the formation of a conjugation bridge and recruiting DNA polymerase (Davison, 1999; Thomas and Nielsen, 2005).

The IncP plasmid is a BHR plasmid that is known to carry antibiotic resistance genes (Schlüter et al., 2003). It is considered to be the most promiscuous of the BHR plasmids that have been studied. The IncP plasmids are also known to encode heavy metal—such as mercury—and chlorinated organic compound resistance (Schlüter et al., 2007). These plasmids are found worldwide and have been detected in enteric bacterial species that had not previously contained IncP plasmids before widespread use of antibiotics (Smith and Thomas, 1987).

The IncP plasmid family is commonly detected in wastewater treatment plants (Dröge et al., 2000). Wastewater treatment plants (WWTPs) are considered hotbeds for ARGs and for plasmids that are capable of the horizontal transfer of these genes across species of gram-negative bacteria, even reaching gram-positive species and eukaryotic species such as yeast (Schlüter et al., 2007; Dröge et al., 2000). These WWTPs are seen as interfaces between different environmental compartments including hospitals, surface waters, and residential wastes. The aerators and clarifiers of WWTPs have high bacterial densities and conditions that promote metabolic activities that facilitate genetic exchange in the treatment facil-

ity (Mancini et al., 1987). Among the mobile genetic materials that can transfer resistance genes, several are BHR plasmids (Schlüter et al., 2007). Although the effluent of WWTPs is treated with a variety of antimicrobial methods, none of the current methods completely destroys all the bacteria or mobile genetic material capable of passing on antibiotic resistance (Asfahl and Savin, 2012).

Asfahl and Savin (2012) detected a 95% reduction in IncP plasmids using an experimental ozonation disinfection procedure. Despite a significant reduction in plasmid copy number, 2.2×10^5 copies per liter of the IncP gene remained in effluent to be discharged into the stream. Any amount of these plasmids that is released into the environment can result in the replication and dissemination of antibiotic resistance genes when they enter bacterial populations. Examples of common antibiotics—some of which are found in Northwest Arkansas (NWA) streams and all of which have resistance genes present on IncP plasmids—are ampicillin, tetracyclines, and sulfonamides (Galloway et al., 2005; Haggard and Bartsch, 2009; Schlüter et al., 2003).

The goal of this research is to determine whether resistance genes of commonly used antibiotics are present in effluent known to contain and demonstrate varying levels of reduction in IncP plasmids following disinfection. Previous research evaluated reduction in IncP plasmid number in effluent as a known carrier of resistance determinants; however, destruction of antibiotic resistance genes was not measured directly and needs to be confirmed or refuted. The objective of this experiment was to determine the presence of these resistance genes in the samples both before and after disinfection by chlorination, ozonation, and UV radiation.

Based on the amount of DNA left after disinfection in the effluent of the WWTPs and the concentration of resistance genes on IncP plasmids (Asfahl and Savin, 2012), genes that code for resistance to the antibiotics mentioned above—ampicillin, tetracyclines, and sulfonamides—were expected to be found. Since ampicillin and tetracycline genes were detected more frequently in WWTPs (Yang et al., 2012; Liu et al., 2012), those resistance genes were expected to be more prevalent in the samples than the sulfonamide resistance genes.

MATERIALS AND METHODS

The DNA used in this experiment was extracted using a bead-beating protocol from water samples before and after three disinfection techniques (ultraviolet light in Fayetteville, chlorination in Springdale, and ozonation pilot study in Springdale) in NWA WWTPs (Asfahl and Savin, 2012). Single one-liter samples were taken three different days from the Fayetteville West WWTP and six different days from the Springdale WWTP. There were three replications for Fayetteville's upstream and ultraviolet-treated samples, three replications for Springdale's ozone-treated samples, and six replications for Springdale's upstream and chlorine-treated samples. The DNA was cleaned by ethanol precipitation, quantified by Nanodrop spectrophotometry and has been maintained in a -77 °C freezer. Polymerase chain reaction (PCR) was used to determine the presence of the resistance genes *ampC* and *oxa2* (resistance to ampicillin), *tetA* (resistance to tetracyclines), and *sul1* (resistance to sulfanomides). Template DNA was amplified in 20-µL volumes in a PTC-200 thermocycler (MJ Research, Waltham, Mass.) in the presence of 1X PCR buffer,1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Promega Corporation, Madison, Wis.), appropriate primer (Table 1), 0.04 % bovine serum albumin (Merck KGaA, Darmstadt, Germany), and GoTaq DNA polymerase (Promega, Madison, Wis.).

Positive and negative controls were run with every batch of PCR reactions. A plasmid (RP4) in the *IncP* family was used as the positive control for the *tetA*, *oxa2*, and *sul1* genes, while an environmental sample that was found to consistently amplify the correct fragment size was used for the *ampC* gene. The negative controls consisted of all the reagents used in the PCR batch without the addition of template DNA. Amplification was confirmed using ethidium bromide agarose gel electrophoresis and

Table 1. Polymerase chain reaction primer sequences used successfully with environmental DNA samples.

Gene	Primer Sequence	Annealing Temperature (°C)	Expected Product size (bp) (Source)
ampC	FW- CCT CTT GCT CCA CAT TTG CT	58	189 (Yang et al., 2012)
	RV- ACA ACG TTT GCT GTG TGA CG		
oxa2	FW- TCT TCG CGA TAC TTT TCT CCA	60	177 (Yang et al., 2012)
	RV- ATC GCA CAG GAT CAA AAA CC		
tetA	FW- GCT ACA TCC TGC TTG CCT TC	61	210 (Liu et al., 2012)
	RV- CAT AGA TCG CCG TGA AGA GG		
sul1	FW- CGC ACC GGA AAC ATC GCT GCA C	56	163 (Pei et al., 2006)
	RV- TGA AGT TCC GCC GCA AGG CTC G		

the Kodak EDAS 290 (Eastman Kodak, Rochester, N.Y.). The size of the products was determined using the mass molecular standard ruler (bands from 1000 bp to 100 bp), which also can be used to determine DNA concentrations of the PCR products by comparing the intensity of the bands to the standard's band intensities. Reduction, or the absence of PCR amplification for a particular gene fragment after disinfection, following the presence of PCR amplification upstream of the disinfection procedure was determined to be statistically significant by conducting a T-test.

RESULTS AND DISCUSSION

Three of the four antibiotic resistance genes (*oxa2*, *tetA*, and *sul1*) were amplified across all replications before and after disinfection across all three disinfection protocols utilized in both WWTPs (Table 2, Figs. 1-2). Qualitatively, the intensity of the *sul1* band fragments indicates a concentration of 10-20 ng/μL DNA amplifed in the upstream site (i.e. prior to disinfection) with no reduction of intensity, or DNA concentration amplified, downstream of disinfection (Fig. 1). Similar results were obtained for the *tetA* tetracycline resistance gene fragment (Fig. 2). The *oxa2* gene also amplified in all replications both before and after all three disinfection methods (Table 2).

The fourth antibiotic resistance gene, *ampC*, was only amplified in 33% of upstream replications in the Fayetteville WWTP and 66% of upstream replications in the Springdale WWTP (Table 2). The replications that did contain *ampC* amplification still contained bands after disinfection for the UV-treated Fayetteville effluent, while half of the chlorinated Springdale replications that initially contained *ampC* no longer amplified the gene after disinfection (Table 2). All of the ozonation replications at the Springdale WWTP no longer contained *ampC* amplification after disinfection.

Table 2. Polymerase chain reaction results for presence of antibiotic resistance genes.

WWTP[a]	Number of Replications	Disinfection Method	Amplification (%)			
			ampC[c]	*oxa2*	*tetA*	*sul1*
Fayetteville	3	Upstream[b]	33	100	100	100
	3	UV	33	100	100	100
Springdale	6	Upstream	66	100	100	100
	6	Chlorination	33[d]	100	100	100
	3	Ozonation	0[d]	100	100	100

[a]Wastewater treatment plant.
[b]Upstream samples are for effluent collected prior to disinfection. UV (ultraviolet radiation), chlorination, and ozonation are for effluent collected after disinfection.
[c]The *ampC* and *oxa2* genes code for ampicillin resistance. The *tetA* gene codes for tetracycline resistance and the *sul1* gene codes for resistance to sulfonamides.
[d]These differences by chlorination and ozonation at the Springdale WWTP were significant at $P < 0.05$.

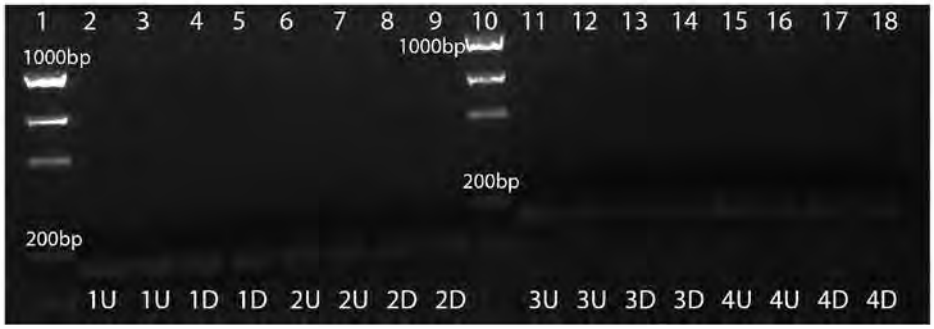

Fig. 1. Selected agarose gel showing polymerase chain reaction products. The gene amplified was *sul1* with an expected product size of 163 bp. Samples from Springdale wastewater treatment plant upstream (U, before) and downstream (D, after) disinfection. Lanes 1, 10 are mass molecular standard. Lanes 2-5 are from replication 1, lanes 6-9 are replication 2, lanes 11-14 are replication 3, and lanes 15-18 are replication 4.

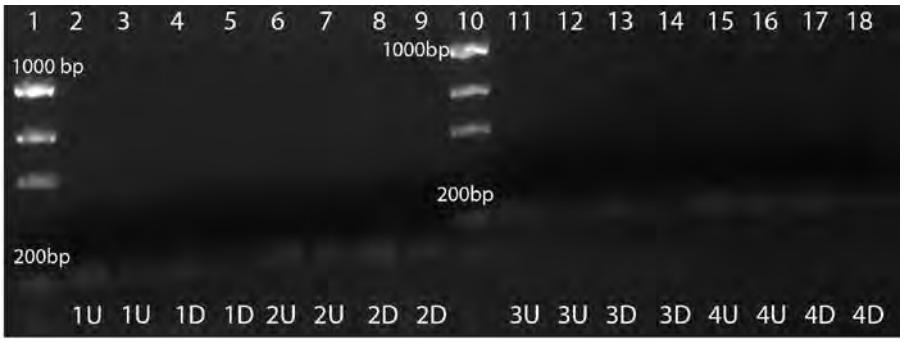

Fig. 2. Selected agarose gel showing *tetA* gene amplification. The expected product size for *tetA* was 210 bp. Samples are from Springdale wastewater treatment plant upstream (U, before) and downstream (D, after) disinfection. Lanes 1, 10 are mass molecular standard. Lanes 2-5 are from replication 1, lanes 6-9 are from replication 2, lanes 11-14 are from replication 3, and lanes 15-18 are from replication 4.

The UV and chlorination treatments of WWTP effluent yielded insignificant reduction levels of IncP plasmid DNA (Asfahl and Savin, 2012). At the Fayetteville WWTP using UV disinfection, 2.7 (± 8.2) × 10^6 copies per liter were detected before disinfection and 0.8 (± 2.2) × 10^6 copies per liter were detected after disinfection (Asfahl and Savin, 2012). Springdale's chlorination disinfection was also not significant with 2.7 (± 1.8) × 10^7 copies per liter detected upstream and 4.2 (± 1.5) × 10^6 copies per liter after disinfection (Asfahl and Savin, 2012). The amplification of *sul1*, *tetA*, and *oxa2* reveal that these were not eliminated from the water samples collected after each of the disinfection methods, and thus may persist and disseminate into new hosts either because of their mobility or facilitated mobility by other genetic elements. These genes are likely to be located either on mobile genetic elements that contain a sequence that facilitates the formation of a junction between two bacteria during the conjugation process or on genetic elements that are replicated through an existing conjugation bridge (Davison, 1999; Thomas and Nielsen, 2005). The IncP plasmids are notorious for both initiating the conjugation process for their own dissemination and facilitating the replication of non self-mobilizing genetic elements (Schlüter et al., 2007). That leaves a possibility for antibiotic resistance genes to be released and spread throughout the environmental populations of bacteria.

The PCR results for *tetA*, *sul1*, *oxa2*, and *ampC* are consistent with the continued detection of total DNA and specifically IncP plasmids upstream and downstream of disinfection (Asfahl and Savin, 2012). From the quantitative PCR results in Asfahl and Savin (2012), it is clear that UV radiation, chlorination, and ozonation do not effectively eliminate mobile genetic elements that carry antibiotic resistance genes from the WWTP effluent. Even following a significant reduction after ozonation in IncP plasmids as indicated by 95% reduction in amplified gene fragment, there were 2.2 (± 0.7) × 10^5 copies per liter remaining. With 45 million liters of water discharged daily from Springdale WWTP, the potential load into Spring Creek is 9.9 × 10^{12} IncP plasmid copies per day. If each IncP plasmid contained one copy of each resistance gene *oxa2*, *sul1*, and *tetA*, then it becomes easier to understand how WWTPs can influence resistance in the environment.

With the exception of the low detection of *ampC*, these results are in agreement with the growing database of antibiotic resistance genes detected in WWTP effluent. Studies involving sulfonamide, tetracycline, and ampicillin resistance genes across a wide range of locations in East Asia and North America have detected many of these genes in the WWTP and associate effluent, specifically *oxa2*, *ampC*, *tetA*, and *sul1* (Liu et al., 2012; Pei et al., 2006; Yang et al., 2012). The *tetA* gene and many other genes conferring tetracycline resistance were detected before and after treatment of antibiotic production wastewater at a manufacturing plant in Hebei Province, China (Liu et al., 2012). The ampicillin resistance gene *ampC* was detected in 100% of WWTPs, and *oxa2* was detected in 93% of the Asian and North American WWTPs tested in a previous study (Yang et al., 2012). The *sul1* gene was detected in sediments of the Poudre River in northern Colorado across all sampling sites, including one downstream from an urban WWTP (Pei et al., 2006).

The results are also consistent with the presence in Northwest Arkansas streams of the antibiotics for which the genes encode resistance. Sulfamethoxazole (part of the sulfonamide group of antibiotics) and, to a lesser extent, tetracycline, have been detected downstream of Fayetteville WWTPs effluent discharge into Mud Creek

(Galloway et al., 2005; Haggard and Bartsch, 2009). The presence of these drugs in the waters can put selective pressure on the environmental populations of bacteria to develop or acquire resistance genes. These genes can be spread in WWTPs, which have been known to have conditions conducive for conjugation, transduction, and transformation (Schlüter et al., 2007; Dröge et al., 2000).

Furthermore, the PCR results showing persistence of antibiotic resistance genes following disinfection are consistent with the linkage of ampicillin, tetracycline, and sulfonamide resistance genes to the IncP plasmid family. The genes *sul1*, *tetA*, and *oxa2* have all been sequenced and linked to multiple plasmids belonging to the IncP family (Schlüter et al., 2007). The absence of the *ampC* gene in many of the replications was unexpected considering the gene's detection in effluent of 100% of WWTPs studied in Asia and North America and the gene's presence on multiple plasmids that carry resistance (Yang et al., 2012). However, these results are interesting since *ampC* is not expected to be associated with IncP plasmids (Yang et al., 2012). The *ampC* gene is located on an assortment of conjugative plasmids including pGC-1 and pGC-2 (Bou et al., 2000). The *oxa2* ampicillin resistance gene is more frequently found on mobile genetic elements than ampC, however, because mobile *ampC* genes are derived from the chromosomal DNA and are not as prevalent on plasmids (Bou et al., 2000).

The nonexistent reduction in the number of samples in which *oxa2*, *sul1*, and *tetA* genes were detected may reflect the results and lack of IncP plasmid DNA destruction detected in Asfahl and Savin (2012) because of the previously stated connection of these genes with IncP plasmids and other DNA. Even though the ozone treatment reduced IncP levels by 95%, it only reduced the total DNA concentrations by 80%. That leaves a possibility for other antibiotic resistance genes that are not associated with IncP plasmids specifically to be released and spread throughout the environmental populations of bacteria.

CONCLUSIONS

Antibiotic resistance gene detection both upstream and downstream of UV irradiation, chlorination, and ozonation treatments of wastewater shows persistence and thus suggests the potential for dissemination of these genes in streams through WWTP effluent. The results from this and similar studies suggest that new methods of disinfection or different policies concerning regulation of DNA concentrations in WWTP effluent need to be developed to avoid the spread of antibiotic resistance genes. The need to at least slow the dissemination of these genes is exemplified by the growing trend of multidrug-resistant, pathogenic species of bacteria. By continuing to allow antibiotic resistance genes to be released into the environment, the reservoir of resistance genes increases and there is a greater chance of pathogenic bacteria acquiring resistance. This creates problems for disease control in clinical, agricultural, and industrial settings. Future research opportunities with this study should involve quantifying antibiotic resistance gene reduction resulting from disinfection using a quantitative PCR analysis.

ACKNOWLEDGEMENTS

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LITERATURE CITED

Aminov, R.I. and R.I. Mackie.2007. Evolution and ecology of antibiotic resistance genes. FEMS Microbiol. Lett. 271: 147-161.

Asfahl, K.L. and M.C. Savin. 2012. Destruction of *Escherichia coli* and broad-host-range plasmid DNA in treated wastewater by dissolved ozone disinfection under laboratory and field conditions. Adv. Microbiol. 2:1-7.

Bou, G., A. Oliver, M. Ojeda, C. Monzón, and J. Martínez-Beltrán. 2000. Molecular characterization of FOX-4, a new AmpC-type plasmid-mediated β-lactamase from an *Escherichia coli* strain isolated in Spain. Antimicrob. Agents Ch. 44(9):2549-2553.

Cantón, R. 2009. Antibiotic resistance genes from the environment: A perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin. Microbiol. Infect. 15(1):20–25.

Davison, J. 1999. Genetic exchange between bacteria in the environment. Plasmid 42(2):73–91.

Dröge, M., A. Pühler, and W. Selbitschka. 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Molec. Genet. Genom. 263(3):471-482.

Galloway, J.M., B.E. Haggard, M.T. Meyers, and W.R. Green. 2005. Occurrence of Pharmaceuticals and Other

Organic Wastewater Constituents in Selected Streams in Northern Arkansas, 2004 (Science Investigations Report 2005-5140). U.S. Geological Survey, Reston, Va.

Haggard, B. and L. Bartsch. 2009. Net changes in antibiotic concentrations downstream from an effluent discharge. J. Environ. Qual.. 38(1):343-352.

Karlowsky, J.A., D.C. Draghi, M.E. Jones, C. Thornsberry, I.R. Friedland, and D.F. Sahm. 2003. Surveillance for antimicrobial susceptibility among clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2001. Antimicrob. Agents Ch. 47(5):1681-1688.

Liu, M., Y. Zhang, M. Yang, Z. Tian, L. Ren, and S. Zhang. 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environ. Sci. Technol. 46(14):7551-7557.

Mancini, P., S. Fertels, D. Nave, and M.A. Gealt. 1987. Mobilization of plasmid pHSV106 from *Escherichia coli* HB101 in a laboratory-scale waste treatment facility. Appl. Environ. Microbiol. 53(4):665-671.

Pei, R., S.C. Kim, K.H. Carlson, and A. Pruden. 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (arg). Water Res. 40(12):2427-2435.

Schlüter, A., H. Heuer, R. Szczepanowski, L.J. Forney, C.M. Thomas, A. Pühler, and E.M. Top. 2003. The 64 508 bp IncP-1b antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1b group. Microbiology, 149(11):3139-3153.

Schlüter, A., R. Szczepanowski, A. Pühler, and E.M. Top. 2007. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. FEMS Microbiol. Rev. 31(4):449-477.

Smith, C.A. and C.M. Thomas. 1987. Comparison of the organization of the genomes of phenotypically diverse plasmids of incompatibility group P: members of the IncP beta sub-group are closely related. Molec. Genet. Genom. 206(3):419–427.

Thomas, C.M. and K.M. Nielsen. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3(9):711–721.

Wenzel, R.P., D.F. Sahm, C. Thornsberry, D.C. Draghi, M.E. Jones and J.A. Karlowsky. 2003. In vitro susceptibilities of Gram-negative bacteria isolated from hospitalized patients in four European countries, Canada, and the United States in 2000-2001 to expanded-spectrum 40 cephalosporins and comparator antimicrobials: Implications for therapy. Antimicrob. Agents Ch. 47(10):3089-3098.

Yang, Y., T. Zhang, X.X. Zhang, D.W. Liang, M. Zhang, D.W. Gao, H.G. Zhu, Q.G. Huang, and H.P. Fang. 2012. Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. Appl. Microbiol. Biot. 95(5):1351-1358.

Alteration of host cell ubiquitination by the intracellular bacterial pathogen *Coxiella burnetii*

Lindsey E. Pritchett* and Daniel E. Voth†

ABSTRACT

The intracellular bacterial agent of Q fever, *Coxiella burnetii*, replicates within a phagolysosome-like parasitophorous vacuole (PV) in human macrophages and delivers effector proteins to the host cytosol via a Dot/Icm type IV secretion system (T4SS). The T4SS effectors are critical for PV formation and prevention of host cell death that allows sufficient time for bacterial replication. Recruitment of ubiquitin-related components to the *C. burnetii* PV is also predicted to be involved in PV formation and bacterial replication and is likely controlled by effector proteins. In this study, we assessed the role of the Dot/Icm T4SS in regulating ubiquitination by comparing subcellular localization of ubiquitinated proteins between cells infected with *C. burnetii* and a mutant that lacks a functional T4SS. Fluorescence microscopy showed ubiquitinated proteins surrounding wild-type *C. burnetii* PV but not phagosomes harboring T4SS-defective organisms. Immunoblot analysis showed altered ubiquitinated protein profiles throughout infection, suggesting *C. burnetii* impacts post-translational modification of host cell and/or bacterial proteins. Future studies will determine how T4SS-mediated recruitment of ubiquitinated proteins impacts *C. burnetii*-host cell interactions and eventual development of disease.

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MEET THE STUDENT-AUTHOR

Lindsey Pritchett

I am a 2011 graduate of Bergman High School and a 2014 graduate of the University of Arkansas, Fayetteville with a bachelor's degree in poultry science. I graduated Magna Cum Laude with Honors and received a silver medal in the Outstanding Honors Thesis competition. During my undergraduate career, I was involved in the Poultry Science Club and Sigma Alpha, a professional agricultural sorority. I also worked as a lab technician at the Tyson Food Safety Research Laboratory and at George's Inc. in their microbiology lab. In my free time I enjoy playing with my dog Roxie, reading a good book, and taking kickboxing lessons.

This summer, I will begin research rotations in the Ph.D. program in Molecular Pathogenesis & Therapeutics at the University of Missouri in Columbia. After I obtain my Ph.D., I would like to work either at an academic or government institution conducting biodefense-related research.

This research project was carried out in conjunction with the Arkansas IDeA Network for Biomedical Research Excellence (INBRE) at the University of Arkansas for Medical Sciences under the mentorship of Dr. Daniel Voth. I'd like to thank all members of the Voth laboratory for their assistance throughout this project.

INTRODUCTION

Ubiquitination is a post-translational protein modification used by eukaryotic cells to control protein fate and location. This three-step process begins with free ubiquitin binding to the E1 ubiquitin-activating enzyme followed by transfer of activated ubiquitin to the E2 ubiquitin-conjugating enzyme. Finally, the ubiquitin molecule is either tethered to its target substrate directly by the E3 ubiquitin-protein ligase or using an E3 adaptor that binds to E2 and the substrate. Eukaryotes encode two E1 proteins, 37 E2 proteins, and over 600 E3 ligases, providing specificity (Komander, 2009). Two general types of ubiquitination can occur and have distinct effects on protein activity. Mono-ubiquitination acts as a protein-targeting signal and often guides plasma membrane-associated proteins to endosomes (Hicke and Dunn, 2003; Patel et al., 2009; Collins and Brown, 2010). Poly-ubiquitination targets proteins to the 26S proteasome or lysosomes for degradation (Thrower et al., 2000; Chen and Sun, 2009). Poly-ubiquitination is typically controlled by the Skp1-Cul1-F-box (SCF) complex that functions as an E3 ligase (Jackson and Eldridge, 2002).

Many intracellular pathogens exploit host ubiquitination to alter host or bacterial protein stability and function. For example, *Listeria monocytogenes* secretes a pore-forming toxin known as listeriolysin O (LLO) that lyses endosomal membranes, allowing bacterial escape to the host cell cytoplasm and subsequent replication. The LLO is then ubiquitinated for degradation to avoid destroying the host cell (Collins and Brown, 2010). *Salmonella typhimurium* uses a needle-like type III secretion system (TTSS) to secrete effector proteins, such as SopB, into the host cytosol. In the cytosol, SopB promotes bacterial replication following mono-ubiquitination (Collins and Brown, 2010). *Legionella pneumophila* also secretes effector proteins, via a Dot/Icm type IV secretion system (T4SS), which interact with ubiquitination machinery. Poly-ubiquitinated proteins also accumulate on wild-type *L. pneumophila* vacuoles (Ivanov and Roy, 2009).

Coxiella burnetii is an intracellular, Gram-negative bacterium that causes the zoonosis human Q fever. Humans are typically infected with *C. burnetii* following inhalation of contaminated aerosols while working with infected livestock (Marrie, 1990). Q fever presents acutely as a flu-like illness, but can also persist chronically, causing life-threatening endocarditis. Due to the flu-like nature of acute Q fever symptoms, many cases are misdiagnosed. However, *C. burnetii* has the potential for use as a biological weapon due to a low infectious dose (<10 organisms), aerosol-mediated transmission, and pronounced environmental stability (Williams, 1991; Gilk et al., 2009). As a United States Center for Disease Control and Prevention category B select agent, the organism warrants extensive research. Additionally, the number of Q fever cases is on the rise worldwide and a better under-

standing of pathogenic mechanisms is needed to identify new therapeutics.

In vivo, *C. burnetii* displays tropism for alveolar macrophages and thrives within a lysosome-like compartment termed the parasitophorous vacuole (PV). The PV formation and maturation occurs through the normal phagolysosomal pathway. The phagosome fuses with host autophagosomes, recruits GTPases (guanosine triphosphate hydrolases) involved in vesicle fusion (Rab5 and Rab7), and eventually acquires lysosomal hydrolases following lysosome fusion with the expanding vacuole. After maturation to a phagolysosome, the PV expands and is maintained by proteins that interact with the host cell following T4SS-mediated translocation to the cytosol (Gilk et al., 2009).

Two recently identified T4SS effectors co-localize with ubiquitinated proteins when expressed in human epithelioid carcinoma (HeLa) cells, suggesting the pathogen interacts with host ubiquitin machinery. CpeC (*Coxiella* plasmid effector protein C) is an effector protein that contains an F-box domain, which is part of the three-component SCF ubiquitination complex in eukaryotic cells (Voth et al., 2011). CpeL (*Coxiella* plasmid effector protein L) was also recently identified as an effector protein that co-localizes with ubiquitinated proteins (Maturana et al., 2013). To test the hypothesis that *C. burnetii* uses T4SS effectors, such as CpeC and CpeL, to exploit host cell ubiquitin machinery, we compared subcellular localization of ubiquitinated proteins between cells infected with wild-type *C. burnetii* and a mutant that lacks a functional T4SS.

MATERIALS AND METHODS

Bacteria and Mammalian Cell Culture. Avirulent *C. burnetii* (Nine Mile phase II, clone RSA439) and a mutant strain with a disrupted *icm*D gene (Beare et al., 2011) were used for infections in this study. Cells infected with the IcmD mutant were grown in the presence of kanamycin (375 μg/ml) for selection. The THP-1 human monocytic cells and HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Prior to infection, THP-1 cells were differentiated into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (200 nM) for 24 h as previously described (Voth et al., 2007). In the final portion of this study, HeLa cells were grown in the presence of lactacystin (5 mM), a known proteasome inhibitor.

Immunofluorescence Microscopy. After infection of THP-1 or HeLa cells with *C. burnetii* at a multiplicity of infection ~10 for 4, 24, 48, 72, or 96 h, cells were fixed and permeabilized with 4% paraformaldehyde for 15 min, then blocked for 1 h in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100 at room temperature. Cells were then incubated at room temperature with either mouse anti-FK1 antibody (1:100) or mouse anti-FK2 antibody (1:250) and rabbit anti-*C. burnetii* antibody (1:1000) for 1 h. Cells were then washed three times with ice-cold PBS and incubated for 1 h in 0.3% Triton X-100-0.5% BSA-PBS containing AlexaFluor-488 or -594-conjugated secondary antibodies at room temperature. Cells were then washed twice with ice-cold PBS and incubated with DAPI for 5 min at room temperature to detect host and bacterial DNA. Fluorescence microscopy was performed using a Nikon Ti-U microscope with a 60X oil immersion objective. Images were obtained with a D5-QilMc digital camera and analyzed using NIS-Elements software. The PV were quantified from 5-10 fields, containing at least 10 cells/field. Wild-type PV were compared to mutant-containing phagosomes at each time point. Graphpad software was used for statistical analyses, and significance of differences was assessed using a Student's t-test where $P < 0.01$.

Immunoblot Analysis. After infection of THP-1 cells for 4, 24, 48, or 72 h, cells were harvested in lysis buffer containing 1% sodium dodecyl sulfate (SDS) and total protein

Table 1. Antibody concentrations for western blot analysis.

Antibody	Host	Specificity
anti-FK1	mouse	poly-ubiquitinated conjugates
anti-FK2	mouse	mono- and poly-ubiquitinated conjugates
anti-Skp1	rabbit	endogenous levels of total Skp1
anti-UBC3	rabbit	endogenous levels of total UBC3 and UBC3B
anti-K48	rabbit	poly-ubiquitin chains formed by Lys48 linkage
anti-K63	rabbit	poly-ubiquitin chains formed by Lys63 linkage

concentration was determined using a detergent-compatible (DC) protein assay. Nine micrograms of total protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane, and immunoblotting was performed using mouse antibodies directed against ubiquitin-related components (1:1000; Table 1). Samples were also probed for equal protein loading using a mouse antibody directed against β-tubulin (1:1000). Reacting proteins were detected using either an anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2000) and enhanced chemiluminescence following exposure to film. Densitometric analysis was performed to quantify the amount of proteins resolved by SDS-PAGE.

RESULTS AND DISCUSSION

Recruitment of Ubiquitinated Proteins to the C. burnetii Parasitophorous Vacuole. To determine if *C. burnetii* infection influences host ubiquitin processes, we first assessed whether ubiquitinated proteins are recruited to the PV membrane similar to reports for other intracellular pathogen replication vacuoles (Ivanov and Roy, 2009; Huang et al., 2012). Cells were assessed for ubiquitinated protein localization over the course of a 96-h infection using fluorescence microscopy (Fig. 1). Samples were incubated with FK1 antibody to detect poly-ubiquitinated proteins or FK2 antibody to detect mono- and poly-ubiquitinated proteins. The FK1- and FK2-labeled proteins

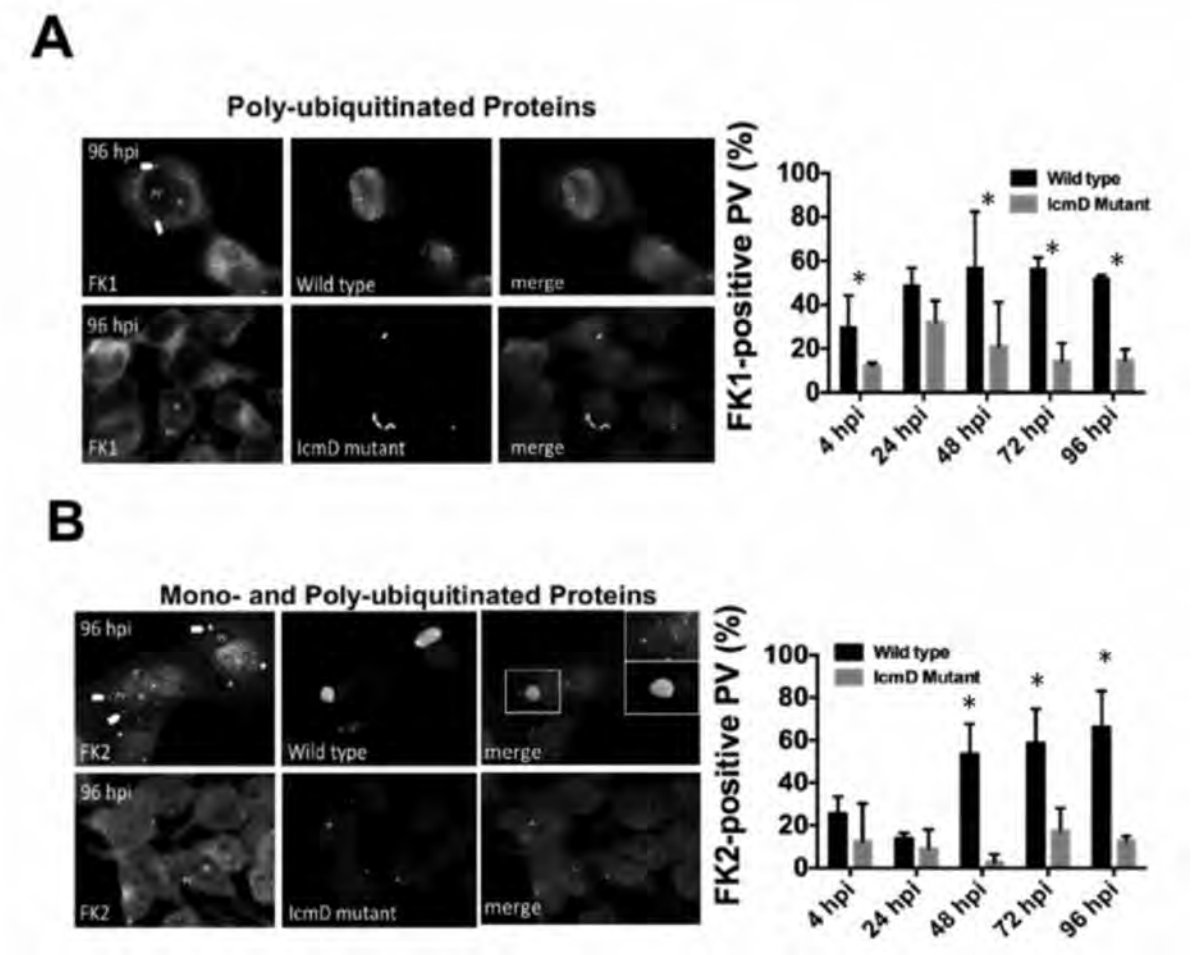

Fig. 1. Recruitment of ubiquitinated proteins to the parasitophorous vacuole (PV). HeLa cells were infected with wild type or T4SS-deficient (IcmD mutant) *C. burnetii* and prepared for fluorescence microscopy at 4, 24, 48, 72, and 96 h post infection (hpi). 4',6-diamidino-2-phenylindole (DAPI) was used to stain DNA. FK1- (A) and FK2- (B) labeled proteins were present on wild type PV (markers) from 24-96 hpi and fewer mutant-containing phagosomes. Statistical analysis was performed using a Student's t-test. Asterisks indicate significant differences where $P < 0.01$.

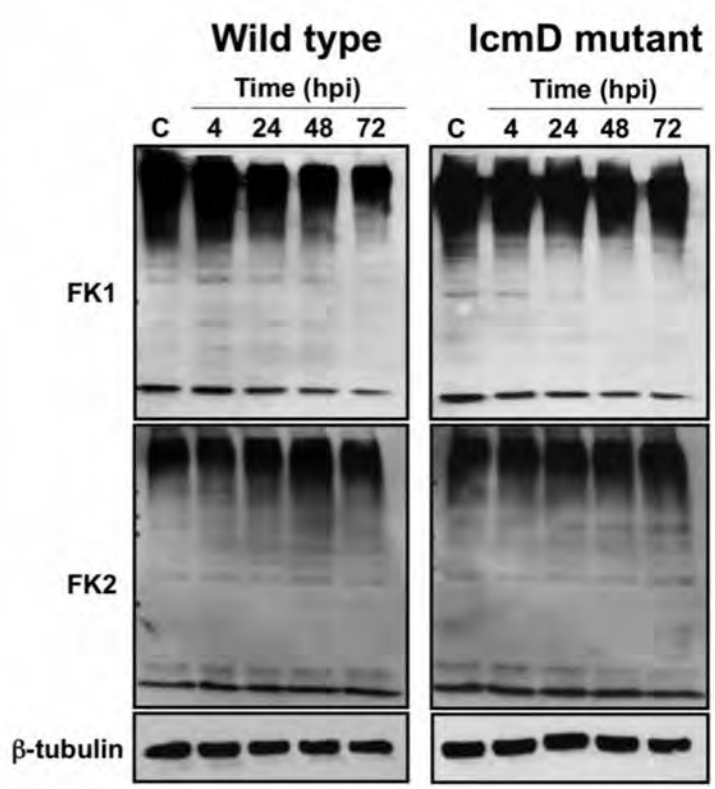

Fig. 2. Alteration of ubiquitinated protein levels in C. burnetii-infected cells. THP-1 human monocytic cells were infected with wild type or IcmD mutant C. burnetii and cellular lysates harvested at 4, 24, 48, and 72 hpi. Lysates were assessed for ubiquitinated protein levels by immunoblot, and β-tubulin was probed to ensure equal protein loading.

were present on wild-type PV at all time points of infection with 40-60% of wild-type PV decorating with FK1- and FK2-labeled proteins by 96 h post infection (hpi) on average. In contrast, when averaged over all time points, only 10-20% of IcmD mutant-containing phagosomes associated with these proteins. These results indicate that mono- and poly-ubiquitinated proteins are recruited to the PV membrane in a T4SS-dependent fashion.

Of note, among wild-type *C. burnetii*-infected cells, there was a high prevalence of large aggregations of ubiquitinated proteins. Similar structures, termed dendritic cell aggresome-like structures (DALIS), have been previously observed around *L. pneumophila*-containing vacuoles (Ivanov and Roy, 2009). Thus, future studies will examine the formation and potential importance of DALIS in *C. burnetii*-infected cells.

Alteration of Ubiquitinated Protein Levels During C. burnetii Infection. Because ubiquitinated proteins were recruited to the wild-type PV membrane, we next assessed whether *C. burnetii* alters ubiquitinated protein profiles using immunoblot analysis (Fig. 2). Levels of poly-ubiquitinated and mono-ubiquitinated proteins were analyzed using FK1 and FK2 antibodies, respectively. Over the course of infection (4-72 hpi), overall levels of FK1-labeled proteins decreased while levels of FK2-labeled proteins increased in wild-type *C. burnetii*-infected cells. In contrast, levels of FK1- and FK2-labeled proteins remained constant in IcmD mutant-infected cells. These results suggest *C. burnetii* uses T4SS effectors to promote accumulation of mono-ubiquitinated proteins at 48 hpi and beyond.

We next evaluated levels of Skp1, a critical component of the SCF complex, and UBC3 throughout intracellular growth (Fig. 3). Density-based analysis confirmed that levels of Skp1 decreased over the course of infection, while levels of UBC3 increased (data not shown). These results correlate with accumulation of mono-ubiquitinated proteins at later times post-infection. We also probed the presence of K48- and K63-linked proteins during infection (Fig. 4). Differentiating these linkages is important because they exert differing effects on target protein function. The K48-linked poly-ubiquitin chains serve proteolytic functions, while K63-linked chains are involved in non-proteolytic functions similar to mono-

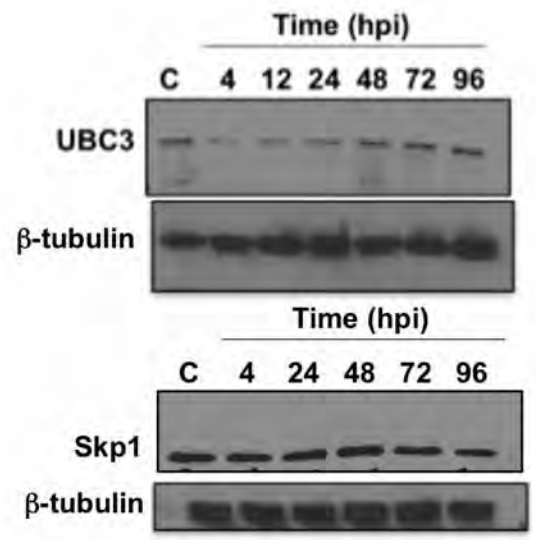

Fig. 3. Alteration of ubiquitin-related components in *C. burnetii*-infected cells. THP-1 human monocytic cells were infected with wild type *C. burnetii* and cellular lysates harvested at various time points post-infection. Lysates were assessed for UBC3 and Skp1 levels by immunoblot.

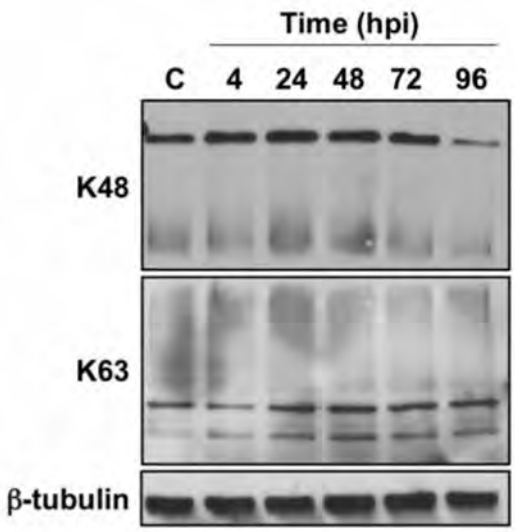

Fig. 4. K48- and K63-linked protein levels in *C. burnetii*-infected cells. THP-1 human monocytic cells were infected with wild type *C. burnetii* and whole cell lysates were taken at 4, 24, 48, 72, and 96 hpi. Lysates were assessed for levels of K-48 and K-63 linkages using immunoblot, and β-tubulin was probed to ensure equal protein loading.

ubiquitinated proteins. As infection progressed, K48-linked protein levels decreased while K63-linked protein levels increased. These results further suggest that *C. burnetii* promotes accumulation of proteins involved in cellular localization processes (mono-ubiquitination), rather than proteasome-mediated degradation.

Proteasome inhibition does not affect infection progression. Previous results suggested that *C. burnetii* interacts with mono-ubiquitinated proteins. To assess the potential importance of poly-ubiquitination during intracellular growth, we treated *C. burnetii*-infected cells with lactacystin, a known proteasome inhibitor, and monitored PV formation. When comparing treated cells to untreated cells, there was no substantial change in PV formation or the progression of infection (Fig. 5), further suggesting mono-ubiquitination events are more important for *C. burnetii* manipulation of host cells.

In the current study, we show that a functional T4SS is necessary for recruitment of ubiquitinated proteins to the *C. burnetii* PV and alteration of ubiquitinated protein levels. Ubiquitinated proteins label the PV only in wild-type *C. burnetii*-infected cells, suggesting ubiquitination is involved in pathogenesis. Additionally, mono-ubiquitinated proteins and K63-linked poly-ubiquitin chains accumulate during infection, suggesting *C. burnetii* manipulates host cell and/or bacterial protein trafficking. Future studies will define a mechanism for T4SS-mediated recruitment of ubiquitin machinery and the impact of this event on *C. burnetii*-host interactions.

The decrease observed in poly-ubiquitinated proteins from 24-72 hpi could be attributed to infection-specific degradation. However, treatment of infected cells with the proteasome inhibitor lactacystin does not alter PV formation or bacterial replication. These results correspond to the presence of DALIS in wild-type *C. burnetii*-infected cells. A DALIS can extend the half-life of poly-ubiquitinated proteins by preventing proteasome-directed degradation (Lelouard et al., 2004), and this may benefit *C. burnetii*. DALIS contain masses of ubiquitinated proteins (Ivanov and Roy, 2009) and sequester defective ribosomal products (DRiPs) targeted for degradation (Lelouard et al., 2004). Previous studies also found the ubiquitin-related proteins E1 and E2 and the C-terminus of an E3 ligase in DALIS. Cells infected with closely-related T4SS-deficient *L. pneumophila* produce DALIS, while organisms with a functional T4SS fail to trigger DALIS formation. The T4SS-deficient *L. pneumophila*, however, fails to avoid lysosome fusion and is degraded (Ivanov and Roy, 2009). In contrast to *L. pneumophila*, DALIS-like structures are present in wild-type *C. burnetii*-infected cells, suggesting they are not detrimental to pathogen viability. Future studies will define the role of DALIS formation in PV formation and *C. burnetii* parasitism of host cells.

In this study, we assessed the role of the Dot/Icm T4SS in regulating ubiquitination by comparing subcellular lo-

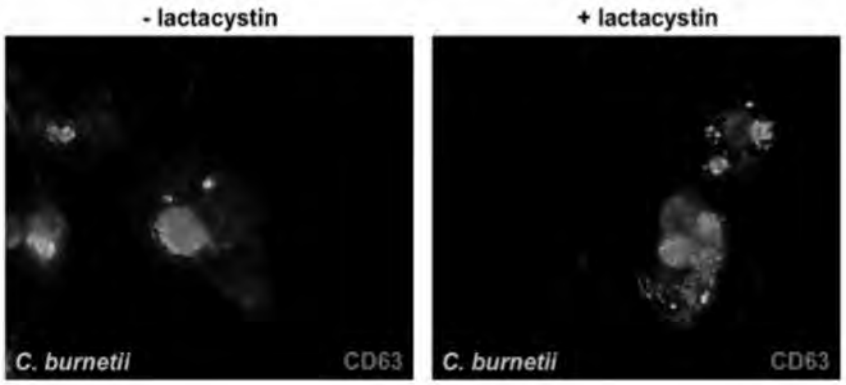

Fig. 5. Lactacystin treatment of wild type *C. burnetii*-infected cells. HeLa cells were infected with wild type *C. burnetii* and prepared for fluorescence microscopy at 4, 24, 48, 72, and 96 hours post infection (hpi). DAPI was used to stain DNA and CD63 was used to label the PV.

calization of ubiquitinated proteins between cells infected with *C. burnetii* and a mutant that lacks a functional T4SS. Using immunofluorescence assays (IFA) we found the wild-type *Coxiella burnetii* PV were labeled with ubiquitinated proteins at a higher frequency than mutant-containing phagosomes. This indicates that recruitment of ubiquitinated proteins to the PV is T4SS-dependent. Additionally, we used immunoblot analysis to look at ubiquitinated protein profiles. A decrease in FK1-labeled proteins and an increase in levels of FK2-labeled proteins in wild-type PV led us to the conclusion that *C. burnetii* potentially promotes accumulation of mono-ubiquitinated proteins during cellular growth. Assessing other ubiquitin-related components further suggested that mono-ubiquitinated proteins were being accumulated on wild-type PV. Treating infected cells with lactacystin, a known proteasome inhibitor, had no visible effect on the ability of *C. burnetii* to establish an infection. This even further suggests that mono-ubiquitinated proteins are more important for proper *C. burnetii infection*.

Overall, the data obtained from this study contribute to increased understanding of the *C. burnetii*-host cell dynamic and provide new ubiquitin-related hypotheses for future testing. Specifically, the presence of DALIS in infected cells represents a novel area of research in *C. burnetii* pathogenesis. Additionally, linking ubiquitin modulation to a specific T4SS effector(s) will establish novel bacterial protein activity that could be targeted in design of new therapeutic strategies. Designing new therapeutic techniques is of utmost importance as *C. burnetii* is a potential bioterror agent.

ACKNOWLEDGMENTS

This research was supported by the Arkansas INBRE program and grants from the National Center for Research Resources (P20RR016460), the National Institute of General Medical Sciences (P20 GM103429), and the National Institute of Allergy and Infectious Diseases (R01AI087669 to D.E.V.).

LITERATURE CITED

Beare, P.A., S.D. Gilk, C.L. Larson, J. Hill, C.M. Stead, A. Omsland, D.C. Cockrell, D. Howe, D.E. Voth, and R.A. Heinzen. 2011. Dot/Icm type IVB secretion system requirements for Coxiella burnetii growth in human macrophages. mBio. 2(4):1-10.

Chen, Z.J. and L.J. Sun. 2009. Nonproteolytic functions of ubiquitin in cell signaling. Mol. Cell. 33(3):275-286.

Collins, C.A. and E.J. Brown. 2010. Cytosol as battleground: ubiquitin as a weapon for both host and pathogen. Trends Cell Biol. 20(4):205-213.

Gilk, S.D., D.E. Voth, and R.A. Heinzen. 2009. *Coxiella burnetii*. *In* Schaible, U.E. and A. Haas, editors. Intracellular Niches of Microbes: A Pathogens Guide Through the Host Cell. Wiley-VCH, Weinheim. pp. 287-300.

Hicke, L. and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin binding proteins. Annu. Rev. Cell Dev. Biol. 19:141-172.

Huang, B., N. Ojogun, S.A. Ragland, and J.A. Carlyon. 2012. Monoubiquitinated proteins decorate the Anaplasma phagocytophilum-occupied vacuolar membrane. FEMS Immunol. Med. Microbiol. 64(1):32-41.

Ivanov, S.S. and C.R. Roy. 2009. Modulation of ubiquitin dynamics and suppression of DALIS formation by the *Legionella pneumophila* Dot/Icm system. Cell Microbiol. 11(2):261-278.

Jackson, P.K. and A.G. Eldridge. 2002. The SCF ubiquitin

ligase: an extended look. Mol. Cell. 9(5):923-925.
Komander, D. 2009. The emerging complexity of protein ubiquitination. Biochem. Soc. Trans. 37(5):937-953.
Lelouard, H., V. Ferrand, D. Marguet, J. Bania, V. Camosseto, A. David, E. Gatti, and P. Pierre. 2004. Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. J. Cell Biol. 164(5):667-675.
Marrie, T.J. 1990. Epidemiology of Q Fever. *In* T.J. Marrie, editor. Q Fever Volume 1: The Disease. CRC Press, Boca Raton, Fla. pp. 49-70.
Maturana, P., J.G. Graham, U.M. Sharma, and D.E. Voth. 2013. Refining the plasmid-encoded type IV secretion system substrate repertoire of *Coxiella burnetii*. J. Bacteriol. 195(14):3269-3276.
Patel, J.C., K. Hueffer, T.T. Lam, and J.E. Galan. 2009. Diversification of a *Salmonella* virulence protein function by ubiquitin-dependent differential localization. Cell. 137(2):283-294.
Thrower, J.S., L. Hoffman, M. Rechsteiner, and C.M. Pickart. 2000. Recognition of the polyubiquitin proteolytic signal. EMBO J. 19(1):94-102.
Voth, D.E., D. Howe, and R.A. Heinzen. 2007. *Coxiella burnetii* inhibits apoptosis in human THP-1 cells and monkey primary alveolar macrophages. Infect. Immun. 75(9):4263-4271.
Voth, D.E., P.A. Beare, D. Howe, U.M. Sharma, G. Samoilis, D.C. Cockrell, A. Omsland, and R.A. Heinzen. 2011. The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. J. Bacteriol. 193(7):1493-1503.
Williams, J.C. 1991. Infectivity, Virulence, and Pathogenicity of *Coxiella burnetii* for Various Hosts. *In* J.C. Williams and H.A. Thompson, editors. Q Fever: The Biology of *Coxiella burnetii*. CRC Press, Boca Raton, Fla., pp. 21-71.

Growth and mortality of Ozark bass (*Ambloplites constellatus*) in streams of the Ozark Highlands

Ashley R. Rodman* and Kristofor R. Brye†

ABSTRACT

The Ozark bass (*Ambloplites constellatus*) is endemic to the Upper White River Basin, and a limited amount of information exists on the Ozark bass population, including growth and mortality characteristics. The purpose of this study was to determine growth and mortality of Ozark bass in the Upper White River Basin, compare growth of Ozark bass to other *Ambloplites* species, and compare growth and mortality of Ozark bass between sample sites. Sampling occurred in Crooked Creek and in the Lower Wilderness Area (LWA) of the Buffalo River, with multiple collections from each body of water. Sampling occurred during summer 2013 via electroshocking from a boat. Length and weight data were recorded while sampling, and fish ages were determined through otolith retrieval. Ozark bass exhibited similar growth patterns to Shadow bass (*Ambloplites ariommus*); however, Rock bass (*Ambloplites rupestris*) grew faster and larger. Growth of Ozark bass appeared to be similar between Crooked Creek and the LWA of the Buffalo River until 5 years of age. After age 5, the growth of fish collected from the LWA of the Buffalo River slowed compared to 5 and older fish collected from Crooked Creek. Ozark bass of the LWA of the Buffalo River had an overall greater mortality rate than those in Crooked Creek; however, one of two sites sampled on Crooked Creek had a comparable fish mortality rate to that measured in the LWA of the Buffalo River. Results indicated that size-selected mortality may have occurred in the LWA of the Buffalo River and at least one location sampled in Crooked Creek, possibly due to fishing mortality and angler popularity at the sites. Data collected in this study were part of a long-term attempt by the Arkansas Game and Fish Commission to gather baseline data on the Ozark bass population and to determine the efficacy of current harvest regulations for that species in the Upper White River Basin. Baseline data will be used in the future to determine whether local fish populations respond to climate change or other impacts to the watershed.

* Ashley R. Rodman is a 2014 graduate with a major in Environmental, Soil, and Water Science and a minor in Wildlife Habitat.
† Kristofor R. Brye is the faculty mentor and a professor in the Department of Crop, Soil, and Environmental Sciences.

MEET THE STUDENT-AUTHOR

Ashley Rodman

Working in Yellowstone National Park the summer after graduating from Viola High School sparked my interest in environmental issues. Becoming a charter member and president of an Arkansas Game and Fish Commission Stream Team while attending Arkansas State University-Mountain Home (ASUMH) contributed to my decision to focus my education on water quality. After receiving my Associate of Arts Degree from ASUMH, I transferred to the University of Arkansas into the Crop, Soil, and Environmental Sciences (CSES) Department to pursue a Bachelor of Science (B.S) in Environmental, Soil, and Water Science. Through the CSES Department, I have been able to compete in national competitions, become involved in the CSES Club, as well as receive college credit and research experience through an internship with the Arkansas Game and Fish Commission. I will graduate with my B.S. this summer after returning from a study abroad trip to Mozambique, Africa where I will be conducting water quality analyses to aid a local poultry operation. Soon after returning from Africa, I will begin my graduate studies in the CSES Department with Thad Scott, focusing on stream water quality.

Numerous people have given me the guidance and encouragement to allow me to be where I am today, and I am truly grateful to each and every one of them.

INTRODUCTION

Prior to 1977, the Ozark bass, Rock bass, and Shadow bass were classified as a single fish species, *Ambloplites rupestris*. In 1977, *Ambloplites rupestris* was split into three different species, and the Ozark bass was renamed *Ambloplites constellatus* (Cashner and Suttkus, 1977). Minimal published data exist on growth or other population characteristics due to the relatively recent species split and the limited range of the Ozark bass. Whisenant and Maughan (1989) have reported some of the only available information for the Ozark bass, including mean size at age. One of the objectives of the Whisenant and Maughan (1989) study was to collect baseline data for the Ozark bass to see if increased recreational pressures and angling effort had impacted the sport fisheries of the Buffalo River. The Arkansas Game and Fish Commission (AGFC) has collected electroshocking data on Ozark bass since 1992; however, to date, no age data have been collected (Stan Todd, Arkansas' District 2 Fisheries Management Biologist, pers. comm.).

Growth and mortality rates are important metrics of any fish population and are useful for comparing different fish populations and evaluating harvest regulations (Ricker, 1975). Age distribution data are collected to aid in the determination of length-at-age, which is required for the calculation of growth and mortality rates (Ricker, 1975). Length-at-age is an estimate of mean size of fish at annual increments. Sagittal otoliths have been used to determine the age of fish species due to ease of readability and accuracy compared to other aging methods (Maceina et al., 2007). Otoliths increase in size with fish size and leave dense rings during periods of slow growth. These annular rings can be used to determine fish age (Jearld, 1985).

Conductivity, pH, water temperature, and dissolved oxygen are often measured in association with electroshocking data collection. If differing growth or mortality rates occur between sampled sites, measured water quality indicators could potentially disclose reasons for conflicting results. Conductivity correlates with total dissolved solids and nutrient availability (Uwidia and Ukulu, 2013). Nutrient availability is a determining factor in fish growth (Keller et al., 1990). Temperature also impacts growth rates due to the direct relationship between temperature and chemical reaction rates (Whiteledge et al., 2006). Extreme levels of dissolved oxygen and pHs that are too acidic or too basic can cause stress to fish, causing more energy expenditure and less energy available for growth (Breitburg et al., 1997; Magnuson et al., 1984).

According to Buchanan and Robison (1988), the Ozark bass is endemic to the Upper White River Basin. However, the Upper White River Basin Foundation (UWRBF, 2012)

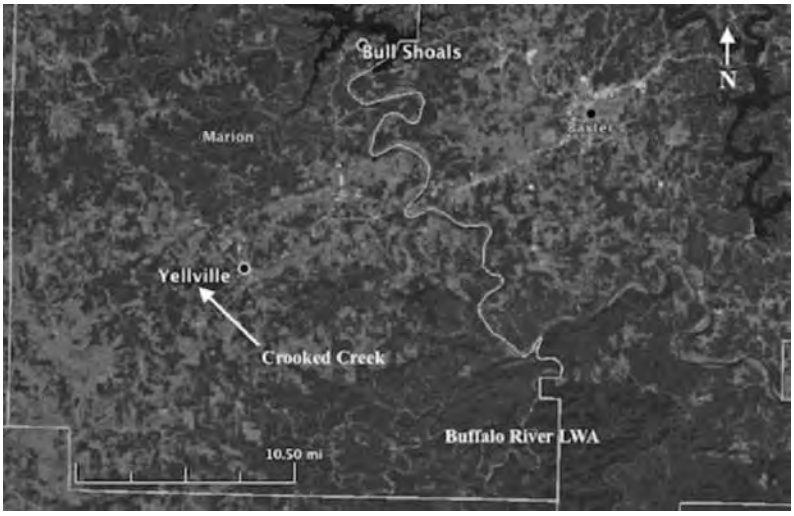

Fig. 1. Location of Crooked Creek and the Lower Wilderness Area of the Buffalo River in Marion County, Ark.

points out several concerns in the White River watershed, including the ease of groundwater contamination in the karst geology of the area, increased urbanization and alteration of watershed hydrology, and increasing confined animal feeding operations. Climate change models for the southeastern United States predict an increase in number and duration of droughts, greater mean annual temperature, as well as heavy rain events with shorter durations (USEPA, 2013). Changes in watershed hydrology, whether from increased urbanization or climate change, could potentially alter stream morphology, and therefore impact habitat of the Ozark bass and other endemic fish species.

Population data aid fisheries biologists in evaluating current regulations to ensure that overfishing does not occur. Recent limitations on harvest pertaining to other sport fishes in Ozark Highland waterways may have increased harvest of the Ozark bass in some streams. Currently, there is a creel limit of 10 fish and no size limit for Ozark bass, Rock bass, and Shadow bass combined (AGFC, 2013). Since the Ozark bass has a limited range, monitoring current population characteristics of the Ozark bass will be beneficial in evaluation of current and potential future impacts on the Ozark bass population. Therefore, the objective of this study was to determine population information and growth and mortality rates for Ozark bass in the Upper White River Basin. Growth rates were compared to that for other *Ambloplites* species and mortality rates were compared among sample locations. It was hypothesized that growth rates would be similar among *Ambloplites* species and mortality rates would be similar among sampled streams in the Ozark Highlands.

METHODS AND MATERIALS

Upper White River Basin Characteristics

The Upper White River Basin is located in southern Missouri and in north central and northwestern Arkansas over karst topography (UWRBF, 2012). Ozark bass generally inhabit stream pools that have high dissolved oxygen, continuous flow, and low turbidity (Buchanan and Robison, 1988), all of which are characteristics present in the Upper White River Basin.

The mean annual precipitation throughout northwest Arkansas is 126.6 cm (USGS, 2005; Climate Zone, 2013). The mean annual air temperature is 16.6 °C, with mean summer-month temperatures as follows: 25.8 °C in June, 27.7 °C in July, and 27.0 °C in August (USGS, 2005; Climate Zone, 2013).

Fish Collection

Ozark bass were collected by boat electroshocking in the Upper White River Basin, including in the Lower Wilderness Area (LWA) of the Buffalo River and in Crooked Creek (Fig. 1). Six runs (i.e., the length of actual shocking time between measuring fish) occurred near Middle Creek, five above Leatherwood Creek, and five below Leatherwood Creek in the LWA of the Buffalo River for a total electroshocking time of 257 minutes (Fig. 2a). In Crooked Creek, five runs occurred at both the Education Center location and at the Pyatt site for a total electroshocking time of 61 and 47 minutes, respectively (Fig. 2b).

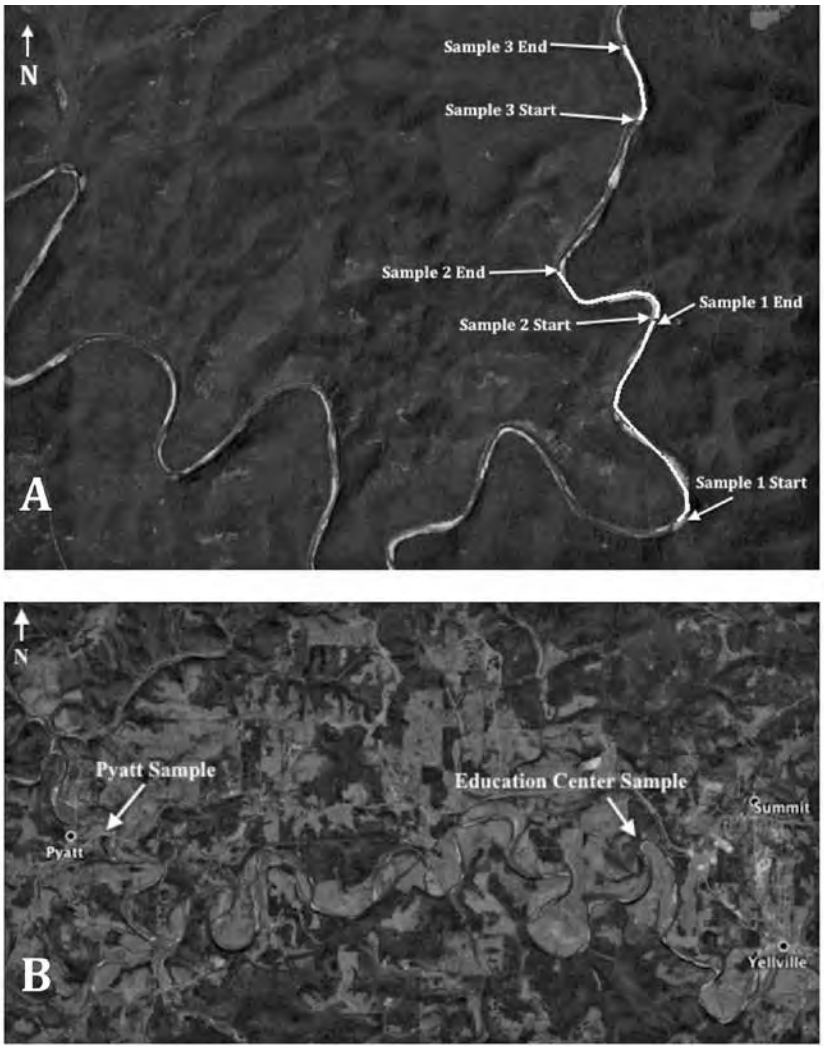

Fig. 2. Sample sites on the Lower Wilderness Area of the Buffalo River (A) and Crooked Creek from 2013 (B).

During the electroshocking process, pulsed direct current (DC) electricity was directed through the water via cathodes and anodes (Kolz et al., 2000). The cathode can either be a metal boat hull or separate electrodes used near the boat when using a fiberglass hull (Kolz et al., 2000). With DC electroshocking, the cathodes repel the fish, while the anodes attract the fish causing temporary electro-narcosis when the fish encounters the current (Kolz et al., 2000). While the fish are temporarily disoriented, they float to the surface where they can be more easily collected with nets.

Ozark bass collections took place during summer 2013 when the water flow was at a level that permitted safe sampling. The Buffalo River was sampled on June 10, 11, and 12, while Crooked Creek was sampled on June 21 and 24. Water levels had to be less than the 1.5-m (~5 ft) stage at Highway 65 in the LWA of the Buffalo River and less than the 3-m (~10 ft) stage at Kelly's Crossing on Crooked Creek for safe sampling to occur. Summer was chosen for the study because water levels are generally lower and fish are more concentrated, more active, and easily caught during the warmer months of the year.

Ozark bass sampling was conducted in pools and other deep habitat reachable by boat. Each run was conducted for approximately 10 minutes. Weights, to the nearest ±2 grams, and total lengths, to the nearest millimeter, of each fish collected were recorded in the field. Ten fish per size class, if available, were saved for otolith retrieval. Size classes ranged from 0 to 270 mm with 10-mm intervals. If the maximum of 10 fish in any given size class were collected, extra fish collected in the same size class thereafter were weighed, measured, and released. The gender

of each Ozark bass collected from the Buffalo River was also recorded so that comparisons of growth rates by sex could be made. Since fish growth can be affected by water quality, a suite of water quality indicators, including pH, conductivity, water temperature, and dissolved oxygen, were measured and recorded in the field. These data were used to help explain results if there were dramatic conflicts in results or unexpected results from stream to stream.

Once fish numbers, lengths, and weights had been recorded, numerous additional calculations were made. The catch-per-unit-effort (CPUE; i.e., fish per hour) was calculated from the number of Ozark bass collected on the Buffalo River and Crooked Creek. The relative stock density (RSD) was determined by grouping fish into various size classes based on the length of the current world record fish. Since there are minimal data for Ozark bass, the RSD size classes for Rock bass were used and included Stock (≥100 mm in length), Quality (≥180 mm), Preferred (≥230 mm), Memorable (≥280 mm), and Trophy (≥330 mm). The relative weight (Wr) was also calculated to compare Ozark bass collected from the two main waterways. Relative weight is a comparison between the weight of a sampled fish compared to a standard weight generated from a standard weight equation for that species. Relative weight gives an index of condition or plumpness for the sampled fish. Relative weight was calculated using the following equation from Anderson and Gutreuter (1983):

$$W_r = \left(\frac{\text{weight of fish}}{\alpha * \text{length of fish}^{\beta}} \right) * 100 \qquad \text{Eq. 1}$$

A Shadow bass standard weight equation was used due to the unavailability of standards for Ozark bass. The parameters used were α = -5.1461 and β = 3.2110 (Mareska and Jackson, 2002). The Shadow bass are a different species of fish, and the Ozark bass may not have the exact same standards; however, Shadow bass and Ozark bass are in the same genus. Thus, the Shadow bass data were assumed sufficient as a comparison tool between the two sampled areas.

Sagittal otoliths, which are two disc-shaped bones in the heads of boney fish that are used to estimate age, were collected from the brain cavities of the Ozark bass (UAF, 2013). Although the goal for collecting otoliths was up to 10 fish per size class, in some instances, 10 fish were not possible in each size class due to a lack of availability during collection. Otoliths were transported to an AGFC laboratory for aging. Otoliths were glued to glass slides and, if needed, sectioned with a low-speed saw. Age data were recorded after microscopic observation and counting of the number of annuli present on each otolith. The population age distribution was estimated based on population length frequency and an age-length key generated from fish with known lengths and ages based on previous otolith observation (Quist et al., 2012). Growth and mortality rates were estimated from the population age distribution (Ricker, 1975). Lengths-at-age were estimated using the von Bertalanffy equation and growth rates were then calculated from those estimates. The von Bertalanffy equation is as follows:

$$L_t = L_\infty \left[1 - e^{-k(t-t_o)} \right] \qquad \text{Eq. 2}$$

where L_t is the length at a certain age, L_∞ is the longest length that fish in the population will ever attain, k is a growth constant, t_o is the hypothetical age of the fish at a length of 0, and t is the age of the fish (Pine et al., 1983). Mortality rates for the LWA of the Buffalo River and Crooked Creek were calculated using the Chapman-Robson method (Robson and Chapman, 1961).

Data Analyses

Mortality rates were compared with similar data from different locations and growth rates were compared among species. A Student's t-test was performed using Microsoft Excel to evaluate whether the slope of the relationship between the log-transformed lengths and weights for the Ozark bass differed between locations (i.e., Buffalo River and Crooked Creek). A Student's t-test was also performed to evaluate the effect of sample location (i.e., Buffalo River and Crooked Creek, combined across the two locations and separately by location) on calculated mortality rates in each age category. The threshold at which significance was judged was α = 0.05.

RESULTS AND DISCUSSION

Ozark bass were present in both Ozark Highland waterways sampled. One hundred and thirty-nine Ozark bass were collected in the LWA of the Buffalo River, 57 near Middle Creek, 45 above Leatherwood Creek, and 37 below Leatherwood Creek. A total of 188 Ozark bass were collected in Crooked Creek, 86 at the Education Center site and 102 at the Pyatt site.

Mean CPUE of Ozark bass in the Buffalo River was 31.2 fish per hour, with the 95% confidence interval of 26.3 to 36.2 fish per hour (Fig. 3). The shortest fish collected from the Buffalo River was 61 mm long, while the longest fish was 256 mm long. There was a noticeable decline in the number of fish from the Buffalo River caught per hour over 220 mm long, with no fish collected in the 240-mm to 249-mm size class.

The mean CPUE of Ozark bass in Crooked Creek was 101.5 fish per hour (Fig. 3). The 95% confidence interval

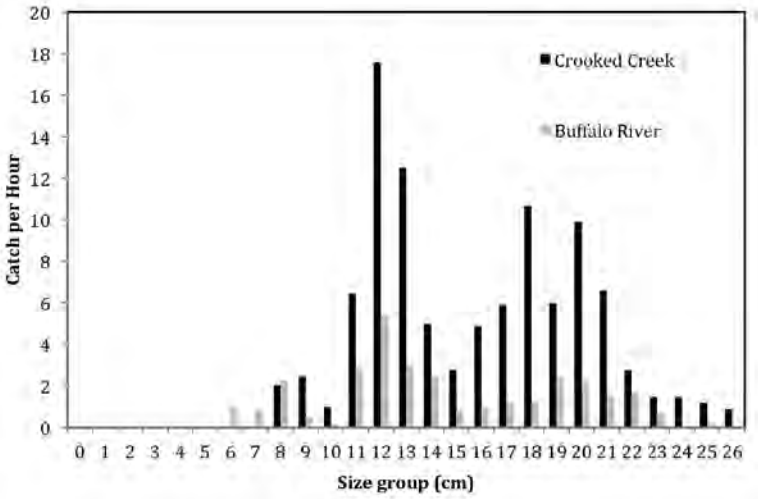

Fig. 3. Catch-per-unit-effort (CPUE, catch per hour) per size group of Ozark bass for the Lower Wilderness Area of the Buffalo River and Crooked Creek, June 2013.

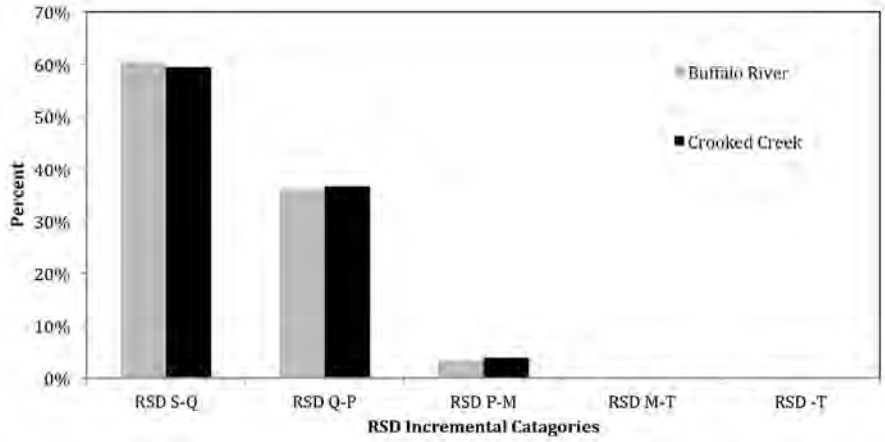

Fig. 4. Relative stock density (RSD) for Ozark bass in the Lower Wilderness Area of the Buffalo River and Crooked Creek, June 2013. S-Q = 100-179 mm, Q-P = 180-229 mm, P-M = -230-279 mm, M-T = 280-329 mm, T = ≥330 mm.

for the population's mean was 71.9 to 131.1 fish per hour. Sizes of fish collected from Crooked Creek ranged from a minimum of 88 mm to a maximum of 260 mm long. There was also a decrease in the number of fish caught per hour above 210 mm long in Crooked Creek. Figure 3 clearly depicts that there was a greater number of fish caught per hour from Crooked Creek than the Buffalo River. Since CPUE is directly related to density, there was also a greater density of fish in Crooked Creek than in the Buffalo River.

The relative stock density (RSD; Fig. 4) offers a different way to represent the size distribution of the Ozark bass that were collected than simply the length of fish collected. Fish under Stock size (<100 mm long) were not included in Fig. 4 because those fish were not significant when considering the impacts of angling pressure on the Ozark bass population. The mean length of Ozark bass greater than Stock size collected from the LWA of the Buffalo River was 164 mm, with a 95% confidence interval of 156 mm to 171 mm. The minimum length of fish greater than Stock size was 107 mm, while the maximum length over Stock size was 256 mm. The mean length of Ozark bass in Crooked Creek that were greater than Stock size was 164 mm, with a 95% confidence interval of 158 mm to 169 mm. The smallest-sized fish greater than Stock size was 103 mm long, while the largest fish was 260 mm long in Crooked Creek.

According to Fig. 4 and Table 1, the percentage of fish present in each size category decreased with increasing size category in the Buffalo River and Crooked Creek. The RSD graph depicts that there is a similar size dis-

Table 1. Relative stock density (RSD) for the Buffalo River and Crooked Creek.

	Buffalo River		Crooked Creek	
RSD Size Category	Relative Abundance (%)	95% Confidence Interval (%)	Relative Abundance (%)	95% Confidence Interval (%)
Stock size (< 100 mm)	14.4	13.9 to 14.9	4.3	4.0 to 4.5
Stock to Quality (100 mm to 179 mm)	60.5	59.7 to 61.3	59.4	58.9 to 60.0
Quality to Preferred (180 mm to 229 mm)	36.1	35.3 to 36.9	36.7	36.1 to 37.2
Preferred to Memorable (230 mm to 279 mm)	3.4	3.1 to 3.7	3.9	3.7 to 4.1
Memorable to Trophy (280 mm to 329 mm)	-[a]	-	-	-
Trophy (≥330 mm)	-	-	-	-

[a] No fish collected in size class.

Table 2. Growth data for Ozark bass in the Buffalo River and in Crooked Creek.

	Water Body/Growth Parameter					
	Buffalo River			Crooked Creek		
Fish Age (years)	Mean Length	Variance	Number of Fish	Mean Length	Variance	Number of Fish
1	76.3	109	21	83.6	14.8	5
2	130	189	46	128	156	40
3	183	117	20	173	81.9	32
4	207	117	9	195	76.7	8
5	216	81.3	16	205	60.3	23
6	214	443	3	206	124	3
7	208	-[a]	1	230	113	2
8	-	-	0	238	32	2
9	258	-	1	253	40.5	2

[a] No data.

tribution of fish between the Buffalo River and Crooked Creek (Fig. 4).

Differential growth patterns between sexes have been observed in some fish, such as long-eared sunfish (*Lepomis megalotis*) present in the Buffalo River (S. Todd, pers. comm.). However, both genders of Ozark bass appeared to grow at a similar rate (data not shown); thus, the subsequent results for fish size characteristics are combined across genders. As the length of Ozark bass increased, the weight of Ozark bass increased logarithmically. Since there was a similar logarithmic relationship between the weight and length of Ozark bass from both the Buffalo River and Crooked Creek, all data were combined to determine a single weight-length relationship for Ozark bass in Ozark Highland waterways. In this study, 99% of the variability in length of Ozark bass was explained by weight variations.

The mean relative weight (Wr) of Ozark bass in the Buffalo River was 85, with a variance of 36.0. Crooked Creek's relative weight was 92, with a variance of 50.4. The minimum relative weight of Ozark bass in the Buffalo River was 70, with a maximum of 100. The minimum relative weight of Ozark bass in Crooked Creek was 72, with a maximum of 114.

Based on the relative weight results, Ozark bass in Crooked Creek appeared to be in slightly better condition than those in the Buffalo River. This would suggest that food availability was greater in Crooked Creek than in Buffalo River, which may partially explain the greater density of Ozark bass in Crooked Creek than in the Buffalo River.

Otoliths were retrieved and aged from 117 Ozark bass from the Buffalo River and from 127 Ozark bass from Crooked Creek. From the age data collected, an age-length key was derived, which allowed for the calculation of the age of sampled fish that were not kept for otolith retrieval. Then, the lengths of fish in each individual age category were averaged. Table 2 summarizes the mean length, variance, and number of fish in each age category from both streams sampled in this study. Comparing our

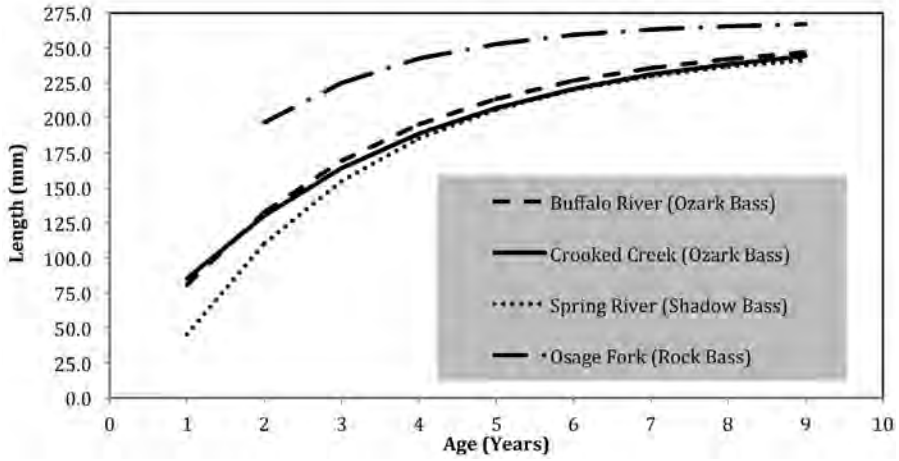

Fig. 5. von Bertalanffy growth curves for Ambloplites sp. (i.e., Ozark bass, Shadow bass, and Rock bass) in the Buffalo River, Crooked Creek, Osage Fork, and the Spring River.

results to data from Whisenant and Maughan (1989), their length data appear to be classified under the wrong age classes. For example, if all of length data from the Whisenant and Maughan (1989) study were shifted one age class older, then both data sets would show similar growth among Ozark bass. Fish scales were used to determine age of Ozark bass reported in Whisenant and Maughan (1989), which could be the reason for a potentially inaccurate age classification. The number of Ozark bass collected in the Whisenant and Maughan (1989) study was drastically greater (n = 1090 in 1980 sampling) than that collected in this study. Therefore, it appears that there were fewer Ozark bass in the Buffalo River in 2013 than in the early 1980s. The National Park Service has reported that the number of visitors to the Buffalo River has doubled since the early 1980s (unpublished data, S. Todd, pers. comm.) when the Whisenant and Maughan (1989) study was conducted. This observation suggests that increased visitation to the Buffalo River may have led to a decrease in the number of Ozark bass in the river.

The length-at-age data for both sampled water bodies depicted similar trends (Fig. 5). The terms in the von Bertalanffy equation for the Buffalo River were 258 mm for maximum attainable length (L_∞), 0.346 for the growth constant (k), and -0.087 for the length-at-age 0 (t_o). The terms in the equation for Crooked Creek were 260 mm for maximum attainable length (L_∞), 0.299 for the growth rate (k), and -0.328 for the length at zero 0 (t_o). Figure 5 portrays that as the age of Ozark bass increased, the growth rates slowed and became more similar in both sample waterways; therefore, growth rates between Crooked Creek and Buffalo River sample sites were comparable.

Growth data for Rock bass collected from Osage Fork by the Missouri Department of Conservation and for Shadow bass collected from the Spring River by the AGFC were compiled and presented in Fig. 5 to compare growth among species (Johnson et al., 2010; unpublished data). Shadow bass were depicted as initially growing slower; however this might have been due to errors in measurement of small fish or time of year fish were collected. As age increased, the growth rate of Shadow bass became more analogous to that of Ozark bass. Rock bass, however, grew faster and larger than both Ozark bass and Shadow bass (Fig. 5).

The growth models for the Buffalo River and Crooked Creek indicated that growth rates for Ozark bass were similar between water bodies; however, Ozark bass in the Buffalo River initially grew faster than those in Crooked Creek (Table 3). Growth rates slowed in both water bodies at approximately age 5; and the growth rate of Crooked Creek fish slightly surpassed that of the Buffalo River fish after age 5. Size-selective mortality (i.e., angling) might have been a factor in the slower growth rate estimates of the older fish in the Buffalo River, as anglers tend to keep the faster growing, better conditioned fish, leaving only the slower growing fish in the larger sizes. Growth rates between the Pyatt and Education Center sampling sites on Crooked Creek were also compared, and, like the growth rates estimated for Ozark bass in Crooked Creek and in the Buffalo River, the estimated growth rates for Ozark bass were similar between the two Crooked Creek sample sites. In addition, water quality indicators measured in the Buffalo River and Crooked Creek were similar between locations.

Mortality rates were calculated for three different age categories: 2 to 9 year olds, 2 to 5 year olds, and 5 to 9 year olds. The population was divided into different age groups due

Table 3. Length-at-age and growth rates for Ozark bass in the Buffalo River and in the combined Crooked Creek sample sites for 2012-2013.

	Buffalo River		Crooked Creek	
Fish Age (Years)	Length-at-age (mm)	Growth Rate (mm/year)	Length-at-age (mm)	Growth Rate (mm/year)
1	80.8	51.8	86.2	44.1
2	132.6	36.7	130.3	32.9
3	169.3	25.9	163.2	24.6
4	195.2	18.4	187.8	18.3
5	213.6	13	206.1	13.7
6	226.6	9.2	219.8	10.2
7	235.8	6.5	230.0	7.6
8	242.3	4.6	237.6	5.7
9	246.9	-[a]	243.3	-

[a] No data.

to the Buffalo River's length-at-age relationship indicating a deviation from the von Bertalanffy growth model after age 5. One-year-old fish were not included in the calculations since fish younger than age 2 were not fully recruited to the sampling gear, and therefore their numbers were probably underestimated. Table 4 shows the mortality rate, variance, and number of fish sampled for each age category at both sampled areas. The two sampling sites on Crooked Creek were further broken down to show individual site mortality rates because there were differences in size distribution at the two sampling sites. More and larger fish were collected at the Pyatt site, implying that there was a greater mortality rate at the Education Center site on Crooked Creek. It was suspected that the Education Center site on Crooked Creek had greater angling pressure than at the Pyatt site.

There was a significant difference between mortality rates in all age categories on the Buffalo River and Crooked Creek, between the Pyatt site and Education Center site on Crooked Creek, and between the Buffalo River and the Pyatt site. Comparison between the Buffalo River and the Education Center site on Crooked Creek revealed no difference in mortality rate of Ozark bass in the 2 to 5 year old age category. A significant difference existed in the other age categories when comparing the Buffalo River and the Education Center site on Crooked Creek.

The lowest overall mortality rate of Ozark bass observed among the sampled areas was at the Pyatt sample site on Crooked Creek. The Education Center site on Crooked Creek had the greatest mortality rate, except when comparing the site's 2 to 5 year old age category to that from the Buffalo River. The Buffalo River's mortality rate was intermediate between that estimated at the Education Center and the Pyatt sites on Crooked Creek, except in the 2 to 5 year old age category when compared to that at the Education Center site. The mortality rates for the Buffalo River and the Education Center site on Crooked Creek in the 2 to 5 year old age category did not differ. Mortality rates were greater at all sites in the >5 year old age category compared to the other age categories.

Total mortality is made up of fishing mortality (e.g., anglers harvesting fish and fish death due to hooking) and natural mortality (e.g., predators, disease, and water quality issues). A possible reason for low mortality at the Pyatt sample site on Crooked Creek was less total mortality than at the other sampled areas, which could be due to lower angling pressure. Greater mortality at the Education Center site on Crooked Creek might have been due to greater levels of fishing mortality (e.g., angling pressure) due to the ease of accessibility and popularity of the site among anglers. Increased mortality in the >5 year old age category at all sampled areas indicated that anglers are harvesting larger, better conditioned fish. There has been a two-fold increase in visitors to the Buffalo River since 1980, and the number of Ozark bass has likely decreased since the Whisenant and Maughan (1989) study. Hence, angling pressure is likely responsibility for the elevated mortality of the >5 year old fish. Since little data have been collected to know for certain that anglers are the main cause for differing mortalities between streams and increased mortality in the >5 year old age category, creel surveys are planned for the Buffalo River and possibly for Crooked Creek in the near future to assess angler impacts to the fisheries. The creel surveys will gather data about the number of anglers utilizing the water bodies and other valuable data when considering angling pressures.

In this study, the growth of Ozark bass were compared to existing data for Shadow bass and Rock bass and mortality rates were compared between sampled sites on the Buffalo River and on Crooked Creek. Data collected in

Table 4. Mortality rates, variance and number of fish for Ozark bass sampled in the Lower Wilderness Area of the Buffalo River, in Crooked Creek combined across sample locations, and in each Crooked Creek sample location.

Water Body/Age Range (Years)	Mortality (%)	Variance	Number of Fish
Buffalo River			
2 to 9	49.3	0.001	119
2 to 5	47.0	0.003	111
5 to 9	65.0	0.007	23
Crooked Creek (sample sites combined)			
2 to 9	45.8	0.001	184
2 to 5	41.0	0.002	172
5 to 9	60.6	0.003	43
Crooked Creek at Education Center site			
2 to 9	51.7	0.002	80
2 to 5	47.0	0.004	78
5 to 9	77.1	0.008	18
Crooked Creek at Pyatt site			
2 to 9	39.6	0.001	79
2 to 5	23.0	0.007	72
5 to 9	57.0	0.008	19

Notes: There was a significant difference between mortality rates in all age categories on the Buffalo River and Crooked Creek, between the Pyatt site and Education Center site on Crooked Creek, and between the Buffalo River and the Pyatt site. The Buffalo River and Education Center site on Crooked Creek had a significant difference in the 2 to 9 age range as well as the 5 to 9 range.

this study were part of a long-term attempt by the AGFC to gather baseline data on the Ozark bass population and to determine the efficacy of current harvest regulations for that species in the Upper White River Basin. Baseline data will be used in the future to determine whether local fish populations respond to climate change or other impacts to the watershed.

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LITERATURE CITED

Anderson, R.O., and S.J. Gutreuter. 1983. Length, weight and associated structural indices. Nielsen, L. and Johnson, D., eds., Fisheries Techniques. Southern Printing Company, Inc., Blacksburg, Virginia, Chapt. 15.

(AGFC) Arkansas Game and Fish Commission. 2013. Statewide Lengths and Daily Limits. <http://www.agfc.com/fishing/Pages/FishingDailyLimits.aspx> Accessed 2 August 2013.

Breitburg, D. L., T. Loher, C.A. Pacey, and A. Gerstein. 1997. Vary effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecological Society of America 67(4):489-507.

Buchanan, T. and H. Robison. 1988. Fishes of Arkansas. The University of Arkansas Press, Fayetteville, Arkansas. 544 pp.

Cashner, R.C. and R.D. Suttkus. 1977. *Ambloplites constellatus*, a new species of rock bass from the Ozark upland of Arkansas and Missouri with a review of western rock bass populations. American Midland Naturalist 8:147-161.

Climate Zone. 2013. Little Rock. <http://www.climate-zone.com/climate/united-states/arkansas/little-rock/> Accessed 18 June 2013.

Jearld, A. 1985. Age determination. Nielsen, L. and Johnson, D., eds., *In* Fisheries Techniques. Southern Printing Company, Inc., Blacksburg, Virginia, pp. 301-324.

Johnson, R.L., S.D. Henry, and S.W. Barkley. 2010. Distribution and population characteristics of Shadow bass of two Arkansas Ozark streams. North Amer. J. Fisheries Mgmnt. 30:1522-1528.

Keller, A.A., P.H. Doering, S.P. Kelly, and B.K. Sullivan. 1990. Growth of juvenile Atlantic Menhaden, *Brevoortia tyrannus* (Pisces: Clupeidae) in MERL mesocosms: effects of eutrophication. Limnology and Oceanography 35(1):109-122.

Kolz, A.L., J. Reynolds, A. Temple, J. Boardman, and D. Lam. 2000. Principles and Techniques of Electrofishing. U.S. Fish and Wildlife Service, Shepherdstown, West Virginia, pp. 3-1, 10-1, 10-2, 10-3, 11-2.

Maceina, M.J., J. Boxrucker, L.D. Buckmeier, R.S. Gangl, D.O. Lucchesi, D.A. Isermann, J.R. Jackson, and P.J. Martinez. 2007. Current status and review of freshwater fish aging procedures used by state and provincial fisheries agencies with recommendations for future direction. Fisheries 32:329-340.

Magnuson, J.J., J.P. Baker, E.J. Rahel. 1984. A critical assessment of effects of acidification on fisheries in North America. Transac. Royal Soc. London 305 (1124):501-516.

Mareska, J.F., and D.C. Jackson. 2002. Use of shadow bass stock characteristics to evaluate natural and scenic waterways in Mississippi. Proc. Ann. Conf. Southeastern Assoc. Fish Wildlife Agencies 54:167-178.

Pine, W.E., J.E. Hightower, L.G. Coggins, M.V. Lauretta, and K.H. Pollock. 1983. Design and analysis of tagging studies. L. Nielsen and D. Johnson, eds., *In* Fisheries Techniques. Southern Printing Company, Inc., Blacksburg, Virginia, pp. 521-564.

Ricker, W.E. 1975. Computation and interpretation of biological statistics of fish populations. Bulletin of Fisheries Research Board of Canada, Ottawa, Canada, pp. 29-74.

Robson, D. S., and D.G. Chapman (1961) Catch curves and mortality rates. Transac. Amer. Fisheries Soc. 90(2):181-189.

Quist, M.C., M.A. Pegg, and D.R. DeVries. 2012. Age and growth. A.V. Zale, D.L. Parrish, and T.M. Sutton, eds., *In* Fisheries Techniques, 3rd ed. American Fisheries Society, Bethesda, Maryland. pp. 677-721.

(U.S EPA) United States Environmental Protection Agency. 2013. Climate Impacts in the Southeast. <http://www.epa.gov/climatechange/impacts-adaptation/southeast.html#impacts> Accessed 6 June 2013.

(USGS) United States Geological Survey. 2005. Arkansas. <http://www.nationalatlas.gov/printable/images/pdf/precip/pageprecip_ar3.pdf> Accessed 18 June 2013.

(UAF) University of Alaska-Fairbanks. 2013. What Is An Otolith? <http://elearning.uaf.edu/cc/otolith/whatis.htm> Accessed 18 June 2013.

(UWRBF) Upper White River Basin Foundation. 2012. The Upper White River: An Overview. <http://www.whiteriverbasin.org/basinoverview.php> Accessed 18 June 2013.

Uwidia, I.E. and H.S. Ukulu. 2013. Studies on electrical conductivity and total dissolved solids concentration in raw domestic wastewater obtained from an estate in Warri, Nigeria. Greener J. Phys. Sci. 3(3):110-114.

Whisenant, K.A. and O.E. Maughan. 1989. Smallmouth bass and Ozark bass in Buffalo National River. Western Region National Park Service Department of the Interior, San Francisco, Calif. pp. 1-58.

Whiteledge, G.W., C.F. Rabeni, G. Annis, and S.P. Sowa. 2006. Riparian shading and groundwater enhance growth potential for Smallmouth bass in Ozark streams. Ecological Applications 16(4):1461-1473.

The potential release of phosphorus in floodplains

Maria S. Rossetti[*], *Nicole K. Ownby*[†], *Erin Scott*[§], *and Brian E. Haggard*[‡]

ABSTRACT

In the Illinois River Watershed, there has been growing concern over elevated phosphorus concentrations in the water column. This study evaluated how much phosphorus is contributed from floodplain soils into surface waters, examining the relationship between the flux of phosphorus released and the amount of phosphorus stored in the soil. This was investigated by artificially inundating soil cores from four sites and determining the soluble reactive phosphorus concentrations of the overlying water and the levels of Water and Mehlich-3 extractable phosphorus in the soil. The flux of phosphorus to the overlying water ranged from 0.43 to 6.61 mg m^{-2} hr^{-1} within the short-term (16.5-hr incubation) and 0.06 to 1.26 mg m^{-2} hr^{-1} over the long term (282.5-hr incubation). Phosphorus flux to the overlying water was significantly correlated with the amount of phosphorus stored in the soil. This study showed that riparian soils with elevated phosphorus content have the potential to release phosphorus when flooded.

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‡ Brian E. Haggard is a professor in the Department of Biological and Agricultural Engineering and the director of the Arkansas Water Resources Center.

MEET THE STUDENT-AUTHORS

Maria Rossetti

I am from Fayetteville, Arkansas and I graduated from Fayetteville High School in 2013. I began my studies in the fall of 2013, and I am majoring in Chemical Engineering and minoring in Sustainability and Math. I am also a Julian and Nana Stewart Honors College Fellow. This project was result of the 6th annual Honors Research Symposium, which is part of the Freshman Engineering Program. The symposium allows freshman students to learn about different kinds of research in the field of engineering and gives freshman research experience. As a result of this research project, I've learned more about the environmental research opportunities in the field of engineering and I am particularly interested in issues with water quality and I hope to be able to conduct more research about these topics during the remainder of the my time as an undergraduate. After I complete my undergraduate studies, I plan on attending graduate school and becoming a professor. I would like to thank Brian Haggard and Erin Scott for their guidance during this project, without them it would not have been possible.

I am from St. Louis, Missouri, where I graduated from Lafayette High School in May, 2013. I just completed my first year at the University of Arkansas, where I recently declared my major as biomedical engineering. These past two semesters have allowed to me gain experience in the research field of biological and agricultural engineering throughout the process of writing the following paper and the field work required to do so with my research partner and departmental mentor. This coming fall I plan to perform undergraduate biomedical engineering research under the supervision of a biomedical engineering professor at the university, specifically with a focus on tissue engineering either with muscle cells or in dealing with traumatic brain injury. After graduation, I plan to attend graduate school in St. Louis, most likely at St. Louis University, for biomedical engineering and ultimately develop a career in an industry setting to further my knowledge and understanding of the field of biomedical engineering.

Nicole Ownby

INTRODUCTION

Over the last half century, there has been growing concern over elevated phosphorus concentrations and its effect on the quality of surface waters. In Northwest Arkansas, this issue has garnered more attention in the past twenty years due to litigation revolving around phosphorus concentrations in the Illinois River Watershed. In 2005, the state of Oklahoma sued 13 poultry companies in the state of Arkansas over water quality in the Illinois River and its other scenic rivers. This lawsuit was based on the premise that phosphorus (P) in poultry litter contributed to water quality problems in the Illinois River Watershed. Each year an estimated 312,978 metric tons of chicken manure and bedding (i.e., poultry litter) is produced in this area (Flynn, 2009). The poultry litter produced is frequently used as fertilizer on pastures and when applied to meet nitrogen (N) needs of the forage, it often results in the buildup of P in soils (Eghball and Power, 1999).

While a vital nutrient for plants and aquatic organisms, P can result in water quality problems when applied or available in excess of plant needs. Phosphorus inputs are a potential cause of eutrophication, which is the process in which a body of water becomes enriched with nutrients that stimulates the growth of aquatic plant life. Elevated concentrations of P can cause an increase in algal blooms which can lead to large diurnal swings in oxygen, possible fish kills from lack of dissolved oxygen in water, taste and odor issues in drinking water, and even algal toxins (Daniel et al., 1998).

Phosphorus can come from many different sources including point and non-point sources. Point sources are identifiable, confined sources from which a pollutant is discharged or emitted; examples include wastewater treatment plants, storm conveyance outlets, and even large confined agricultural operations. Non-point sources are diffused in nature and transported into bodies of water from the landscape during excess rainfall when all the water does not infiltrate the soil and instead runs off (i.e. a rainfall-runoff event).

Phosphorus in poultry litter has been applied to the landscape for many decades in the Illinois River Watershed, as well as other watersheds across the U.S. This P is stored in the soil and it is transported downhill toward the streams and rivers with each runoff event. Furthermore, P can be stored in riparian areas and floodplains, and there is a potential for the riparian soils to release P into surface waters during runoff events or inundation. Several researchers have shown the potential for P in soils to be released (via surface runoff and even subsurface flow) at levels that have the potential to cause eutrophication (Fuchs et al., 2009; Pote et al., 1999). Extensive research has been done regarding how point and non-point sources affect P concentrations in surface water. For instance, research has been conducted on how the application of poultry litter—which can contain elevated amounts of P relative to N—increases P concentrations in runoff water and contributes to non-point source pollution (Haggard et al., 2005). In addition, other studies have shown that effluent discharges from wastewater treatment plants can increase P concentrations in streams and rivers for several kilometers downstream (Haggard, 2010; Scott et al., 2011).

It is important to understand all the potential sources of P in a watershed to better understand how to improve watershed management and water quality. The historic application of P to the soils and introduction to streams from wastewater treatment plants and agriculture represents a legacy source in the riparian floodplains and within the stream channel (Jarvie et al., 2013). In order to better understand all sources of P and provide more information on how P might be released from these soils when inundated during rainfall-runoff events, we evaluated how P is released to the overlying water from soils during inundation and related the amount of P stored in riparian soils to P flux into the overlying water.

MATERIALS AND METHODS

In order to determine the amount of P released from inundated riparian soils, four sites were chosen at the Water Research and Education Center at the Arkansas Agricultural Research and Extension Center, Fayetteville, Ark., that have varying levels of soil P based on historic records (Fig. 1). Then three Plexiglas cores (~6 cm inside diameter) were pushed into the ground at each site and removed with an intact soil core. The cores were transported back to campus and allowed to equilibrate to room temperature (~20 °C). After temperature equilibration, the bottoms of the cores were sealed with a stopper and weatherproof tape and then wrapped in aluminum foil to limit light in order to prevent algal growth in the overlying water during incubation. The cores were flooded with 0.75 L of tap water and monitored approximately daily to maintain a constant volume of overlying water during the incubation period.

Approximately three times a week, water samples were taken from each core. The water samples (~20 mL) were pulled from the overlying water, then filtered through a syringe filter (0.45-µm pore size) and acidified to pH < 2 using concentrated HCl. The samples were analyzed for soluble reactive phosphorus (SRP) using the ascorbic acid method (APHA, 2012). After two weeks, each core was drained and the top 5 cm of the soil was removed and analyzed for water extractable P (WEP) and Mehlich-3 extractable P (M3P), which are two different fractions of P stored in soils. The WEP represents the P that is easily released to the water, and it is measured using a 1:10 soil (dry weight)-to-water extraction ratio and the sample is filtered through a 0.45-

Fig. 1. Locations of sites where soil samples were collected from the Watershed Research and Education Center (WREC, black boundary) at the Arkansas Agricultural Research and Extension Center (AAREC), Fayetteville, Arkansas. The provided values are the experimentally determined average Mehlich-3 phosphorus values.

μm pore size filter with a vacuum and analyzed using Inductively Coupled Plasma Optical Emissions Spectrometry (ICP OES); WEP is reported as mg kg^{-1} dry soil. The M3P represents the P that is available to support plant growth and it is a typical agronomic soil test in Arkansas; it is also measured using a 1:10 extraction ratio and ICP OES (Pierzynski, 2000).

The mass of P released to the overlying water in each core was determined by multiplying the SRP concentration by the volume of overlying water in each core (0.75 L). The mass of P (mg) released to the overlying water was compared against time (h) using simple linear regressions, and the slope (mg h^{-1}) was divided by the surface area of the soil in the cores to give the flux or rate of release per unit area (mg m^{-2} h^{-1}). The data from the 282.5-h inundation period was analyzed using linear regression to estimate "long-term flux"; whereas the slope between two points (i.e., 16.5-h incubation) was used to estimate the "short-term flux". The individual flux rates were used in an analysis of variance with means separated using least significant difference (ANOVA LSD) to determine differences between sites. The soil data, i.e. M3P and WEP content, was compared to the flux data using linear regression. In addition, the M3P and WEP content was compared across the sites using ANOVA LSD. All statistical comparisons were made using $\alpha = 0.05$ to determine significant differences between means.

RESULTS AND DISCUSSION

The linear regression for the long-term SRP flux was significant ($P < 0.05$) in almost all cases, except for one or more replicates at sites 2, 3, and 4. The mean flux rates for each site ranged from 0.06 mg m^{-2} h^{-1} at site 3 to 1.26 mg m^{-2} h^{-1} at site 1. Sites 1 and 2 (on average 1.26 and 0.83 mg m^{-2} h^{-1}, respectively) had significantly greater P flux than the other two sites at the Watershed Research and Education Center ($P = 0.0042$; Table 1), and these sites also had the highest soil P content.

However, it was observed that the mass of SRP released to the overlying water was not necessarily linear across the long-term inundation, thus indicating that this model was not always the best fit for our data (Fig. 2). The mass of SRP in the overlying water increased faster in the beginning and then tended to reach a plateau over time. For this reason, the data was analyzed using a power function to reflect how the amount of SRP released by the soil reaches a plateau concentration that is at equilibrium with the overlying water column by the end of the two-week period (Fig. 2).

Table 1. Mean soluble reactive phosphorus (SRP) flux, standard deviation, and homogenous groups (based on LSD groupings) over the long-term incubation (282.5 h).

Site†	Mean Flux ($mg\ m^{-2}\ h^{-1}$)	Standard Deviation ($mg\ m^{-2}\ h^{-1}$)	LSD Groupings‡
1	1.26	0.03	A
2	0.83	0.60	A
3	0.06	0.09	B
4	0.13	0.12	B

† See Fig. 1 for site information.

‡ Least significant difference (LSD) groupings with the same letter aren't statistically different ($P = 0.0042$).

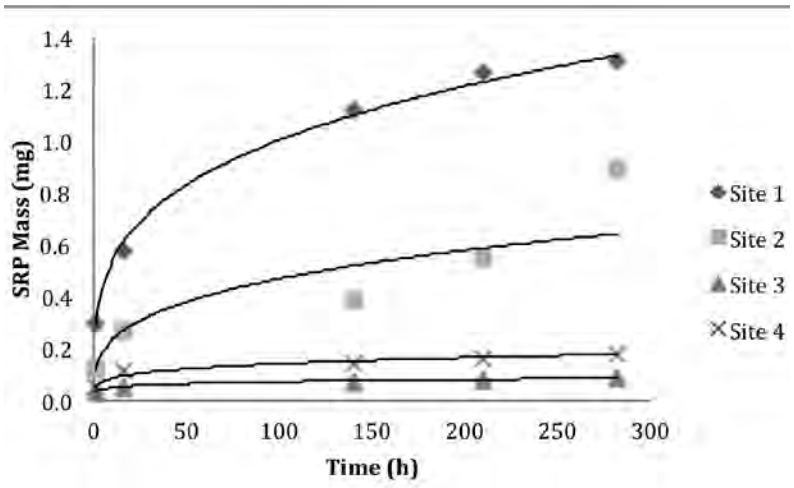

Fig. 2. Mass of soluble reactive phosphorus (SRP) released from the soil to the overlying water over the long-term incubation (282.5 h), fit to a power function. (n = 4).

Floodwaters typically do not inundate riparian soils for more than a few days, especially at the Watershed Research and Education Center where the soil cores were collected. As a result it was decided that since overlying waters remain for only a few days, the flux should be calculated based on our first two samples taken over the first 16.5 hours of inundation. This short-term flux (based on a 16.5-h inundation) represents maximum potential release, which could possibly occur during short-term inundation of riparian soils. This is consistent with a study by Aldous et al. (2005) where net SRP flux was greater after 1 day compared to 4 days.

The initial change in SRP concentration (from 1-h to 16.5-h) was used to approximate how much P would be released during inundation for a short duration, showing that short-term flux varied from an average of 0.43 to 6.61 mg $m^{-2}\ h^{-1}$ across the four sites (Table 2). The short-term P fluxes in this study were greater than the rates reported for a study of sediments of river impoundments, where P flux from aerobic cores ranged from 0.02 to 0.15 mg $m^{-2}\ h^{-1}$ (Haggard and Soerens, 2006; Haggard et al. 2012). Differences in dynamics between floodplain soils and reservoir sediments likely led to the large differences in flux rates measured in this study. Studies of P flux from lake-bottom sediments generally find that data are linear over a 10-14 d incubation, while the P concentrations through time did not follow a linear trend in the current study. Additionally, the soils used in the current study (up to 297 mg M3P kg $soil^{-1}$) may have had far more stored P than lake-bottom sediments.

The short-term flux rates were an order of magnitude greater than that observed over the long-term inundation, showing that the amount of SRP released from the soil to the overlying water was much greater in the beginning and then only smaller incremental increases occurred with time. During several days of soil inundation, P release to the water column may reach an equilibrium concentration, where P is neither released nor retained by sediments (Lottig and Stanley, 2007) or soils (Taylor and Kunishi, 1971). Whenever sediments and soils are incubated with overlying water under aerobic conditions, then the phosphate is released to a plateau concentration, representing an equilibrium between the solid and aqueous phases.

The sites had M3P contents ranging from 41 mg kg^{-1} at site 3 to 297 mg kg^{-1} at site 1, and WEP contents ranging from 9.0 mg kg^{-1} at site 3 to 61.0 mg kg^{-1} at site 1. The sites had significantly different M3P contents ($P = 0.0001$); site 1 had the greatest mean M3P content (297 mg kg^{-1}), and site 3

Table 2. Mean soluble reactive phosphorus (SRP) flux, standard deviation, and homogenous groups (based on LSD groupings) over the short-term incubation (16.5 h).

Site†	Mean Mass at 1 h (mg)	Mean Mass at 16.5 h (mg)	Mean Flux (mg m^{-2} h^{-1})	Standard Deviation (mg m^{-2} h^{-1})	LSD groupings‡
1	0.294	0.581	6.61	2.29	A
2	0.121	0.269	3.40	1.39	B
3	0.034	0.053	0.43	0.68	B
4	0.048	0.114	1.52	1.92	B

† See Fig. 1 for site information.

‡ Least significant difference (LSD) groupings with the same letter aren't statistically different (P = 0.0094).

Table 3. Mean Mehlich-3 phosphorus (M3P) and water extractable phosphorus (WEP) in soils, standard deviation and homogenous groups (based on LSD groupings).

Site†	Mean M3P (mg kg^{-1})	Standard Deviation (mg kg^{-1})	LSD Groupings	Mean WEP (mg kg^{-1})	Standard Deviation (mg kg^{-1})	LSD Groupings‡
1	297	35	A	61	5	A
2	225	28	B	34	3	B
3	41	28	D	9	8	C
4	96	24	C	25	7	B

† See Fig. 1 for site information.

‡ Least significant difference (LSD) groupings for each soil-test P type with the same letter aren't statistically different (P < 0.001).

had the lowest M3P content (41 mg kg^{-1}) (Table 3). Also, the WEP contents across the sites showed significant differences (P = 0.0002); WEP content was greatest at site 1 (61 mg kg^{-1}), slightly lower at sites 2 and 4 (34 and 25 mg kg^{-1}, respectively), and lowest at site 3 (9 mg kg^{-1}). Differences in soil-test P between sites complemented those differences observed in mean SRP flux rates across these sites. The mean flux rate at site 1 was significantly greater relative to sites 2, 3, and 4 (P = 0.0094; Table 2). The flux rate at site 1 was 6.60 mg m^{-2} h^{-1}; whereas the lowest flux was 0.43 mg m^{-2} h^{-1} at site 3, which reflects the pattern seen in the soil-P content data.

The M3P and WEP contents were compared to the short-term flux data using linear regression, showing that (1) soil M3P content was positively related to short-term SRP flux (r^2 = 0.76, P < 0.0001), and (2) WEP content was also significantly related to short-term flux (r^2 = 0.75, P < 0.0001) (Fig. 3). Other studies have shown that P concentrations in runoff waters (from rainfall simulation studies) are positively correlated to WEP and M3P content of soils (Pote et al., 1999), especially with the amount of P near the soil surface (0-5 cm) (Torbert et al., 2002). This suggests that as the amount of P stored in the riparian soils (e.g., WEP and M3P) increases, so does the potential for SRP to be released to the overlying water during runoff events and especially when inundated during flooding.

In conclusion, the data analyses show that P was released to the overlying water when soils were inundated to simulate flooding. There was variability in the amount of P released due to the amount of P stored in the soil (e.g., WEP and M3P) at the different sites at the Watershed Research and Education Center. There was a significant positive relationship between the amount of P released into the overlying water in each core and the amount of P (M3P and WEP) measured in the upper 5 cm of the soil in each core. Hence, there was a significant increase in SRP flux with increase in soil P measured as either M3P or WEP. Riparian soils, which have stored large amounts of P from upstream sources or direct application, have the potential to be a P source when inundated during flood events. In addition, comparing the long-term versus short-term SRP flux demonstrates that the rate with which P is released from the soil tends to increase rapidly in the beginning and then level off over time.

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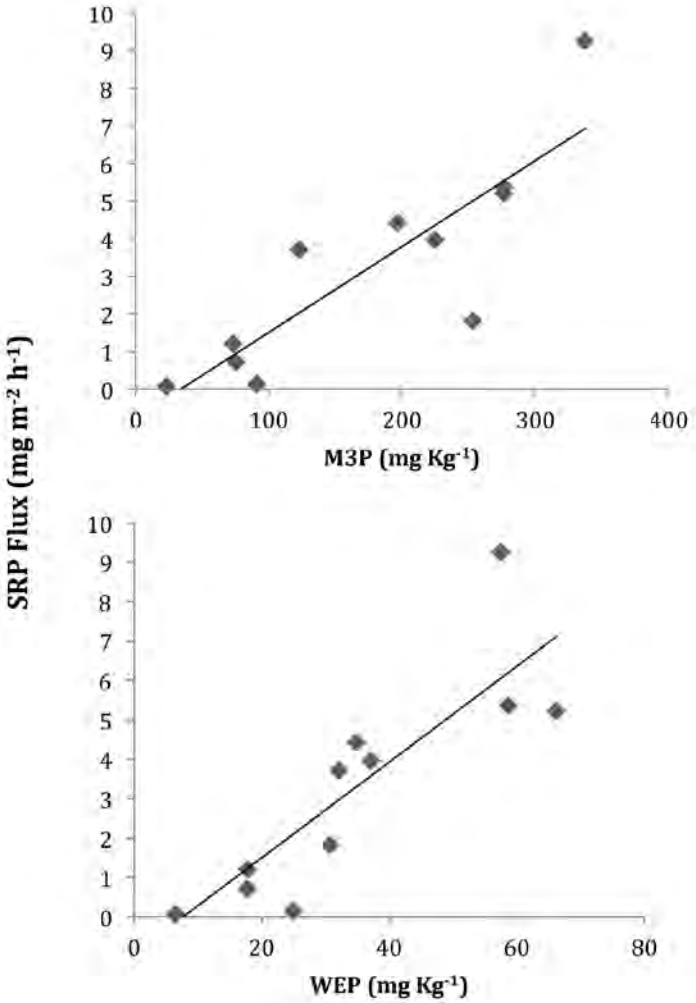

Fig. 3. Soil Mehlich-3 phosphorus (M3P) and water extractable phosphorus (WEP) content compared to short-term (16.5 h) soluble reactive phosphorus (SRP) flux to the overlying water column during inundation for each replicate.

LITERATURE CITED

APHA. 2012. American Public Health Association. Standard Methods for the Examination of Water and Wastewater, 22nd edition. E.W. Rice, R.B. Baird, A.D. Eaton, and L.S. Clesceri, eds. Washington, D.C. 1469 pp.

Aldous, A., P. McCormick, C. Ferguson, S.Graham, and C. Craft. 2005. Hydrologic regime controls soil phosphorus fluxes in restoration and undisturbed wetlands. Restoration Ecology 13:341-347.

Carlson, R.E. and J. Simpson. 1996. A Coordinator's Guide to Volunteer Lake Monitoring Methods. North American Lake Management Society.

Daniel, T. C., A. N. Sharpley, and J. L. Lemunyon. 1998. Agricultural phosphorus and eutrophication: A symposium overview. J. Environ. Qual. 27(2): 251-257.

Eghball, B., and J.F. Power. 1999. Phosphorus-and Nitrogen-Based Manure and Compost Applications Corn Production and Soil Phosphorus. Soil Sci. Soc. Amer. J. 63(4): 895-901.

Flynn, D. "Biomass Energy: 'Poultry Litter' Solution." Food Safety News. Food Safety News, 25 Sept. 2009. <www.foodsafetynews/2009/09/biomass-energy-a-poultry-litter-solution/#.u7BG.PldV8E> Accessed 5 Dec 2013.

Fuchs, J.W., G.A. Fox, D.E. Storm, C.J. Penn, and G.O. Brown. 2009. Subsurface transport of phosphorus in riparian floodplains: Influence of preferential flow paths. J. Environ. Qual. 38(2): 473-484.

Haggard, B.E. 2010. Phosphorus concentrations, loads, and sources within the Illinois River drainage area, northwest Arkansas, 1997–2008. J. Environ. Qual. 39 (6): 2113-2120.

Haggard, B.E., P.B. DeLaune, D. R. Smith, and P.A. Moore Jr. 2005 Nutrient and β17Estradiol Loss in Runoff Water from Poultry Litters. J. Amer. Water Res. Assoc. 41(2): 245-256.

Haggard, B.E. and T.S. Soerens. 2006. Sediment phosphorus release at a small impoundment on the Illinois River, Arkansas and Oklahoma, USA. Ecol. Engineer. 28:280-287.

Haggard, B.E., J.T. Scott, and S. Patterson. 2012. Sediment phosphorus flux in an Oklahoma reservoir suggests reconsideration of watershed management planning. Lake Reserv. Mngmnt. 28:59-69.

Jarvie, H. P., A. N. Sharpley, P.J. Withers, J.T. Scott, B.E. Haggard, and C. Neal. 2013. Phosphorus Mitigation to Control River Eutrophication: Murky Waters, Inconvenient Truths, and "Postnormal" Science. J. Environ. Qual. 42(2): 295-304.

Lottig, N.R. and E.H. Stanley. 2007. Benthic Sediment Influence on Dissolved Phosphorus Concentrations in a Headwater Stream. Biogeochemistry 84.3 (2007): 297-309.

Pierzynski, G.M. 2000. Methods of phosphorus analysis for soils sediments, residuals and waters. North Carolina State University, Raleigh.

Pote, D. H., T.C. Daniel, D.J. Nichols, A. N. Sharpley, P.A. Moore Jr., D. M. Miller, and D.R. Edwards. 1999. Relationship between Phosphorus Levels in Three Ultisols and Phosphorus Concentrations in Runoff. J. Environ. Qual. 28 (1): 170. Print.

Scott, J. T., B. E. Haggard, A.N., Sharpley, and J.J. Romeis. 2011. Change point analysis of phosphorus trends in the Illinois River (Oklahoma) demonstrates the effects of watershed management. J. Environ. Qual. 40 (4): 1249-1256.

Taylor, T.W., and H.M. Kunishi. 1971. Phosphate equilibira on stream sediment and soil in a watershed draining an agricultural region. J. Agric. Food Chem. 19(5): 827-831.

Torbert, H. A., T. C. Daniel, J. L. Lemunyon, and R. M. Jones.2002. Relationship of Soil Test Phosphorus and Sampling Depth to Runoff Phosphorus in Calcareous and Noncalcareous Soils. J. Environ. Qual. 31 (4): 1380-387.

Safety of improved Milbond-TX mycotoxin binder when fed to broiler breeders above recommended levels

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ABSTRACT

An increasing concern in poultry nutrition is the effects of mycotoxins in contaminated grain. Several new products have come onto the market that chemically bind these toxins preventing mycotoxicosis. However, many of these products have not been tested for safety if accidently overfed to broiler breeders. In order to simulate a feed mixing error at a feed mill, Improved Milbond-TX® was overfed to broiler breeders to see if this would cause any negative effects on bird performance. A typical corn-soybean based diet supplemented with Milbond-TX mycotoxin binder at three different levels of inclusion (0%, 0.5%, and 1%) was fed to 300 broiler breeder hens. Data were collected on egg production, egg weights, hatchability, fertility, and chick weights from 24 to 35 weeks of age. Eggs per hen housed were not significantly different between the three treatments. The differences in egg weights, hatchability, fertility, and chick weights were also insignificant among the three treatments. We were able to conclude that overfeeding Improved Milbond-TX had no negative effect on bird performance and is safe to feed at a level of up to 1%.

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‡ Dr. Coon is the mentor and a professor of Poultry Nutrition in the Department of Poultry Science Center of Excellence for Poultry Science.

MEET THE STUDENT-AUTHORS

Michael Schlumbohm

I am the son of Aron and Marie Schlumbohm of Leipsic, Ohio and I am a senior Poultry Science major. I have a background with exhibition poultry and I continue to work with our extension service in judging poultry shows and organizing the state fair poultry show. However, my other interest is with commercial poultry production and particularly with poultry nutrition. I am a member of the Poultry Science Club and I currently work for Dr. Coon at the poultry research farm.

Last summer I had the opportunity to study abroad in Nampula, Mozambique where I worked with a broiler and layer farm for three weeks. After earning my B.S. degree I intend to go to graduate school and earn at least my master's degree in poultry nutrition. I would like to thank Dr. Coon for giving me this research trial to work on. I would also like to thank Judy England and Justina Caldas for giving me advice on writing and revising the paper, and helping me interpret the data.

My name is Ruben Kriseldi. I am a May 2014 graduate with a Poultry Science major. I am originally from Indonesia and I came to the U.S. for the first time in 2010 to get my bachelor degree. I chose University of Arkansas because of the poultry science program. The influence came from my father who has been involved in the industry for several years. In the past, he used to have several research projects in chicken and I often helped him. From there, I developed an interest in poultry science which led me to study here. My future plan is to get my Ph.D in poultry nutrition. I would like to work as a poultry nutritionist. After that, I hope that I can have my own company and wish to help feed the people in need.

Rueben Kriseldi

INTRODUCTION

Mycotoxins are caused by fungi that grow on grain either in the field or during storage of complete feed (Fig. 1). Dangers of mycotoxin infections are especially high during drought years or if grain is improperly stored. These fungal infections are hard to avoid and exist in virtually all feed that livestock and poultry consume. Previous studies of mycotoxin levels in poultry feeds have revealed the presence of a number of different toxins. Many recent samples contain at least 10 contaminants (Croubels, 2013). The most prevalent mycotoxins include toxins from the genera of *Fusarium, Aspergillus*, and *Penicillium* (Croubels 2013). Most of the time there is no marked effect on animal performance. However, when exceptionally high levels occur in poultry feeds it can lead to mycotoxicosis. Mycotoxicosis can be a serious threat to poultry performance as it can cause lesions in the gastrointestinal tract, and oral cavity as well as erosion of the gizzard, inflammation of the proventriculus and epithelial mucosa of the intestinal wall (Fig. 2). These conditions can cause reduced uptake of nutrients and can leave the bird susceptible to further infection from other pathogens. Mycotoxicosis also results in reduced flock uniformity.

Because of the ill-effects of mycotoxicosis, there have been several methods employed to reduce the amount of mycotoxins ingested by animals. Among these is the use of genetically resistant crops (Wu et al., 2006; Kabak et al., 2006). Proper crop rotation and management, use of biological and chemical agents, and irradiation are also ways to prevent mycotoxin growth in grains (Kabak et al., 2006). Recently there has been research into the use of clay-based adsorbents. Improved Milbond-TX® is an inert montmorillonite clay-based hydrated sodium calcium aluminosilicate (HSCAS) adsorbent that originates from natural clay deposits (Miles and Henry, 2007a). Though there has been research testing the safety of Improved Milbond-TX in broiler and layer diets, there has not been as much research done with broiler breeders. The basic principle of clay-based adsorbents is similar to a chemical reaction and therefore, the release of free energy is the driving force of every adsorption (Huwig et al., 2001). Physical structure of the adsorbent such as total charge and charge distribution and surface area are important features as well. Along with this the properties of the adsorbate molecules, the mycotoxins, such as polarity, solubility, size, shape and—in case of ionized compounds—charge distribution and dissociation constants play a significant role. Because of the variability of these properties, it is important to investigate the effectiveness of each product when it interacts with the mycotoxins (Huwig et al., 2001).

However, it was not certain how higher than recommended levels of Improved Milbond-TX would affect egg production, egg weight, hatchability, fertility, or chick weights of broiler breeders. The goal of this study was to determine if or how bad the effect of higher than recommended levels of this product would affect bird performance. Based on previous studies done with this product, it was hypothesized that the effect would be slight if at all.

MATERIALS AND METHODS

The birds raised for this experiment were Cobb 500 females and MX males (Cobb-Vantress, Inc., Siloam Springs, Ark.). All of the feed that was fed from 0 to 21 weeks of age also was supplied by Cobb-Vantress. The birds were fed on

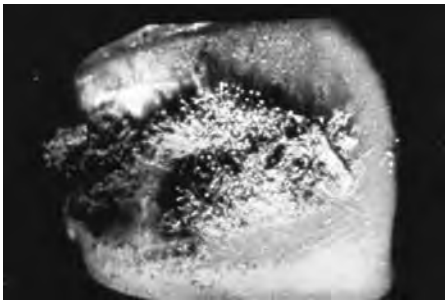

Fig. 1. Corn kernel infected by *Aspergillus flavus*. Infection by this mold can lead to mycotoxicosis. Photo courtesy of Dr. William Huff, USDA.

Fig. 2. Aflatoxin affected liver (left) vs healthy liver (right). Photo courtesy of Dr. William Huff, USDA.

a skip-a-day schedule and reared to the body weight growth curve recommended by the primary breeder.

At 21 weeks, the birds were divided between 12 pens. There were 4 pens per treatment. In each treatment there were a total of 100 hens making for a total of 300 hens used. The hens were housed in floor pens with 25 hens and two cocks per pen.

Also at 21 weeks, the birds were switched over from the grower diet to the breeder diets that included the Milbond-TX mycotoxin binder; it was also at this age that they were light stimulated. There were three treatments, each with a different level of inclusion of Improved Milbond TX. Treatment one had an inclusion of 0%, treatment two, 0.5%, and treatment three, 1.00% (Table 1). In all three treatments, birds were fed the same amount of feed daily (g/bird/day). The amount of feed was adjusted for pens that had mortality. The males were also fed according to the recommendations of the primary breeder.

All diets were formulated to ideal protein profile, 15.5% crude protein (CP) and to contain 2915 apparent metabolizable energy (AME) kcal/kg (Table 2). Hens were fed according to the primary breeder's recommendations for feeding hens into production. At peak feed consumption, hens were receiving 154 g feed/bird/day. At peak consumption, hens were consuming 450 kcal/bird/day and 23.9 g CP/bird/day. Feed was withdrawn post-peak egg production. Samples of the feed were submitted to the Central Analytical Laboratory (University of Arkansas Poultry Science Center, Fayetteville, Ark.) for analysis (Table 3).

Eggs were gathered three times daily from the start of production until peak production. After the hens reached peak, eggs were picked up twice daily. At each egg gathering, the number of eggs was recorded. All eggs were saved for hatching; a marker was used to write the pen number on each egg in order to track the hatchability and fertility by pen.

Table 1. Experimental Breeder Diets.

Ingredient†, %	Treatment 1 (0%)	Treatment 2 (0.5%)	Treatment 3 (1.00%)
Corn 9.5% CP	64.30	63.70	62.10
Soybean meal 47.1% CP	17.10	17.20	17.60
Wheat Shorts	5.00	5.00	5.00
Poultry Fat	3.12	3.30	3.82
Limestone	7.51	7.51	7.51
Dicalcium Phosphate	1.51	1.51	1.52
Salt	0.33	0.33	0.33
Sodium Bicarbonate	0.20	0.20	0.20
Methionine 98.5%	0.30	0.30	0.30
Lysine	0.20	0.20	0.20
Choline Chloride 60%	0.12	0.12	0.12
Vitamin Premix	0.20	0.20	0.20
Trace Mineral	0.08	0.08	0.08
Selenium Premix 0.6%	0.02	0.02	0.02
Ethoxoquin	0.02	0.02	0.02
Milbond-TX	0.00	0.50	1.00

† These were the formulas used to provide the necessary nutrients for optimum bird performance based on primary breeder recommendations, plus the addition of the Milbond-TX mycotoxin binder. CP = crude protein.

Table 2. Calculated nutrient content.

Calculated Nutrient†	Diets		
	0%	0.5%	1.00%
AME, Kcal/Kg	2,915.00	2,915.00	2,915.00
CP, %	15.50	15.50	15.50
Calcium, %	3.25	3.25	3.25
Available Phosphorous, %	0.41	0.41	0.41
Digestible Lysine, %	0.75	0.75	0.75
Digestible TSAA, %	0.68	0.68	0.68
Digestible Threonine, %	0.42	0.42	0.42

† Nutrient content of each diet based on mathematic calculation. AME = Apparent metabolizable energy, CP = crude protein, TSAA = Total sulfur amino acids.

Table 3. Actual nutrient analysis.

Analyzed Nutrient †	Diets		
	0% (treatment 1)	0.5% (treatment 2)	1.00% (treatment 3)
DM, %	89.80	89.40	89.90
CP, %	15.30	16.00	15.30
Ash, %	11.39	11.56	12.42
Fat, %	5.45	5.48	6.27
Calcium, %	3.27	3.37	3.38
Total Phosphorous, %	0.60	0.64	0.60
Calories/g	3,788.00	3,787.00	3,772.00

† Nutrient content of the diets as determined by laboratory analysis.
DM = dry matter, CP = crude protein.

Egg weights were recorded two days of each week. The weight was recorded by gathering all the eggs in a pen and weighing all the eggs on a digital scale and then dividing by the number of eggs gathered.

Starting the week after the hens came into production, the eggs were set in an incubator weekly for 12 weeks total. At transfer on the 18th day, the eggs were candled for infertile, contaminated, and early dead embryos. These eggs were then broken open to determine if it was infertile, contaminated, or died early during incubation. When the hatch was pulled each chick was weighed individually using a digital scale. The remaining unhatched eggs were broken open to determine when the embryo died. At the end of the trial, the percent hatch and fertility were compared between the treatments. The number of eggs, egg weights, and chick weights were also analyzed and compared.

Data were analyzed using JMP Pro 10 (SAS Institute, Inc., Cary, N.C.) using standard least square analysis reduced maximum likelihood (REML) method. Pen was considered as a random effect. When significant differences were found, means were separated using Tukey's honestly significant difference (HSD), $\alpha = 0.05$.

RESULTS AND DISCUSSION

Overall the birds used stayed in good health with total hen mortality at only 1.7%, not due to the inclusion of Improved Milbond-TX mycotoxin binder. Similarly, in trials done with broilers and commercial egg-type layers, mortality has been kept below 3% in one case and below 2% in the other. Mortality in these trials was not due to the inclusion of Improved Milbond-TX mycotoxin binder (Miles and Henry, 2007a,b).

Egg Production. In the current trial, the number of eggs per hen housed was not significantly different between the treatments. The numbers of eggs per hen housed were 55.5, 57.8, and 56.9 for treatments 1, 2, and 3, respectively (P = 0.4233, SEM =1.2).

In an experiment done by Miles and Henry, 2007a with Leghorn hens fed Improved Milbond TX, there was a significant difference between hens fed a diet with 0% and 1% inclusion. Hens fed diets with no added Improved Milbond TX had a higher average daily production from weeks 9-12. However, they were using hens from two different genetic lines, one selected for poor shell quality and one for good shell quality. When this was taken into account, there was no effect of the Improved Milbond TX on egg production (Miles and Henry, 2007a). Improved Milbond TX had no effect on feed intake and it appeared that no other nutrients relating to egg production were tied up by the binder.

Egg Weights. Egg weights were not significantly different among treatments (Table 4, P = 0.759, SEM = 0.689). The egg weights at 35 weeks of age were within 0.6 grams between treatment one and treatments two and three. Treatments two and three were exactly the same. It would seem that no nutrients were tied up by the binder that may affect egg weight or synthesis of protein.

Likewise, Miles and Henry, 2007a found no significant differences ($P > 0.10$) between egg weights among treatments except for during two separate weeks of their trial. During weeks 16 and 20, they did find a significant difference between treatments which received 0%, 1%, and 2% inclusion of Improved Milbond-TX mycotoxin binder, with the weights of the treatment given 2% being the lowest ($P > 0.05$; Miles and Henry 2007a)

Hatchability. Hatchability in this trial was calculated from total eggs set for the week. Overall hatchability was not significantly different among treatments (P = 0.3152). Only in one week's hatch was there a significant difference. Between treatment 2 (0.5%) and treatments 1 (0%) and 3 (1%) there was a difference. Due to this occurring only one week of the whole trial, it is unlikely that this was because of feeding Improved Milbond TX. Some possibilities for this reduced hatchability could have been a result of a hatchery error that affected the trays containing eggs from this treatment or rough handling, cracked eggs etc. Besides this one week with significant difference (P = 0.0306), hatchability was consistent throughout the trial and was not affected by feeding Improved Milbond TX (Table 5).

Table 4. Egg weights (g) each week for each treatment. Average weight of eggs collected each week during the trial.

Treatments	24	25	26	27	28	29	30	31	32	33	34	35
1	47.8	47.5	50.8	55.2	54.6	55.3	56.8	60.0	59.3	60.7	62.2	62.8
2	44.8	50.5	51.5	53.8	54.4	55.5	56.8	58.6	56.0	60.6	61.7	62.2
3	46.0	47.0	51.7	53.6	56.0	57.3	57.4	60.2	58.4	61.2	62.4	62.2
P-value	0.264	0.175	0.795	0.595	0.168	0.418	0.432	0.475	0.335	0.742	0.710	0.759
SEM		1.313	1.057	1.173	0.562	1.126	0.384	0.994	1.547	0.561	0.595	0.689

P-value less than 0.05 indicates significant differences. SEM is the standard error of the mean.

Table 5. Hatchability of all eggs set (%). Percent of all eggs set that hatched.

Treatments	26	27	28	29	30	31	32	33	34	35	Overall
1	85.0	88.0	90.1	88.9	90.2	90.2	90.0[a‡]	89.7	89.7	87.6	89.0
2	82.3	83.3	87.6	88.2	87.8	88.2	85.2[b]	91.3	91.3	88.9	87.4
3	85.1	85.8	87.3	88.2	87.7	88.0	91.6[a]	86.7	86.7	87.0	87.7
P-value†	0.8481	0.3549	0.2958	0.9613	0.6483	0.5746	0.0306	0.1272	0.1272	0.5107	0.3152

† Standard least square reduced maximum likelihood (REML) analysis (pen treated as a random effect). Means separated by student's *t* test α = 0.05.

‡ Values followed by the same letter are not significantly different.

Fertility. The fertility among the treatments remained consistent throughout the trial and there was no significant difference between any treatments ($P = 0.2630$). Overall fertility was consistent, there was a moderate increasing trend as the birds aged in treatments 2 and 3 (Table 6). It appears that feeding higher than recommended levels of Improved Milbond TX has no effect on sperm production or sperm quality of the males and does not reduce mating activity. Likewise, it had no effect on the ability of the female to store sperm and did not interfere with fertilization in the female.

Chick Weights. The chick weights were unaffected by feeding higher levels of Improved Milbond TX (Table 7) and there was no significant difference among the treatments ($P = 0.6738$). The increase of chick weights over the trial period is normal. Older hens lay larger eggs and as a result the chick weights increased. In regard to egg weights, this same phenomenon was found in a trial done by Miles and Henry (2007a) with commercial egg-type laying hens. On average, chick weights increased 6.3 grams per chick over 10 weeks. As far as overall weights are concerned, there was only a 0.3 gram difference between treatment 3 and treatments 1 and 2. Treatments 1 and 2 were exactly the same.

Since chick weight is closely linked to egg weight (Halbersleben and Mussehl, 1922) it can be asserted that if egg weight is not affected by feeding higher than recommended levels of Improved Milbond-TX mycotoxin binder, then chick weight will also be unaffected.

SUMMARY

In conclusion, the inclusion of Improved Milbond-TX mycotoxin binder at levels up to 1% in broiler breeder diets has no negative effect on bird performance in terms of egg production, egg weight, hatchability, fertility, and chick weight, and is therefore safe to use up to this level.

ACKNOWLEDGMENTS

I would like to thank Cobb-Vantress for providing the birds and feed used in this trial.

LITERATURE CITED

Croubels, S. 2013. Mycotoxins and their effects on the intestinal health of poultry. The Poultrysite Digital. 5M Publishing. <http://www.thepoultrysite.com/>. Accessed 10 November 2013.

Huwig, A., S. Freimund, O. Käppeli, and H. Dutler. 2001. Mycotoxin detoxication of animal feed by different adsorbents. Toxicol. Lett., 122: 179–188.

Kabak B, A.D Dobson, and I. Var. 2006. Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review. Crit. Rev. Food Sci. Nutr. 46:593-619.

Miles, R.D. and P.R. Henry. 2007a. Safety of improved Milbond-TX when fed to laying hens at higher-than-recommended levels. J. Appl. Poultry Res. 16:404-411.

Miles, R.D. and P.R. Henry. 2007b. Safety of Improved Milbond-TX when fed in broiler diets at higher-than-recommended levels. Animal Feed Sci. Technol. 138:309-317.

Wu, F. 2006. Mycotoxin Reduction in Bt Corn: Potential Economic, Health, and Regulatory Impacts. Transgenic Research 15:277-289.

Halbersleben, D.L. and F.E. Mussehl. 1922. Relation of Egg Weight to Chick Weight at Hatching. Poultry Science 1(4): 143-144.

Table 6. Fertility of all eggs set (%). Percent of eggs set that were fertilized each week.

Treatment	26	27	28	29	30	31	32	33	34	35	Overall
1	98.2	99.1	97.2	97.8	98.7	97.8	98.6	100	99.4	99.1	98.4
2	94.7	97.2	94.9	97.8	98.1	98.6	97.3	100	98.6	99.6	97.5
3	96.9	98.6	97.9	98.8	99.2	98.7	99.6	100	99.3	98.4	98.6
P-value†	0.2754	0.3089	0.1153	0.7397	0.6167	0.8704	0.4142	-	0.3397	0.2241	0.2630

† Standard Least Square reduced maximum likelihood (REML) analysis (pen treated as a random effect).

Table 7. Chick Weights (g). Day old chick weights each week.

Treatments	26	27	28	29	30	31	32	33	34	35	Overall
1	37.1	37.0	38.7	38.7	39.6	41.3	42.1	42.8	42.8	42.3	40.1
2	35.3	37.2	38.4	39.2	39.5	41.3	42.8	42.9	42.9	42.5	40.1
3	36.3	37.4	39.5	39.6	39.7	41.7	42.4	43.3	43.3	42.8	40.4
P-value†	0.5104	0.6654	0.1284	0.2850	0.9421	0.4847	0.3948	0.5099	0.5099	0.7445	0.6738

† Standard Least Square reduced maximum likelihood (REML) analysis (pen treated as a random effect).

Reducing water extractable phosphorus in poultry litter using chitosan treatment

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ABSTRACT

Phosphorus (P) is an important factor in the eutrophication of freshwater, and watershed sources include effluent discharges and the landscape. Poultry litter applied to the landscape can be a potential source of P, which is dependent on rainfall, runoff and dissolution. Chitosan, the deacetylated form of the biopolymer chitin, has been shown to have an effect on reducing water extractable phosphorus (WEP) in poultry litter when applied as a powder. The intent of this study was to measure the effect that poultry litter treatment (PLT), acetic acid and incubation time have on chitosan's ability to reduce WEP in poultry litter. The results were that (1) the presence of PLT in the litter inhibits chitosan's ability to reduce WEP; (2) chitosan dissolved in acetic acid (0.005, 0.01, 0.02, and 0.05 g mL^{-1}) does not decrease WEP at any point during a 7 week incubation period; and (3) chitosan in a powder form reaches its full effectiveness after three weeks of incubation. Chitosan could be an effective coamendment to poultry litter with other treatments in order to reduce WEP.

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MEET THE STUDENT-AUTHOR

Zachary Simpson

I am from Vilonia, Arkansas and graduated from Vilonia High School in 2010. I had an interest in environmental health and sustainability which led to joining the biological engineering program at the University of Arkansas. In May 2014, I graduated with a B.S. in Biological Engineering with a minor in Sustainability. During my time at the university, I was active in the Biological Engineering Student Club and am a member of the American Society of Agricultural and Biological Engineers. I plan to pursue my interests in water quality and watershed management by attending graduate school in Biological Engineering at the University of Arkansas in the fall of 2014.

I would like to thank Dr. Brian Haggard, the project mentor, for making this possible and for his teaching. I would also like to thank Brina Smith, an associate of the Arkansas Water Resources Center, who guided me through all of the laboratory work for this project and provided a great amount of support.

INTRODUCTION

Phosphorus (P) has been a concern for water quality because it is considered to be one of the primary factors limiting algal growth and influencing eutrophication (Parry, 1998; Correll, 1998). The enrichment of freshwaters causes increased primary production (i.e. algal growth), leading to changes in aquatic communities (Smith, 1998; Swingle, 1966), diurnal changes in dissolved oxygen (Alabaster, 1959; Alabaster, 1961; Floyd, 1992), anoxic bottom waters during lake and reservoir stratification (Diaz and Rosenberg, 2008; Floyd, 1992), and even taste and odor issues in drinking water supplies (Walker, 1983). Phosphorus and other nutrients enter freshwaters through defined discharges and diffuse sources from the landscape.

The diffuse sources are transported during rainfall-runoff events from the landscape, including agricultural fields and urban development. The agricultural sources include P stored in soils and what is applied to the landscape in fertilizers and animal manures. In northwest Arkansas, poultry production and application of poultry litter (manure plus bedding) represent an important diffuse source of P in watersheds. Several studies have shown that the water extractable phosphorus (WEP) content of poultry litter is positively correlated to P concentrations in runoff during rainfall simulation studies (Haggard et al., 2005; Kleinman and Sharpley, 2003; Kleinman et al., 2007; Vadas et al., 2004). This relation has prompted research on ways to minimize the WEP content of poultry litter; for example, aluminum sulfate (alum) has been shown to reduce WEP in poultry litter (Dao, 1999) and therefore reduce P concentrations in runoff from field plots (Moore et al., 2000; Shreve et al., 1995; Smith et al., 2001).

A biologically derived coamendment, in the form of chitosan, has also been researched for its ability to reduce WEP in animal manures (Bailey, 2012) among its other uses (Garcia et al., 2009; Kumar and Majeti, 2000; Rabea et al., 2003; Rinaudo, 2006). The preliminary lab studies have shown that WEP in poultry litters was reduced when chitosan was applied at 1-10% rates (as is basis), and chitosan was as effective as alum at the 1-5% application rates (Bailey et al., 2014). To further understand the ability of chitosan to reduce WEP content in poultry litter, the goal of this study is to evaluate factors that alter WEP reduction in poultry litter treated with chitosan. We hypothesized that chitosan delivered in acetic acid solution, which is commonly used to dissolve chitosan in other applications, will produce a significantly greater reduction of WEP content in poultry litter than dry application of chitosan powders. We also hypothesized that there is greater reduction of WEP content in poultry litter as incubation time progresses, especially with the dry application of chitosan powders.

MATERIALS AND METHODS

Poultry litter was collected from the stacking barn and compost at University of Arkansas poultry facilities, which grows birds under contract for Simmons Foods (Siloam Springs, Ark.). These poultry facilities used Poultry Litter Treatment (PLT, sodium bisulfate, $NaHSO_4$) during bird production to reduce ammonia (NH_3) volatilization, and PLT also influences litter chemistry (Pope and Cherry, 2000; Sweeney et al., 1996). In the first experiment using PLT treated litter, a control and four different application rates (percent on dry weight basis) were used for each delivery method, which is delivery as a powder or dissolved in dilute (2%) acetic acid solution. The PLT treated litter was homogenized and divided into 20-g samples (dry weight equivalent), mixed with the treatment, and incubated at room temperature for two weeks. The treatments consisted of a control (untreated), a control treated with only dilute acetic acid, four application rates of ChitoClear® chitosan (90% or more pure) in powder form (i.e., 0.5%, 1.5%, 3%, and 5% dry weight equivalent, g chitosan g^{-1} poultry litter), provided by Dr. Zaharoff, and then chitosan delivered as dissolved in acetic acid (0.05%, 0.1%, 0.2%, and 0.5% dry weight equivalent, g chitosan g^{-1} poultry litter); for each treatment, 4 replicates were used. After incubation, the poultry litter samples were extracted for water extractable phosphorus (WEP) using a 1:100 dry litter-to-water ratio (Kleinman et al., 2007) and then the filtrate was analyzed using the inductively coupled argon plasma optical emission spectrometry (ICP-OES) at the University of Arkansas Soil Diagnostic Lab, Fayetteville, Ark. The WEP_{ICP} content was compared across treatments using analysis of variance (ANOVA) with mean separation (least significant difference, LSD) at $\alpha = 0.05$. The filtrate was also analyzed using the ascorbic acid method for soluble reactive P (SRP) to measure WEP_{SRP}.

In the second experiment, a new source of poultry litter that was not treated with PLT was collected from the University of Arkansas experimental poultry facilities at the Arkansas Agricultural Research and Extension Center in Fayetteville. This litter was handled as previously described in experiment one, and then both litters (PLT and non-PLT amended) were used in the next experiment. Four different types of chitosan (all at 90% or greater purity) were used in this experiment (Table 1), including the one used in first experiment and the same three used in a previous study (Bailey, 2012; Bailey et al., 2014). A control and four treatments (each chitosan form applied at 10% on dry weight basis) were used for each litter source, where the chitosan was applied in powder form not dissolved in dilute acetic acid. Five replicates were used for each control and treatment, where 6-g dry weight equivalent poultry litter was incubated. The treatments were applied; the litter was mixed, incubated for 7 weeks, and then WEP was measured on subsamples after 1, 4, and 7 weeks. After the selected incubation time, up to 2 g (dry weight) of the samples were extracted to measure WEP (Kleinman et al., 2007) as modified in the first experiment. The WEP solutions were filtered using a Whatman-40 filter (GE Healthcare Life Sciences, Pittsburgh, Pa.) via gravity filtration (primary filtration) and the filtrate as analyzed for SRP using the modified ascorbic acid reduction method, which is analogous to WEP_{SRP}. Analysis of variance with mean separation (LSD) at $\alpha = 0.05$ was used to compare treatments. Calculation of WEP_{SRP} removed across all chitosan treatments compared to the control was also done for each incubation period.

In the third experiment, only the non-PLT litter source was used based on the results from experiments one and two. Approximately, 8 g of poultry litter (dry weight equivalent) were separated into containers. This experiment featured the following treatments: a control, a control with just acetic acid (approximately 0.8 mL), 10% (dry weight basis) chitosan in powder form, and varying application rates of chitosan delivered in a dilute acetic acid solution (i.e., 0.05%, 0.10%, 0.20%, and 0.50% chitosan on a dry weight basis, g chitosan g^{-1} poultry litter). The chitosan used was the medium molecular weight chitosan, and incubation times were set ranging from 1 to 7 weeks for all treatments. The treatments were sampled at the selected incubation times, and then extracted following the same process as in experiment two and analyzed for WEP_{SRP}. The same statistical analysis as for the previous two experiments was repeated in the third experiment to compare treatment means.

Table 1. A list of chitosan types used in experiment 2.

Number	Type of Chitosan
1	ChitoClear®, provided by Dr. Zaharoff.
2	≥75% deacetylated chitosan†
3	Practical grade chitosan‡
4	Medium molecular weight chitosan§

† Sigma-Aldrich, C3646-25G.
‡ Sigma-Aldrich, 417963-25G.
§ Sigma-Aldrich, 448877-50G.

RESULTS AND DISCUSSION

Experiment 1. The results from the first experiment were unexpected since the WEP_{ICP} content of the poultry litter samples treated with chitosan in powder form and the samples treated with chitosan dissolved in acetic acid were not significantly different from the control (3942 mg kg^{-1}, Table 2). The PLT litter treated with 0.20 (dry weight basis) chitosan dissolved in acetic acid had WEP_{ICP} content (3986 mg kg^{-1}) numerically greater than the control. The PLT litter treated with 0.50% (dry weight basis) chitosan dissolved in acetic acid had WEP_{ICP} content (4143 mg kg^{-1}) numerically greater than the control and was significantly different from WEP_{ICP} content of some of the other chitosan treatments. These results were contrary to the observations made in previous studies (Bailey, 2012; Bailey et al., 2014), which showed that chitosan applied to poultry litter in powder form significantly reduced WEP_{ICP} content.

The first experiment was conducted to follow Bailey (2012), where WEP was measured using ICP-OES at the University of Arkansas System Division of Agriculture Soil Diagnostic Lab (i.e., following Kleinman et al., 2007). However, the filtrate was also analyzed for SRP using a colorimetric method, which is designated as WEP_{SRP}. These two methods differ, where WEP_{ICP} represents the total P measured in the filtrate whereas WEP_{SRP} represents the reactive P measured in the filtrate. However, analysis of the same samples using both analytical methods showed a significant, positive correlation between WEP_{ICP} and WEP_{SRP} (Fig. 1). Since both analyses were comparable and SRP analysis was more practical in the laboratory, SRP using spectrometry analysis was used for the rest of the experiments.

Experiment 2. Since the first experiment showed such unexpected results, several factors were called into question: the source of the poultry litter, the source of chitosan used, and also the length of the incubation. Experiment 1 used poultry litter that had been treated with PLT, or sodium bisulfate ($NaHSO_4$), and is used in commercial poultry production to reduce ammonia volatilization. The bisulfate, HSO_4^-, reduces litter pH which reduces ammonia volatilization and therefore improves bird health (Sweeney et al., 1996). This chemical amendment was suspected to have an effect on chitosan's ability to reduce WEP in the litter. In order to examine its effect, a new source of poultry litter that had not been treated with PLT was obtained for the second experiment.

To test whether the source of chitosan played a role in the first experiment's results, three new sources of chitosan, all used by Bailey (2012), and the source of chitosan in the first experiment were included in the second experiment. The second experiment tested the new sources of chitosan and the original source on both sources of poultry litter (PLT and non-PLT treated) at a rate of 10% (dry weight basis), which was within the range of treatment shown to be effective at reducing WEP_{ICP} (see also Bailey et al., 2014).

For the poultry litter that had been treated with PLT, the results after a 4 week incubation showed that WEP_{SRP} of PLT litter treated by all sources of chitosan were not significantly different than WEP_{SRP} of the control (4172 mg kg^{-1}, Table 3). The samples treated with chitosan had numerically greater amounts of WEP_{SRP} than that of the

Table 2. Water extractable phosphorus (WEP_{ICP}) in poultry litter amended with Poultry Litter Treatment (PLT) after mixing with chitosan delivered as powder or dissolved in acetic acid (n = 4) and incubated at room temperature for two weeks (Experiment 1).

	WEP_{ICP} (mg kg^{-1} dry litter)		
Treatment	Mean	Standard Deviation	Homogeneous Groups[†]
Control	3942	247	AB
AA Control[‡]	3769	77	B
0.5% Powder[§]	3774	32	B
1.5% Powder	3867	95	B
3.0% Powder	3869	244	B
5.0% Powder	3904	167	AB
0.05% Dissolved[¶]	3761	210	B
0.10% Dissolved	3859	165	B
0.20% Dissolved	3986	90	AB
0.50% Dissolved	4143	245	A

[†] Homogenous groups based on means separation using least significant difference, ($\alpha = 0.05$).

[‡] AA designates acetic acid, where this treatment received the same volume of AA without chitosan.

[§] Chitosan applied as a dry powder.

[¶] Chitosan applied dissolved in acetic acid.

control samples. These results showed that none of the sources of chitosan that had been shown to reduce WEP by Bailey (2012) were able to have a similar effect on the litter treated with PLT. This suggests that chitosan is not effective at reducing WEP, when poultry litter is treated with PLT. The addition of PLT to litter adds an excess of sulfate (SO_4^{2-}) ions, which might inhibit chitosan's ability to remove P from solution and reduce WEP due to the competition with phosphate as the anion with which to form electrostatic complexes (Rinaudo, 2006). The results for the poultry litter not treated with PLT were much different. The WEP_{SRP} content of the control non-PLT litter (4448 mg kg^{-1}) was significantly greater than the WEP_{SRP} content of the four chitosan treatments, and the chitosan treatments were not statistically different. These results match with the results seen by Bailey (2012), which showed that WEP_{ICP} was significantly reduced by chitosan application. These results showed that the chitosan source used in the first experimentwas not the factor that resulted in the lack of WEP_{SRP} reduction.

Experiment 2 also showed that incubation time has an effect on chitosan's ability to reduce to WEP_{SRP}. Subsamples from the non-PLT litter source were extracted after 1, 4, and 7 weeks of incubation. The amount of WEP_{SRP}

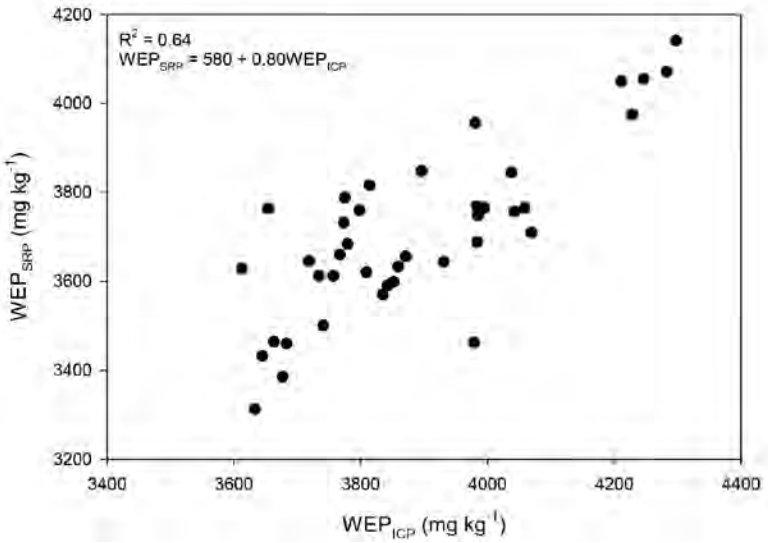

Fig. 1. Comparison of water extractable phosphorus (WEP) content by spectrometry (WEP_{SRP}) and by ICP-OES (WEP_{ICP}) for samples from experiment 1.

Table 3. Water Extractable Phosphorus (WEP_{SRP}) from two sources of poultry litter treatment with various sources of chitosan at a 10% dry weight basis application rate (Experiment 2) following a four week incubation.

			WEP_{SRP} (mg kg^{-1} dry litter)		
Application Rate	Litter Source	Chitosan Source[†]	Mean	Standard Deviation	Homogeneous Groups[‡]
	PLT[§]		4172	393	A
10%	PLT	1	4527	385	A
10%	PLT	2	4466	378	A
10%	PLT	3	4559	170	A
10%	PLT	4	4566	408	A
	Non-PLT[¶]		4448	70	A
10%	Non-PLT	1	3833	68	B
10%	Non-PLT	2	3830	67	B
10%	Non-PLT	3	3841	81	B
10%	Non-PLT	4	3918	42	B

[†] Refer to Table 1 for description of chitosan source.

[‡] Homogenous groups, based on means separation with least significant difference (α = 0.05) within a litter source.

[§] Poultry litter that has been treated with Poultry Litter Treatment (PLT).

[¶] Poultry litter that has not been treated with PLT.

removed across all chitosan treatments compared to the control is illustrated in Fig. 2. While chitosan had some effectiveness after 1 week of incubation, its performance appeared to peak after 4 weeks of incubation and remained about the same for the rest of its incubation. The experiments performed by Bailey (2012) used incubation times that exceeded 4 weeks, based on the time incubated in the lab and then analyzed at the Soil Diagnostic Lab for WEP_{ICP}. So, it can be concluded from experiment 2 that chitosan reduced WEP in poultry litters not treated with PLT and that it needs to be mixed with litter for 4 weeks to maximize the reduction.

Experiment 3. Having determined that the treatment of PLT to poultry litter has an effect on chitosan's ability to reduce WEP_{SRP} in the second experiment, the third experiment was a modified version of the first experiment that excluded the presence of PLT. The source of the litter used was the non-PLT litter from the second experiment. This allowed us to investigate the effect that dissolving chitosan into acetic acid has on its ability to reduce WEP. Since the second experiment showed that the sources of chitosan used did not produce significantly different results, which source of chitosan to use was not heavily considered.

After one week of incubation, the results showed that the chitosan powder (4354 mg kg^{-1}, Table 4) was the only treatment to reduce WEP_{SRP} in comparison to the control (4586 mg kg^{-1}); WEP_{SRP} content in the litter treated with chitosan powder was significantly different from the control, but it was applied at a rate an order of magnitude greater than the chitosan dissolved in acetic acid. Since this experiment (and the first experiment) intended to

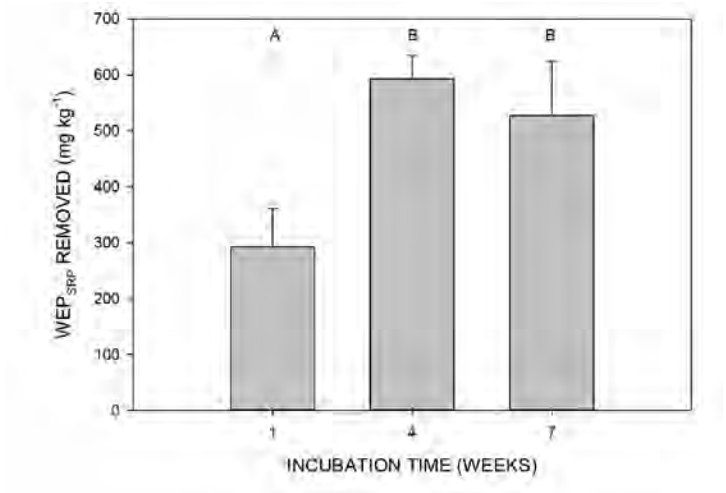

Fig. 2. Comparison of removal ability of WEP_{SRP} for all chitosan treatments compared to the control after various incubation times for experiment 2.

Table 4. Water Extractable Phosphorus (WEP_{SRP}) from poultry litter (without PLT) treated with chitosan as a powder or dissolved in acetic acid (Experiment 3) and incubated at room temperature for 1 week.

	WEP_{SRP} (mg kg^{-1} dry litter)		
Treatment[†]	Mean	Standard Deviation	Homogeneous Groups[‡]
Control	4596	215	B
AA Control[§]	4730	91	AB
10% powder	4354	213	C
0.05% Dissolved[¶]	4895	146	A
0.10% Dissolved	4796	191	AB
0.20% Dissolved	4848	87	A
0.50% Dissolved	4840	159	A

[†] Chitosan used is 4 in Table 1.

[‡] Homogenous groups based on means separation using least significant difference (α = 0.05).

[§] AA designates acetic acid, where this treatment received the same volume of AA without chitosan.

[¶] Chitosan applied dissolved in acetic acid.

use a constant and reasonable amount of acetic acid (1 mL of acetic acid per 10 g of litter) for the chitosan dissolved in acetic acid treatments, the highest concentration that was possible was the 0.50% (dry weight equivalent) treatment. At this concentration, the solution becomes very viscous and especially difficult to dissolve more chitosan into the dilute acetic acid. To apply more chitosan to the litter like the 10% dry weight equivalent powder treatment, the amount of acetic acid added would increase as well to levels that would likely not reflect a reasonable real-world application.

The four chitosan dissolved in acetic acid treatments (0.05%, 4895 mg kg^{-1}; 0.10%, 4796 mg kg^{-1}; 0.20%, 4848 mg kg^{-1}; 0.50%, 4840 mg kg^{-1}) all had WEP_{SRP} contents numerically greater than the control, and only the WEP_{SRP} content of the 0.10% treatment was significantly not different from the control. Interestingly, the control with just acetic acid applied (4730 mg kg^{-1}) was also numerically greater than the control, but not significantly different. The expectation was that dissolving chitosan into dilute acetic acid would increase the effectiveness, or the reduction in WEP. However, the use of acetic acid in poultry litter treatment likely increases WEP. It is also impractical to apply chitosan dissolved in acetic acid at rates equivalent to dry application, because of the volume of acetic acid required.

Three weeks of incubation had results with the same trend as discussed above (Table 5). The 10% powder treatment (4372 mg kg^{-1}) had the least WEP_{SRP} content and was significantly different from all of the treatments. The next lowest WEP_{SRP} content was found in the control (4757 mg kg^{-1}). Of the treatments that involved acetic acid, only the 0.50% chitosan dissolved in acetic acid (4993 mg kg^{-1}) was significantly not different than the control. The 0.50% treatment was also the only one that was significantly different from the control with acetic acid (5334 mg kg^{-1}). The other three chitosan dissolved in acetic acid treatments (0.05%, 5171 mg kg^{-1}; 0.10%, 5306 mg kg^{-1}; 0.20%, 5202 mg kg^{-1}) were not significantly different from the acetic acid control nor the 0.50% treatment. It is possible that the dilute acetic acid might hydrolyze bound P in the litter, resulting in the increase in WEP_{SRP}.

Seven weeks of incubation gave results that differ slightly than the previous incubations (Table 6). The control and the 10% powder treatment (4972 and 4764 mg kg^{-1}, respectively) were not statistically different from each other. This contradicts what was shown in the previous two sets of extractions, where chitosan powder appeared to reduce WEP_{SRP} in comparison to the control. The acetic acid control (5353 mg kg^{-1}) and the other chitosan dissolved in acetic acid treatments (0.05%, 5324 mg kg^{-1}; 0.10%, 5502 mg kg^{-1}; 0.20%, 5342 mg kg^{-1}; 0.50%, 5404 mg kg^{-1}) were not statistically different from each other but were statistically greater in WEP_{SRP} than the control and chitosan powder treatment.

Since the original results after seven weeks of incubation were unexpected with respect to the control and chitosan powder treatment, the data was closely investigated. The control and the powder treatment had one WEP value that was a possible outlier, where it was much lower in the control and then much greater in the powder treatment. Removing the possible outlier from among the powder treatments is supported by the observation that the treatment had visibly less chitosan powder. The alternative results show that, as was predicted, the chitosan powder treatment (4656 mg kg^{-1}) was significantly less in WEP_{SRP} than all other treatments including the control (5076 mg kg^{-1}). This was consistent with the previous extractions in the third experiment, and it also supported that observed in the previous studies on chitosan (Bailey et al., 2014).

Table 5. Water Extractable Phosphorus (WEP_{SRP}) from poultry litter (without PLT) treated with chitosan as a powder or dissolved in acetic acid (Experiment 3) and incubated at room temperature for 3 weeks.

	WEP_{SRP} (mg kg^{-1} dry litter)		
Treatment[†]	Mean	Standard Deviation	Homogeneous Groups[‡]
Control	4757	66	C
AA Control[§]	5334	280	A
10% powder	4372	277	D
0.05% Dissolved[¶]	5171	113	AB
0.10% Dissolved	5306	191	A
0.20% Dissolved	5202	200	AB
0.50% Dissolved	4993	281	BC

[†] Chitosan used is 4 in Table 1.

[‡] Homogenous groups based on means separation using least significant difference (α = 0.05).

[§] AA designates acetic acid, where this treatment received the same volume of AA without chitosan.

[¶] Chitosan applied dissolved in acetic acid.

These results are evidence against the hypothesis that chitosan in acetic acid at a practical application rate would have a greater effect on the reduction of WEP_{SRP} in poultry litter. The presence of acetic acid appears to possibly increase WEP_{SRP}. The highest chitosan dissolved in acetic acid treatment (0.50%) almost appeared to have the desired effect at 3 weeks of incubation, however, the control remained statistically less than the treatment at 7 weeks of incubation. The results of the chitosan powder treatment resemble that of the second experiment; chitosan powder has a peak effectiveness on reducing WEP_{SRP} after 3 weeks. Thus, it does not seem beneficial to dissolve chitosan into acetic acid at these low treatment levels when applying to poultry litter. However, acetic acid would likely reduce litter pH and therefore inhibit ammonia volatilization but it would possibly increase WEP and the potential loss of P during rainfall runoff events.

CONCLUSION

Chitosan's ability to reduce WEP was inhibited by the presence of PLT in the poultry litter. The source of poultry litter must be untreated with PLT in order for chitosan to have its desired effect, i.e. reduce WEP. Application of chitosan dissolved in acetic acid (0.05%, 0.10%, 0.20% and 0.50% dry weight basis, g chitosan g^{-1} poultry litter) was ineffective and the presence of acetic acid alone potentially increases WEP. The time of incubation did have an effect on the reduction of WEP, suggesting chitosan's effectiveness peaks after 3 weeks of incubation. Future studies may find alternative methods of applying chitosan to poultry litter to improve effectiveness, such as using a different acid solution in place of acetic acid. Furthermore, the next step needs to be applying poultry litter treated with chitosan to field plots where rainfall simulation studies can be used to evaluate P transport in runoff waters.

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LITERATURE CITED

Alabaster, J.S. 1959. The effect of a sewage effluent on the distribution of dissolved oxygen and fish in a stream. J. Animal Ecol. 28:283-291.

Alabaster, J.S. 1961. The effect of diurnal changes in temperature, dissolved oxygen and illumination on the behaviour of roach (*Rutilus rutilus* (L.)), bream (*Abramis brama* (L.)) and perch (*Perca fluviatilis* (L.)). Animal Behaviour. 9:187-192.

Bailey, I.M. 2012. Chitosan reduces water solubility of phosphorus in poultry litter. Honors Thesis, Biological Engineering Program, Biol. and Agric. Eng. Dept., University of Arkansas.

Bailey, I.M., D.A. Zaharoff, and B.E. Haggard. 2014. Chitosan reduces water extractable phosphorus and increases nitrogen content of poultry litter. J. Poultry Sci. Accepted.

Table 6. Water Extractable Phosphorus (WEP_{SRP}) from poultry litter (without PLT) treated with chitosan as a powder or dissolved in acetic acid (Experiment 3) and incubated at room temperature for 7 weeks.

	WEP_{SRP} (mg kg^{-1} dry litter)		
Treatment[†]	Mean	Standard Deviation	Homogeneous Groups[‡]
Control	4972	268	B
AA Control[§]	5353	230	A
10% powder	4764	285	B
0.05% Dissolved[¶]	5324	388	A
0.10% Dissolved	5502	166	A
0.20% Dissolved	5342	65	A
0.50% Dissolved	5404	182	A

[†] Chitosan used is 4 in Table 1.

[‡] Homogenous groups based on means separation using least significant difference (α = 0.05).

[§] AA designates acetic acid, where this treatment received the same volume of AA without chitosan.

[¶] Chitosan applied dissolved in acetic acid.

Correll, D.A. 1998. The role of phosphorus in the eutrophication of receiving waters: a review. J. Environ. Qual. 27:261-266.

Dao, T. 1999. Coamendments to modify phosphorus extractability and nitrogen/phosphorus ratio in feedlot manure and composted manure. J. Environ. Qual. 28:1114-1121.

Diaz, R.J. and R. Rosenberg. 2008. Spreading dead zones and consequences for marine ecosystems. Science. 321(5891): 926-929.

Floyd, R.F. 1992. Dissolved oxygen for fish production. University of Florida: Fisheries and Aquatic Sciences Dept. <http://edis.ifas.ufl.edu/fa002> Accessed 14 April 2014.

Garcia, M.C., A.A. Szogi, M.B. Vanotti, J.P. Chastain, and P.D. Millner. 2009. Enhanced solid–liquid separation of dairy manure with natural flocculants. Bioresource Tech. 22:5417-5423.

Haggard, B.E., P.A. Vadas, D.R. Smith, P.B. DeLaune, and P.A. Moore Jr. 2005. Effect of poultry litter to water ratios on extractable phosphorus content and its relation to runoff phosphorus concentrations. Biosystems Eng. 92:409-417.

Kleinman, P. and A. Sharpley. 2003. Effect of broadcast manure on runoff phosphorus concentrations over successive rainfall events. J. Environ. Qual. 32:1072-1081.

Kleinman, P., D. Sullivan, A. Wolf, R. Brandt, Z. Dou, H. Elliot, J. Kovar, A. Leytem, R. Maguire, P. Moore, L. Saporito, A. Sharpley, A. Shober, T. Sims, J. Toth, G. Toor, H. Zhang, and T. Zhang. 2007. Selection of a water-extractable phosphorous test for manures and biosolids as an indicator of runoff loss potential. J. Environ. Qual. 36:1357-1367.

Kumar, R. and N. Majeti. 2000. A review of chitin and chitosan applications. Reactive and Functional Polymers. 46:1-27.

Moore, P.A., D.R. Edwards, and T.C. Daniel. 2000. Reducing phosphorus runoff and inhibiting ammonia loss from poultry manure with aluminum sulfate. J. Environ. Qual. 29: 37-49.

Parry, R. 1998. Agricultural phosphorus and water quality: a U.S. environmental protection agency perspective. J. Environ. Qual. 27:258-261.

Pope, M.J. and T.E. Cherry. 2000. An evaluation of the presence of pathogens on broilers raised on poultry litter treatment-treated litter. Poultry Sci. 79(9): 1351-1355.

Rabea, E., Badawy, M., C. Stevens, G. Smagghe, and W. Steurbaut. 2003. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules. 4:1457-1465.

Rinaudo, Marguerite. 2006. Chitin and chitosan: properties and applications. Progress in Polymer Science. 31(7): 603-632.

Shreve, B.R., D.R. Edwards, D.M. Miller, P.A. Moore Jr., and T.C. Daniel. 1995. Reduction of phosphorus in runoff from field-applied poultry litter using chemical amendments. J. Environ. Qual. 24: 106-111.

Smith, V.H. 1998. Cultural eutrophication of inland, estuarine, and coastal waters. Successes, Limitations and Frontiers in Ecosys. Sci. 7-49.

Smith, D.R., P.A. Moore Jr., C.L. Griffis, T.C. Daniel, D.R. Edwards, and D.L. Boothe. 2001. Effects of alum and aluminum chloride on phosphorus runoff from swine manure. J. Environ. Qual. 30: 992-998.

Sweeney, C.R., S. McDonnell, G.E. Russell, and M. Terzich. 1996. Effect of sodium bisulfate on ammonia concentration, fly population, and manure pH in a horse barn. American J. Vet. Res. 57(12): 1795-1798.

Swingle, H.S. 1966. Fish kills caused by phytoplankton blooms and their prevention. In FAO World Sym. on Warm Water Pond Fish Culture. Auburn, Ala.

Vadas, P.A., P.J.A. Kleinman, and A.N. Sharpley. 2004. A simple method to predict dissolved phosphorus in runoff from surface-applied manures. J. Environ. Qual. 33:749-756.

Walker, W.W. Jr. 1983. Significance of eutrophication in water supply reservoirs. J. Amer. Water Works Assoc. 75: 38-42.

Development of *fad7-1* single mutant *Arabidopsis thaliana* plants that are resistant to aphids

Kaleb L. Vaughn*, Carlos A. Avila†, Carmen S. Padilla-Marcia§, and Fiona L. Goggin‡

ABSTRACT

Aphids are a group of sap-feeding insects that attack most of the world's crops. The loss of function of fatty acid desaturase7 (FAD7) in *Solanum lycopersicum* (tomato plant) induces aphid resistance that is dependent upon the accumulation of plant defense hormones such as salicylic acid (SA). Tomato lacks most of the genetic resources found in the model plant Arabidopsis (*Arabidopsis thaliana*). There is an analogous *fad7-1* line of Arabidopsis; however, the line has a background mutation, the *glabra-1 (gl1)*, that causes the absence of trichomes (small hairs), which are essential to plant defense. In order to study aphid resistance, a single mutant line of *fad7-1* mutants were developed using cross breeding between the *fad7-1/gl1* mutant and wild-type plants. Homozygous *fad7-1* mutants were then identified using the Kasajima DNA extraction method, followed by the use of single nucleotide polymorphism-polymerase chain reaction (SNP-PCR) primers using allele-specific PCR. A phenotypic screening was then performed to screen out the plants with the *glabra-1* mutation using the presence or absence of trichomes. Two single Arabidopsis *fad7-1* mutant lines were identified, and subsequently verified using a bioassay to be aphid resistant relative to other genotypes as seen in tomato.

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MEET THE STUDENT-AUTHOR

Kaleb Vaughn

I was born and raised in Fayetteville and graduated from Har-Ber High School in 2010. I graduated from Harding University with a degree in Molecular and Cellular Biology in spring of 2014 and will be attending medical school in the fall. I plan to continue research as a medical student and physician using the skills I learned in Fiona Goggin's lab.

I could not have completed this research without the members of the Goggin lab, including Dr. Fiona Goggin and Dr. Carlos Avila, who were patient and devoted in teaching me the necessary skills of a researcher. Due to their guidance my poster presentation won best undergraduate presentation at the 98th annual Arkansas Academy of Sciences Conference. I was also able to present this research as my senior seminar at Harding. I also am indebted to Dr. Steven Moore along with other Harding science faculty who saw my potential in research and encouraged me to pursue it.

INTRODUCTION

Organisms must have mechanisms by which they defend against predators. Plants have developed structural, chemical, and protein-based defense mechanisms designed to detect and defend against invading organisms. Aphids (plant lice) are a group of sap-feeding insects that attack most of the world's crops (Goggin, 2007). They reduce yield by stealing nutrients from the plant or by transmitting plant pathogenic viruses. In response to insect attack, plants release hormones that activate plant defensive signaling. A specific hormone is salicylic acid (SA), a signaling hormone that stimulates systemic acquired resistance throughout the plant via the increased expression of defense proteins. It has been shown that the loss of fatty acid desaturase7 (FAD7) in *Solanum lycopersicum* (tomato plant), due to a mutant allele (*fad7-1*), leads to an increased accumulation and signaling of SA along with an increased level of aphid resistance (Avila et al., 2012). However, the molecular mechanisms by which SA accumulation and signaling are enhanced in the *fad7-1* mutant in response to aphids are unknown.

Tomato is an excellent model to study plant-aphid interactions; however it lacks most of the genetic resources found in model plant Arabidopsis (*Arabidopsis thaliana*). There is an analogous *fad7-1* line of Arabidopsis; however, the line has a background mutation, the *glabra-1 (gl1)*, that causes the absence of trichomes (small hairs), which are essential to plant defense (Xia et al., 2010). Therefore, to identify the molecular mechanisms by which SA levels are increased due to the FAD7 mutation using the genetic resources found in Arabidopsis, a single mutant line of *fad7-1* plants must be developed, and aphid resistance must be confirmed in this line.

MATERIAL AND METHODS

Plant Material. We used the F2 segregating population of a previously made cross between Arabidopsis *fad7-1/gl1* with *sid2-2* salicylic acid mutant plant to screen for single mutant lines. The salicylic acid mutant was used for practical reasons that included its availability in the Goggin lab. The *sid2-2* mutation was screened out later in a progeny test.

DNA Extraction. The DNA was extracted from 3-5 mg of fresh tissue using the Extract-N-Amp Plant kit (Sigma-Aldrich, St. Louis, Mo.) or the method reported by (Kasajima et al., 2004) consisting of a buffer solution of 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. Then the buffer was diluted tenfold with TE buffer 910 mM Tris-HCl pH 8 and 1 mM EDTA. In order to speed-up tissue grinding, tissue was homogenized using 3 grinding glass beads in 1.5- or 2-ml tubes in a GenoGrinder (Spex Sample Prep, Metuchen, N.J.) tissue homogenizer at 1750 RPM for 30 s.

fad7-1 screening. The *fad7-1* mutation in Arabidopsis was identified to be a C to T transition in the *FAD7* DNA coding sequence, converting the amino acid at position 253 from proline to leucine (Xia et al., 2010). Single nucleotide polymorphism primers (SNP-primers) were designed by placing the wild-type or mutated base at the 3' end on the forward oligonucleotide primer. Both primer sets share the same reverse primer. Touchdown polymerase chain reaction (TD-PCR; Korbie and Mattick, 2008) was performed to increase amplification sensitivity and specificity using the following conditions for mutated allele: initial denaturation = 95 °C for 5 min; phase I = 95 °C for 45 s, 65/67 °C to 56/58 °C for 45 s (reducing 1 °C per cycle), and 72 °C for 45 s; phase II = 95 °C for 45 s, 55/57 °C for 45 s, and 72 °C for 45 s (20 cycles); and final extension at 72 °C for 5 min.

Gel Electrophoresis. The PCR amplification was run in 1% agarose gel at 250 V for 16 min (Fig. 1).

gl1 Screening. Presence or absence of trichomes was visually assessed with the help of a magnifying glass.

sid2-2 Screening. A progeny test was performed on 12 homozygous *fad7-1* F2-3 plants using a primer set which only amplifies DNA from plants having wild-type *SID2* gene (SID2(F) = 5'-TTCTCAATTGGCAGGGAGAC-3' and SID2(R) = 5'-AAGCCTTGCTTCTTCTGCTG-3') using the following PCR conditions: = 95 °C for 5 min initial denaturation; 95 °C for 45 s, 55 °C to 56 °C for 45 s, and 72 °C for 45 s (30 cycles); and final extension at 72 °C for 5 min.

Screening for fad7-1 Homozygous Mutant. The (*fad7-1/gl1 x sid2-2*) F2 population was first visually selected by the presence of trichomes and then screened by PCR for the presence of the *fad7-1* mutation (*fad7-1/gl1 x sid2-2*) F3 progeny test. Selected homozygous *fad7-1* mutant F2 plants were self-pollinated to obtain the F3 lines.

Aphid Reproduction Bioassay. Thirty-two plants of four genotypes: *fad7-1*, *fad7-1/gl1*, *gl1*, and *wild-type* were grown to maturity and then inoculated with three aphid adults per plant. Aphids were allowed to feed and reproduce for 72 hours before aphid numbers were assayed. Statistical analysis was completed using a Kruskal-Wallis with pairwise comparison post-hoc analysis.

RESULTS AND DISCUSSION

As expected, the wild-type *FAD7* primer set amplified a band from plants carrying the wild-type allele (the *sid2* parental line and one of the F2 plants shown), but not

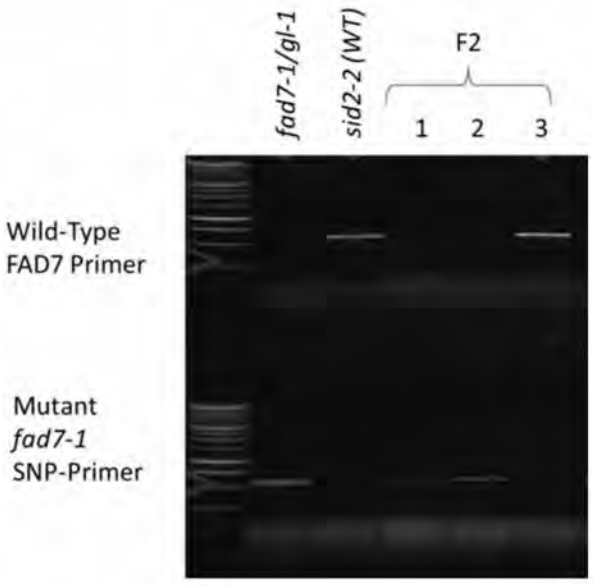

Fig. 1. Single Nucleotide Polymorphism-based polymerase chain reaction (PCR) screening was used to detect the presence of the wild-type and/or mutant alleles of the *FAD7* gene in a segregating population. From the left: lane 1 contains molecular weight markers, lane 2 contains PCR products amplified from a plant that is known to be homozygous for the mutant allele, lane 3 contains PCR products from a plant homozygous for the wild-type allele, and lanes 4 through 6 (labeled F2 1-3) are from 3 separate F2 plants from the *fad7-1/gl1 x sid2-2* cross. In two separate PCR reactions, the samples were amplified with primers for the wild-type allele (top), or the mutant allele (bottom).

from the *fad7-1* mutant plants. Conversely, the primer set designed to be specific to the *fad7-1* mutation amplified a band in the *fad7-1* mutant and two of the segregating F2 plants, but not the *sid2* parental line (Fig. 1). Heterozygous F2 plants show amplification with both primer sets (not shown). Twenty-four F3 plants for each of the 17 homozygous *fad7-1* mutant F2 selected plants were tested for *sid2-2* segregation using wild-type SID2 allele specific primers. We did not obtain a segregation ratio of 1 (*FAD7/FAD7*):2 (*FAD7/fad7-1*):1 (*fad7-1/fad7-1*), instead we observed a 1.1:1.5:1 ratio, respectively (19 *FAD7/FAD7*; 25 heterozygotes; 17 *fad7-1/fad7-1*). Alteration of expected segregation ratio may be due to low F2 sampling number and/or distortion by selecting *GL1* plants only.

Before the *sid2-2* progeny test, which would require a large volume of screening, DNA extraction methods were compared to discern which was the most efficient. The PCR amplification using DNA extracted using the method of Kasajima et al. (2004) yielded brighter bands as compared to the Extract-N-Amp Plant kit from Sigma (Fig. 2). Band intensity varied between different tissue homogenization treatments using Kasajima method, however extracting DNA in 1.5-ml tubes yielded brighter bands than using 2-ml tubes. Although, using rods to crush the DNA gives good DNA yield, using the tissue homogenizer reduced the time of sample preparation.

Two independent F2:3 lines have shown no segregation for the presence of the *SID2* allele (Fig. 3). Therefore, these two lines have a single mutation at the *FAD7* locus and are wild type for *SID2* and *GL1*. These mutant lines of Arabidopsis were aphid resistant relative to other genotypes after conduction an aphid reproduction assay ($P < 0.001$; Fig. 4).

Fig. 2. Comparison of DNA extraction methods. Polymerase chain reaction (PCR) was performed with primers for the wild-type SID2 allelle on DNA samples from 2 wild type plants that were collected by 4 different DNA extraction methods: 1) a commercial DNA extraction and amplification kit, the Extract-N-Amp Plant kit (Sigma-Aldrich, St. Louis, Mo.); 2) a simple, one-step method reported by Kasajima and coworkers (Kasajima et al., 2004) using a GenoGrinder (Spex Sample Prep, Metuchen, N.J.) tissue homogenizer in 1.5-ml tubes; 3) the Kasajima method using tissue homogenizer in 2-ml tubes; and 4) the Kasajima method using a plastic pestle to crush the tissue in 1.5-ml tubes. Samples that gave brighter bands were assumed to contain higher-quality DNA with fewer PCR inhibitors.

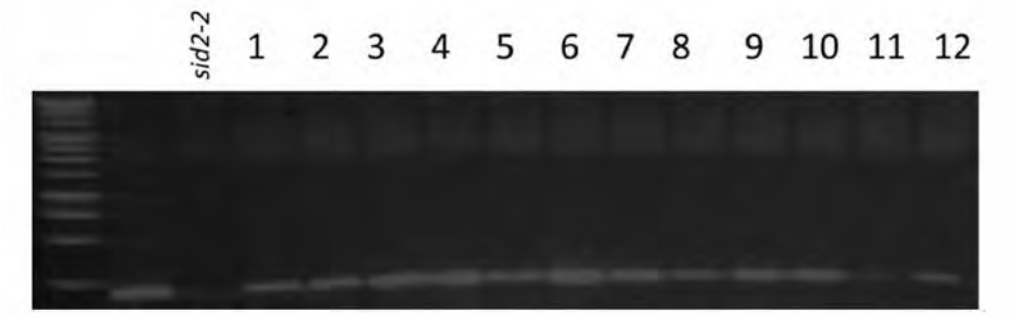

Fig. 3. F3 progeny derived from the *fad7-1gl1 X sid2* cross were screened by polymerase chain reaction (PCR) with primers for the wild-type *SID2* allele in order to to select for a homozygous line that carries the *fad7-1* mutation but not the *sid2* mutation, and that can be used as a control in experiments to analyze the effects of the *fad7-1*, *gl1*, and *sid2* genes singly and in combination. From the left, the first lane contains molecular weight markers, the second contains PCR products amplified from a *fad7-1 gl1* sample, the third is from a homozygous *sid2* mutant, and the remaining lanes (labeled 1 through 12) are from 12 separate T3 progeny collected from the same F2 parent, which was previously confirmed to be homozygous for the *fad7-1* mutation. All F3 progeny had the wild-type *SID2* allele. This gives a high probability that no segregation is present, and that the line is homozygous for the *SID2* allele, and therefore *fad7-1* single mutants.

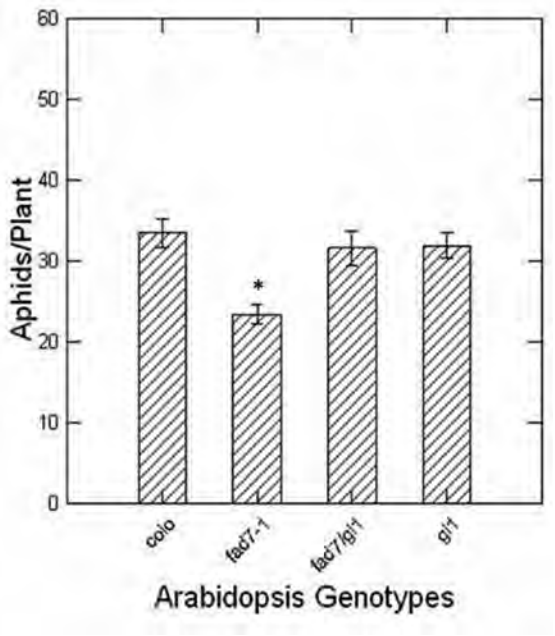

Fig. 4. Aphid population growth on different Arabidopsis genotypes, including wild-type plants (Col0), single mutants (*fad7-1, gl1*) and a double mutant with both the *fad7-1* and *gl1* mutations (*fad7/gl1*). Plants were inoculated with 3 aphids per plant, and then total aphid numbers were counted 72h later (n = 32). The *fad7-1* single mutants had significantly lower aphid numbers relative to the other three genotypes, according to a Kruskal-Wallis test with pairwise comparison ($P < 0.001$).

Because of the significant genetic resources found in Arabidopsis along with its short life cycle and hardy disposition, the development of a *fad7-1* single mutant allows for more efficient research into increased salicylic acid accumulation due to loss of FAD7. The SNP-based PCR screening along with glabra-1 phenotypic screening successfully identified homozygous *fad7-1* mutants without the *gl1* mutation in Arabidopsis. The screening methodology involved was efficient and relatively inexpensive, and can be used in further genotype tracking among plants including developing double mutants carrying the *fad7-1* and the *ssi2* mutations. Identified single *fad7-1* mutant lines will open the opportunity to test the effect of fatty acid desaturation on plant-aphid interaction in Arabidopsis.

The aphid reproduction bioassay was performed to test the effect of the *fad7-1* mutation on aphid reproduction and mortality in Arabidopsis, without the interfering effect of the *glabra1* (*gl1*) mutation. The *fad7-1* single mutant exhibited significantly decreased numbers of aphids relative to the other three genotypes after the 72 hour period. This result confirmed the *fad7-1* line in Arabidopsis to be aphid resistant relative to other genotypes as seen in tomato (Fig. 4).

Further research using single mutant *fad7-1* Arabidopsis will yield information into the mechanism that confers aphid resistance in FAD7 deficient plants through increased accumulation of salicylic acid. The epigenetic silencing of the *FAD7* gene also yields adverse effects to plants that include vulnerability to caterpillars and drought. The ultimate goal is to discover the primary catalyst of increased SA accumulation in plants and to maintain this effect without the loss of function of the FAD7 protein. Aphid resistant crops would lessen the formidable burden of agricultural pests to farmers.

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LITERATURE CITED

Avila, C.A., L. M. Arevalo-Soliz, L. Jia, D. A. Navarre, Z. Chen, G. A. Howe, Q. W. Meng, J. E. Smith, and F. L. Goggin. 2012. Loss of function of Fatty Acid Desaturase 7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. Plant Physiology. 158:2028-2041.

Goggin, F.L. 2007. Plant-aphid interactions: molecular and ecological perspectives. Current Opinion in Plant Biology. 10:399-408.

Kasajima, Y., N. Ide, H. Ohkama-Ohtsu, T. H. Yoneyama, and T. Fujiwara. 2004. A protocol for rapid dna extraction from *Arabidopsis thaliana* for PCR analysis. Plant Molecular Biology Reporter. 22:49-52.

Korbie, D.J. and J.S. Mattick. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nature Protocols. 3:1452-1456.

Xia, Y., K. Yu, D. Navarre, K. Seebold, A. Kachroo and P. Kachroo. 2010. The *glabra1* mutation affects cuticle formation and plant responses in microbes. Plant Physiology. 154:833-846.

Steer stress response as affected by genotype and transportation

Megan Wary[*], *Marites Sales*[†], *Ben Williamson*[§], *Ken Coffey*[‡], *Mike Looper*[¶], *and Charles Rosenkrans*[#]

ABSTRACT

Bovine cytochrome P450 3A28 is responsible for metabolizing ergot alkaloids that cattle ingest when feeding on endophyte-infested tall fescue grass. The objective of this research was to determine associations among genotype, transportation, and stress responses. Angus crossbred steers (n = 47) were genotyped (CC, CG, or GG) for a single-nucleotide polymorphism (C994G) in cytochrome P450 3A28. Genotypes were determined by polymerase chain reaction (PCR) amplification followed by restriction enzyme (Alu1) digestion. Steers were backgrounded on a mixed-cultivar tall fescue pasture. Following the stocker phase, steers were transported to the feedlot for finishing. Stress responses were determined 27 h prior to, and 6 and 20 h after transport. Plasma concentrations of prolactin and cortisol, and white blood cell expression of prolactin, cytochrome P450, tumor necrosis factor-alpha, and short form prolactin receptor were our indicators of stress. Both time and genotypic effects were determined. Time ($P < 0.05$) relative to transportation was associated with expression of all four genes tested. In addition, plasma concentrations of cortisol and prolactin, as well as their ratio were affected ($P < 0.05$) by time. In contrast, neither genotype nor the interaction between genotype and time affected ($P > 0.1$) our stress indicators. In previous studies, C994G genotype has been associated with cattle productivity; however, those effects were not observed in this study.

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† Marites Sales is a post-doctoral research associate in the Department of Animal Science.
§ Ben Williamson is a former Master of Science student in the Department of Animal Science.
‡ Ken Coffey is a professor in the Department of Animal Science.
¶ Michael Looper is Head of the Department of Animal Science.
# Charles Rosenkrans, Jr. is the research mentor and professor in the Department of Animal Science.

MEET THE STUDENT-AUTHOR

Megan Wary

I grew up in Rogers, Arkansas and attended Rogers High School, graduating in 2010. I chose to stay close to home to pursue a degree in Biochemistry at the University of Arkansas with a pre-pharmacy emphasis. After my freshman year, I decided to also declare minors in Mathematics and Animal Science because I have always had a passion for animals and enjoy challenging myself. During my time here at the University of Arkansas, I have been a student ambassador, active in my sorority Kappa Kappa Gamma, and have worked part-time as a pharmacy technician. After graduation in the spring, I will move to Denver, Colorado to pursue a Pharm. D. at the University of Colorado School of Pharmacy.

I enjoyed doing my Honors thesis project in the Animal Science department, where I was able to have hands-on experience in the field and the laboratory. I would like to thank Dr. Charles Rosenkrans for agreeing to be my mentor and for diligently teaching me all I needed to know. He was supportive and directive in the writing of my thesis and preparation for my defense. Special thanks should be given to Dr. Ali Moubarak who provided expert understanding of ergot alkaloids, and to Marites Sales and Jonathan Anthony who taught me technique in the laboratory.

INTRODUCTION

Many cattle producers in the Midwestern and Southern United States use tall fescue, *Lolium arundinaceum*, which is a cool season perennial grass that is typically infected with a fungus, *Neotyphodium coenophialum* (Browning, 2003). When cattle ingest the fungal-infected fescue, they consume ergot alkaloids which results in a condition termed fescue toxicosis. Fescue toxicosis is characterized by poor growth, decreased weight gain, suppressed appetite, reduced conception and calving rates, poor peripheral circulation, uneven hair coats, heat stress, decreased prolactin and milk production (Browning, 2003; Lyons et al., 1986; Paterson et al., 1995; Settivari et al., 2008). Fescue toxicosis is estimated to cost the U.S. beef industry approximately $500 million to $1 billion annually in lost revenue because of reduced growth and reproductive rates in cattle (Browning, 2003).

One enzyme family involved with detoxification of alkaloid toxins is cytochrome P450 (CYP450) hemethiolatemonooxygenases. Those proteins are particularly prevalent in the smooth endoplasmic reticulum of liver cells (Hardin et al., 2012). One of the expressed bovine cytochrome P450 genes is CYP450 3A28. Cattle have a single nucleotide polymorphism (SNP) in the genetic region that codes for CYP450 3A28 and that SNP is related to cattle productivity (Sales et al., 2012).

This study aimed to determine if SNP C994G genotypes and(or) transportation affected prolactin and cortisol concentrations in plasma, and white blood cell expression of CYP3A28, PRL, sPRLR, and TFN-α. Understanding the underlying cellular and metabolic pathways following exposure to ergot alkaloids will allow for pharmacological developments to diminish the symptoms associated with fescue toxicosis.

MATERIALS AND METHODS

Animals and Sample Collection. Forty-seven Gelbvieh × Angus steers were weaned and developed on a pasture of mixed (toxic and non-toxic) tall fescue grass. After 154 d of grazing, steers were transported from Booneville, Ark. to Stillwater, Okla. (approximately 250 miles, and 5 h duration) where they were finished in a feedlot. Blood samples from the 47 steers were collected by venipuncture at 27 h prior to transport (T – 27), 6 h after arrival (T + 6), and 20 h after arrival (T + 20). Blood samples were immediately put on ice, and centrifuged to isolate the buffy coat and plasma.

DNA Preparation and Amplification. Using the blood samples collected, each steer was genotyped at the CYP450 3A28 coding sequence SNP C994G. Genomic DNA was purified from the buffy coat of each steer, and the DNA region that contains SNP C994G was amplified by poly-

merase chain reaction (PCR). Components used for PCR were 43 µL supermix, 1.3 µL reverse primer, 1.3 µL forward primer, and 5 µL template DNA (diluted to 20 ng/mL). Initial denaturing was done at 94 °C for 2 min, followed by denaturing at 94 °C for 30 sec. Annealing occurred at 55 °C for 1 min, with extension subsequent at 68 °C for 1 min. Thirty-five cycles were completed before the final extension at 68 °C for 10 min. The PCR primers for the CYP450 3A28 coding sequence (Forward: 5'-CAACAACATGAATCAGCCAGA-3'; Reverse: 5'-CCTACATTCCTGTGTGTGCAA-3') amplified a 565-base pair DNA fragment.

Genotype Assignment. Amplification products were genotyped by restriction fragment length polymorphism (RFLP). The RFLP reaction consisted of restriction enzyme AluI (5 µL), Alu I buffer (2.5 µL), and amplified DNA product (3 µL) incubated at 37 °C for one hour. Genotype was assigned after digestion products were separated by gel electrophoresis (2% agarose). The polymorphism is a transversion in the SNP; consisting of either a cytosine or a guanine in the CYP450 3A28 region with three possible genotypes, homozygous cytosine (CC) or guanine (GG), or heterozygous (CG).

Stress Indicators. From blood samples that were taken at T – 27, T + 6, and T + 20, plasma concentrations (ng/mL) were determined for cortisol and prolactin using validated radioimmunoassay. White blood cell gene expression was assessed by purifying total RNA from steer buffy coats using RiboPure Blood kit (Life Technologies Corp., Carlsbad, Calif.). Prolactin, short form prolactin receptors (sPRLR), CYP450 3A28, and tumor necrosis factor alpha (TNFα) gene expression was determined by quantitative PCR (StepOnePlus Real-Time PCR System; Life Technologies Corp., Carlsbad, Calif.). To avoid bias, real-time PCR results were normalized to one or more internal control genes, also known as 'housekeeping genes.' Those are genes whose expression are critical for basic cell function and should be expressed during normal and pathological conditions (Nicot et al., 2005). Cyclophilin is a common housekeeping gene, and was used as the reference gene in this study.

Statistical Analyses. Mixed model analysis of variance was used to determine effects of C994G genotype (CC, CG, GG), time (T – 27, T + 6, T + 20), and their interaction on stress indicators. When F-tests were significant ($P < 0.05$), then least squares means were separated using the Tukey-Kramer method. Expression values for transcription levels were computed by a comparative threshold method. Comparative threshold (Ct) is the number of cycles of PCR before the reaction meets threshold. The Ct values for the gene of interest were normalized twice, first to the control gene Ct, cyclophilin in this case, and second to the biological control, which for this experiment was CC genotype at time T – 27, this method is known as δδ-Ct method (Dharmaraj, 2014).

RESULTS AND DISCUSSION

Of the 47 Angus crossbred steers used in this study, 5 were homozygous cytosine (CC) genotype, 31 were homozygous guanine (GG), and 11 were heterozygous (CG) at the SNP in the CYP450 3A28 coding sequence. Allele frequency for the herd was 22% cytosine, and 78% guanine. Genotype at C994G did not affect ($P > 0.1$) any of the traits evaluated in this study (Table 1). The interaction of main effects was not significant for traits evaluated in this study.

Table 1. Steer stress response as affected by CYP450 3A28 genotype.

Item†	CC	GC	GG	SE	*P*-value
WBC Gene Expression					
TNFα	1.07	1.09	1.07	0.07	0.98
sPRLR	1.05	1.06	1.09	0.08	0.93
Prolactin	1.12	1.13	1.23	0.05	0.25
CYP450 3A28	0.97	0.99	1.07	0.05	0.34
Hormones					
Cortisol, ng/mL	70.1	65.5	66.4	4.7	0.83
Prolactin, ng/mL	177.6	241.1	241.1	41.7	0.61
Prolactin:cortisol	2.7	3.9	3.5	0.54	0.31

† Genes were: tumor necrosis factor alpha (TNFα), short form prolactin receptors (sPRLR), prolactin, and cytochrome P540 3A28 (CYP450 3A28). Cortisol and prolactin are plasma concentrations.

Main affect of transportation affected all stress indicators in this study (Table 2). White blood cells (WBC) are not typically known for expression of prolactin or CYP450 3A28; however, this study demonstrates that those two genes are differentially expressed in WBC. Prolactin increased ($P < 0.05$) in relation to transportation; whereas, CYP450 3A28 expression decreased ($P < 0.05$) shortly after transportation but returned to pre-travel expression by 20 h post-travel. Both sPRLR and TNFα expression profiles in WBC were highest ($P < 0.05$) at 20 h post-transportation. Circulating concentrations of cortisol were lowest ($P < 0.05$) at 6 h post-travel. In contrast, prolactin concentrations increased ($P < 0.05$) in relation to transportation and were highest at 20 h after arrival at feedlot. The prolactin:cortisol followed the prolactin profile and was different ($P < 0.05$) at each time point.

A reliable, quantifiable result of fescue toxicosis is decreased serum prolactin concentrations and this can be attributed to ergot alkaloid interaction with dopamine receptors that mediate prolactin production (Paterson et al., 1995). Ergot alkaloids interacting with other neurotransmitter receptors that are responsible for controlling blood flow, especially epinephrine and norepinephrine, are thought to cause vasoconstriction and increased respiration, which are often associated with fescue toxicosis (Paterson et al., 1995).

One enzyme family involved with detoxification of alkaloid toxins is CYP450 heme-thiolatemonooxygenases, which are particularly prevalent in the smooth endoplasmic reticulum of liver cells (Hardin et al., 2012). Previously, our research team has demonstrated that cattle have altered production traits that were associated with genotype of SNP C994G (Sales et al., 2012). In C994G, the SNP results in a point mutation (C994G) with amino acid replacement of leucine with valine at amino acid location 331 for steers of the homozygous guanine genotype.

A few other genes of interest that may be involved in animal response to fescue toxicosis include cytokine tumor necrosis factor-α (TNFα), and short form prolactin receptors (sPRLR). Prolactin receptors are cytokine receptors that interact with the prolactin molecule. Cytokines are small chemical messengers that mediate critical brain endocrine immune responses to infection and are produced by white blood cells (Feuerstein et al., 1993). White blood cells express TNFα for involvement in inflammation, and also for ischemia (restriction of blood supply) and trauma in addition to immune function (Feuerstein et al., 1993). In conclusion, our results confirm that WBC express genes that are essential for immune function, and demonstrate that WBC also express genes related to animal toxicology response.

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LITERATURE CITED

Browning, Jr., R. 2003. Tall fescue endophyte toxicosis in beef cattle: Clinical mode of action and potential mitigation through cattle genetics. Proceedings 35th Annual Research Symposium and Annual Meeting, Beef Improvement Federation:28-31.

Dharmaraj, S. 2014. The Basics: RT-PCR. Life Technologies. Retrieved 3 March 2014, www.lifetechnologies.com/us/en.

Table 2. Steer stress response as affected by transportation stress.

	Time Relative to Transportation				
Item†	T – 27	T + 6	T + 20	SE	*P*-value
WBC Gene Expression					
TNFα	1.02[b]	1.07[b]	1.15[a]	0.04	0.001
sPRLR	1.03[b]	1.06[ab]	1.11[a]	0.05	0.02
Prolactin	1.11[b]	1.17[ab]	1.21[a]	0.03	0.001
CYP450 3A28	1.05[a]	0.96[b]	1.03[a]	0.03	0.005
Hormones					
Cortisol, ng/mL	68.8[a]	57.5[b]	75.6[a]	3.4	0.001
Prolactin, ng/mL	106.7[b]	166.0[b]	387.1[a]	37.7	0.001
Prolactin:cortisol	1.6[c]	3.3[b]	5.2[a]	0.52	0.001

[abc] Least squares means within the same row with different superscripts are different ($P < 0.05$).

† Genes were: tumor necrosis factor alpha (TNFα), short form prolactin receptors (sPRLR), prolactin, and cytochrome P540 3A28 (CYP450 3A28). Cortisol and prolactin are plasma concentrations.

Feuerstein, G.Z., T. Liu, and F.C. Barone. 1993. Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. Cerebrovascular and Brain Metabolism Reviews 6(4):341-360.

Hardin, J., G. Bertoni, L.J. Kleinsmith, and W.M. Becker. 2012. World of the Cell 8th Edition. San Francisco: Pearson Education, Inc.

Lyons, P.C., R.D. Plattner, and C.W. Bacon. 1986. Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. Science 232(4749):487-489.

Nicot, N., J.F. Hausman, L. Hoffmann, and D. Evers. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J. Exper. Botany 56(421):2907-2914.

Paterson, J., C. Forcherio, B. Larson, M. Samford, and M. Kerley. 1995. The effects of fescue toxicosis on beef cattle productivity. J. Animal Sci. 73(3):889-898.

Sales, M.A., M.J. Larson, S.T. Reiter, A.H. Brown, Jr., M.A. Brown, M.L. Looper, C.F. Rosenkrans Jr. 2012. Effects of bovine cytochrome P450 single-nucleotide polymorphism, forage type and body condition on production traits in cattle. J. Animal Physiol. Animal Nutr. 96(4):545-553.

Settivari, R.S., T.J. Evans, E. Rucker, G.E. Rottinghaus, and D.E. Spiers. 2008. Effect of ergot alkaloids associated with fescue toxicosis on hepatic cytochrome P450 and antioxidant proteins. Toxic. Appl. Pharmac. 227(3):347-356.

Dilute-acid-extractable phosphorus, arsenic, and selenium in weathered and fresh coal fly ash

Chris Wilbanks[*], Kristofor R. Brye*[†], and David Miller*[§]

ABSTRACT

Fly ash is a byproduct of the combustion of coal, primarily by coal-fired power plants. Over 97.8 million tonnes of fly ash are produced each year in the United States. Fly ash can contain trace elements in concentrations that can cause health risks. Recent spills have highlighted that fly ash disposal is problematic. The objective of this study was to evaluate the effects of fly-ash type (fresh and weathered) from a local coal-burning power plant and extraction time (2 and 6 h) on dilute-acid extractable concentrations of phosphorus (P), arsenic (As), and selenium (Se). Ash samples were extracted with 0.1 M HCl and shaken for either 2 or 6 h. Extracts were analyzed by inductively coupled argon plasma mass spectrometry. Phosphorus concentrations increased with the longer extraction time, but there was no significant difference between ash type. Arsenic and Se concentrations were greater in fresh ash and decreased with longer extraction time in fresh ash, but no difference between extraction times was observed in weathered ash. It was determined that P concentrations were not related to As and Se concentrations, but were possibly dependent on calcium phosphates because of the high pH of fly ash. The lower As and Se concentration in the fresh ash, 6-h extraction, as compared to the 2-h extraction suggests that a process analogous to environmental weathering occurred during extraction. Research into the behavior and speciation of these insoluble forms will help explain movement and behavior of trace elements in fly ash.

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MEET THE STUDENT-AUTHOR

Chris Wilbanks

I grew up in the suburbs of North Texas and was fascinated by nature documentaries and wanted to help protect natural environments. After graduating from Guyer High School in Denton, Texas, in May of 2010, I came to the U of A to study environmental science. Though the coursework was much more concrete than my lofty ideals of saving the planet, I came to enjoy them. Because I came into the program with a number of AP credits, I was able to focus on environmental coursework, particularly the wide variety of courses offered in the department and across the campus. With environmental issues in particular, different perspectives often collide, and the various backgrounds and teaching styles of the faculty helped me understand how agricultural, industrial, environmentalist, and scientific perspectives interact in real world projects. This is something I hope to carry forward as I pursue a Masters in Ecological Science and Engineering at Purdue University starting in the Fall of 2014. I would like to thank everyone in the Crop, Soil, and Environmental Sciences department for excellent teaching. I would also like to particularly thank Drs. Brye and Miller for helping me gain some very valuable lab experience, which I believe served as a controlled introduction to my future as a graduate student.

INTRODUCTION

In February 2014, a spill of coal wastes into the Dan River in North Carolina occurred. This began because of a leak from a retired power plant's ash landfill and took one week to stop (Shoichet, 2014). Cases such as this in recent years have heightened awareness and concern about the disposal of coal wastes and focused attention on the need for disposal methods that limit the potential for coal wastes to contaminate the air, water, and soil (Swan, 2014).

Fly ash is a subset of coal wastes that is produced during the burning of coal for power generation. Fly ash is precipitated from the smoke produced by coal combustion in order to meet air quality standards. Fly ash is predominantly inert, siliceous and aluminous glasses, which is similar to volcanic ash. Significant levels of base-forming cations are present in fly ash, giving the material a relatively high pH. Lower concentrations of trace elements, such as arsenic (As), selenium (Se), cadmium (Cd), and chromium (Cr), are also present (Ahmaruzzaman, 2010). Table 1 summarizes the composition and chemical properties of a typical Class C fly ash produced from a coal-burning power plant in northwest Arkansas.

Fly ash is disposed of in a variety of ways, the most common being landfilling. Other disposal methods include use in cement and use as a soil amendment. Landfilling is the dominant form of fly ash disposal (Ahmaruzzaman, 2010), but little scientific research has been undertaken into the status of fly ash landfills. Ash landfills were not required by Arkansas state law to include a liner until recently, and there are still no federal restrictions. Siting is also not restricted, which can lead to spills of material, as in the case of the Dan River. Use of fly ash as an additive in cement is common, where fly ash serves

Table 1. Selected properties of Flint Creek Power Plant fresh fly ash (adapted from Cantrell, 2014).

Ash Property	Value
Silicon	29.9%
Aluminum	18.4%
Iron	6.9%
Calcium	29.1%
Magnesium	6.2%
Potassium	0.3%
Phosphorus	1.3%
Carbon	0.1%
Selenium	9.0 mg/kg
Arsenic	< 1 mg/kg
pH	11.5

Elements were determined by weight in a total digest. pH was determined at 25 ˚C (1% slurry). Carbon measured is Total Carbon.

both as a pozzolan that provides structure to concrete and as a source of lime that is required in the Portland cement chemical reaction (Helmuth, 1987). Fly ash has two uses when used as a soil amendment. Fly ash can function as a liming material to raise soil pH due to the high amounts of base-forming cations, particularly calcium (Ca), that are present in the ash. Fly ash can also be used as a fertilizer to supply micronutrients, such as sulfur (S) and boron (B) (Pandey and Singh, 2010).

Use of fly ash is often limited by trace element content. Trace elements, some of which are also heavy metals, occur in fly ash due to coal's organic origin. Miniscule amounts of trace elements that were present in the precursor plant tissue were preserved when the plant tissue underwent lignification to become coal. These elements volatilize when coal is combusted and then condense in the ash that is left over, concentrating the trace elements in coal wastes (Ahmaruzzaman, 2010). These trace elements, such as As, Se, and Cd, can pose environmental health risks. Trace elements differ from other contaminants in soil because, unlike organic compounds or pathogens, they cannot be degraded. Contamination can also persist because trace elements sorb to soil colloids or precipitate into solid phase minerals, which can dissolve and be mobilized with alteration of pH. Both of these mechanisms can replenish the active fraction in the soil solution, which is the fraction that poses the greatest environmental health risk.

Currently, the United States Environmental Protection Agency (USEPA) does not regulate the disposal of coal wastes. The USEPA has considered classifying coal wastes as a hazardous material under the Resource Conservation and Recovery Act of 1976, but in both 1993 and 2000, USEPA determined coal wastes to be non-hazardous (USEPA, 2014a). The USEPA has remained concerned about the potential for coal wastes to cause environmental damage and in 2010 reopened the investigation into classifying coal wastes as hazardous. This investigation is slated to release a final decision by December 2014 (USEPA, 2014a).

In a previous study (Cantrell, 2014) of the same fly ash material used in the present study, As and Se were selected for examination due to their toxicity and similar chemical behavior. Phosphorus (P) was also examined because P and As are both Group 15 elements from the periodic table that exhibit similar chemical behavior and exist as oxyanions in soils. Research has indicated that As and P compete for sorption sites in soil (McDonald et al., 2009). This is of particular concern in northwest Arkansas, as many soils are saturated with P from decades of land application of P-rich poultry litter (Daigh et al., 2010). Research has also suggested that in low pH Ultisols, P is more competitive for soil adsorption sites than As (Violante and Pigna, 2002). However, Se is a Group 16 element and is more mobile than As and P, acting similarly to sulfate in soil, which can threaten groundwater.

The central premise of this study was that land application of fly ash is a viable method of fly ash disposal in northwest Arkansas as long as leaching of As and Se can be shown to be insignificant. It is likely that long-term storage of ash in ash landfills changes the chemical forms and leachability of trace elements, but research on the topic is scarce. The objective of this study was to evaluate the effects of long-term storage and extraction time (2 and 6 h) on dilute-acid extractable concentrations of P, As, and Se in fresh and weathered ash. It was hypothesized that extractable P, As, and Se concentrations would be greater in fresh than weathered ash and that extractable P, As, and Se concentrations would be greater after a 6-h extraction than a 2-h extraction.

MATERIALS AND METHODS

Fly Ash Materials. Samples of fly ash were collected from the Flint Creek Power Plant in Benton County, Ark., which is operated by the American Electric Power-Southwestern Electric Power Company (AEP-SWEPCO; Cantrell, 2014). The plant uses Powder River Basin coal from the Wyodak Beds in Wyoming (Cantrell, 2014). Fresh samples were collected over a 30-d period from 7 July 2013 to 5 August 2013 during normal plant operation from the plant's fly ash collection piping (Cantrell, 2014). Weathered samples were collected from the on-site ash landfill, which has been used to store fly ash since the plant began operation in 1978 (Cantrell, 2014). The actual age of the weathered ash is unknown due to the disposal method, but samples were collected from areas believed to have the oldest ash (Cantrell, 2014).

Ash Extraction Procedures. Ash samples were extracted following procedures similar to those outlined by Daigh et al. (2010) and McDonald et al. (2009), using a solid:solution ratio of 1:10 (mass:vol) or 3 g fly ash in 30 mL of 0.1 M HCl , dispensed into 50-mL plastic centrifuge tubes. Ten samples each of fresh and weathered ash were further divided into a 2- and a 6-h shake time on an end-over-end shaker, for a total of five samples in each ash type-extraction time combination. For each group of samples, three control samples of 30 mL HCl with no fly ash were run under the same experimental conditions to serve as method blanks for quality control. After being removed from the shaker, samples were centrifuged at 6000 revolutions per minute and filtered through 0.45-µm Suppor-450 membrane filter (Pall Life Science, Port Washington, N.Y.) into 20-mL scintillation vials. Immediately following the filtration, samples were stored at 4 °C until they were shipped the following week to the University of Georgia for analysis, where a Perkin-Elmer

Elan 6000 inductively coupled plasma mass spectrometer was used to measure As, Se, and P concentrations.

Statistical Analyses. A two-factor analysis of variance was conducted using SAS (version 9.3, SAS Institute Inc., Cary, N.C.) to evaluate the effects of ash type, extraction time, and their interaction on concentrations of As, Se, and P. Means were separated by least significant difference at the 0.05 level.

RESULTS AND DISCUSSION

Ash type and extraction time affected the extractability of all three elements evaluated in this study (Table 2). Dilute-acid-extractable P varied only by extraction time (P = 0.049), while As and Se concentrations varied by extraction time within ash type (P < 0.001). The mean extractable P concentration was 9.6 mg/kg for the 2-h extraction and 19.3 mg/kg for the 6-h extraction time. One possible cause for this observed trend is the dissolution of calcium phosphates, which are soluble at low pH. With a longer extraction time, it is possible that the reaction was allowed to continue to equilibrium in the 6-h extraction, while in the 2-h extraction, equilibrium had not yet been reached. However, the experimental procedure used in this study did not include measurements of the rate of P release. Phosphorus concentration showed no statistically significant relationship to As or Se with ash type or extraction time (Table 2). Due to the experimental design not including interaction with soil, it is unclear how P amendment from fly ash could affect the solubility of As, particularly in Northwest Arkansas.

Dilute-acid-extractable As concentrations were greatest from the fresh ash with a 2-h extraction (18.2 µg As/kg; Fig. 1), while all other treatment combinations had a lower mean As concentration and did not differ among themselves (Fig. 1). Similar to As, dilute-acid-extractable Se was greatest from the fresh ash with a 2-h extraction time (258 µg Se/kg; Fig. 1). However, in contrast to As, extractable Se was also greater from the fresh ash with a 6-h extraction time than from the weathered ash at either extraction times, which did not differ (Fig. 1).

Table 2. Analysis of variance summary of the effects of ash type, extraction time, and their interaction on dilute-acid extractable phosphorus (P), arsenic (As), and selenium (Se).

Source of Variation	P	As	Se
Ash Type	0.684	< 0.001	< 0.001
Extraction Time	0.049	< 0.001	< 0.001
Ash Type * Extraction Time	0.690	< 0.001	< 0.001

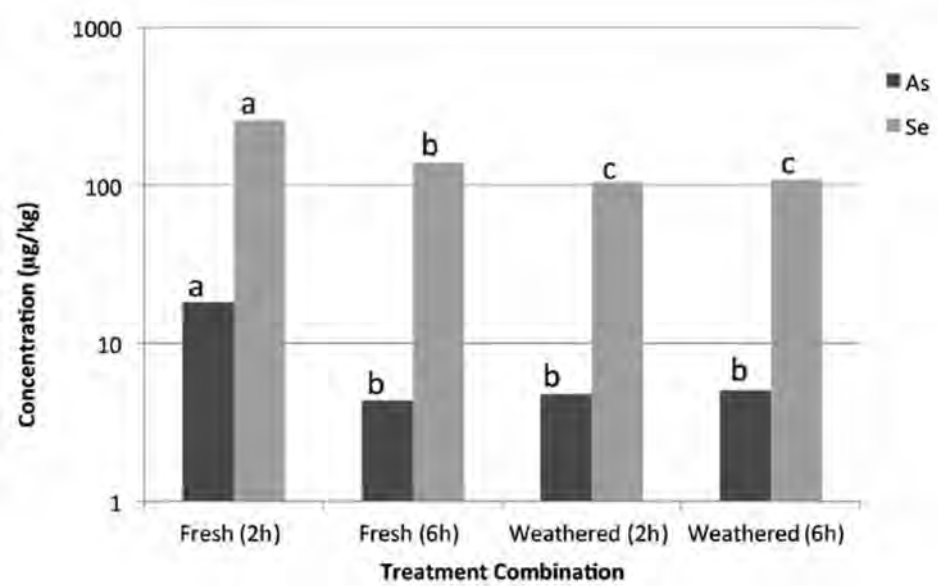

Fig. 1. The effects of ash type (i.e.,freshly collected and weathered for a long period of time in an ash landfill) and extraction time (i.e., 2 and 6 h) on mean concentrations of arsenic (As) and selenium (Se). For a given element, different letters atop bars denote significant differences at the 0.05 level.

Arsenic concentration in the fresh ash 2 h extraction are greater than the USEPA's Maximum Contaminant Level (0.010 ppm) (USEPA, 2013). Additionally, all measured concentrations of Se are greater than the MCL (0.050 ppm) (USEPA, 2014b). However, these are measurements of total element concentrations, and not speciation, which plays a large role in toxicity. The choice of extractant has also elevated results above those of a previous study with the same material (Cantrell, 2014).

The lack of a significant difference between extraction times for weathered ash, as well as a decline with greater extraction time from the fresh ash, indicate that some process is serving to create non-reactive or insoluble forms of As and Se over both extraction and storage times. As weathered ash was exposed to environmental weathering for years prior to the study, it is possible that the mechanism that caused an observed reduction in concentration with extraction time in fresh ash is also involved in the lower extractable concentrations in weathered ash.

Analysis in a prior study (Cantrell, 2014) indicated that calcium selenite ($CaSeO_3$) and hydrated complexes of calcium and aluminum oxides served as a sink for Se. It takes a period of several months to stabilize these compounds under environmental conditions, such as those to which the weathered ash was exposed, during which time selenite (Se^{+4}) can oxidize to selenate (Se^{+6}), a far more mobile form that can easily leach (Cantrell, 2014). This indicates that acid extraction in the fresh ash may have followed a chemical process similar to the formation of $CaSeO_3$ to render a larger fraction of the Se insoluble, giving a similar result to that of the weathered ash.

The dominant form of As in coal wastes is the less soluble arsenate (As^{5+}). Arsenic undergoes similar reactions as Se to form precipitates, but is unlikely to reduce to more mobile forms (Cantrell, 2014). The overall lower level of initial soluble trace elements contributed to the lower observed concentrations, and it is probable that a similar chemical process explains the observed decline in As between fresh ash extractions. Speciation of As and Se was not performed in this study, which would have determined what forms the As and Se existed in, but further research into the chemistry of these trace element species would be helpful.

Results of this study both support and negate the initial hypotheses. The forms of trace elements within the weathered ash are likely dominated by insoluble forms, due to transformations, such as the weathering process of Se discussed above, and leaching of mobile forms to soil and ultimately groundwater over years of infiltration. If the results of the weathered ash are indicative of how fresh ash would behave environmentally, then this fly ash would be suitable for disposal via land application. However, the actual chemical speciation and reactivity of the trace elements are still largely unknown. The wide temporal and spatial variability of soil properties leaves open the possibility that insoluble forms deemed safe could dissolve under conditions not currently understood and eventually pose a health risk. However, concerns about the concentration of P displacing trace elements seem unlikely given the results of this study. Observed P concentrations increased with extraction time, but As and Se were both lower in weathered ash, which has been exposed to environmental conditions similar to those land-applied fly ash might experience. This indicates that processes controlling P are different from those controlling As and Se. Currently, the best usage of fly ash for land application as it is currently understood is to base maximum application rates on total trace element concentration, until such time that the speciation of the stable forms are better understood.

LITERATURE CITED

Ahmaruzzaman, M. 2010. A review on the utilization of fly ash. Prog. in E. Comb. Sci 36: 327-363.

Cantrell, M. 2014. Leaching Characteristics of Selenium from Coal Fly Ash. MS thesis, Univ. of Arkansas, Fayetteville.

Daigh, A.L., K.R. Brye, A.N. Sharpley, D. Miller, and E. Gbur. 2010. Broiler litter composition as affected by water extractant, dilution ratio, and extraction time. Comm. in Soil Sci. Plt. Anal. 41: 2340-2357.

Helmuth, R. 1987. Fly Ash in Cement and Concrete. Portland Cement Association, Skokie, IL.

McDonald, M., K.R. Brye, D.M. Miller, and E.E. Gbur. 2009. Broiler-litter application history and soil-depth effects on arsenic sorption. Soil Sci. 174: 661-675.

Pandey, V.C. and N. Singh. 2010. Impact of fly ash incorporation in soil systems. Agri., Ecosys. Environ. 136: 16-27.

Pezzarossa, B., G. Petruzzelli, H. Selim, and D. Sparks. 2001. Selenium contamination in soil: sorption and desorption processes. p. 191-206. In Selim and Sparks (ed.). Heavy metals release in soils. CRC Press LLC, Boca Raton, Fla.

Shoichet, C.E. 2014. Spill spews tons of coal ash into North Carolina river [Online]. Available at http://www.cnn.com/2014/02/09/us/north-carolina-coal-ash-spill/(accessed 2 April 2014).

Swan, N. 2014. Toxic coal ash poses persistent threat to US waters [Online]. Available at http://www.csmonitor.com/USA/2014/0330/Toxic-coal-ash-poses-persistent-threat-to-US-waters (accessed 2 April 2014).

(USEPA) United States Environmental Protection Agency. 2013. Arsenic in Drinking Water [Online]. Available at http://water.epa.gov/lawsregs/rulesregs/sdwa/arsenic/index.cfm (accessed 20 June 2014).

(USEPA) United States Environmental Protection Agency. 2014a. Fossil Fuel Combustion (FFC) Waste Legislative and Regulatory Time Line [Online]. Available at http://www.epa.gov/osw/nonhaz/industrial/special/fossil/regs.htm (accessed 2 April 2014).

(USEPA) United States Environmental Protection Agency. 2014b. Basic Information about Selenium [Online]. Available at http://water.epa.gov/drink/contaminants/basicinformation/selenium.cfm (accessed 20 June 2014).

Violante, A. and M. Pigna. 2002. Competitive sorption of arsenate and phosphate on different clay minerals and soils. SSA J. 66: 1788-1796.

Encouraging teacher change within the realities of school-based agricultural education: lessons from teachers' initial use of socioscientific issues-based instruction

Amie K. Wilcox[*], *Catherine W. Shoulders*[†], *and Brian E. Myers*[§]

ABSTRACT

Calls for increased interdisciplinary education have led to the development of numerous teaching techniques designed to help teachers provide meaningful experiences for their students. However, methods of guiding teachers in the successful adoption of innovative teaching approaches are not firmly set. This qualitative study sought to better understand how school-based agricultural education teachers decide to adopt or discontinue a teaching innovation when introduced through ready-made lesson plans, which is currently a common practice of teaching method integration in school-based agricultural education (SBAE). Constant comparative analysis unveiled themes within the reactions to the teaching method's use, as well as how teacher actions to those reactions led to their ultimate adoption or discontinuance of the teaching method.

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MEET THE STUDENT-AUTHOR

Amie Wilcox

I am a junior from Waldron, Ark. majoring in Agricultural Education, Communications and Technology (AECT) with a concentration in Agricultural Education. I have served as a Bumpers College ambassador and the Director of Lectures for the Honors Student Advisory Board. In the AECT department, I am a member of Representing Excellence, Pride and Service and am a former President of the Collegiate FFA chapter (previously known as the Future Farmers of America). Throughout my undergraduate education, I have had the opportunity to travel and present leadership workshops to high school students with the National FFA Organization. These experiences piqued my interest in curriculum development and presentation. I look forward to continuing research in my future educational career. I would like to give a special thank you to Dr. Catherine Shoulders, who challenged me and supported me. I will be forever grateful for the opportunities that have been shown to me throughout this research.

INTRODUCTION

The notion of change stemming from school reform has ironically been a constant within the United States public school system for the past 60 years. School-based agricultural education (SBAE) has not been omitted from these calls. The national calls for increased interdisciplinary education and real-world connections (American Association for the Advancement of Science, 1993) have led to the development and delivery of numerous teaching methods designed to help teachers provide meaningful experiences for their students. While these methods are not unique to agricultural education, agriculture teacher educators focus on introducing these interdisciplinary teaching methods to their students (Newcomb, et al., 2004; Phipps, et al., 2008). Recent studies have introduced socioscientific issues (SSI)-based instruction, a method stemming from inquiry-based instruction and commonly highlighted within science education, as an appropriate method for use in SBAE due to its focus on agricultural issues (Shoulders, 2012).

Thus far, one study has examined the impact of SSI-based instruction in agriculture classrooms (Shoulders, 2012). This study found significant gains in student knowledge following the SSI-based instructional unit, which delivered 45 ready-made lesson plans and accompanying PowerPoint presentations and student materials to teachers after they attended a one-hour training session on SSI-based instruction. While the methods of integration, chosen due to teachers' available time to engage in professional development related to the instruction, followed recommendations of researchers in agricultural education, the study experienced an exceptionally high attrition rate; seven out of the 11 original teachers requested to be removed from the study after they began utilizing the materials. The quantitative nature of the study did not lend itself to further investigation into the reasons for this high attrition. Based on the positive impact the SSI-based instructional model had on the students whose teachers remained in the study, the researcher recommended qualitative research be conducted to further understand how SSI-based instruction can impact student learning within the realities of the everyday classroom. The current study served to better understand how teachers made the decision whether to continue utilizing the SSI-based instructional approach when given ready-made materials in an effort to provide recommendations for teaching method adoption approaches appropriate for agriculture teachers, both within SSI-based instruction and for those focused on in the future.

The purpose of this basic qualitative study was to understand how teachers made the decision to continue or discontinue using an SSI-based instructional approach when supplied with ready-made materials. To achieve this purpose, the following research questions guided this study:

1. What are teachers' perceptions regarding SSI-based instruction throughout the unit?
2. What are teachers' perceptions regarding the use of ready-made materials in their classrooms?
3. How do teachers decide whether to fully adopt SSI-based instruction?
4. What teacher concerns and/or actions lead to decisions to discontinue using SSI-based instruction?

MATERIALS AND METHODS

This study followed basic qualitative methodology in order to better understand how teachers made the decision to continue or discontinue using an SSI-based instructional approach when supplied with ready-made materials.

Participants

Teachers were selected to participate in the study using purposeful criterion sampling, in which the cases studied meet specific criteria to ensure richness and quality of data (Patton, 1990). When using criterion sampling, the researchers predetermine the criterion by which participants should be selected, based on aspects which the researchers deem influential on data quality (Patton, 1990). The social desirability of teachers to successfully and easily adopt teaching methods required that only teachers with proven ability to maintain honest communication with the researchers would supply honest evaluation of the lesson plans. Therefore, teachers were purposefully selected based on their history with the researchers; and those with a professional history that exhibited honest, detailed, and consistent communication with at least one of the researchers were invited to participate. The teachers were also selected based on their past willingness to attempt novel teaching approaches on their own. Each teacher taught a variety of animal science, plant science, mechanics, and introductory agriculture courses, and each had high school agricultural education experience.

Data Collection

Two researchers were involved in all aspects of data collection, while one was omitted from data collection in order to enable him/her to analyze the raw data from a perspective alternate to that of the other researchers, whose lens of the data could have been altered by the data collection experience. Data were collected through the use of daily journal prompts, weekly semi-structured interviews, and a focus group (Flick, 2006). Journal prompts included a set of questions for teachers to answer after every lesson, and included items intended to guide teachers through a lesson reflection. Protocols for the semi-structured interviews were different each week, and each included questions that guided teachers through their overall reactions to the lessons, the current state of their classrooms' cultures, aspects of the lessons they had trouble with or altered, classroom preparation, and student behaviors. The planned focus group protocol enabled teachers that successfully entered the confirmation stage of adoption to collectively provide insight into what they saw as the main strengths of the lessons, the main weaknesses of the lessons, their students' reactions to the unit, the alterations they made to the lessons, and their opinions on what changes would enable a greater number of teachers to adopt the innovation.

Daily journals were submitted by teachers via email at the end of each day. Weekly interviews were conducted via telephone and were recorded. The focus group was conducted through a recorded online session which enabled the group to speak together, see one another, and collectively work on a web-based "white board". All recordings were then transcribed and coded. Coded data were first identified by the participant (P1–5), then by the data source, (J = journal, I = interview, E = email), then by the number of the data source, then by line number (L). Mrs. Smith was coded as Participant 1, Mrs. Jones was coded as Participant 2, Mr. Jackson was coded as Participant 3, Mr. East was coded as Participant 4, and Ms. Martin was coded as Participant 5. Using this method, data obtained from, for example, the third participant, on the fourth journal entry, from lines 6–10 would be coded as P3, J4, L6–10.

Data Analysis

Daily written journals and interview and focus group transcriptions were analyzed using the constant comparative method (Lincoln and Guba, 1985), which includes four stages: 1) compare incidents applicable to each category, 2) integrate categories and their properties, 3) delimit the construction, and 4) write the construction. Following this method, the researchers reviewed transcriptions and journals for trends, which were utilized to discover emerging categories within the data. Researchers first used an open coding procedure to discover themes found within fragments of each journal entry or transcription and compare them to the remainder of the journal entry or transcription to determine whether other fragments aligned with the same theme. The researchers then compared fragments from individual texts to determine whether they repeated information or offered new information (Lincoln and Guba, 1985). Those with repeating information were coded to the same theme. Those with new information were initially coded into different themes. Once texts were coded into themes, the researchers sought to label the categories with the most

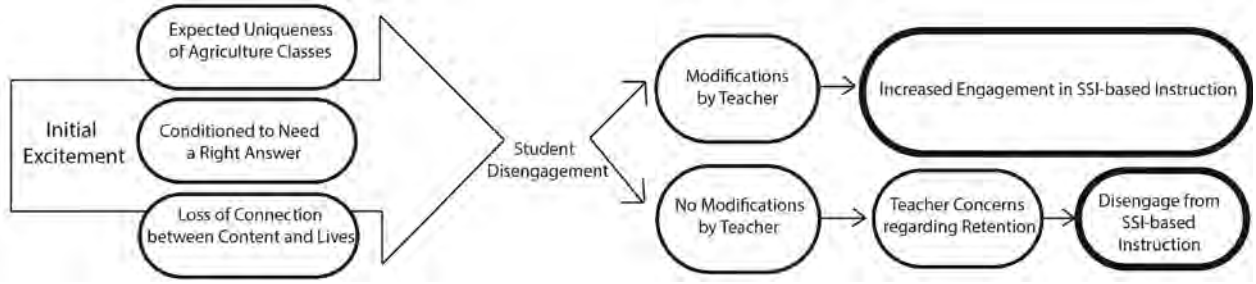

Fig. 1. Evolution of students' reaction to socioscientific-based instruction.

appropriate theme titles. The determination of appropriate theme titles enabled researchers to further distinguish between repetitive themes, overlapping themes, related themes, and separate themes.

RESULTS AND DISCUSSION

To protect participant confidentiality, the five teachers participating in the study were given pseudonyms that accurately reflected their genders. Mrs. Smith, Mrs. Jones, and Mr. Jackson were all high school agricultural education teachers in [State]. Mrs. Jones and Mr. Jackson shared a two-teacher program. Mrs. Smith taught in a three-teacher program, but was the only study participant from her school. Mrs. Smith and Mr. Jackson had both taught for three years in the schools in which they were currently employed. Mrs. Smith, Mrs. Jones, and Mr. Jackson each used the study's lesson plans in their introductory agriculture courses, which consisted primarily of ninth grade students. Mrs. Smith's and Mr. Jackson's classes participated in block scheduling and held class every other day. Mrs. Jones had eight years of teaching experience, all in the school in which she was currently employed. Her classes were 45 minutes long, and students met every day.

Mr. East was a high school agricultural education teacher in a single teacher department in [State] with three years of teaching experience. He had one year of previous teaching experience in [State], and was engaging in his second year teaching in his current school during the study. He utilized the SSI-based lesson plans in his Ag. II course, which is a year-long course for sophomore students. Mr. East's students met for 45 minutes every day. Ms. Martin was a high school agricultural education teacher in a three-teacher department in [State] with three years of teaching experience. She utilized the SSI-based lesson plans in her Survey of Agricultural Systems course, which was comprised of students in grades 10 through 12.

Analysis of the data showed that within each class, after initial excitement, specific factors including the expected uniqueness of agriculture classes, students' conditioned need for a right answer, and a loss of connection between lesson content and students' lives, led to student disengagement from SSI-based instruction. Teachers' actions following this disengagement resulted in either increased engagement in SSI-based instruction or ultimate disengagement from the innovation, which caused the teacher to drop out of the study. This process of adoption or discontinuance as found within the data is displayed in Fig. 1, and is discussed more fully below.

Although all teachers expressed that students were initially excited about the new and modern material, three factors emerged as causes leading to student disengagement in all classes. These factors are the expected uniqueness of an agriculture class, the students' conditioned need for a right answer, and a loss of connection between content and the students' lives. Teachers each noticed the disengagement, but took one of two paths to reengage their students. Mrs. Smith, Mrs. Jones, and Mr. Jackson opted to discontinue use of SSI-based instruction and readopt their traditional methods of teaching, confidently feeling as though their previous methods of instruction would align with students' expected uniqueness of agriculture classes, their conditioned need for a right answer, and the relevance they needed to see between content and their lives. This action aligns with Rogers' (1995) position that adopters of an innovation may discontinue the use of an innovation during the implementation stage, and reflects the pattern of discontinuance seen in Shoulders' (2012) study. Mr. East and Ms. Martin chose to alter lesson plans in order to better align the innovation of SSI-based instruction with the students' expectations of the agriculture classroom and need for connections between content and their lives. This reinvention of the innovation was noted by Rogers (1995) to be a common occurrence within the implementation stage before adopters entered the confirmation stage. As Rogers suggested, the reinvention of the innovation enabled both Mr. East and Ms. Martin to continue to use the innovation throughout the study. Supporting the critical need for reinvention before entering the confirmation stage, both Mr.

East and Ms. Martin recommended that, because of the differences in their classes, the ability to make their own activities to teach the content was a key component to the engagement they saw from their students. It was the reengagement displayed by students that gave them the confirmation they needed in order to fully adopt the innovation and make the lessons their own.

Recommendations

Based on the themes identified through examination of the data, the researchers agree upon the following recommendations for teacher educators interested in introducing agriculture teachers to curricular innovations. First, teacher reaction to student disengagement is key to the long-term success and implantation of a new curriculum innovation. Students and teachers expressed an initial positive reaction to the SSI-based instruction. However, once the realities of the SBAE classroom were realized, student disengagement and frustration with the new approach were witnessed. This finding is consistent with Moore and Moore's (1984) position that the ideals of novel teaching methods may clash with the realities of the classroom, setting them up for failure when implemented. In this study, the reaction observed was reinvention of the innovation to overcome the causes of student disengagement. The teachers in this study who made modifications during the implementation stage by assessing student disengagement and making lesson alterations were rewarded with student behaviors that led to confirmation of the adoption. Rogers (1995) noted that modification is common during the implementation stage; this study suggests that personal modification may be necessary for teachers to reach the confirmation stage when deciding whether to adopt innovative teaching strategies. Teacher educators should therefore create easily adaptable materials for teachers; and during professional development, help teachers distinguish between the components of the curricular innovation that are crucial to its implementation and those that can be altered to best meet the needs of the teachers' students.

Secondly, factors beyond just the classroom component of the complete SBAE program impact the adoption of instructional innovations, and it is recommended that curriculum innovations incorporate FFA (formerly known as Future Farmers of America), supervised agricultural experience (SAE), and classroom instruction. A number of teachers in this study struggled with the opportunity costs of teaching strictly in the classroom and spending less time focusing on the laboratory, FFA, and SAE components of the SBAE program. Ironically, agriculture teachers' year-round responsibilities in each of these three areas may keep them from being able to engage in professional development opportunities designed to help them tailor SSI-based instruction to fit their programs (Anderson, et al., 1992). Therefore, it is recommended that teacher educators give attention to all aspects of the total SBAE program in any new curriculum innovation both during its development and during professional development with teachers.

Participants noted that students expected the culture of the SBAE classroom/program to be different than that of the other classes in the school. Teachers perceived that SSI-based instruction ran counter to the students' expected SBAE program culture and was too similar to what the students expected to find in other courses in the school. In reaction to student perceptions, teachers noted concern on how continuing with the SSI-based instruction would impact future student enrollment in the SBAE program. This teacher implementation concern is unique to literature regarding SSI-based instruction adoption. Previous studies investigating this topic were conducted in academic courses where teachers were not as concerned with student recruitment and retention (Dawson, 2011; Klosterman and Sadler, 2011; Osborne, et al., 2004; Sadler, et al., 2011; Yager, et al., 2006; Zeidler, et al., 2009). This phenomenon deserves further attention to assist teachers in implementing strategies to allow for new curricular innovations while mitigating any negative enrollment impacts. These strategies should include ways teachers can assist students' transitions from known or perceived cultural norms for the SBAE program/classroom to those the teacher is attempting to implement.

Finally, this study yielded a recommendation for future research. While researchers discovered the process through which teachers proceeded in order to move from implementation to either discontinuance or confirmation, the reasons why teachers decided to either discontinue the innovation or reinvent it were not uncovered. Interviews with participants did not include any suggestions or recommendations regarding teachers' possible options to proceed when they expressed difficulties with the innovation; two teachers requested to alter lesson plans and three requested to discontinue their use. The researchers recommend that further investigation be carried out to determine the factors that lead teachers to make the decisions they choose with regard to reinventing or discontinuing an innovation.

As this study implies, teachers of SBAE may be faced with unique circumstances that impact their abilities to adopt innovative teaching techniques designed for the traditional science classroom. However, unyielding calls for educational reform require that teachers and teacher educators continue to experiment with different instructional techniques in an effort to improve student learning. Through trials, adaptations, and recognition of the unique circumstances of SBAE, teachers and teacher educators can continue to meet the needs of today's students.

LITERATURE CITED

American Association for the Advancement of Science (1993). Project 2061–Science for All Americans. Washington, DC: Authors.

Anderson, T.J., R.K. Barrick, and M. Hughes. (1992). Responsibilities of teacher education vocational teacher professional development programs. J. Agric. Edu., 33(2), 43-50. doi: 10.5032/jae.1992.02043

Dawson, V.M. (2011). A case study of the impact of introducing socio-scientific issues into a reproduction unit in a catholic girls' school. In T. Sadler (Ed.), Socioscientific issues in the classroom (pp. 311-344). New York, NY: Springer Publication.

Flick, U. (2006). An introduction to qualitative research. London, UK: SAGE Publications.

Klosterman, M.I. and T.D. Sadler. (2011). Multi-level assessment of scientific content knowledge gains associated with socioscientific issues-based instruction. International Journal of Science Education, 32(8), 1017-1043. doi:10.1080/09500690902894512

Lincoln, Y.S. and E.G. Guba. (1985). Naturalistic inquiry. Newbury Park, CA: Sage Publications.

Moore, G.E. and B.A. Moore. (1984). The problem solving approach to teaching: Has it outlived its usefulness? J. Amer. Assoc. Teacher Educ. Agric., 25(2), 3-10.

Newcomb, L.W., J.D. McCracken, J.R. Warmbrod, and M.S. Whittington. (2004). Methods of teaching agriculture. (3rd ed.) Upper Saddle River, NJ: Pearson Prentice Hall.

Osborne, J., S. Erduran, and S. Simon. (2004). Enhancing the quality of argumentation in school science. J. Res. Sci. Teach., 41(10), 1004-1020. doi:10.1002/tea.20035

Patton, M. (1990). Qualitative evaluation and research methods. Beverly Hills, CA: Sage

Phipps, L.J., E.W. Osborne, J.E. Dyer, and A. Ball. (2008). Handbook on Agricultural Education. (6th ed.). Clifton Park, NY: Thomson Delmar Learning.

Rogers, E.M. (1995). Diffusion of Innovations. (4th ed.). New York, NY: Simon and Schuster Inc.

Sadler, T.D., M.I. Klosterman, and M.S. Topeu. (2011). Learning science content and sociosientific reasoning through classroom explorations of global climate change. In T.D. Sadler (Ed.), Socioscientific issues in the classroom: Teaching, learning and research (pp. 45-47). New York, NY: Springer.

Shoulders, C.W. (2012). The effects of a socioscientific issues instructional model in secondary agricultural education on students' content knowledge, scientific reasoning ability, argumentation skills, and views of the nature of science (Doctoral dissertation). Retrieved from ProQuest (No. 2012.3569446)

Yager, S.O., G. Lim, and R. Yager. (2006). The advantages of an STS approach over a typical textbook dominated approach in middle school science. School Science and Mathematics, 106, 248-260.

Zeidler, D. L., T.D. Sadler, S. Applebaum, and B.E. Callahan. (2009). Advancing reflective judgment through socioscientific issues. J. Res. Sci. Teach., 46(1), 74-101. doi:10.1002/tea.20281

Bermudagrass growth in soil contaminated with hydraulic fracturing drilling fluid

Douglas C. Wolf and Kristofor R. Brye†*

ABSTRACT

Hydraulic fracturing is the process of injecting aqueous solutions at high pressure to break apart rock formations and increase the extraction of natural gas. The solutions are recovered and have been land-applied as one disposal technique. Excessive fluid application can result in increased soil salinity that can inhibit plant growth. The objective of this greenhouse study was to evaluate the effects of inorganic fertilizer, broiler litter, and Milorganite® and soil depth interval (0-15 cm or 0-30 cm) on the growth of bermudagrass [*Cynodon dactylon* (L.) Pers] in soil that was collected from a site that had been contaminated with fracturing fluid and was initially devoid of vegetation. Amendment rates were added to provide 60 mg of plant-available N/kg. Bermudagrass was sprigged and harvested after nine weeks and shoot, root, and total biomass were determined. Addition of inorganic fertilizer, broiler litter, or Milorganite® resulted in greater shoot biomass compared to unamended soil. Plants grown in 0-30-cm-depth soil had greater root biomass compared to the 0-15-cm soil depth. The addition of recommended plant nutrients and mixing of the contaminated surface soil with the subsurface soil enhanced bermudagrass growth.

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MEET THE STUDENT-AUTHOR

Douglas Wolf

I graduated summa cum laude with a Bachelor of Science in the Crop, Soils, and Environmental Sciences Department and was a member of the Agricultural, Food, and Life Sciences Honors Program. I have had the opportunity to participate in study abroad programs in three countries—Belize, Scotland, and New Zealand—with funding from the Honors College, American Society of Agronomy Cross Cultural Experience Program Scholarship, and Phi Kappa Phi. In 2012, I was 2012 National Golden Opportunity Scholar with the Soil Science Society of America and also received an Arkansas State Undergraduate Research Fellowship, which was used to fund this study. I am also a recipient of a Morris K. Udall Fellowship Honorable Mention.

After completing my internship at the National Center for Toxicology Research this summer, I will be attending the University of California, Riverside Environmental Toxicology Doctoral Graduate Program. I will be funded by the University of California, Riverside Chancellor's Distinguished Fellowship and the National Science Foundation Graduate Research Fellowship.

I would like to express deep gratitude and appreciation to Dr. Kristofor Brye for his guidance and support throughout my academic career at the University of Arkansas as a mentor, advisor, and friend. I would also like to thank my committee members, Dr. Mary Savin, Dr. David Miller, and Dr. Curt Rom, for revisions on my thesis.

INTRODUCTION

Throughout the 21st century, the world has experienced an ever-increasing demand for petroleum to power an exponentially growing population (Cunningham and Cunningham, 2008). The United States' response to the depletion of petroleum is to explore other domestic energy sources such as natural gas. In 2011, natural gas consumption accounted for approximately 23% of the total United States energy use, which is expected to increase to 27% by 2040 (USEIA, 2013). In its 2013 Annual Energy Outlook, the U.S. Energy Information Administration (USEIA) estimated that natural gas consumption would increase from 6.91×10^{11} m^3 in 2011 to 8.35×10^{11} m^3 in 2040. The USEIA 2013 report also predicted that a 57% increase in natural gas production (1.02×10^{12} m^3) would occur by 2035. The increased production would be due primarily to the advancements in drilling technology and techniques such as a process called hydraulic fracturing that can extract natural gas in previously unconventional reservoirs.

Unconventional gas reservoirs typically contain concentrations of natural gas over large areas that have a lower permeability or porosity than conventional gas reservoirs, such as sandstone and carbonate reservoirs (Vidas and Hugman, 2008). An unconventional gas reservoir, called the Fayetteville Shale, is located in the Arkoma Basin of north-central Arkansas where approximately 5,357 natural gas producing wells have been constructed (AOGC, 2014a). The Fayetteville Shale contains approximately 6.5×10^4 km^2 of black, fissile, concretionary, dense clay shale that has very low permeability. However, extraction has recently become economically feasible due to advances in horizontal drilling and hydraulic fracturing (Kresse et al., 2012).

Hydraulic fracturing has been utilized in combination with horizontal drilling for multiple decades of shale gas extraction and is a stimulation technique to enhance the flow rate of natural gas wells by increasing the gas-bearing rock permeability (USEPA, 2004). The hydraulic fracturing process begins with the building of site infrastructure, such as well pads, holding ponds, and access roads, that generally encompasses 1 to 2 ha (USEPA, 2011). Production wells are then drilled to a depth of 2130 to 3040 m depending on gas-containing rock formations and can be vertical, horizontal, or S-shaped (Wang et al., 2014). Currently, horizontal wells are the most common well formation because horizontal wells provide more exposure to the rock containing natural gas compared to vertical wells. Horizontal drilling increases the recovery of natural gas and the cost effectiveness of

the natural gas well in addition to allowing multiple wells drilled on a single well pad to access natural gas resources (Arthur et al., 2008).

After the hydraulic fracturing well is constructed, the gas-containing formation is hydraulically fractured in a series of stages by pumping fracturing fluid into the wellbore under high pressures that fill the voids in the geologic formation until the formation can no longer accommodate the fracking fluid, causing the rock to fracture and release natural gas to the well bore and surface (Wang et al., 2014). The fracturing fluids, comprised primarily of water mixed with chemical additives, are used throughout the natural gas operation to increase the performance and efficiency of extraction by creating fractures of ample width that maximize fluid injection rates. In addition to these optimized fractures, a proppant, typically sand, is also used to keep the induced hydraulic fractures from closing when the injection ceases (Arthur et al., 2008). Hydraulic fracturing operations in the Fayetteville Shale use an estimated 1.1×10^7 L per well (Satterfield et al., 2008). Because of the large volume of water used per well, large amounts of chemical additives are being disposed of as waste products.

After hydraulic fracturing and the gas extraction process is complete, the internal pressure of the geologic formation causes the drilling fluids to rise to the surface and is referred to as flowback or "produced" water (Wang et al., 2014). Rahm (2011) and Wang et al. (2014) reported that as much as 15% to 100% of the "produced" water may be returned to the surface and require disposal. Historically, flowback was disposed of by deep injection into underground injection control (UIC) class II wells or through land application. However, the controlled injection disposal of used fracking fluids has been associated with a 4.7-magnitude earthquake in central Arkansas in 2011 (Ellsworth, 2013). Although north-central Arkansas is partially located on the New Madrid seismic zone, 98% of 157 earthquakes occurred within 6 km of one of the three Arkansas UIC class II wells during flowback injection from 2010 to mid-2011(Horton, 2012; King, 2012). Because of this apparent correlation, the Arkansas Department of Environmental Quality (ADEQ) has closed all disposal wells and suspended any new disposal wells within the Fayetteville Shale (Arkansas Hydraulic Fracturing State Review, 2012).

The ADEQ also regulates the land application process of water-based drilling fluids based upon the physical landscape (i.e., slope, storage capacity, and weather), physical and chemical soil properties (i.e., electrical conductivity and pH), and the drilling waste characteristics (ADEQ, 2009). In addition to the chemical additives, the flowback also contains high concentrations of total dissolved solids and the potential for high concentrations of hydrocarbons and heavy metals (Wang et al., 2014). At some natural gas drilling sites, over-application of drilling fluid waste has resulted in contaminated soil that could not adequately support vegetation to meet ADEQ requirements for natural gas site closure. Site closure requires vegetative coverage of 75% or more, or equivalent to the surrounding landscape, whichever is less, within six months of site closure (AOGC, 2014b). Previous studies have been conducted concerning the impact of land application of hydraulic fracturing drilling fluid on vegetation (Nelson et al., 1984; Adams, 2011); however, no information regarding revegetation has been discussed.

Since little is known about the optimum approach to revegetate a site that has been contaminated by surface-applied hydraulic fracturing fluid, much benefit can be gained from the evaluation of potential inorganic and organic soil amendments to enhance plant establishment and growth. Therefore, the objective of this 9-wk greenhouse study was to evaluate the effects of inorganic fertilizer, broiler litter, and Milorganite® and soil depth interval (i.e., 0-15 cm or 0-30 cm) on the growth of bermudagrass [*Cynodon dactylon* (L.) Pers] in soil that was collected from a site that had been contaminated with hydraulic fracturing fluid and was initially devoid of vegetation. It was hypothesized that adding required plant nutrients through soil amendments in addition to a dilution effect created by mixing soil from a lower depth with highly contaminated topsoil would increase bermudagrass growth in soil contaminated with fracturing fluid.

MATERIALS AND METHODS

Initial Soil Collection and Analyses

Contaminated soil was collected using a shovel during December 2011 from the 0-15 cm (Ap horizon) and 0-30 cm (deep plow) depths from a 3-ha drilling-fluid-contaminated field near a natural gas drilling site located in the Fayetteville Shale in Branch, Ark. Soil collected from each of the two depths was air-dried for 5 days at approximately 22 °C, crushed, and sieved through a 2-mm, stainless steel mesh screen. Representative sub-samples of the soil were analyzed for soil physical and chemical properties (Table 1).

Soil particle-size distribution and textural classification were determined using the 12-hr hydrometer method (Gavlak et al., 2003). Soil electrical conductivity (EC) were determined potentiometrically in a 1:1 and 1:2 soil (m)-to-water (v) suspension (U.S. Salinity Laboratory Staff, 1954; Donohue, 1992). The initial EC values (μmhos/cm) were converted to saturated-paste values (EC_e) using the relationship described by Zhang et al. (2005). The soil pH was determined potentiometrically in a 1:2 soil (m)-to-water (v) suspension (Donohue,

Table 1. Initial soil physical and chemical properites of the two soil depth intervals used in the 9-wk study. Dry-weight means ± standard deviation are reported based on three replications.

	Soil Depth (cm)	
Soil Characertistic	**0-15**	**0-30**
Sand (%)	12 ± 0.9	9 ± 0.5
Silt (%)	59 ± 0.5	64 ± 2.3
Clay (%)	29 ± 0.4	27 ± 1.7
Electrical Conductivity (1:2) (dS/m)	4.23 ± 0.874	3.65 ± 0.125
Saturated Paste Extract (EC_e) (dS/m)	14.5 ± 0.42	14.1 ± 1.32
pH	7.99 ± 0.0170	8.00 ± 0.051
Mehlich-3 Extractable		
P (mg/kg)	6.0 ± 0.59	4.3 ± 0.18
K (mg/kg)	303 ± 4.39	276 ± 5.53
Na (mg/kg)	2994 ± 114.8	2550 ± 40.59
Ca (mg/kg)	2390 ± 72.64	2176 ± 31.59
Mg (mg/kg)	332 ± 6.33	285 ± 3.20
S (mg/kg)	161 ± 6.61	99 ± 2.7
Cu (mg/kg)	7.0 ± 0.03	5.7 ± 0.13
Zn (mg/kg)	10.1 ± 0.087	7.99 ± 0.102
Water-extractable Cl^- (mg/kg)	5603 ± 76.38	5020 ± 264.4
Total N (%)	0.1215 ± 0.0013	0.1081 ± 0.0046
Total C (%)	2.347 ± 0.0370	1.839 ± 0.0300
NO_3-N (mg/kg)	10.6 ± 0.351	6.9 ± 0.20
NH_4-N (mg/kg)	1.7 ± 0.30	2.1 ± 0.23

1992). Mehlich-3 extractable nutrients (i.e., P, K, Na, Ca, Mg, S, Cu, and Zn) were determined from a 1:10 soil (m)-to-extractant-solution (v) ratio (Tucker, 1992) using a SPECTRO ARCOS inductively coupled, argon-plasma (ICP) spectrophotometer (SPECTRO Analytical Instruments, Inc., Mahwah, N.J.). Water-extractable chloride (Cl^-) was determined by axially viewed ICP spectrometry at a wavelength of 134.7 nm, the most sensitive wavelength that is viable for high Cl^- concentrations, in a 1:2 soil (m)-to-water (v) suspension (Wheal and Palmer, 2010). Total soil N and C were analyzed by high-temperature combustion using an Elementar Vario MAX C/N instrument (Elementar Americas, Inc., Mt. Laurel, N.J.) (Bremner, 1996). Inorganic nitrate (NO_3-N) and ammonium (NH_4-N) were determined by steam distillation of a 2 *M* KCl soil extract with the additions of MgO and Devarda's alloy (Mulvaney, 1996).

Organic Soil Amendments and Analyses

Broiler litter and Milorganite® were the two organic soil amendments evaluated in this study. One-year-old broiler litter from a rice (*Oryza sativa* L.)-hull-bedding-cleanout material following five flock cycles from a commercial broiler operation in Lincoln, Ark. was used in this experiment. Milorganite® is a commercially available, activated wastewater sewage sludge that has undergone secondary aerobic microbial degradation and dried in a rotary kiln at 450 to 600 °C for 40 min. and is sold nationally as a lawn fertilizer (Cogger et al., 2011). Both organic soil amendments were characterized for their initial physical and chemical properties (Table 2). Initial water contents were determined gravimetrically. Similar to the soil analyses, EC and pH were determined potentiometrically in a 1:1 and 1:2 soil (m)-to-water (v) suspension, respectively. Total N and C were determined by high-temperature combustion. The NO_3-N and NH_4-N were determined by 2 *M* KCl extraction using a Skalar Continuous Flow Analyzer (Skalar Analytical Instruments, Breda, Netherlands). Total metals (i.e., P, K, Na, Ca, Mg, Cu, and Zn) were determined by ICP spectrometry following USEPA Method 3050B after a 6-h digestion at 95 °C in concentrated nitric acid (HNO_3), 30% hydrogen peroxide (H_2O_2), and concentrated hydrochloric acid (HCl) (USEPA, 1996).

Treatment Preparation

In addition to contaminated soil from two depth intervals, four soil amendments (i.e., inorganic fertilizer, broiler litter, Milorganite®, and an unamended control) were evaluated in this study. Five hundred grams (dry weight) of contaminated soil were placed into a bag and the appropriate amendments were added and thoroughly mixed. Based on the University of Arkansas System Division of Agriculture's Cooperative Extension Service's fertilization recommendation for establishment and maintenance of bermudagrass (Espinoza et al., 2006), a rate of 60 mg plant available N (PAN)/kg soil was selected to be added by the soil amendments to the contaminated soil . The inorganic fertilizer used was ammonium nitrate (34-0-0), which was added at a rate of 176 mg/kg soil (338 kg/ha). To balance the nutrient additions for plant growth in the inorganic fertilizer treatment, P was added at a rate of 131 mg/kg soil (251 kg/ha) as triple super phosphate (0-46-0). Because of the above-optimal, initial soil-test K concentration (Table 2), no K fertilizer was needed to support optimal plant growth, thus no K was added. The poultry litter treatment received 4.16 g poultry litter (dry weight)/kg soil based upon an estimated 30% mineralization rate. Total N mineralization rates for poultry litter have been calculated from previous research studies to range from 16% to 66% (Hadas et al., 1983; Chescheir et al., 1986; Sims, 1986; Gilmour et al., 1987; Bitzer and Sims, 1988; Golden et al., 2006). The Milorganite® treatment received 3.25 g Milorganite® (dry weight)/kg soil based upon an estimated 30% mineralization rate (Gilmour et al., 2000; Cogger et al., 2011).

Once the soil plus amendment mixture was prepared, the mixture was added to 6.4-cm diameter by 25-cm long Conetainers® (Stuewe and Sons Inc., Corvallis, Ore.) that had been sealed at the bottom with a plug to prevent soil and water loss (Kirkpatrick et al., 2006). The soil-amendment mixture was added to the Conetainers® to achieve a bulk density of 1.28 g/cm^3.

Greenhouse Experiment

Prior to plant establishment and to achieve approximate field capacity moisture conditions, 100 mL of distilled water was added to each Conetainer® to achieve a gravimetric soil water content of 20% and an approximate soil water potential of -33 kPa (Brady and Weil, 2002). Two, 7-cm long Bermudagrass sprigs, collected from the same natural gas drilling site where the contaminated soil was collected, were planted per Conetainer® to constitute a vegetation treatment. A no-vegetation treatment was also prepared. The Conetainers® were placed randomly in one of four racks (i.e., blocks), each containing one replication of all treatment combinations (n = 16), in a climate-controlled greenhouse. Each Conetainer® was weighed daily for nine weeks and soil moisture was gravimetrically adjusted to 20% by daily application of distilled water. During the 9-wk greenhouse experiment, the maximum, minimum, and average greenhouse air temperatures were 37, 21, and 28.5 °C, respectively. Ber-

Table 2. Physical and chemical characterization of the two organic amendments used in the 9-wk greenhouse study. Dry-weight means ± standard deviation are reported based on two replications.

	Organic Amendment	
Parameter	**Broiler Litter**	**Milorganite®**
Moisture Content (%)	31.80 ± 0.3182	7.44 ± 0.184
Electrical Conductivity (1:2) (dS/m)	13.98 ± 0.4596	7.01 ± 0.014
pH	8.8 ± 0.0	5.6 ± 0.07
Total N (%)	4.50 ± 0.066	7.10 ± 0.074
Total C (%)	37.30 ± 0.3040	38.63 ± 0.0424
NO_3-N (mg/kg)	304 ± 4.24	36 ± 0.98
NH_4-N (mg/kg)	11803 ± 18.384	1760 ± 5.472
Total Zn (mg/kg)	706 ± 31.8	411 ± 1.31
Total Cu (mg/kg)	289 ± 5.66	236 ± 3.54
Total P (%)	2.53 ± 0.212	2.28 ± 0.021
Total K (%)	4.18 ± 0.127	0.64 ± 0.00
Total Na (%)	0.99 ± 0.04	0.17 ± 0.00
Total Ca (%)	4.08 ± 0.827	2.04 ± 0.007
Total Mg (%)	0.85 ± 0.04	0.54 ± 0.01

mudagrass growth was facilitated on a12-h day length by metal halide, high-intensity discharge lights.

Vegetation Analyses

After nine weeks, bermudagrass shoots were cut at the soil surface, rinsed with distilled water, dried to a constant weight at 55 °C, and were ground to pass a 2-mm, stainless-steel mesh screen using a Wiley Mill Grinder (Thomas Scientific, Swedesboro, N.J.). Bermudagrass shoots were digested in concentrated HNO_3, 30% H_2O_2, and concentrated HCl on a heating block for elemental analysis (Kirkpatrick et al., 2006). Analyses for total P, K, Cu, and Zn were conducted by ICP spectrometry. Total N was determined by high-temperature combustion using an Elementar Rapid N III (Elementar Americas, Inc., Mt. Laurel, N.J.) (Donohue, 1992).

Soil was transferred from the Conetainers® to a tray and the roots were manually collected using forceps. Plant roots were placed on a 500-µm, stainless-steel sieve and rinsed with distilled water to remove any soil adhering to the roots. The rinsed roots were stained with a 10% ethanol solution containing 0.1 g methylene blue/L. Root length, volume, and surface area were determined with Win/Mac RHIZO version 5.0® image analysis system (Regent Instruments, Inc., Quebec City, Canada) (Thompson et al., 2008). Root biomass was determined by drying the roots to a constant weight at 55 °C following the root scanning.

Statistical Analyses

The experiment was designed and analyzed as a randomized complete block design with four blocks arranged in a 4 × 2 factorial treatment structure [i.e., soil amendment (four levels; inorganic fertilizer, broiler litter, Milorganite®, and an unamended control) and soil depth (two levels; 0-15 cm and 0-30 cm)]. Blocks were treated as a random effect and the two experimental factors were treated as fixed effects. Least squares means for significant effects were separated using a protected least significant difference (LSD) procedure at $\alpha = 0.05$. All statistical analyses were conducted using SAS® Version 9.2 (SAS Institute, Inc., Cary, N.C.).

RESULTS AND DISCUSSION

Soil

Initial soil chemical analyses determined that soil from the 0-15-cm depth contained high salinity and Na and Cl^- concentrations compared to the 0-30-cm depth (Table 1). The soil previously contaminated with hydraulic fracturing drilling fluid lacked optimal N and P to support plant growth, but had excessive K. Chemical analyses also verified that the initial soil contained low concentrations of the toxic trace metals Cu and Zn. Soil particle-size analyses determined that both soil depths had a silty-clay-loam soil texture (Table 1).

Vegetation

Of the total of 64 bermudagrass sprigs planted at the beginning of the experiment, 11 died during the greenhouse study. For the unamended, inorganic-fertilizer-, broiler-litter-, and Milorganite®-amended soils, 1, 1, 7, and 2 plants, respectively, did not survive. Replacement bermudagrass sprigs were transplanted to the Conetainers® containing plants that died during the initial 2 wks of the study. Bermudagrass total ($P = 0.0032$) and shoot biomass ($P = 0.0032$) production did not differ among the inorganic fertilizer, broiler litter, and Milorganite® treatments, but were greater in all amended soils than in the unamended control soil during the 9-wk greenhouse study (Table 3). Root biomass was greater ($P = 0.0256$) in the inorganic fertilizer amendment compared to the unamended control and Milorganite® treatments. The root biomass of the broiler-litter-amended soil did not differ compared to the unamended control, inorganic fertilizer, Milorganite® treatments.

Table 3. Influence of four soil amendments on bermudagrass shoot, root, and total biomass following a 9-wk greenhouse study.

Soil Amendment	Root	Shoot	Total
	----------mg/plant----------		
None	46.9 b†	222.5 b	269. 4 b
Inorganic Fertilizer	146.0 a	868. 5 a	1014.5 a
Broiler Litter	107.4 ab	758.9 a	864.9 a
Milorganite®	74.7 b	606.1 a	680.8 a
LSD with broiler litter	63.8	372.7	421.5
LSD without broiler litter	60.7	358.6	405.1

†Means for a given parameter followed by the same letter do not differ ($P > 0.05$).
Notes: n = 32 for None, Inorganic Fertilizer, and Milorganite®; n = 31 for Broiler Litter.

Bermudagrass grown in soil from the 0-30-cm depth resulted in increased root length (P = 0.0057), surface area (P = 0.0103), volume (P = 0.0287), and root biomass (P = 0.0117) compared to bermudagrass grown in the 0-15-cm-depth soil (Table 4). The addition of the broiler litter or inorganic fertilizer resulted in greater (P = 0.0320) bermudagrass root volume compared to the unamended control treatment (Table 5). Roots in the broiler-litter-amended soil had greater (P = 0.0483) root surface area compared to the unamended control. The inorganic-fertilizer- and Milorganite®-amended treatments did not differ from the unamended control.

Bermudagrass shoot Na and Cl concentrations were unaffected by soil depth interval or the addition of soil amendments and averaged 3905 mg/kg and 12596 mg/kg, respectively, across all treatment combinations. Vegetation from the 0-15-cm-depth soil had a greater (P = 0.0167) shoot N concentration (1.46%) than vegetation grown in soil from the 0-30-cm-depth soil (1.21%). Shoot N concentrations were greater (P = 0.0219) in the Milorganite®-amended soil compared to inorganic fertilizer, broiler litter, and unamended control soils, which did not differ (Table 5).

The greatest (P < 0.0001) P concentration in the bermudagrass shoots resulted from the application of broiler litter to the soil, which was greater than that from inorganic fertilizer and Milorganite®, which were both greater than that from the unamended control (Table 5). Bermudagrass shoot P concentration in the broiler-litter-amended soils was two times greater than in the unamended control soil. Shoot K concentration was greater (P = 0.0019) in the Milorganite® and inorganic fertilizer treatments than in the unamended control (Table 5). Broiler-litter-amended treatments did not differ from the unamended control or inorganic fertilizer treatments, but contained lower shoot K concentration compared to the Milorganite® treatments.

Bermudagrass shoot Cu concentrations were greater (P = 0.0084) in the unamended control and Milorganite®-amended soils compared to those in the inorganic-fertilizer-amended soil while the broiler-litter-amended soil did not differ from all treatments (Table 5). Bermudagrass grown in the unamended soil resulted in the greatest (P < 0.0001) concentration of Zn in the bermudagrass shoots (Table 5). Vegetation grown in the 0-15-cm soil depth had greater (P = 0.0431) Cu concentration (9.3

Table 4. Influence of drilling-fluid-contaminated-soil depth on bermudagrass root length, surface area, volume, and biomass following a 9-wk greenhouse study.

Soil Depth	Length	Surface Area	Volume	Biomass
cm	cm/plant	cm^2/plant	cm^3/plant	mg/plant
0-15	471.9 b†	53.1 b	0.490 b	63.6 b
0-30	788.1 a	87.7 a	0.790 a	123.9 a
LSD	263.25	29.93	0.3085	41.71

† Means for a given parameter followed by the same letter do not differ (P > 0.05).
Notes: n = 32.

Table 5. Comparison of four soil amendments on bermudagrass root surface area and volume, bermudagrass shoot N, P, K, Cu, and Zn concentrations following a 9-wk greenhouse study.

	Roots		Shoot				
Soil Amendment	Surface Area	Volume	N	P	K	Cu	Zn
	cm^2/plant	cm^3/plant	%			mg/kg	
None	48.1 b†	0.377 b	1.22 b	0.059 c	1.26 c	9.7 a	105.1 a
Inorganic fertilizer	83.4 ab	0.804 a	1.26 b	0.102 b	1.49 ab	7.8 b	48.2 b
Broiler Litter	94.2 a	0.888 a	1.26 b	0.142 a	1.43 bc	7.9 ab	47.0 b
Milorganite®	56.0 ab	0.490 ab	1.61 a	0.097 b	1.69 a	9.7 a	65.3 b
LSD with broiler litter	40.09	0.4163		0.0215	0.230	1.85	23.76
LSD without broiler litter	38.55	0.4000		0.0204	0.222	1.80	22.83
LSD Inorg. and Milorg.®			0.2705				
LSD Litter and none			0.2945				
LSD Other			0.2828				

† Means for a given parameter followed by the same letter do not differ (P > 0.05).
Notes: Root n = 32 for None, Inorganic Fertilizer, and Milorganite®; n = 31 for Broiler Litter; Shoot N n = 30; Shoot P, K, Cu, and Zn n = 32 for None, Inorganic Fertilizer, and Milorganite®; n = 31 for Broiler Litter.

mg/kg) than plants grown in the 0-30-cm-depth soil (8.3 mg/kg).

Soil Amendments

Since the addition of plant nutrients from synthetic or organic soil amendments resulted in greater shoot biomass, soil amendments would likely increase the vegetative surface coverage to meet the 75% vegetation coverage required by the Arkansas Oil and Gas Commission (AOGC, 2014b). Total bermudagrass biomass was largely determined by shoot biomass and thus followed the same trend. Adams (2011) reported damaging symptoms and acute and chronic mixed hardwood trees, mixed shrub subcanopy, and ground vegetation mortality by the land application of hydraulic fracturing drilling fluid. In her study, the maximum Na and Cl concentrations were 805 and 746 mg/kg, respectively. Land application of drilling fluids to agriculturally productive lands has also shown similar harmful impacts. Nelson et al. (1984) reported ryegrass (*Lolium perenne* L.) and swiss chard (*Beta vularis* L.) yield reductions in drilling-fluid-amended soils resulting from increased concentrations of soluble salts. Miller and Pesaran (1980) and Miller et al. (1980) reported decreased growth in green beans (*Phaseolus vulgaris* L.) and sweet corn (*Zea mays* var. *Saccharata Sturt*.) when studying the effects of land-applying individual drilling fluid components.

Tucker (1985) concluded that the Na and Cl constituents of the hydraulic fracturing drilling fluid most adversely affected plant growth. Bermudagrass ion toxicity and mortality under saline soil conditions occurs due to the accumulation of Na^+ and Cl^- concentrations and subsequent decrease in K^+ concentrations in plant tissue (Chen et al., 2014; Adavi et al., 2006). However, the addition of soil amendments did not result in decreased bermudagrass shoot Na and Cl concentrations and lead to an increase in bermudagrass shoot K concentration in the inorganic-fertilizer and Milorganite®-amended soil. Bermudagrass continued to uptake K from the soil in a process known as "luxury consumption", where the plant accumulates nutrients above levels that are necessary for optimum growth (Burton and Jackson, 1962). Considering the effects of the soil amendment applications, the successful growth of bermudagrass was likely due to the plant's salt tolerance and other benefits of the amendments such as plant-available nutrients. The salt-tolerance range of bermudagrass has been classified as moderate (Marcum, 1999) to very tolerant (Devitt, 1989; U.S. Salinity Laboratory Staff, 1954) due to bermudagrass shoot's highly active salt gland excretion of Na^+ cations and shoot exclusion of excessive Na^+ and Cl^- (Marcum, 2006; Marcum and Pessarakli, 2006), which allows plant growth under saline soil conditions. Increased Na (356%) and Cl (498%) concentrations in bermudagrass shoots over an annual growing period due to increased salinity levels have been reported by Adavi et al. (2006). Other studies have also reported increased Na^+ and Cl^- ions in bermudagrass shoots under saline soil conditions (Ackerson and Youngner 1975; Chen et al., 2009; Chen et al., 2014; Thomas and Langdale, 1980).

The reduced plant survival in the broiler-litter-amended soil could be related to NH_3 toxicity due to the high level of NH_4-N and uric acid in the broiler litter, the initial soil pH of 8.0 (Table 1), and the average greenhouse temperature of 28.5 °C for the 9-wk study. Losada and Arnon (1963) and van der Eerden (1982) reported plant necrosis caused by ammonia toxicity due to the inhibition of photosynthetic phosphorylation that subsequently decreased carbohydrate production and plant growth. Vines and Wedding (1960) reported that ammonia toxicity occurred in alkaline soils because the pH level controlled the forms of ammonia present in the soil, where the undissociated ammonia was the form of ammonia that caused inhibition of plant tissue respiration (Eq. 1). Using composted broiler litter would avoid NH_3 toxicity issues (Kelleher et al., 2002).

$$NH_{3(g)} + H^+ \leftrightarrow NH_{4\,(aq)}^+ \qquad \text{Eq. 1}$$

By applying the amount of broiler litter to provide 60 mg PAN/kg soil, excessive amounts of P were added. Broiler litter has one of the highest P concentrations of all animal manures (Sims and Wolf, 1994). The greatest plant uptake of P, calculated as the shoot biomass × shoot %P, was 1.4 mg/pot and occurred in the broiler-litter and inorganic fertilizer-amended soils (Tables 3 and 5). The greatest plant uptake of N, calculated as the shoot biomass × shoot %N, was 18.2 mg N/pot, which occurred in the inorganic fertilizer-amended soil due to the soluble inorganic N readily available for plant uptake (Tables 3 and 5).

Broiler-litter-amended soils typically contain elevated Cu and Zn concentrations because these trace elements are added to broiler feed as dietary supplements to improve weight gain, prevent diseases, and manage fungi in the broiler feed (Han et al., 2000). Because broiler litter and Milorganite® contain trace elements, caution must be taken to ensure that long-term application of these amendments does not increase trace element concentrations to toxic levels. Pederson et al. (2002) reported that short- and long-term application of poultry litter increased soil Cu and Zn concentrations, especially in the 5-10-cm soil depth interval. However, the addition of soil amendments resulted in similar or lower Cu and Zn concentrations in bermudagrass shoots compared to

the unamended control, which can possibly be attributed to the trace elements not being readily plant available. Increased metal retention in the soil would be expected in highly alkaline soils because trace-element solubility generally decreases with increasing pH (Pepper et al., 2011). Han et al. (2000) reported low micronutrient removal by bermudagrass as a result of plant uptake. This observation agrees with Bates (1988) who reported low bermudagrass uptake of Zn in a reserve-pit-fluid-contaminated silt-loam soil.

Soil Dilution Effect

In addition to inadequate nutrient levels in the contaminated soil (Table 1), excessive EC, Na, and Cl concentrations inhibit vegetation growth. Soil EC and water-extractable Cl^- concentration were greater in the 0-15-cm compared to the 0-30-cm soil depth (Table 1). Since the hydraulic fracturing drilling fluid and flowback water were surface applied following natural gas extraction, mixing of the less contaminated 15-30-cm-depth soil (i.e., deep plow region) with the 0-15-cm-depth surface soil resulted in a dilution effect. Ahmad et al. (2012) and Hseu et al. (2010) recommended soil dilution for remediation of metal-contaminated surface soils.

All root properties increased (biomass, length, volume, and surface area) when bermudagrass was grown in the 0-30-cm-depth compared to the 0-15-cm-depth soil (Table 4). Increased root growth benefits vegetation by increasing drought stress tolerance, nutrient uptake, and vegetative growth (Brady and Weil, 2002). Ackerson and Youngner (1975), Adavi et al. (2006), and Dudeck et al. (1983) reported that increased root growth and decreased shoot growth allowed bermudagrass vegetation to tolerate the osmotic and ionic stresses associated with increasing soil salinity levels.

CONCLUSIONS

Based on the results of this study, the addition of the recommended plant nutrients enhanced bermudagrass growth. In addition, the mixing of the surface-applied hydraulic fracturing fluid with the 0-30-cm soil depth resulted in a dilution effect that decreased detrimental soil salinity concentrations. The results from this study can be used to develop an effective management strategy to establish vegetation in soil previously contaminated with hydraulic fracturing fluid and should include:

1. Plowing the soil to a depth of 30 cm to create a dilution effect caused by the mixing of the less contaminated, deep plow soil region with the 0-15-cm-depth surface soil since the hydraulic fracturing drilling fluid and flowback water were surface applied following natural gas extraction.
2. Applying inorganic fertilizer, broiler litter, or Milorganite® to the contaminated soil to provide adequate plant nutrients. In this study, the soil lacked optimal levels of N and P, but contained excessive levels of K. Organic amendments such as broiler litter and Milorganite® can utilize a waste product to provide similar plant nutrient concentrations as inorganic fertilizer.
3. Sprigging the contaminated soil with bermudagrass, a salt-tolerant plant.
4. Providing adequate moisture for plant growth.

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LITERATURE CITED

Ackerson, R. C., and V. B. Youngner. 1975. Responses of bermudagrass to salinity. Agron. J. 67:678-681.

Adams, M. B. 2011. Land application of hydrofracturing fluids damages a deciduous forest stand in West Virginia. J. Environ. Qual. 40:1340-1344.

Adavi, Z., K. Razmjoo, and M. Mobli. 2006. Salinity tolerance of bermudagrass (*Cynodon* spp. L. C. Rich) cultivars and shoot Na, K and Cl contents under a high saline environment. J. Hortic. Sci. Biotechnol. 81:1074-1078.

Ahmad, M., L.S. Soo, J.E. Yang, H.M. Ro, Y. Han Lee, and Y. Sik Ok. 2012. Effects of soil dilution and amendments (mussel shell, cow bone, and biochar) on Pb availability and phytotoxicity in military shooting range soil. Ecotoxicol. Environ. Saf. 79:225-231.

(ADEQ) Arkansas Department of Environmental Quality. 2009. Regulation no. 8: Administration <http://www.adeq.state.ar.us/regs/files/reg08_final_090228.pdf> Accessed 10 March 2012.

Arkansas Hydraulic Fracturing State Review. 2012. Arkansas hydraulic fracturing state review. <http://www.aogc.state.ar.us/notices/AR_HFR_FINAL.pdf> Accessed 4 April 2014.

(AOGC) Arkansas Oil and Gas Commission. 2014a. B-43–well completions 04-03-14. <http://www.aogc2.state.ar.us/FayettevilleShaleInfo/regularly%20updated %20docs/B-43%20Field%20-%20Well%20Completions.pdf> Accessed 27 March 2014.

(AOGC) Arkansas Oil and Gas Commission. 2014b. General rules and regulation. <http://www.aogc.state.ar.us/onlinedata/forms/rules%20and%20regulations.pdf> Accessed 2 April 2014.

Arthur, J. D., B. Bohm, B.J. Coughlin, and M. Layne. 2008. Hydraulic fracturing considerations for natural gas wells of the Fayetteville Shale. ALL Consulting.

Bates, M. H. 1988. Land farming of reserve pit fluids and sludges-fates of selected contaminants. Wat. Res. 22: 793-797.

Bitzer, C.C. and J. T. Sims. 1988. Estimating the availability of nitrogen in poultry manure through laboratory and field studies. J. Environ. Qual. 17:47-54.

Brady, N.C. and R.R. Weil. 2002. The Nature and Properties of Soils. Prentice Hall, Upper Saddle River, N.J.

Bremner, J.M. 1996. Nitrogen–total. pp. 1085-1121. *In*: D.L. Sparks (ed.). Methods of Soil Analysis Part 3–Chemical Methods. SSSA Book Series no. 5. ASA, CSSA, and SSSA, Madison, Wis.

Burton, G.W. and J.E. Jackson. 1962. Single vs. split potassium applications for coastal bermudagrass. Agron. J. 54:13-14.

Chatterjee, A. and T. Desutter. 2012. Soil salinity can impair the tile drainage system. <https://www.agronomy.org/publications/crops-and-soils/4505> Accessed 3 April 2014.

Chen, J. B., J. Yan, Y.L. Qian, Y.Q. Jiang, T.T. Zhang, H.L. Guo, A.G. Guo, and J.X. Liu. 2009. Growth responses and ion regulation of four warm season turfgrasses to long-term salinity stress. Sci. Hortic. 122:620-625.

Chen, J., Q. Jiang, J. Zong, Y. Chen, X. Chu, and J. Liu. 2014. Variation in the salt-tolerance of 13 genotypes of hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. X C. *Transvaalensis* Burtt-Davy] and its relationship with shoot Na^+, K^+, and Cl^- ion concentrations. Hortic. Sci. Biotechnol. 89:35-40.

Chescheir III, G., P. Westerman, and L. Safley Jr. 1986. Laboratory methods for estimating available nitrogen in manures and sludges. Agric. Wastes. 18:175-195.

Cogger C.G., A.I. Bary, and E.A. Myhre. 2011. Estimating nitrogen availability of heat-dried biosolids. Appl. Environ. Soil Sci. 2011:1-7.

Cunningham, W. P. and M.A. Cunningham. 2008. Principles of Environmental Science: A Global Concern. McGraw-Hill, Boston, Mass.

Devitt, D. 1989. Bermudagrass response to leaching fractions, irrigation salinity, and soil types. Agron. J. 81: 893-901.

Donohue, S.J. 1992. Reference soil and media diagnostic procedures for the southern region on the United States. Southern Coop. Ser. Bull. 374. Virginia Agric. Exp. Stn., Blacksburg, Va.

Dudeck, A., S. Singh, C. Giordano, T. Nell, and D. Mcconnell. 1983. Effects of sodium chloride on *Cynodon* turfgrasses. Agron. J. 75:927-930.

Ellsworth, W.L. 2013. Injection-induced earthquakes. Science. 341:142-149.

Espinoza, L., M. Mozaffari, and N. Slaton. 2006. Soil testing, lime and fertilizer recommendations handbook. Misc. Publ. 463. Univ. of Arkansas Coop. Ext. Serv. Little Rock, Ark.

Gavlak R., D. Horneck, R.O. Miller, and J. Kotuby-Amacher. 2003. Soil, plant and water reference methods for the western region, 2nd edn. (WCC-103 Publication). Ft. Collins: Colorado State University.

Gilmour, J., D.C. Wolf, and P. M. Gale. 1987. Estimating potential ground and surface water pollution from land application of poultry litter. Publ. 128. Arkansas Water Resour. Res. Center, Fayetteville, Ark.

Gilmour, J.T., C.G. Cogger, L.W. Jacobs, S.A. Wilson, G.K. Evanylo, and D.M. Sullivan. 2000. Estimated plant-available nitrogen in biosolids. Project Report 97-REM-3. Water Environ. Res. Foundation. Alexandria, Va.

Golden, B.R., N.A. Slaton, R.J. Norman, E.E. Gbur Jr., K.R. Brye, and R. E. DeLong. 2006. Recovery of nitrogen in fresh and pelletized poultry litter by rice. Soil Sci. Soc. Am. J. 70:1359-1369.

Hadas, A., B. Bar-Yosef, S. Davidov, and M. Sofer. 1983. Effect of pelleting, temperature, and soil type on mineral nitrogen release from poultry and dairy manures. Soil Sci. Soc. Am. J. 47:1129-1133.

Han, F., W. Kingery, H. Selim, and P. Gerard. 2000. Accumulation of heavy metals in a long-term poultry waste-amended soil. Soil Sci. 165:260-268.

Horton, S. 2012. Disposal of hydrofracking waste fluid by injection into subsurface aquifers triggers earthquake swarm in central Arkansas with potential for damaging earthquake. Seismological Research Letters. 83:250-260.

Hseu, Z. Y., S.W. Su, H.Y. Lai, H.Y. Guo, T.C. Chen, and Z. S. Chen. 2010. Remediation techniques and heavy metal uptake by different rice varieties in metal-contaminated soils of Taiwan: New aspects for food safety regulation and sustainable agriculture. Soil Sci. Plant Nutr. 56:31-52.

King, G. E. 2012. Hydraulic fracturing 101: What every representative environmentalist regulator reporter investor university researcher neighbor and engineer should know about estimating frac risk and improving frac performance in unconventional gas and oil wells. SPE Hydraulic Fracturing Technology Conference. Society of Petroleum Engineers.

Kirkpatrick, W. D., P.M. White Jr, D.C. Wolf, G.J. Thoma, and C. M.Reynolds. 2006. Selecting plants and nitrogen rates to vegetate crude-oil–contaminated soil. Inter. J. Phytorem. 8:285-297.

Kelleher, B. P., J.J. Leahy, A.M. Henihan, T.F. O'Dwyer, D. Sutton, and M. J. Leahy. 2002. Advances in poultry litter disposal technology–a review. Bioresour. Technol. 83:27-36.

Kresse, T., N. Warner, P. Hays, A. Down, A. Vengosh, and R. Jackson. 2012. Shallow groundwater quality and geochemistry in the Fayetteville Shale gas-production area, north-central Arkansas, 2011. US Geol. Surv. Scient. Invest. Rep. 5273.

Losada, M. and D. Arnon. 1963. Selective inhibitors of photosynthesis. Metab. Inhib. 2:503-611.

Marcum, K.B. 1999. Salinity tolerance mechanisms of grasses in the subfamily *Chloridoideae*. Crop Sci. 39:1153-1160.

Marcum, K. B. 2006. Use of saline and non-potable water in the turfgrass industry: Constraints and developments. Agric. Water Manage. 80:132-146.

Marcum, K. B. and M. Pessarakli. 2006. Salinity tolerance and salt gland excretion efficiency of bermudagrass turf cultivars. Crop Sci. 46:2571-2574.

Miller, R. W. and P. Pesaran. 1980. Effects of drilling fluids on soils and plants: II. Complete drilling fluid mixtures. J. Environ. Qual. 9:552-556.

Miller, R. W., S. Honarvar, and B. Hunsaker. 1980. Effects of drilling fluids on soils and plants: I. Individual fluid components. J. Environ. Qual. 9:547-552.

Mulvaney, R.L. 1996. Nitrogen–inorganic forms. pp. 1123-1184. *In*: D.L. Sparks (Ed.). Methods of Soil Analysis Part 3–Chemical Methods. SSSA Book Series no. 5. ASA, CSSA, and SSSA, Madison, Wis.

Nelson, D. W., S.L. Liu, and L. E. Sommers. 1984. Extractability and plant uptake of trace-elements from drilling-fluids. J. Environ. Qual. 13:562-566.

Pederson, G. A., G.E. Brink, and T. E. Fairbrother. 2002. Nutrient uptake in plant parts of sixteen forages fertilized with poultry litter. Agron. J. 94:895-904.

Pepper, I. L., C.P. Gerba, and M. L. Brusseau. 2011. Environmental and Pollution Science. Academic Press, Waltham, Mass.

Rahm, D. 2011. Regulating hydraulic fracturing in shale gas plays: The case of Texas. Energy Policy. 39:2974-2981.

Satterfield, J., M. Mantell, D. Kathol, F. Hiebert, K. Patterson, and R. Lee. 2008. Managing water resources challenges in select natural gas shale plays. Chesapeake Energy Corp. Presented at the GWPC Annual Meeting. Cincinnati, Ohio.

Sims, J. 1986. Nitrogen transformations in a poultry manure amended soil: Temperature and moisture effects. J. Environ. Qual. 15:59-63.

Sims, J. and D. Wolf. 1994. Poultry waste management: Agricultural and environmental issues. Adv. Agron. 52:2-72.

Thomas, J. R. and G. W. Langdale. 1980. Ionic balance in coastal bermudagrass influenced by nitrogen fertilization and soil salinity. Agron. J. 449-452.

Thompson, O. A., D.C. Wolf, J.D. Mattice, and G.J. Thoma. 2008. Influence of nitrogen addition and plant root parameters on phytoremediation of pyrene-contaminated soil. Water, Air, and Soil Pollut. 189:37-47.

Tucker, B. 1985.Soil application of drilling wastes. Proceedings of National Conference on Disposal of Drilling Wastes. Environmental and Ground Water Institute. Norman, Okla.

Tucker, M.R. 1992. Determination of phosphorus by Mehlich 3 extraction. Soil and Media Diagnostic Procedures for the Southern Region of the United States. S. J. Donohue (ed.). Vir. Agric. Exp. Stn. Ser. Bull. 374: 6-8.

(USEIA) United States Energy Information Administration. 2013. Annual Energy Outlook 2013. Energy Information Administration United States Department of Energy, Washington, D.C. <http://www.eia.gov/forecasts/aeo> Accessed 1 April 2014.

(USEPA) United States Environmental Protection Agency. 1996. Method 3050b. <http://www.epa.gov/wastes/hazard/testmethods/sw846/pdfs/3050b.pdf> Accessed 5 January 2014.

(USEPA) United States Environmental Protection Agency. 2004. Evaluation of impacts to underground sources of drinking water by hydraulic fracturing of coalbed methane reserves. United States Environmental Protection Agency Office of Water, Washington, D.C. <http://nepis.epa.gov/Adobe/PDF/P100A99N.PDF> Accessed 30 March 2014.

(USEPA) United States Environmental Protection Agency. 2011. Draft plan to study the potential impacts of hydraulic fracturing on drinking water resources. United States Environmental Protection Agency Office of Research and Development, Washington, D.C. <http://fracfocus.org/sites/default/files/publications/hf-studyplandraft_sab_020711.pdf> Accessed 30 March 2014.

United States Salinity Laboratory Staff. 1954. Diagnosis and Improvement of Saline and Alkali Soils. United States Department of Agriculture Handbook 60. U.S. Gov. Printing Office, Washington, D.C.

Van der Eerden, L. 1982. Toxicity of ammonia to plants. Agric. Environ. 7:223-235.

Vidas, H. and B. Hugman. 2008. Availability, economics, and production potential of North American unconventional natural gas supplies. The INGAA Foundation. Fairfax, Va.

Vines, H. M. and R. Wedding. 1960. Some effects of ammonia on plant metabolism and a possible mechanism for ammonia toxicity. Plant Physiol. 35:820-825.

Wang, Q., X. Chen, A.N. Jha, and H. Rogers. 2014. Natural gas from shale formation–the evolution, evidences and challenges of shale gas revolution in United States. Renewable and Sustainable Energy Rev. 30:1-28.

Wheal, M.S. and L. T. Palmer. 2010. Chloride analysis of botanical samples by ICP-OES. J. Anal. At. Spectrom. 25:1946-1952.

Zhang, H., J.L. Schroder, J.J. Pittman, J.J. Wang, and M.E. Payton. 2005. Soil salinity using saturated paste and 1:1 soil to water extracts. Soil Sci. Soc. Am. J. 69:1146-1151.

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The title page should include the following:

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Abstract

The *Abstract* summarizes the purpose, procedures, and main findings in 250 words or less.

Introduction

The *Introduction* states the purpose of the study, the hypothesis, and pertinent background information.

Materials and Methods

The *Materials and Methods* section describes the experimental design, materials used, statistical analysis **(required)**, and any other details needed for another researcher to reproduce the study and to confirm the validity of findings and conclusions.

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The *Results and Discussion* section presents appropriate data, but not all data, in text, tables, and figures and places the findings in context with other research in the field. The discussion emphasizes new and important aspects of the research and conclusions that follow from them. Include implications and impact of the findings. Relate your findings to observations of other studies. State new hypotheses when warranted, but avoid unqualified statements not supported by your data.

Acknowledgments

The *Acknowledgment* section recognizes financial support and other assistance. Note support by any companies or parties with a vested interest in the research results. Please thank your advisor, other professors, co-authors, and other individuals who helped with your research in the *Meet the Student-Author* section *NOT* in Aknowledgments.

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