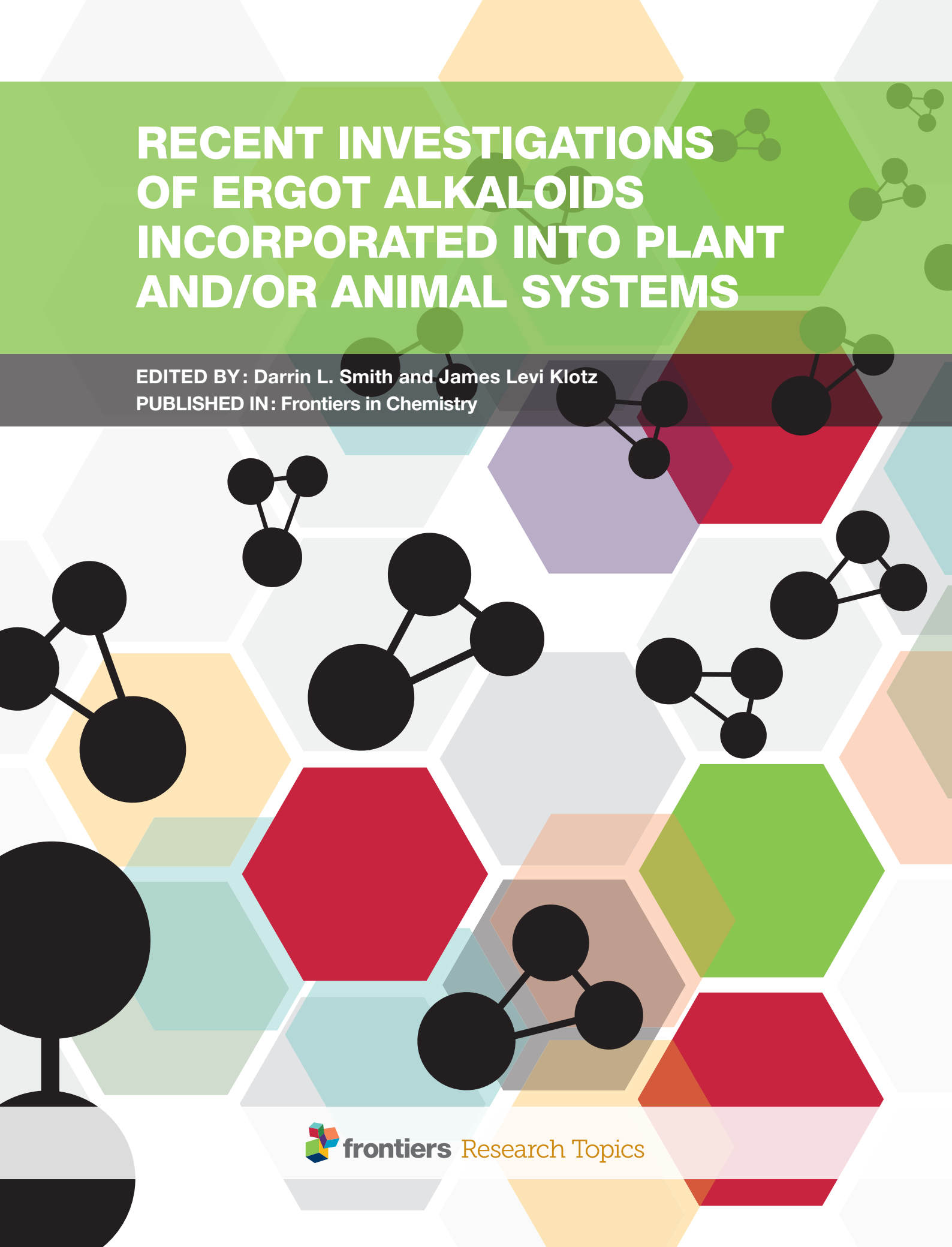
RECENT INVESTIGATIONS OF ERGOT ALKALOIDS INCORPORATED INTO PLANT AND/OR ANIMAL SYSTEMS

EDITED BY: Darrin L. Smith and James Levi Klotz

PUBLISHED IN: Frontiers in Chemistry



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88919-551-0
DOI 10.3389/978-2-88919-551-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

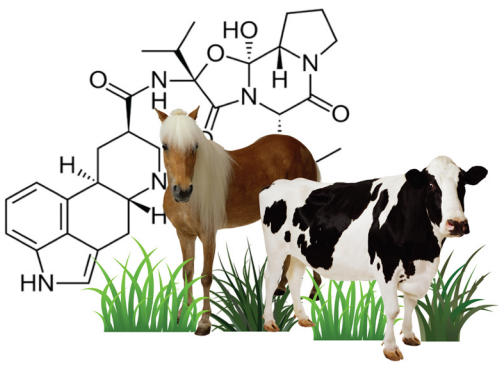
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

RECENT INVESTIGATIONS OF ERGOT ALKALOIDS INCORPORATED INTO PLANT AND/OR ANIMAL SYSTEMS

Topic Editors:
Darrin L. Smith, Eastern Kentucky University, USA
James Levi Klotz, United States Department of Agriculture, USA



Ergot alkaloids produced by symbiotic endophytic fungus located within plants can trigger detrimental physiological responses in forage animals (i.e., cattle, horses, sheep, and goats) when ingested resulting in production losses.

Ergot alkaloids produced by fungi have a basic chemical structure but different chemical moieties at substituent sites result in various forms of alkaloids that are distinguishable from one another. Since the ergoline ring structure found in ergot alkaloids is similar to that of biogenic amines (neurotransmitters), a variety of physiological effects can result after ingestion. Research involving ergot alkaloids is an increasing important global issue as more governments pass laws that limit permissible levels of ergot alkaloids in both foodstuffs and feedstuffs. Regardless of whether these compounds are found directly in foodstuffs or in feed/plants given to forage animals (i.e., cattle, horses, sheep, and goats), introduction of these compounds can complicate the food supply. In addition, toxicosis resulting from alkaloids can be a costly hindrance, with mounting annual production losses associated with forage-animal production systems that impact other agricultural and food based industries. Recent advances for the analysis of these compounds in different matrices as well as the understanding the role these compounds play in distinct biological pathways have begun to help address the issue.

This Research Topic "Recent Investigations of Ergot Alkaloids Incorporated into Plant and/or Animal Systems" has developed a novel platform where different groups share recent data in their investigations with ergot alkaloids. The presented collection of articles emphasizes the complexity of this issue and the multiple approaches necessary to resolve the global ergot alkaloid challenges.

Citation: Smith, D. L., Klotz, J. L., eds. (2015). Recent Investigations of Ergot Alkaloids Incorporated into Plant and/or Animal Systems. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-551-0

Table of Contents

frontiers in Chemistry

EDITORIAL
published: 25 March 2015
doi: 10.3389/fchem.2015.00023

Recent investigations of ergot alkaloids incorporated into plant and/or animal systems

James L. Klotz[1]* *and Darrin L. Smith*[2]

[1] Forage-Animal Production Research Unit, United States Department of Agriculture-Agricultural Research Service, Lexington, KY, USA, [2] Department of Chemistry, Eastern Kentucky University, Richmond, KY, USA

Keywords: ergot alkaloids, livestock, animal systems, plant systems, fungus

Ergot alkaloids have been associated with endophyte-infected grasses (e.g., the *Epichloë*, Bacon et al., 1977 and *Balansia*, Porter et al., 1979 spp.) with examples including tall fescue and fescue toxicosis in the United States (Yates et al., 1985) as well as perennial ryegrass in New Zealand (Rowan and Shaw, 1987) and Ireland (Canty et al., 2014). In addition to animals grazing these grasses being affected by alkaloid toxicities, these regions also provide hay for parts of the world where sufficient feedstuff cannot be grown to support existing livestock. The result has been increased occurrences of ergot alkaloid issues arising in areas not typically associated with pasture-based agriculture. To illustrate, weight loss in camels in the United Arab Emirates consuming an imported ergovaline-containing endophyte-infected perennial ryegrass straw (Alabdouli et al., 2014) along with issues associated with import and feeding of perennial ryegrass straw to Japanese black cattle (Miyazaki et al., 2001) have been documented. In addition to these incidents, grasses can also become infested with *Claviceps purpurea* where the alkaloids, typically ergotamine and ergocristine, are responsible for the resultant ergotism associated with *C. purpurea*. The presence of these toxins can compound livestock issues with the concomitant consumption of ergovaline produced by the endophyte. In terms of livestock production systems, associated ergot alkaloid toxicities are not limited to pasture or feeding pasture products. While *Claviceps Africana* is widespread throughout Africa and Asia, the first reported case of toxicity was in Australian sorghum in 1996 (Ryley et al., 1996). The *C. Africana*-infested sorghum has been demonstrated to be detrimental to steer performance in Australian feedlots that utilize this feedstuff (Blaney et al., 2011) and is an example of how ergot-contaminated feed can distress intensive livestock production.

While ergot alkaloid incidences are rare in humans resulting from increased regulation of grain processing (Flieger et al., 1997; EFSA, 2012), reports are still present from occasional pharmacological overdose or accidental exposure (e.g., Stange et al., 1998). More broad aspects of alkaloid-derived problems still persist in intensive and extensive livestock systems. The impact of ergot alkaloids has a global footprint and a large economic influence on agricultural industries. While difficult to place an exact dollar amount on the global cost from ergot alkaloids, several estimates regarding the cost of ergot alkaloids (as fescue toxicosis) have been projected in the southern United States. Hoveland (1993) estimated over $600 million in annual beef cattle losses from reduced calf births and lower weaning weights. Strickland et al. (2011) expanded this estimate to exceed $1 billion annually with the inclusion of the negative impact to small ruminant and equine industries. The human population is estimated to climb and stabilize at ~9 billion by 2050 (Lutz and Samir, 2010). As prices and global demand for meat and other animal products continue to rise, concentration of livestock production systems will also rise. If unchecked, financial losses and vulnerability of the food supply to toxins (including ergot alkaloids) will also increase proportionally (Bryden, 2012).

If fungi that synthesize ergot alkaloids pre-date the human race, and knowledge of ergot properties has been recorded as far back as 1100 BC (Schiff, 2006), why have associated problems

OPEN ACCESS

Edited and reviewed by:
John D. Wade, Florey Institute of Neuroscience and Mental Health, Australia

***Correspondence:**
James L. Klotz, james.klotz@ars.usda.gov

Specialty section:
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry

Received: *26 February 2015*
Accepted: *09 March 2015*
Published: *25 March 2015*

Citation:
Klotz JL and Smith DL (2015) Recent investigations of ergot alkaloids incorporated into plant and/or animal systems. Front. Chem. 3:23. doi: 10.3389/fchem.2015.00023

with ergot alkaloid consumption not been solved? The primary aspect limiting progress can be attributed to the number of interactions associated with alkaloid production. The plant and fungus (endophytic or parasitic) have an interaction that is still being defined. The plant-alkaloid symbiont interacts with the ambient environment and environmental influences can impact alkaloid production. In addition to plant–fungus–environment interaction variations, the grazing animal will also influence alkaloid production. Consumption of ergot alkaloid-containing feedstuff will interact with the gut microbiome prior to the animal and likely influences the level of exposure to ergot alkaloids by the animal (De Lorme et al., 2007; Ayers et al., 2009). Biological activity of ergot alkaloids absorbed by the animal is defined by the structural similarity of these compounds to biogenic amines (Berde, 1980) allowing ergot alkaloids to interact with serotonergic, adrenergic, and dopaminergic receptors that exist in varying populations throughout the body and results in variable negative effects. In addition, limited progress can be attributed to the availability of standard reference materials or validated methods/tools to accurately extract and measure ergot alkaloids from biologic matrices. In the case of ergovaline, analytical standards for this compound are not readily available; therefore, this compound must be custom synthesized. If pure standards are not available, then actual quantities cannot be obtained and only relative responses between data sets can be generated. If pure standards can be acquired, then validation of extraction and analytical methods (using specific equipment and/or chemical instrumentation) for ergot alkaloids found in different biological matrices must be performed to ensure results are reliable and reproducible while any potential matrix effects are minimized (Smith et al., 2009).

A multi-disciplinary approach will be needed to solve most ergot alkaloid related issues. This research topic, Recent Investigations of Ergot Alkaloids Incorporated into Plant and/or Animal Systems, epitomizes that reality through diverse scientific approaches addressing the core issue of ergot alkaloids in agriculture. Innovative research articles highlight the numerous effects that ergot alkaloids can have on livestock (Aiken and Flythe, 2014; Duckett et al., 2014; Egert et al., 2014; Eisemann et al., 2014), improved characterizations of fungal endophytes (Young et al., 2014), clarification of the alkaloid variation within the plant (Mace et al., 2014), and how fungal infestations and subsequent alkaloid concentrations interact with the environment (McCulley et al., 2014). Furthermore, challenges such as alkaloid stability in collected samples (Lea et al., 2014), the generation of a large alkaloid source in the absence of a consistent supply for animal studies (Ji et al., 2014), a perspective on interpreting alkaloid concentrations and level of animal response (Craig et al., 2015), and rapid screening of livestock are addressed (Rosenkrans and Ezell, 2015). This collection of articles highlights both the complexity of the problem and the diverse approaches necessary to address these issues with the hope that future interest will be cultivated to solve global ergot alkaloid challenges.

Acknowledgments

The editors would like to thank all of the authors and reviewers for their dedication and time that generated the valuable contributions found in this Frontiers Research Topic.

References

Aiken, G. E., and Flythe, M. D. (2014). Vasoconstrictive responses by the carotid and auricular arteries in goats to ergot alkaloid exposure. *Front. Chem*. 2:101. doi: 10.3389/fchem.2014.00101

Alabdouli, K. O., Blythe, L. L., Duringer, J. M., Elkhouly, A., Kassab, A., Askar, M., et al. (2014). Physiological effects of endophyte-infected perennial ryegrass straw on female camels in the Middle East. *Emir. J. Food Agric.* 26, 82–92. doi: 10.9755/ejfa.v26i1.16473

Ayers, A. W., Hill, N. S., Rottinghaus, G. E., Stuedemann, J. A., Thompson, F. N., Purinton, P. T., et al. (2009). Ruminal metabolism and transport of tall fescue ergot alkaloids. *Crop Sci.* 49, 2309–2316. doi: 10.2135/cropsci2009.01.0018

Bacon, C. W., Porter, J. K., Robbins, J. D., and Luttrell, E. S. (1977). *Epichloë typhina* from toxic tall fescue grasses. *Appl. Environ. Microbiol.* 35, 576–581.

Berde, B. (1980). "Ergot compounds: a synopsis," in *Ergot Compounds and Brain Function: Neuroendocrine and Neuropsychiatric Aspects*, eds M. Goldstein, A. Lieberman, D. B. Calne, and M. O. Thorner (New York, NY: Raven Press), 4–23.

Blaney, B. J., McLennan, S. R., Kidd, J. F., Connell, J. A., McKenzie, R. A., and Downing, J. A. (2011). Effect of sorghum ergot (*Claviceps africana*) on the performance of steers (*Bos taurus*) in a feedlot. *Anim. Prod. Sci.* 51, 156–166. doi: 10.1071/AN10086

Bryden, W. L. (2012). Food and feed, mycotoxins and the perpetual pentagram in a changing animal production environment. *Anim. Prod. Sci.* 52, 383–397. doi: 10.1071/AN12073

Canty, M. J., Fogarty, U., Sheridan, M. K., Ensley, S. M., Schrunk, D. E., and More, S. J. (2014). Ergot alkaloid intoxication in perennial ryegrass (*Lolium perenne*): an emerging animal health concern in Ireland? *Ir. Vet. J.* 67:21. doi: 10.1186/2046-0481-67-21

Craig, A. M., Klotz, J. L., and Duringer, J. M. (2015). Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed. *Front. Chem*. 3:8. doi: 10.3389/fchem.2015.00008

De Lorme, M. J., Lodge-Ivey, S. L., and Craig, A. M. (2007). Physiological and digestive effects of *Neotyphodium coenophialum*-infected tall fescue fed to lambs. *J. Anim. Sci.* 85, 1199–1206. doi: 10.2527/jas.2005-430

Duckett, S. K., Andrae, J. G., and Pratt, S. L. (2014). Exposure to ergot alkaloids during gestation reduces fetal growth in sheep. *Front. Chem*. 2:68. doi: 10.3389/fchem.2014.00068

Egert, A. M., Klotz, J. L., McLeod, K. R., and Harmon, D. L. (2014). Development of a methodology to measure the effect of ergot alkaloids on forestomach motility using real-time wireless telemetry. *Front. Chem*. 2:90. doi: 10.3389/fchem.2014.00090

Eisemann, J. H., Huntington, G. B., Williamson, M., Hanna, M., and Poore, M. (2014). Physiological responses to known intake of ergot alkaloids by steers at environmental temperatures within or greater than their thermoneutral zone. *Front. Chem*. 2:96. doi: 10.3389/fchem.2014.00096

European Food Safety Authority. (2012). Scientific opinion on ergot alkaloids in food and feed. *EFSA J.* 10:2798. doi:10.2903/j.efsa.2012.2798

Flieger, M., Wurst, M., and Shelby, R. (1997). Ergot alkaloids—sources, structures, and analytical methods. *Folia Microbiol.* 42, 3–29. doi: 10.1007/BF02898641

Hoveland, C. S. (1993). Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. *Agric. Ecosyst. Environ.* 44, 3–12. doi: 10.1016/0167-8809(93)90036-O

Ji, H., Fannin, F., Klotz, J., and Bush, L. (2014). Tall fescue seed extraction and partial purification of ergot alkaloids. *Front. Chem*. 2:110. doi: 10.3389/fchem.2014.00110

Lea, K., Smith, L., Gaskill, C., Coleman, R., and Smith, S. R. (2014). Ergovaline stability in tall fescue based on sample handling and storage methods. *Front. Chem*. 2:76. doi: 10.3389/fchem.2014.00076

Lutz, W., and Samir, K. C. (2010). Dimensions of global population projections: what do we know about future population trends and structures. *Philos. Trans. R. Soc. B.* 365, 2779–2791. doi: 10.1098/rstb.2010.0133

Mace, W. J., Lunn, K. L., Kaur, N., and Lloyd-West, C. M. (2014). Variation in the expression of ergot alkaloids between individual tillers of perennial ryegrass. *Front. Chem*. 2:107. doi: 10.3389/fchem.2014.00107

McCulley, R. L., Bush, L. P., Carlisle, A. E., Ji, H., and Nelson, J. A. (2014). Warming reduces tall fescue abundance but stimulates toxic alkaloid concentrations in transition zone pastures of the U.S. *Front. Chem*. 2:88. doi: 10.3389/fchem.2014.00088

Miyazaki, S., Fukumura, M., Yoshioka, M., and Yamanaka, N. (2001). Detection of endophyte toxins in the imported perennial ryegrass straw. *J. Vet. Med. Sci.* 63, 1013–1015. doi: 10.1292/jvms.63.1013

Porter, J. K., Bacon, C. W., and Robbins, J. D. (1979). Lysergic acid amide derivatives from *Balansia epichloë* and *Balansia Claviceps* (Clavicipitaceae). *J. Nat. Prod.* (Lloydia*)* 42:309. doi: 10.1021/np50003a015

Rosenkrans, C. F., and Ezell, N. S. (2015). Relationships among ergot alkaloids, cytochrome P450 activity, and beef steer growth. *Front. Chem*. 3:16. doi: 10.3389/fchem.2015.00016

Rowan, D. D., and Shaw, G. J. (1987). Detection of ergopeptine alkaloids in endophyte-infected perennial ryegrass by tandem mass spectrometry. *N.Z. Vet. J.* 35, 197–198. doi: 10.1080/00480169./1987.35448

Ryley, M. J., Alcorn, J. L., Kochman, J. K., Kong, G. A., and Thompson, S. M. (1996). Ergot on *Sorghum* spp. in Australia. *Australas. Plant Pathol.* 25, 214. doi: 10.1071/AP96038

Schiff, P. L. (2006). Ergot and its alkaloids. *Am J. Pharm. Educ*. 70:98. doi: 10.5688/aj700598

Smith, D. L., Smith, L. L., Shafer, W. D., Klotz, J. L., and Strickland, J. R. (2009). Development and validation of a LC/MS method for quantitation of ergot alkaloids in lateral saphenous vein tissue. *J. Agric. Food Chem*. 57, 7213–7220. doi: 10.1021/jf901086q

Stange, K., Pohlmeier, H., Lübbesmeyer, A., Gumbinger, G., Schmitz, W., and Baumgart, P. (1998). Ergotamine-induced vascular spasms through chronic inhalation of ergotamine during the preparation of rye flour. *Dtsch. Med. Wochenschr.* 123, 1547–1550. doi: 10.1055/s-2007-1024221

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). St. Anthony's Fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci.* 889, 1603–1626. doi: 10.2527/jas.2010-3478

Yates, S. G., Plattner, R. D., and Garner, G. B. (1985). Detection of ergopeptine alkaloids in endophyte infected, toxic KY-31 tall fescue by mass spectrometry/mass spectrometry. *J. Agric. Food Chem*. 33, 719–722. doi: 10.1021/jf00064a038

Young, C. A., Charlton, N. D., Takach, J. E., Swoboda, G. A., Trammell, M. A., Huhman, D. V., et al. (2014). Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal? *Front. Chem*. 2:95. doi: 10.3389/fchem.2014.00095

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 21 October 2014
doi: 10.3389/fchem.2014.00088

Warming reduces tall fescue abundance but stimulates toxic alkaloid concentrations in transition zone pastures of the U.S.

Rebecca L. McCulley*, Lowell P. Bush, Anna E. Carlisle, Huihua Ji and Jim A. Nelson

Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA

Edited by:
James Levi Klotz, United States Department of Agriculture, USA

Reviewed by:
Chuck West, Texas Tech University, USA
Jonathan A. Newman, University of Guelph, Canada

****Correspondence:***
Rebecca L. McCulley, Department of Plant and Soil Sciences, University of Kentucky, N-222D Ag. Science North, Lexington, KY 40546-0091, USA
e-mail: rebecca.mcculley@uky.edu

Tall fescue pastures cover extensive acreage in the eastern half of the United States and contribute to important ecosystem services, including the provisioning of forage for grazing livestock. Yet little is known concerning how these pastures will respond to climate change. Tall fescue's ability to persist and provide forage under a warmer and wetter environment, as is predicted for much of this region as a result of climate change, will likely depend on a symbiotic relationship the plant can form with the fungal endophyte, *Epichloë coenophiala*. While this symbiosis can confer environmental stress tolerance to the plant, the endophyte also produces alkaloids toxic to insects (e.g., lolines) and mammals (ergots; which can cause "fescue toxicosis" in grazing animals). The negative animal health and economic consequences of fescue toxicosis make understanding the response of the tall fescue symbiosis to climate change critical for the region. We experimentally increased temperature (+3°C) and growing season precipitation (+30% of the long-term mean) from 2009–2013 in a mixed species pasture, that included a tall fescue population that was 40% endophyte-infected. Warming reduced the relative abundance of tall fescue within the plant community, and additional precipitation did not ameliorate this effect. Warming did not alter the incidence of endophyte infection within the tall fescue population; however, warming significantly increased concentrations of ergot alkaloids (by 30–40%) in fall-harvested endophyte-infected individuals. Warming alone did not affect loline alkaloid concentrations, but when combined with additional precipitation, levels increased in fall-harvested material. Although future warming may reduce the dominance of tall fescue in eastern U.S. pastures and have limited effect on the incidence of endophyte infection, persisting endophyte-infected tall fescue will have higher concentrations of toxic alkaloids which may exacerbate fescue toxicosis.

Keywords: climate change, *Epichloë coenophiala*, ergot alkaloids, loline alkaloids, *Lolium arundinaceum*, pasture sustainability

INTRODUCTION

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh, a.k.a. *Festuca arundinacea* (Schreb.), and *Schedonorus arundinaceus* (Schreb.) Dumort.] is a C_3 physiology, cool-season perennial grass that was introduced to North America from Eurasia in the late 1800s, but today covers more than 14 million hectares, primarily in the eastern half of the United States (Ball et al., 1993; Hoveland, 2009; Young et al., 2013). In this region, tall fescue is widely utilized as a forage in pasture-based livestock systems, in part due to its ease of establishment, environmental hardiness, and ability to persist and produce forage under a range of management regimes (Roberts et al., 2009). Many of these attributes are linked to a symbiotic relationship the plant can form with an asymptomatic, asexual fungal endophyte—*Epichloë coenophiala* (a.k.a., *Neotyphodium coenophialum*; Clay, 1990; Schardl et al., 2004; Leuchtmann et al., 2014).

Similar grass—fungal endophyte relationships are common (Arnold et al., 2000), occurring on every continent except Antarctica and within 20–30% of grass species (White, 1987; Leuchtmann, 1992). The nature of these relationships varies across the symbiotic continuum (Saikkonen et al., 1998), depending on plant and fungal genetics and biotic and abiotic environmental parameters (Cheplick and Faeth, 2009). While the relationship between tall fescue and *E. coenophiala* is generally considered mutualistic (Clay, 1990), prior work suggests that the benefits of fungal endophyte infection to the plant might be most pronounced under conditions of high herbivory (Bouton et al., 1993; Clay et al., 2005), high or low nutrient availability (Malinowski and Belesky, 2000; Rahman and Saiga, 2005), and/or at times of extreme heat or water limitation, such as commonly occurs during summer droughts (Arachevaleta et al., 1989; Elbersen and West, 1996; Marks and Clay, 1996; Assuero et al., 2006).

Tall fescue is thought to provide food, shelter, and a means of reproduction for the fungus, as *E. coenophiala* can only infect new tall fescue individuals through vertical, mother-daughter

transmission (Clay and Schardl, 2002; Schardl et al., 2004). While in return, the fungus produces a suite of alkaloids, including peramines, lolines, and ergots—some of which are known to deter herbivory (Bush et al., 1997). Because these fungal-produced alkaloids are toxic to some herbivores, their presence can alter herbivory [e.g., cause increased consumption of surrounding endophyte-free (E−) individuals or other plant species] such that a significant competitive advantage is conferred to endophyte-infected (E+) tall fescue (Clay and Holah, 1999; Clay et al., 2005). Unfortunately, grazing livestock are susceptible to these alkaloids, and significant negative animal health issues arise when animals are forced to graze infected tall fescue material, particularly during times of temperature stress (i.e., fescue toxicosis in the southeastern U.S.; Stuedemann and Hoveland, 1988; Hoveland, 1993; Strickland et al., 2011).

However, the tall fescue—*E. coenophiala* symbiosis is not just a defensive mutualism (Clay and Schardl, 2002). While studies have shown that in situations where herbivory levels are high, the percentage of fungal endophyte infected individuals within a tall fescue community can increase over time (Clay et al., 2005), fungal endophyte infection frequencies (EIF) have also been shown to vary in relation to environmental stress (Lewis et al., 1997). Higher EIF are frequently encountered in harsher environments (West et al., 1993), and for tall fescue, survival, and recovery following exposure to high temperatures and water limitation are often enhanced by endophyte infection (Elmi and West, 1995; Elbersen and West, 1996; Marks and Clay, 1996; Belesky and West, 2009). Such results suggest that endophyte infection may improve tall fescue's ability to resist and adapt to environmental perturbations that are likely to result from climate change—a supposition made for many symbiotic associations (Compant et al., 2010; Redman et al., 2011; Kivlin et al., 2013).

Only a few studies to date have experimentally evaluated this possibility for tall fescue and results are mixed. Endophyte infection sometimes alters tall fescue response to elevated atmospheric CO_2 (Newman et al., 2003) but not always (Chen et al., 2007). Effects of endophyte infection on plant recovery and survival following drought and temperature stress, while somewhat variable, tend to be positive for tall fescue (Arachevaleta et al., 1989; Marks and Clay, 1996; Worchel et al., 2013). Elevated CO_2 has been shown to reduce, while drought and increased temperature stimulate, alkaloid concentrations in E+ material (Agee and Hill, 1994; Brosi et al., 2011b; Ryan et al., 2014). Long-term field manipulations of multiple climate change factors are rare, and yet they are essential to understanding how complex systems will respond (Backlund et al., 2008). In one such study, Brosi et al. (2011b) found that elevated CO_2 led to a higher EIF within the tall fescue community of a mixed species, unmanaged old field after 5 years of experimental manipulation, but surprisingly, measured no change in EIF under drought or elevated temperature treatments or their interactions. Unfortunately, this study could not capture changes in tall fescue—endophyte dynamics over time and was not representative of pasture conditions (species- or management-wise); therefore, implications from it for tall fescue pastures are limited.

Given that tall fescue is a dominant component of pastures across extensive acreage in the eastern U.S., and serves as the forage base for a multi-million dollar animal industry, which suffers substantially from the negative effects of fungal-produced alkaloids, it is important to understand how potential changes in climate will impact the fescue—endophyte symbiosis. This project evaluated the consequences of warming (+3°C, day and night, year-round) and increased growing season precipitation (+30% of the long-term mean), conditions that are representative of current climate projections for this region (Brunjes, 2004; Karl et al., 2009), on tall fescue abundance, EIF, and alkaloid concentrations over a 5 years period in a hay-managed, mixed species pasture in central Kentucky. Because tall fescue is a cool season grass and the warming treatment was expected to increase the intensity and duration of seasonal dry periods normally experienced at the site, we anticipated that warming would decrease tall fescue abundance, especially in mid-summer and early fall, and that this environmental stress would select for E+ individuals and thereby increase the level of endophyte infection within the tall fescue community over time. We also hypothesized that warming would stimulate alkaloid concentrations in E+ individuals and that the addition of precipitation would ameliorate some of these effects of warming on the plant and its symbiotic partner.

MATERIALS AND METHODS

SITE DESCRIPTION

The climate change project was established in an existing pasture, on relatively deep (>1 m) Maury silt loam soils, located at the University of Kentucky's Spindletop Farm, Lexington, KY, USA (38.1081°N; 84.4916°W). The site is situated in a transition zone between subtropical and continental climates, experiencing long-term mean annual summer and winter temperatures of 23.8 and 1.6°C, respectively (Ferreira et al., 2010), with rainfall relatively evenly distributed throughout the year, averaging 1137 mm a year. Average annual temperatures (°C) and total annual rainfall (mm) were as follows, respectively, over the 5 years project: 2009—12.7, 1323; 2010—13.2, 1048; 2011—13.4, 1656; 2012—14.4, 1019; and 2013—12.8, 1495 (Kentucky Mesonet—Lexington).

In March and April 2008, the pasture was sprayed with glyphosate, plowed, and disked to remove existing vegetation and to prepare a seedbed. A seed mixture consisting of cool season, C_3 physiology forage species common to the area were planted on April 8, 2008 and consisted of: Kentucky bluegrass (*Poa pratensis*, cultivar "Ginger"), tall fescue (cultivar "Kentucky-31"), red clover (*Trifolium pretense*, cultivar "Freedom"), and white clover (*Trifolium repens*, cultivar "Patriot"). For tall fescue, 50% of the seed was infected with the common toxic strain of *E. coenophialum* and 50% was not infected (i.e., was *Epichloë*-free). On August 22, 2008, the warm-season, C_4 physiology forage grass, Bermuda (*Cynodon dactylon*, cultivar "Wrangler"), was plugged throughout the establishing stand. Plugs originated from an existing adjacent field. For more details on site characteristics and stand establishment see Brosi (2011a), Slaughter (2012), and Bourguignon (2013).

EXPERIMENTAL DESIGN

Within the newly established stand (~1500 m^2), five replicate blocks, similar in vegetative composition and large enough to contain four, 3 m diameter (5.8 m^2) plots, were

identified. Plots within a block were randomly assigned to one of four climate treatments: +heat (+3°C above ambient temperature); +precip (+30% long-term normal precipitation; +343 mm); +heat+precip (the combination of the first two treatments); and an untreated, ambient control. The +heat treatment was achieved by following the approach of Kimball et al. (2008). Salamander infrared ceramic heaters (Mor Electric Heating Assoc., Comstock Park, MI) were positioned around each plot, and heat was applied as needed to maintain the desired +3°C difference between the +heat plot and its paired ambient temperature comparison (control plots for +heat treatment and +precip plots for the +heat+precip treatment). To control for potential shade effects from the heaters, all plots were surrounded by the housing units used to mount them. For the +precip treatment, rainfall collected on-site was applied using a metered wand, twice a month, on rainy days, during the growing season only (April to September). Quantities of additional precipitation applied each month were based on long-term rainfall trends for the site and were constant across years of the experiment (April +50.8 mm; May +50.8 mm; June +61.7 mm; July +67.0 mm; August +61.7 mm; September +50.8 mm). Aluminum sheeting was inserted into the soil to 50 cm depth around individual plots to minimize movement of soil moisture into or out of the plots. Climate treatments began May 1, 2009 and ran continuously until November 12, 2013.

MEASUREMENTS

Effects of the climate treatments on air temperature and soil moisture were measured continuously throughout the experiment. Air temperature was measured every 15 s at 30 cm above the ground surface with shielded Type T thermocouples (FW3648, TE Wire, Saddle Brook, NJ). Volumetric water content of the top 15 cm of soil was measured every 15 min using time domain reflectrometers (CS-616, Campbell Scientific, Logan, UT). Daily, monthly, and seasonal averages of these data for each treatment were computed.

The experiment was managed as a hay field, meaning that all vegetation was mowed to a height of 7.6 cm, and cut material was removed off site. For all years of the project, mowing occurred three times per growing season. Climate treatment effects on total aboveground plant biomass production (data not shown) and the biomass and relative abundance of tall fescue were obtained from seasonal sub-plot harvests that immediately preceded whole-plot mowing events. All vegetation located within two, 0.25 m^2 permanent sub-plots per plot was cut to 7.6 cm height, removed, and sorted by hand to species. This material was then oven-dried (55°C for 3–4 days) and weighed. The relative abundance of tall fescue was calculated on a biomass basis: tall fescue biomass/total biomass per sub-plot. Sub-plot values were averaged for a plot value for subsequent statistical analyses. Harvests occurred on the following dates: 2009 (June 1, July 21, Sept. 22); 2010 (May 17, July 26, Oct. 5); 2011 (May 23, July 25, Oct. 5); 2012 (May 21, July 30, Oct. 8); and 2013 (May 21, Aug. 5, Oct. 14).

In order to determine whether the climate treatments were affecting the occurrence of E+ and E− individuals within the tall fescue population, ∼40 tall fescue tillers per plot were harvested by hand, at 7.6 cm above ground level, with a razor blade, immediately preceding all spring and fall harvests. These tillers were kept cold in a freezer (−4.0°C) until they could be processed for endophyte presence (usually within 1–2 weeks). We utilized an enzyme-linked, endophyte-specific immunosorbent assay to determine whether individual tillers were E+ or E− (Hiatt et al., 1999). Each tiller was individually labeled and double-blotted onto nitrocellulose paper that was then assayed for the presence of the endophyte-specific immunoprotein. If the endophyte protein was present, a color reaction would occur, and tiller blots would turn pink. They remained colorless if no endophyte protein was detected. Plot-level tall fescue EIF was calculated as the number of tillers testing positive for the presence of the endophyte divided by the total number of tillers tested per plot. One person scored all the blots as either positive or negative for the entire project, and positive and negative controls were run with each batch. Because, like others (Ju et al., 2006), we detected regular seasonal patterns in EIF (spring values usually lower than fall values, across all treatments; data not shown), we present only the initial June 2009 and all following fall EIF data. Our treatment-related EIF results do not change if all data are included in the statistical models. Fescue tillers were lyophilized once the blots were scored.

Because only the fungal endophyte can produce the insect and mammal toxic alkaloids we were interested in measuring (Bush et al., 1997), the immunoblot results were used to separate the E+ tillers from the E− before alkaloid analysis. Only E+ tillers were composited per plot, ground to pass through a 1 mm screen using a Cyclotec 1093 mill, and analyzed for loline and ergot alkaloid concentrations. Due to lab error, we did not measure alkaloids on the first fescue tiller harvest (June 2009), but they were measured on all E+ tillers collected thereafter. Gas chromatography (GC) was used to identify and quantify three loline compounds (*N*-formyl loline, *N*-acetyl loline, and *N*-acetyl norloline), according to the protocol of Blankenship et al. (2001). A 0.3 g sub-sample of ground material was extracted in sodium bicarbonate and methylene chloride containing quinoline (15 $\mu g\ mL^{-1}$; an internal standard) via shaking for an hour. Extracts were filtered and analyzed on a GC (Perkin Elmer Clarus 500) equipped with a flame ionized detector and using an SPB-1 fused silica capillary column (15 m × 0.53 mm, 0.5 μm film thickness; Supelco). The GC temperature program increased from 80 to 160°C at 20°C min^{-1}, was held for 2 min, was increased to 290°C at 45°C min^{-1}, and held for 5 min. Injector and detector temperatures were set at 250 and 275°C, respectively.

High performance liquid chromatography (HPLC) with florescence detection was employed to quantify ergovaline and ergovalinine concentrations, as developed by Yates and Powell (1988). A 0.1 g ground sample was extracted in 80% methanol via shaking for 2 h. The extractant was then forced, by syringe, through a PreSep column (SPE, C18 disposable) fitted with a 0.2 μm polytetrafluoroethylene filter. The third mL aliquot of extractant was isolated and eluted with the following solutions: (A) 0.1 M ammonium acetate: acetonitrile, 97:3 v/v and (B) 100% acetonitrile. A reverse phase Kinetex XB-C18 column (100 × 4.6 mm with 2.6 μm particle size) was used to separate compounds at a flow of 1.2 mL min^{-1} with the following gradient conditions: initial 22% mobile phase B increased linearly to 35% over 20 min and then further linearly to 58% B in 8 min before being increased

to 100% B and held for 5 min. After this period, re-equilibration was achieved by reducing to 22% B for 9 min. Ergovaline and ergovalinine were identified by excitation at 310 and emission measurement at 420 nm and had retention times of 14.1 and 24.4 min, respectively. Endophyte-free samples were included as checks periodically and consistently had non-detectable levels of all the compounds of interest. Independently verified E+ material, not associated with this project, was run during all analyses as a lab standard.

STATISTICAL ANALYSIS

The randomized complete block design of the project included five replicates of four climate change treatments that were implemented in a 2 × 2 factorial design (+heat × +precip), with measurements taken over a 5-years period of time. Therefore, we statistically analyzed majority of our data with a repeated measures analysis of variance mixed linear model using the restricted maximum likelihood method within Proc Mixed procedures of SAS version 9.3 (SAS Institute Inc., Cary, NC). Main, fixed effects were designated as heat, precip, time, and all interactions therein. Block was the random effect, and the repeated subject was block by climate treatment. All parameters were tested for normality and homogeneity of variance and adjusted, if necessary, as follows.

The time variable, and therefore the repeated measure, differed across parameters. For air temperature and soil moisture, average seasonal values were calculated for each year (Spring—March, April, May; Summer—June, July, August; Fall—September, October, November; Winter—December, January, February). Because we knew years varied significantly in these parameters, we analyzed this seasonal data by year (2009–2013) using a multiple analysis of variance model (Proc GLM; SAS v.9.3), with block, heat, and precipitation as main effects and season as the repeated variable. For tall fescue relative abundance, harvest period (Spring, Summer, and Fall) and year were included as the time variables in the models. For alkaloid concentrations, material sampled was from two seasons (Spring and Fall) over the 5 years, so both season and year were included in the models.

To determine whether changes in EIF occurred over time in the climate treatments, we used time as a continuous measure (months since experiment began), and calculated the difference in EIF between the month of measure and the initial infection frequency on a per plot basis, which was then analyzed with the same model as the other parameters. To assess whether differences in EIF existed at the start of the experiment, the June 2009 EIF data were angular transformed, to meet assumptions of the model, and run with heat, precip, and their interaction as main effects, block as the random effect, and no repeated statement. There were no significant differences in EIF across plots at the initial measurement period (all $p > 0.4$), with the site averaging 41.1% (±3.3) of fescue tillers infected.

For significant main effects and interactions ($p < 0.05$), mean comparisons were performed with lsmeans using either the pdiff or the Dunnett multiple comparison adjustment (for EIF data only). Because we did not measure alkaloids the first spring (2009), we ran means comparison tests on these data with year 2009 excluded. For air temperature and soil moisture data, Type I sums of squares were used to generate p-values for the climate treatment effects (heat, precip, and their interaction), and for season and its interactions with the climate treatments, Hotelling-Lawley Trace method was used to generate the *p*-values.

RESULTS

AIR TEMPERATURE AND SOIL MOISTURE

As expected, given the intention of the climate manipulations, air temperatures were increased by 3.04 ± 0.17°C in +heat plots vs. the ambient temperature treatments, averaged (±SE) across the entire 5-years project (**Figure 1**). Increases in air temperature due to warming were greater during the summer (3.87 ± 0.22°C) than the winter (1.63 ± 0.13°C), explaining the significant season × heat effect in all years of the project (**Table 1**), but the magnitude of this seasonal effect varied across years (**Figure 1**).

Warming reduced soil moisture by 13.9% averaged over the duration of the project, but this effect was strongest during the summer period, especially in the drier years—2010 and 2012 (**Figure 1**, **Table 1**). The +precip treatment mediated, to some degree, the effects of warming during the summer dry-down period: +heat+precip plots averaged 24.3% more soil moisture during the summer than +heat plots. However, +heat+precip plots rarely had greater soil moisture then ambient control plots (**Figure 1**). Plots receiving only the +precip treatment were the wettest, especially during the summer, when the additional water was being applied (averaging 15.2–50.7% more soil moisture than ambient controls or +heat treatments in all summers of the project). Soils were generally wettest in the winter and driest during the summer, though considerable year-to-year variability in soil moisture was encountered [average annual (or less than annual in some years) soil moisture across treatments ± SE: 31.1 ± 0.12%—2009 (June–Dec.); 24.8 ± 0.14%—2010; 28.7 ± 0.27%—2011; 23.3 ± 0.30%—2012; and 27.6 ± 0.14%—2013 (Jan.–Oct.)]. In 2011, the wettest year of the project, additional precipitation had no effect on soil moisture, even during the summer (although the season × precip interaction was significant, means comparison tests failed to identify a significant comparison), but slightly cooled the plots receiving this treatment (**Table 1**).

TALL FESCUE RELATIVE ABUNDANCE AND ENDOPHYTE INFECTION FREQUENCY

Warming significantly reduced tall fescue relative abundance across the entirety of the project, except for the Spring 2012 harvest period when there was no significant difference between +heat and non-heated treatments (**Figure 2**). Even in June (Spring) 2009, after only a month of +heat treatments, tall fescue relative abundance was depressed by ∼35% compared to non-heated plots. This immediate treatment effect did not reflect pre-existing differences in tall fescue abundance across plots, as visual cover estimates made in October 2008 indicated treatments were similar: 23.6% tall fescue ± 3.8—control; 26.3% ± 9.0—+heat; 27.6% ± 4.3—+precip; 24.0% ± 4.3—+heat+precip; and 25.6% vs. 25.2% for ambient temperature treatments vs. heated (average ± SE). Tall fescue relative abundance increased for both +heat and non-heated plots after

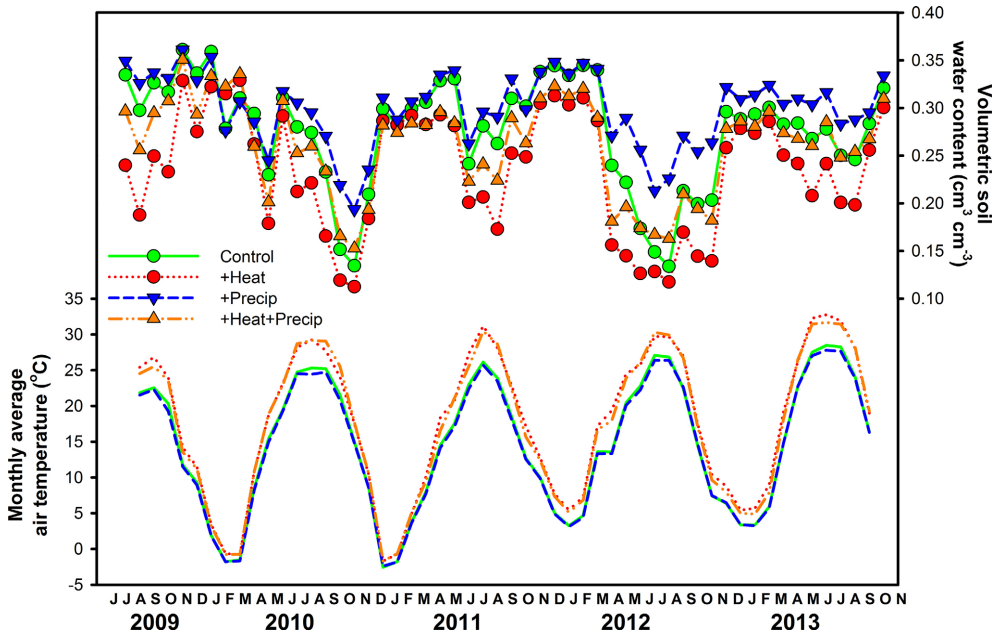


FIGURE 1 | Average (±SE) monthly air temperature (lines only) and volumetric soil moisture (0–15 cm; lines plus symbols) measured in each plot of the four climate treatments (ambient control, +heat, +precip, +heat+precip) across the 5 years study period.

Table 1 | Statistical results of the effects of season, the climate treatments (+heat and +precip), and their interactions on air temperature and soil moisture across the 5-years project period, 2009–2013.

Effect	2009			2010			2011			2012			2013		
	df	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*
AIR TEMPERATURE															
Heat	1	102.92	**<0.0001**	1	195.18	**<0.0001**	1	260.61	**<0.0001**	1	160.31	**<0.0001**	1	87.20	**<0.0001**
Prec	1	3.04	0.1069	1	0.00	0.9839	1	5.13	**0.0429**	1	1.41	0.2577	1	0.70	0.4180
Heat*Prec	1	0.23	0.6386	1	2.30	0.1556	1	0.62	0.4447	1	0.20	0.6638	1	0.03	0.8603
Seas	2,11	10848.5	**<0.0001**	3,10	15135.3	**<0.0001**	3,10	11160.9	**<0.0001**	3,10	14452.7	**<0.0001**	2,11	5012.8	**<0.0001**
Seas*Heat	2,11	31.75	**<0.0001**	3,10	37.98	**<0.0001**	3,10	31.96	**<0.0001**	3,10	22.92	**<0.0001**	2,11	4.95	**0.0294**
Seas*Prec	2,11	2.12	0.1666	3,10	0.51	0.6815	3,10	1.86	0.2005	3,10	1.97	0.1826	2,11	2.65	0.1150
Seas*Heat*Prec	2,11	0.55	0.5918	3,10	3.25	0.0682	3,10	0.71	0.5684	3,10	5.49	**0.0172**	2,11	0.22	0.8040
SOIL MOISTURE															
Heat	1	33.07	**<0.0001**	1	14.82	**0.0023**	1	11.26	**0.0057**	1	17.06	**0.0014**	1	7.77	**0.0164**
Prec	1	11.03	**0.0061**	1	6.60	**0.0246**	1	1.03	0.3302	1	9.85	**0.0086**	1	5.79	**0.0331**
Heat*Prec	1	5.80	**0.0330**	1	0.01	0.9323	1	0.09	0.7749	1	0.44	0.5215	1	0.04	0.8459
Seas	2,11	65.76	**<0.0001**	3,10	1218.62	**<0.0001**	3,10	357.86	**<0.0001**	3,10	622.09	**<0.0001**	2,11	103.96	**<0.0001**
Seas*Heat	2,11	76.26	**<0.0001**	3,10	9.40	**0.0029**	3,10	11.67	**0.0013**	3,10	44.67	**<0.0001**	2,11	7.19	**0.0101**
Seas*Prec	2,11	11.76	**0.0019**	3,10	37.58	**<0.0001**	3,10	7.66	**0.0060**	3,10	22.32	**<0.0001**	2,11	22.74	**0.0001**
Seas*Heat*Prec	2,11	2.78	0.1053	3,10	9.98	**0.0024**	3,10	0.85	0.4996	3,10	3.36	0.0633	2,11	3.10	0.0855

Significant p-values are bolded.

the first year (by 217 and 132%, respectively) and remained relatively stable across years thereafter (averaging 36.4% across treatments). Tall fescue was most abundant in the Spring harvests for all treatments, and the difference between ambient temperature and +heat plots increased as the growing season progressed from Spring to the Summer and Fall harvests across all years of the study. Surprisingly, despite the +precip treatment increasing soil moisture by 8.5, 21.7, and 12.9% over control, +heat, and +heat+precip, respectively, over the course of the study, tall fescue relative abundance was not significantly affected by the addition of precipitation alone ($p = 0.1029$; **Table 2**). Although added precipitation increased soil moisture in the +heat+precip treatment over that of the +heat alone (by 11.2% over the course of the study), especially in summer, only a marginally significant

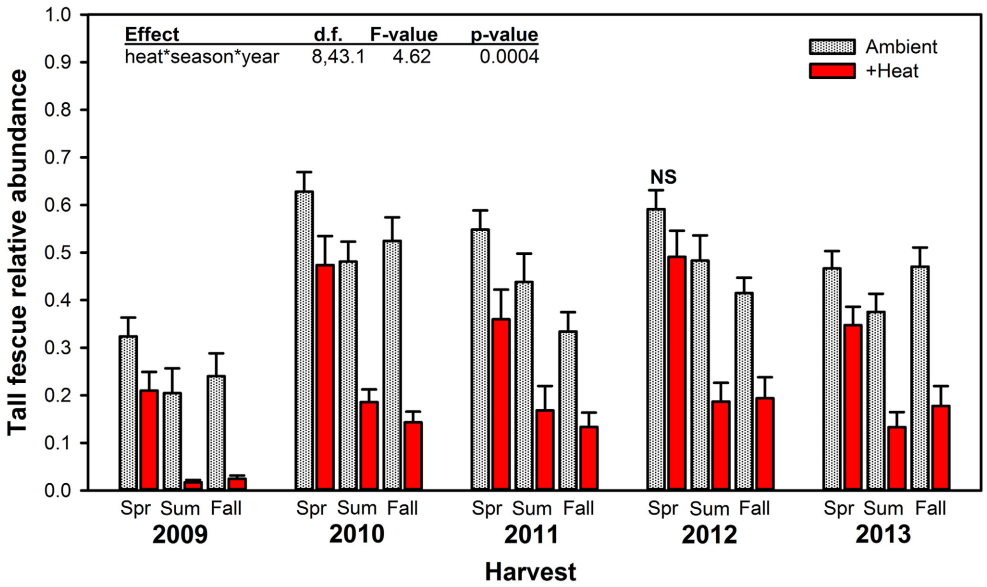


FIGURE 2 | Average (±SE) relative abundance of tall fescue (g tall fescue biomass/g total biomass) measured in heated (+3°C) vs. non-heated, ambient temperature treatments at every harvest period (Spring, Summer, and Fall) for all 5 years of the study. The highest order significant interaction among the main effects of heat, season, and year is provided (see **Table 2** for full statistical results). NS indicates the only harvest period where significant effects of heat were not observed.

Table 2 | Statistical results of the effects of the climate treatments (+heat and +precip), season, year, and their interactions on tall fescue relative abundance in a mixed species pasture.

Effect	Num,Den *df*	*F*-value	Pr > *F*
Heat	1,33.7	51.68	**<0.0001**
Precip	1,33.7	2.81	0.1029
Heat*Precip	1,33.7	3.96	0.0546
Season	2,24.4	75.29	**<0.0001**
Heat*Season	2,24.4	9.33	**0.0010**
Precip*Season	2,24.4	1.89	0.1729
Heat*Precip*Season	2,24.4	1.16	0.3317
Year	4,26.6	48.10	**<0.0001**
Heat*Year	4,26.6	1.87	0.1449
Precip*Year	4,26.6	1.49	0.2344
Heat*Precip*Year	4,26.6	0.21	0.9283
Season*Year	8,43.1	12.51	**<0.0001**
Heat*Season*Year	8,43.1	4.62	**0.0004**
Precip*Season*Year	8,43.1	1.70	0.1269
Heat*Precip*Season*Year	8,43.1	1.41	0.2197

Significant main effects and interactions are bolded.

Table 3 | Statistical results of the effects of the climate treatments (+heat and +precip), month since the project began, and their interactions on the change in tall fescue endophyte infection frequency from the initial harvest June 1, 2009.

Effect	Num,Den *df*	*F*-value	Pr > *F*
Heat	1,76	1.49	0.2260
Precip	1,76	0.06	0.8073
Heat*Precip	1,76	0.09	0.7713
Month	4,76	3.13	**0.0194**
Heat*Month	4,76	2.31	0.0651
Precip*Month	4,76	0.09	0.9844
Heat*Precip*Month	4,76	6.49	**0.0002**

Bolding indicates significant main effects and interactions.

interactive treatment effect ($p = 0.0546$ for heat × precip) on tall fescue abundance was identified (**Table 2**).

In contrast to our hypothesis that +heat treatments would favor the survival and dominance of E+ individuals over E− within the tall fescue population, we found relatively limited effects of warming on changes in EIF over time (**Table 3**, **Figure 3**).

While a significant heat × precip × month since experiment began interaction was found, comparison tests identified only one significant mean separation. In October 2012, tall fescue within the +heat plots tested 20% more endophyte infected than in the beginning of the project, while the control plot EIF appeared to have not changed. However, this apparent warming effect disappeared the next year, as there were no significant treatment differences identified in October 2013 samples. Differences in EIF across time within a temperature treatment did occur (e.g., for +heat, changes in EIF in October 2012 were greater than in October 2010; for +precip, changes in EIF was greater in October 2012 than in October 2013; and for +heat+precip plots, changes in EIF was greater in October 2011 than in October 2009, 2010, and 2012).

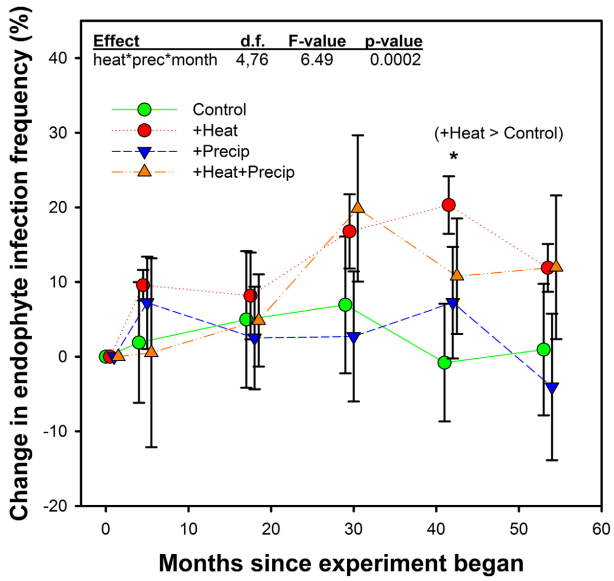


FIGURE 3 | Average (±SE) change in endophyte infection frequency within the tall fescue population in each plot over the 5 years study in heated and non-heated, ambient temperature treatments. Points are slightly offset to clarify presentation, but measurements occurred at the same time for all treatments. Initial endophyte infection frequency averaged 41.1% (±3.3) across the site. Plot-level initial (June 2009) individual values were set as the zero point in this figure, and change from these values were calculated for measurements which occurred in Octobers from 2009–2013. The highest order significant interaction between heat, precipitation, and month since project began is shown, along with the only significant treatment comparison. See **Table 2** for full statistical results. $^*p = 0.0346$.

ALKALOIDS

Warming significantly increased concentrations of both measured ergot alkaloids and their sum, but this effect was primarily limited to the Fall harvested material (**Figure 4**, **Table 4**). Except for Spring 2013, the last year of the study, no warming effect was observed in Spring harvested material. The Spring 2013 trend was similar to Fall effects: +heat treatments had higher concentrations of ergot alkaloids than the non-heated, ambient temperature treatments. The magnitude of the warming induced increase in ergot alkaloids varied over time from +20.4 to +74.1% for ergovaline and +31.6 to +61.8% for ergovalinine. For both compounds, warming effects were strongest in Fall 2010, 2011, and 2012.

For the measured loline alkaloids, Fall harvested material tended to have significantly greater concentrations than Spring material (**Figure 5**, **Table 5**). The magnitude of difference between the seasons varied across years (hence the significant season × year interactions) and for the different compounds measured. NFL was the only loline alkaloid that had similar concentrations in Spring and Fall harvested material, and this only occurred in one year—2010. For all three loline compounds, Fall 2012 had the highest measured concentrations.

Fall harvested material was also the only material that exhibited significant climate treatment effects on loline concentrations (**Figure 6**, **Table 5**). Across all 5 years of the study, in Fall harvested material, the combination of +heat and +precip produced significantly higher concentrations of all three measured loline compounds and their sum. None of the other three climate treatments (+heat alone, +precip alone, or the ambient control) differed from each other.

DISCUSSION

Our hypothesis that warming would reduce the relative abundance of tall fescue in this mixed species pasture, in part by increasing summer water limitation, a condition which is known to be stressful for this cool-season, C_3 physiology grass (Belesky and West, 2009), was supported. Warming did reduce soil moisture, particularly in the summer and fall, and in every year of the study, warmed plots had significantly less tall fescue than ambient temperature plots. However, water limitation is only one potential factor explaining the warming associated decline in tall fescue abundance: competition from other plant species was most likely also important. Indeed, the increase in tall fescue abundance that was observed at the start of the second growing season (2010) across all treatments was associated with a dramatic decline in red clover, which was the dominant species in all treatments at the start of the project (data not shown). Additionally, in every year of the project, as seasonal temperatures increased from spring to summer and fall, warm-season, C_4 physiology grasses (initially crabgrass, *Digitaria* spp., which recruited naturally from the seed bank, and later on in the project, Bermuda, which was planted) increased in dominance. These species were most abundant in the warmed treatments (data not shown) and were most likely strong competitors with tall fescue. Given the stressful conditions (both abiotic and biotic) that warming created for tall fescue, it is remarkable that it persisted in these plots for the duration of the study, at relatively stable levels.

One hypothesis that might explain how tall fescue managed to persist and effectively compete in the stressful warmed conditions of our project is that competitively superior, more environmental stress tolerant, E+ individuals were being selected for over time in these treatments, as E− individuals perished. However, this is not what we observed. Only at one point in time in our study, October 2012, after 4 years of experimental conditions, did we identify a significant treatment effect on the change in EIF over time. This effect was in the direction of what we hypothesized (the +heat treatment had 20% more E+ individuals than at the start of the experiment, whereas the EIF in the control treatment had not changed), but the significance of the effect disappeared the following year. In general, changes in EIF over time tended to be positive for +heat and +heat+precip treatments but were relatively small in magnitude (averaging 11.5% increase over time), while +precip and control treatments averaged almost no change (3.0% increase). The lack of a substantial change in EIF over time observed in this study, despite warming increasing abiotic and biotic stress, concurs with the results of Brosi et al. (2011b), who found that neither warming (+3°C) nor water limitation caused significant change in tall fescue EIF in a constructed, multi-species old field in Tennessee.

These results suggest that the importance of endophyte infection to tall fescue's ability to withstand and recover from elevated temperatures and water-limiting conditions may be less

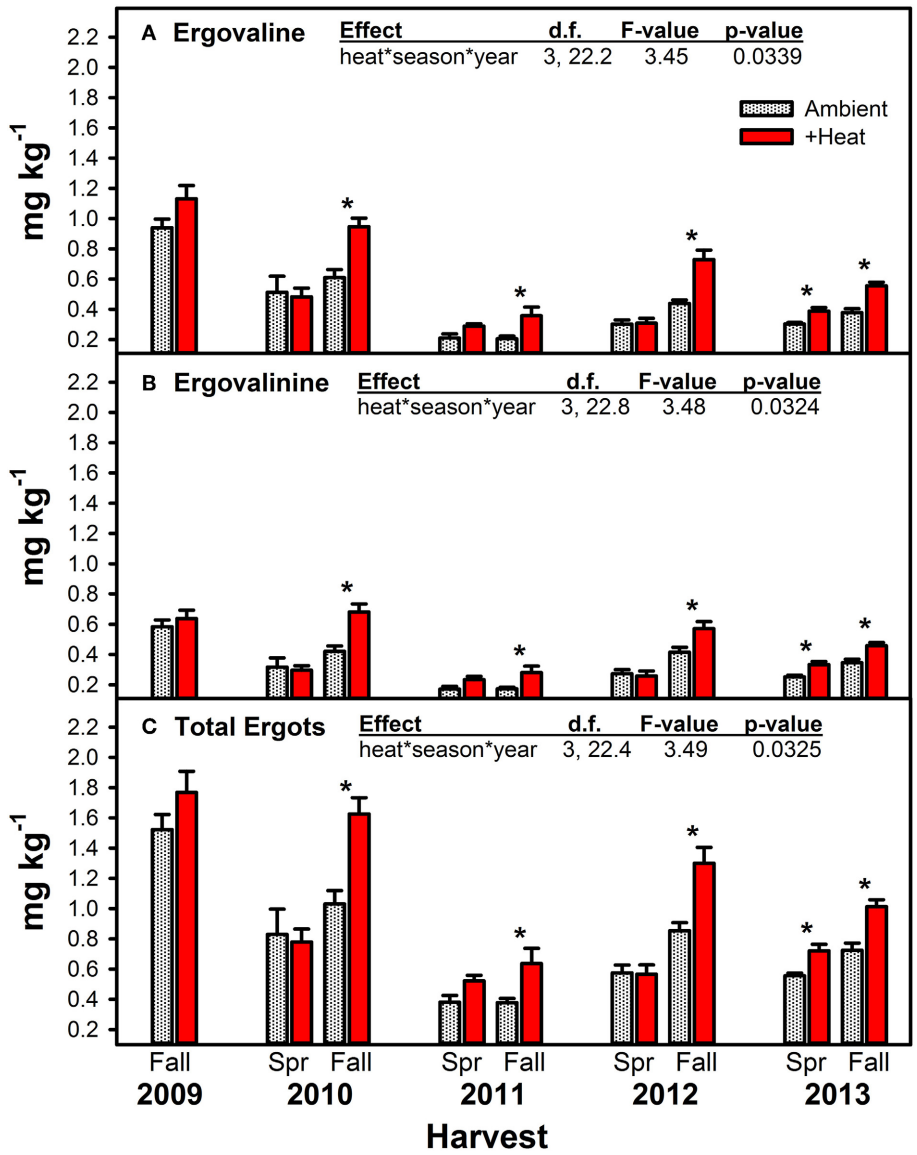


FIGURE 4 | Average (±SE) concentrations of ergovaline (A), ergovalinine (B), and the sum of these two ergot alkaloids (C) measured in endophyte-infected tall fescue tillers harvested from heated and non-heated, ambient temperature treatments in Spring and Fall of all years of the study. Spring 2009 is missing due to lab error. The highest order significant interaction between heat, season, and year is shown (see **Table 4** for full statistical results). Harvest periods with significant differences between treatments are indicated with an "*."

than prior work suggests. It is possible that our experimental treatments were not harsh enough to evoke the symbiotic benefits; however, both 2010 and 2012 were drier than normal years, with 2012 having one of the driest early growing seasons on record at the site (Mallya et al., 2013). Therefore, it is difficult to believe that the +heat treatment would not have produced harsh conditions for tall fescue at least at that time. These environmental conditions may have played into the Fall of 2012 being the only time period when significant treatment differences in the change in EIF over time were identified. It is also possible that endophyte benefits are conferred under more intense, but shorter duration, abiotic "extreme" events—rather than the long, continuous nature of our experimental climate manipulations. Additional work could further explore these possibilities.

Our results of relatively limited importance of endophyte infection to tall fescue abiotic stress tolerance are not unheard of: not all studies have shown benefits of endophyte infection to tall fescue under stressful conditions (MacLean et al., 1993; Richardson et al., 1993; Elbersen and West, 1996; Buck et al., 1997). Some have shown no effects (Arachevaleta et al., 1989; Belesky et al., 1989; Hill et al., 1996), and work performed in this same project found that E+ tall fescue experienced more mortality than E− after 1 month of +heat treatments (Brosi, 2011a) and that tall fescue's response to the treatments is under plant genetic control, in addition to being impacted by *Epichloë*

Table 4 | Statistical results of the effects of the climate treatments (+heat and +precip), season, year, and their interactions on the concentrations of ergovaline, ergovalinine, and their sum (total ergots measured in this study) in endophyte infected tall fescue tillers.

Effect	Ergovaline			Ergovalinine			Total ergots		
	Num,Den			Num,Den			Num,Den		
	df	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*
Heat	1,17.2	20.9	**0.0003**	1,26.2	13.66	**0.0010**	1,20.5	18.67	**0.0003**
Precip	1,17.2	0.84	0.3727	1,26.2	0.40	0.5302	1,20.5	0.68	0.4207
Heat*Precip	1,17.2	1.60	0.2226	1,26.2	1.07	0.3095	1,20.5	1.45	0.2427
Season	1,20.4	57.14	**<0.0001**	1,20.0	67.68	**<0.0001**	1,20.2	65.08	**<0.0001**
Heat*Season	1,20.4	18.89	**0.0003**	1,20.0	12.94	**0.0018**	1,20.2	17.07	**0.0005**
Precip*Season	1,20.4	0.00	0.9807	1,20.0	0.08	0.7788	1,20.2	0.02	0.8911
Heat*Precip*Season	1,20.4	0.01	0.9062	1,20.0	0.03	0.8720	1,20.2	0	0.9940
Year	4,17.4	35.83	**<0.0001**	4,18.1	22.3	**<0.0001**	4,17.5	30.65	**<0.0001**
Heat*Year	4,17.4	0.21	0.9319	4,18.1	0.80	0.5382	4,17.5	0.32	0.8638
Precip*Year	4,17.4	0.65	0.6368	4,18.1	0.42	0.7937	4,17.5	0.56	0.6951
Heat*Precip*Year	4,17.4	1.37	0.2858	4,18.1	0.78	0.5532	4,17.5	1.12	0.3801
Season*Year	3,22.2	10.24	**0.0002**	3,22.8	11.36	**<0.0001**	3,22.4	10.93	**0.0001**
Heat*Season*Year	3,22.2	3.45	**0.0339**	3,22.8	3.48	**0.0324**	3,22.4	3.49	**0.0325**
Precip*Season*Year	3,22.2	0.56	0.6465	3,22.8	0.80	0.5087	3,22.4	0.59	0.6228
Heat*Precip*Season*Year	3,22.2	0.64	0.5951	3,22.8	0.05	0.9843	3,22.4	0.29	0.8338

Bolding indicates significant main effects and interactions.

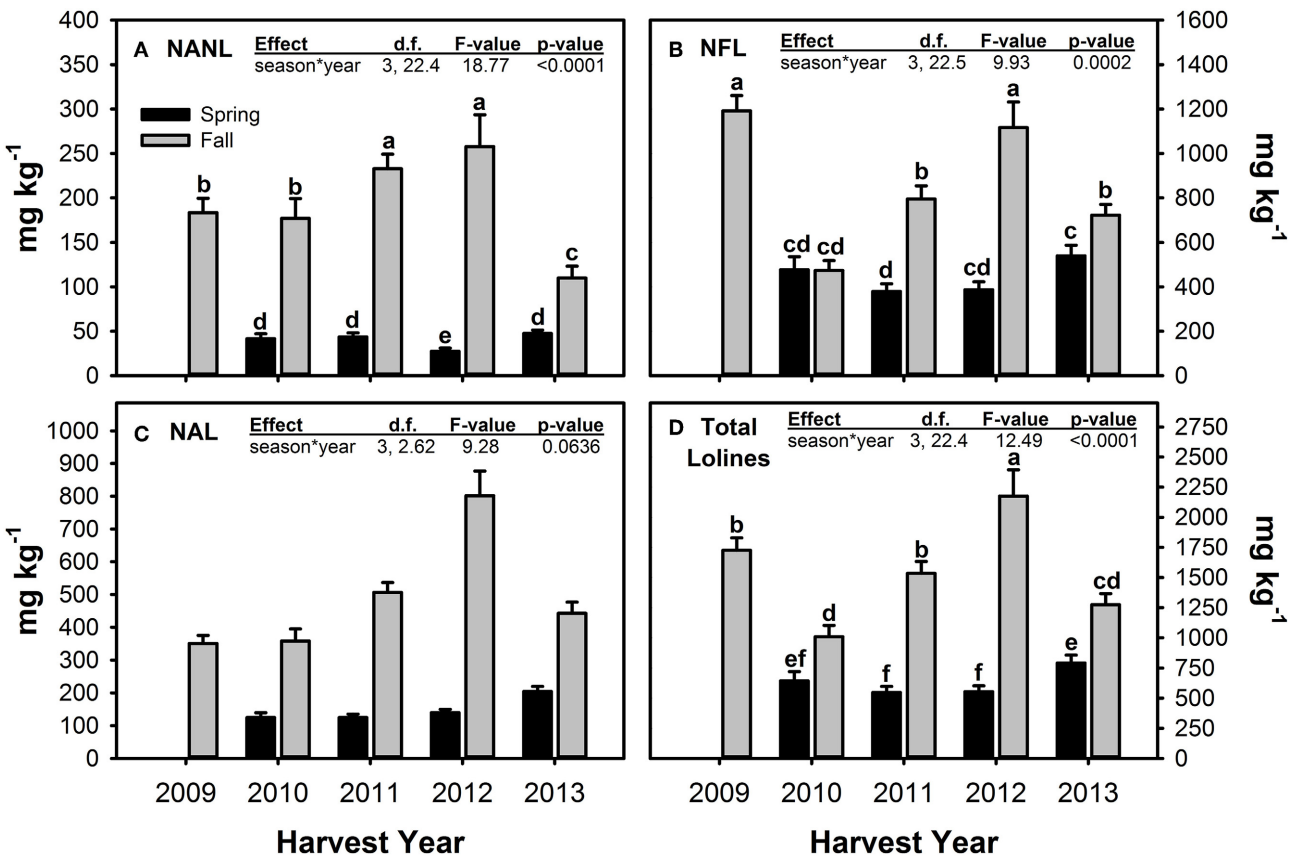


FIGURE 5 | Concentrations of *N*-acetyl norloline—NANL (A), *N*-formyl loline—NFL (B), *N*-acetyl loline—NAL (C), and the sum of these three loline alkaloids (D) measured in endophyte-infected tall fescue tillers harvested in Spring and Fall of all years of the study and averaged (±SE) across all climate treatments. Spring 2009 is missing due to lab error. For loline alkaloids with a significant season × year interaction term, different letters indicate significant differences across all seasons and years of the study.

Table 5 | Statistical results of the effects of the climate treatments (+heat and +precip), season, year, and their interactions on concentrations of *N*-acetyl norloline (NANL), *N*-formyl loline (NFL), *N*-acetyl loline (NAL), and their sum (total lolines measured in this study) in endophyte infected tall fescue tillers.

Effect	NANL			NFL			NAL			Total lolines		
	df	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*	*df*	*F*-value	Pr > *F*
Heat	1,21.5	18.51	**0.0003**	1,17.3	6.94	**0.0173**	1,8.75	16.85	**0.0028**	1,20.9	16.01	**0.0007**
Prec	1,21.5	1.54	0.2274	1,17.3	3.49	0.0788	1,8.75	0.28	0.6109	1,20.9	2.74	0.1127
Heat*Prec	1,21.5	2.77	0.1107	1,17.3	1.53	0.2322	1,8.75	5.55	**0.0436**	1,20.9	3.91	0.0614
Seas	1,22.7	175.47	**<0.0001**	1,22.6	90.85	**<0.0001**	1,2.13	397.27	**0.0018**	1,22.9	223.34	**<0.0001**
Heat*Seas	1,22.7	11.58	**0.0025**	1,22.6	3.64	0.0691	1,2.13	12.90	0.0632	1,22.9	9.07	**0.0062**
Prec*Seas	1,22.7	2.34	0.1403	1,22.6	10.45	**0.0037**	1,2.13	4.84	0.1510	1,22.9	8.84	**0.0068**
Heat*Prec*Seas	1,22.7	6.40	**0.0188**	1,22.6	16.78	**0.0005**	1,2.13	19.42	**0.0425**	1,22.9	19.53	**0.0002**
Yr	4,17.5	8.94	**0.0004**	4,17.6	14.71	**<0.0001**	4,3.34	8.18	**0.0474**	4,16.9	7.07	**0.0016**
Heat*Yr	4,17.5	0.52	0.7228	4,17.6	0.33	0.8546	4,3.34	0.90	0.5506	4,16.9	0.46	0.7637
Prec*Yr	4,17.5	1.20	0.3457	4,17.6	0.64	0.6410	4,3.34	0.69	0.6438	4,16.9	0.61	0.6615
Heat*Prec*Yr	4,17.5	1.11	0.3850	4,17.6	1.16	0.3610	4,3.34	1.54	0.3629	4,16.9	1.56	0.2312
Seas*Yr	3,22.4	18.77	**<0.0001**	3,22.5	9.93	**0.0002**	3,2.62	9.28	**0.0636**	3,22.4	12.49	**<0.0001**
Heat*Seas*Yr	3,22.4	1.39	0.2708	3,22.5	1.74	0.1875	3,2.62	2.21	0.2843	3,22.4	1.97	0.1472
Prec*Seas*Yr	3,22.4	1.36	0.2806	3,22.5	0.07	0.9747	3,2.62	0.08	0.9635	3,22.4	0.11	0.9550
Heat*Prec*Seas*Yr	3,22.4	1.19	0.3369	3,22.5	0.10	0.9596	3,2.62	0.38	0.7769	3,22.4	0.22	0.8798

Bolding indicates significant main effects and interactions.

presence and strain type (Bourguignon, 2013). We did not control for plant genetic background in this experiment, as we were interested in measuring changes to the population, and it is possible that differing fescue genotypes in our project responded to both climate treatments and endophyte presence in contrasting ways (West, 2007), which might have hampered our ability to detect an endophyte-associated response to the climate treatments. Complicated symbiotic responses to abiotic perturbations and their resulting effects on various ecological parameters are consistent with the growing body of knowledge regarding controls of grass—*Epichloë* interactions (Faeth and Saikkonen, 2007; West, 2007; Rudgers et al., 2010; Yurkonis et al., 2014), and will need to be factored into predictions regarding population-level grass symbiotic responses to climate change.

It is possible that the primary importance of the fungal endophyte symbiosis for tall fescue under altered climatic conditions will be conferred through interactions with herbivory. Field manipulations of herbivory have clearly demonstrated that endophyte presence confers an advantage under high herbivore pressure, such that significant increases in EIF of the tall fescue community were observed over time (Clay et al., 2005). In our project, in E+ individuals, we measured significant increases in both ergot and loline alkaloids in fall plant material from treatments representative of predicted future climates for the region. Because these compounds are toxic to herbivores, these results suggest that those herbivores capable of selecting for E− fescue or utilizing other plant species will do so, potentially altering plant competitive dynamics.

While assessing the response of herbivores to the climate change treatments was not the primary focus of our study, concurrent projects that monitored aphids, slugs, and small mammal seed predation and population dynamics, at varying times throughout the 5 years project, found few changes in herbivore numbers or feeding patterns related to the biotic and abiotic alterations that the climate change treatments produced. Rua et al. (2014) monitored aphids in May 2010 and found that there were more aphids in the +heat plots than the other treatments, but was unable to test for differences in aphid numbers between E+ and E− tall fescue due to low numbers of aphids being found on this material—a result similar to that of Brosi (2011a). Three years of slug manipulations and trapping at the site indicated that slug herbivory levels were low and that climate treatment effects on these herbivores were minor (Weber, 2014). Small mammal, primarily vole (*Microtus pennsylvanicus*, *M. ochrogaster*), population dynamics varied from year-to-year, as is common for these organisms (Chitty, 1960; Krebs and Myers, 1974), but seed predation was generally similar across plots (A.E. Carlisle, unpublished data). It is possible that the mowing regime or other environmental or site factors created conditions that minimized herbivory levels at our site, and may be one reason why changes in EIF were not observed, despite climate treatments increasing alkaloid concentrations and significantly altering the abiotic environment and plant communities. Changes in tall fescue EIF may have been more dramatic had selective, heavy grazing, rather than non-selective mowing, been practiced on site, though it should be noted that an experiment in southern Illinois found that mowing increased water limitation, which the authors hypothesized selected for higher EIF of the tall fescue in mowed compared to adjacent not mowed plots (Spyreas et al., 2001). The factors causing the observed lack of change in EIF in this experiment remain unknown.

Our project failed to incorporate increases in atmospheric CO_2 concentrations, due to financial and infrastructure limitations,

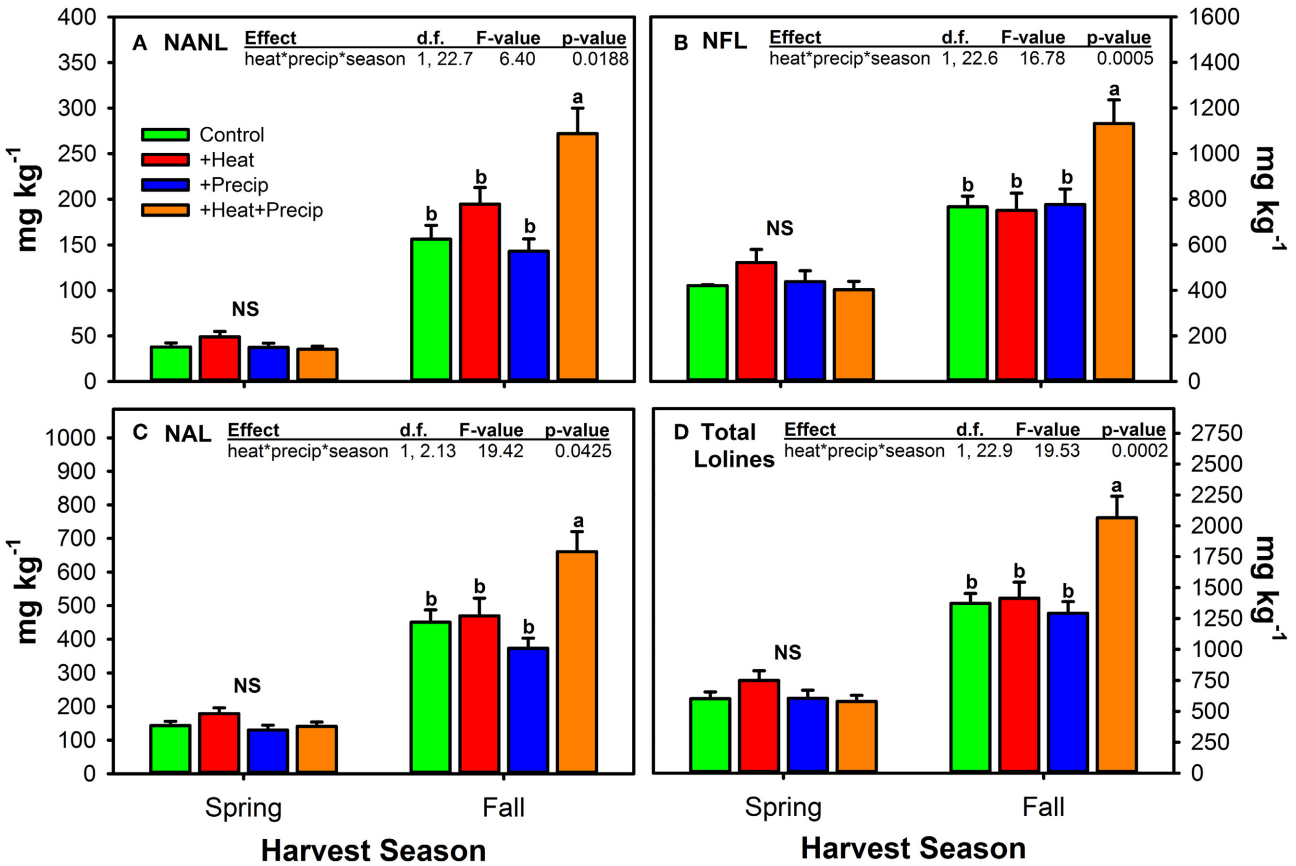


FIGURE 6 | Concentrations of *N*-acetyl norloline—NANL (A), *N*-formyl loline—NFL (B), *N*-acetyl loline—NAL (C), and the sum of these three loline alkaloids (D) measured in endophyte-infected tall fescue tillers from each of the four climate treatments averaged (±SE) by season of harvest (Spring or Fall) and across years of the study. The highest order significant interaction between heat, precip, and season is shown (see **Table 5** for full statistical results). NS indicates seasons when there were no statistically significant differences between the climate treatments. Otherwise, different letters illustrate significant differences between climate treatments.

which is unfortunate as this factor will be an important component of future environmental conditions and may modify effects of warming and altered precipitation on tall fescue (Yu et al., 2012), and has been shown to be important in governing the fescue-endophyte symbiosis. Brosi et al. (2011b) found that elevated CO_2 increased the occurrence of E+ tall fescue individuals in a mixed species stand but reduced the concentrations of both ergot and loline alkaloids. Ryan et al. (2014) also reported reduced alkaloid concentrations in E+ tall fescue in response to elevated CO_2, despite measuring increased fungal DNA concentrations in these same individuals. In contrast to the Brosi et al. (2011b) work, we measured significant increases in ergot alkaloids in response to warming (Brosi measured no response of ergots to warming), and we found that lolines increased in concentration only when warming was accompanied by additional precipitation (Brosi found warming alone stimulated lolines, that dry, not wet conditions, increased loline concentrations, and there was no interaction between warming and water availability in that study). Differences in site conditions or experimental designs and treatments may account for these discrepancies, as nitrogen availability has been shown to alter the response of alkaloid concentrations to elevated CO_2 in perennial ryegrass when infected with *E. festucae* var. *lolii* (Hunt et al., 2005).

Clearly, the *Epichloë*—tall fescue symbiosis will be affected by and respond to climate change factors. Given that all research performed to date on the subject has shown alkaloids to be responsive to the effects of elevated atmospheric CO_2, warming, and drought/additional precipitation, and these biological compounds play key roles in governing herbivory and animal production in pastures, it is imperative that future work attempts to better characterize the interactive effects of these various climate change factors on alkaloid concentrations. Brosi et al. (2011b) is the only paper to date able to assess the interactive effects, and they found no significant interactions on any of the measured compounds, suggesting that the individual climate treatment main effects cancel each other out. However, replication was limited in the Brosi work ($n = 3$), and the above-mentioned differences between our results and theirs for warming and precipitation manipulations suggest that additional factors may be governing tall fescue—endophyte alkaloid responses. If elevated atmospheric CO_2 does not mitigate the effects of warming and changes in precipitation on alkaloid concentrations in E+ tall

fescue, our data suggest that those herbivores that are incapable of altering forage selection (e.g., cattle grazing highly infected tall fescue dominated pastures) will likely consume higher levels of these toxic compounds, and they will do so at an already hot time of the year in many places (late Summer/Fall), when there are significant challenges to maintaining ideal thermal conditions for optimal animal functioning (Spiers et al., 2005). Additional research evaluating pasture management options to reduce consumption of toxic alkaloids (e.g., the use of novel, non-toxic endophyte infected material, specific grazing strategies, and/or other forage species, especially warm season grasses; Aiken and Strickland, 2013) while sustaining pasture-based animal production under future climatic conditions seems warranted.

AUTHOR CONTRIBUTIONS

Rebecca L. McCulley and Jim A. Nelson conceived the research idea and designed the experiment. All authors were involved in aspects of data acquisition, analysis, and interpretation. All authors contributed to writing the manuscript, approved the final version, and are accountable for the data presented and interpretation therein.

ACKNOWLEDGMENTS

This work was supported by a grant to Rebecca L. McCulley (US Department of Energy 08-SC-NICCR-1073), a cooperative agreement with the USDA-ARS Forage Animal Production Research Unit (58-6440-7-135), the Kentucky Agricultural Experiment Station (KY006045), and the University of Kentucky's College of Agriculture, Food, and the Environment. The following people deserve thanks for the hours of time they contributed to the planting, harvesting, sorting, grinding, and blotting required: Maria Almagro, Sara Arthur-Paratley, Marie Bourguignon, Luke Box, Alexis Breton, Glade Brosi, Cody Burton, Allison Cooke, Dawn DeColibus, Jarred Earnest, Amael Gy, Sarah Hall, Javed Iqbal, Eric Kalosa-Kenyon, Ben Leffew, Marléne Mauras, Michael Payton May, Lindsey Slaughter, Rachel Smith, and Dan Weber. We also thank Kristen McQuerry and the UK Department of Statistics for statistical analysis assistance.

REFERENCES

Agee, C. S., and Hill, N. S. (1994). Ergovaline variability in acremonium-infected tall fescue due to environment and plant genotype. *Crop Sci.* 34, 221–226. doi: 10.2135/cropsci1994.0011183X003400010040x

Aiken, G. E., and Strickland, J. R. (2013). Forages and pasture management symposium: managing the tall fescue-fungal endophyte symbiosis for optimum forage-animal production. *J. Anim. Sci.* 91, 2369–2378. doi: 10.2527/jas.2012-5948

Arachevaleta, M., Bacon, C. W., Hoveland, C. S., and Radcliffe, D. E. (1989). Effect of the tall fescue endophyte on plant-response to environmental-stress. *Agron. J.* 81, 83–90. doi: 10.2134/agronj1989.00021962008100010015x

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., and Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecol. Lett.* 3, 267–274. doi: 10.1046/j.1461-0248.2000.00159.x

Assuero, S. G., Tognetti, J. A., Colabelli, M. R., Agnusdei, M. G., Petroni, E. C., and Posse, M. A. (2006). Endophyte infection accelerates morpho-physiological responses to water deficit in tall fescue. *New Zeal. J. Agr. Res.* 49, 359–370. doi: 10.1080/00288233.2006.9513726

Backlund, P., Schimel, D., Janetos, A., Hatfield, J., Ryan, M., Archer, S., et al. (2008). *Introduction: the Effects of Climate Change on Agriculture, Land Resources, Water Resources, and Biodiversity*. Washington, DC: U.S. Climate Change Science Program and the Subcommittee on Global Change Research.

Ball, D. M., Pedersen, J. F., and Lacefield, G. D. (1993). The tall-fescue endophyte. *Am. Sci.* 81, 370–379.

Belesky, D. P., Stringer, W. C., and Hill, N. S. (1989). Influence of endophyte and water regime upon tall fescue accessions. 1. Growth-characteristics. *Ann. Bot.* 63, 495–503.

Belesky, D. P., and West, C. P. (2009). Abiotic stresses and endophyte effects. Tall fescue for the twenty-first century. *Agron. Monogr.* 53, 49–64. doi: 10.2134/agronmonogr53.c4

Blankenship, J. D., Spiering, M. J., Wilkinson, H. H., Fannin, F. F., Bush, L. P., and Schardl, C. L. (2001). Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media. *Phytochemistry* 58, 395–401. doi: 10.1016/S0031-9422(01)00272-2

Bourguignon, M. (2013). *Ecophysiological Responses of tall Fescue Genotypes to Endophyte Infection and Climate Change*. Theses and Dissertations, Plant and Soil Sciences, University of Kentucky. Paper 28. Available online at: http://uknowledge.uky.edu/pss_etds/28.

Bouton, J. H., Gates, R. N., Belesky, D. P., and Owsley, M. (1993). Yield and persistence of tall fescue in the southeastern coastal-plain after removal of its endophyte. *Agron. J.* 85, 52–55. doi: 10.2134/agronj1993.00021962008500010011x

Brosi, G. B. (2011a). *The Response of Tall Fescue and its Fungal Endophyte to Climate Change*. Theses and Dissertations, Plant and Soil Sciences, University of Kentucky. Paper 126. Available online at: http://uknowledge.uky.edu/gradschool_theses/126

Brosi, G. B., McCulley, R. L., Bush, L. P., Nelson, J. A., Classen, A. T., and Norby, R. J. (2011b). Effects of multiple climate change factors on the tall fescue-fungal endophyte symbiosis: infection frequency and tissue chemistry. *New Phytol.* 189, 797–805. doi: 10.1111/j.1469-8137.2010.03532.x

Brunjes, J. (2004). *Action Plan to Respond to Climate Change in Kentucky: a Strategy of Resilience*. Frankfort, KY: Kentucky Department of Fish and Wildlife.

Buck, G. W., West, C. P., and Elbersen, H. W. (1997). "Endophyte effect on drought tolerance in diverse Festuca species," in *Neotyphodium/Grass Interactions*, eds C. W. Bacon and N. S Hill (NewYork, NY: Plenum Press), 141–143.

Bush, L. P., Wilkinson, H. H., and Schardl, C. L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol.* 114, 1–7.

Chen, X., Tu, C., Burton, M. G., Watson, D. M., Burkey, K. O., and Hu, S. (2007). Plant nitrogen acquisition and interactions under elevated carbon dioxide: impact of endophyte and mycorrhizae. *Glob. Change Biol.* 13, 1238–1249. doi: 10.1111/j.1365-2486.2007.01347.x

Cheplick, G. P., and Faeth, S. H. (2009). *Ecology and Evolution of the Grass-Endophyte Symbiosis*. New York, NY: Oxford University Press.

Chitty, D. (1960). Population processes in the vole and their relevance to general theory. *Can. J. Zool.* 38, 99–113. doi: 10.1139/z60-011

Clay, K. (1990). Fungal endophytes of grasses. *Annu. Rev. Ecol. Evol. Syst.* 21, 275–297. doi: 10.1146/annurev.es.21.110190.001423

Clay, K., and Holah, J. (1999). Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285, 1742–1744. doi: 10.1126/science.285.5434.1742

Clay, K., Holah, J., and Rudgers, J. A. (2005). Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12465–12470. doi: 10.1073/pnas.0503059102

Clay, K., and Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am. Nat.* 160, S99–S127. doi: 10.1086/342161

Compant, S., Van Der Heijden, M. G. A., and Sessitsch, A. (2010). Climate change effects on beneficial plant–microorganism interactions. *FEMS Microbiol. Ecol.* 73, 197–214. doi: 10.1111/j.1574-6941.2010.00900.x

Elbersen, H. W., and West, C. P. (1996). Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. *Grass Forage Sci.* 51, 333–342. doi: 10.1111/j.1365-2494.1996.tb02068.x

Elmi, A., and West, C. (1995). Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. *New Phytol.* 131, 61–67. doi: 10.1111/j.1469-8137.1995.tb03055.x

Faeth, S. H., and Saikkonen, K. (2007). "Variability is the nature of the endophyte-grass interaction," in *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses*, eds A. J. Popay and E. R. Thom (Christchurch: New Zealand Grassland Association (no. 13)), 37–48.

Ferreira, W. P. M., Priddy, T. K., Souza, C. F., and Mathews, J. (2010). "Trends in precipitation and air temperature time series in Lexington/KY - USA," in *ASABE Annual International Meeting. Paper # 1009768* (Pittsburgh, PA), 3–13.

Hiatt, E., Hill, N., Bouton, J., and Stuedemann, J. (1999). Tall fescue endophyte detection: commercial immunoblot test kit compared with microscopic analysis. *Crop Sci.* 39, 796–799.

Hill, N., Pachon, J., and Bacon, C. (1996). *Acremonium coenophialum*-mediated short-and long-term drought acclimation in tall fescue. *Crop Sci.* 36, 665–672. doi: 10.2135/cropsci1996.0011183X003600030025x

Hoveland, C. S. (1993). Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. *Agr. Ecosyst. Environ*. 44, 3–12. doi: 10.1016/0167-8809(93)90036-O

Hoveland, C. S. (2009). Origin and history. Tall fescue for the twenty-first century. *Agron. Monogr.* 53, 3–10. doi: 10.2134/agronmonogr53.c1

Hunt, M. G., Rasmussen, S., Newton, P. C. D., Parsons, A. J., and Newman, J. A. (2005). Near-term impacts of elevated CO2, nitrogen and fungal endophyte-infection on *Lolium perenne* L. growth, chemical composition and alkaloid production. *Plant Cell Environ.* 28, 1345–1354. doi: 10.1111/j.1365-3040.2005.01367.x

Ju, H. J., Hill, N. S., Abbott, T., and Ingram, K. T. (2006). Temperature influences on endophyte growth in tall fescue. *Crop Sci.* 46, 404–412. doi: 10.2135/cropsci2005.0282

Karl, T. R., Melillo, J. M., and Peterson, T. C. (2009). *Global Climate Change Impacts in the United States*. New York, NY: Cambridge University Press.

Kimball, B. A., Conley, M. M., Wang, S., Lin, X., Luo, C., Morgan, J., et al. (2008). Infrared heater arrays for warming ecosystem field plots. *Glob. Change Biol.* 14, 309–320. doi: 10.1111/j.1365-2486.2007.01486.x

Kivlin, S. N., Emery, S. M., and Rudgers, J. A. (2013). Fungal symbionts alter plant responses to global change. *Am. J. Bot.* 100, 1445–1457. doi: 10.3732/ajb.1200558

Krebs, C. J., and Myers, J. H. (1974). Population cycles in small mammals. *Adv. Ecol. Res.* 8, 267–399. doi: 10.1016/S0065-2504(08)60280-9

Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106, 202–215. doi: 10.3852/106.2.202

Leuchtmann, A. (1992). Systematics, distribution, and host specificity of grass endophytes. *Nat. Toxins* 1, 150–162. doi: 10.1002/nt.2620010303

Lewis, G. C., Ravel, C., Naffaa, W., Astier, C., and Charmet, G. (1997). Occurrence of *Acremonium* endophytes in wild populations of *Lolium spp*. in European countries and a relationship between level of infection and climate in France. *Ann. Appl. Biol.* 130, 227–238. doi: 10.1111/j.1744-7348.1997.tb06828.x

MacLean, B., Matthew, C., Latch, G., and Barker, D. (1993). "The effect of endophyte on drought resistance in tall fescue," in *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*, eds D. E. Hume, G. C. M. Latch, and H. S. Easton (Palmerston North: AgResearch), 165–169.

Malinowski, D. P., and Belesky, D. P. (2000). Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci.* 40, 923–940. doi: 10.2135/cropsci2000.404923x

Mallya, G., Zhao, L., Song, X., Niyogi, D., and Govindaraju, R. (2013). 2012 Midwest drought in the United States. *J. Hydrol. Eng.* 18, 737–745. doi: 10.1061/(ASCE)HE.1943-5584.0000786

Marks, S., and Clay, K. (1996). Physiological responses of *Festuca arundinacea* to fungal endophyte infection. *New Phytol.* 133, 727–733. doi: 10.1111/j.1469-8137.1996.tb01941.x

Newman, J. A., Abner, M. L., Dado, R. G., Gibson, D. J., Brookings, A., and Parsons, A. J. (2003). Effects of elevated CO_2, nitrogen and fungal endophyte-infection on tall fescue: growth, photosynthesis, chemical composition and digestibility. *Glob. Change Biol.* 9, 425–437. doi: 10.1046/j.1365-2486.2003.00601.x

Rahman, M., and Saiga, S. (2005). Endophytic fungi (*Neotyphodium coenophialum*) affect the growth and mineral uptake, transport and efficiency ratios in tall fescue (*Festuca arundinacea*). *Plant Soil* 272, 163–171. doi: 10.1007/s11104-004-4682-6

Redman, R. S., Kim, Y. O., Woodward, C., Greer, C., Espino, L., Doty, S. L., et al. (2011). Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLOS ONE* 6:e14823. doi: 10.1371/journal.pone.0014823

Richardson, M. D., Hoveland, C. S., and Bacon, C. W. (1993). Photosynthesis and stomatal conductance of symbiotic and nonsymbiotic tall fescue. *Crop Sci.* 33, 145–149. doi: 10.2135/cropsci1993.0011183X003300010026x

Roberts, C. A., Lacefield, G. D., Ball, D., and Bates, G. (2009). Management to optimize grazing performance in the northern hemisphere. Tall fescue for the twenty-first century. *Agron. Monogr.* 53, 85–99. doi: 10.2134/agronmonogr53.c6

Rua, M. A., McCulley, R. L., and Mitchell, C. E. (2014). Climate drivers, host identity and fungal endophyte infection determine virus prevalence in a grassland ecosystem. *J. Ecol.* 102, 690–699. doi: 10.1111/1365-2745.12238

Rudgers, J. A., Fischer, S., and Clay, K. (2010). Managing plant symbiosis: fungal endophyte genotype alters plant community composition. *J. Appl. Ecol.* 47, 468–477. doi: 10.1111/j.1365-2664.2010.01788.x

Ryan, G. D., Rasmussen, S., Xue, H., Parsons, A. J., and Newman, J. A. (2014). Metabolite analysis of the effects of elevated CO_2 and nitrogen fertilization on the association between tall fescue (*Schedonorus arundinaceus*) and its fungal symbiont *Neotyphodium coenophialum*. *Plant Cell Environ.* 37, 204–212. doi: 10.1111/pce.12146

Saikkonen, K., Faeth, S., Helander, M., and Sullivan, T. (1998). Fungal endophytes: a continuum of interactions with host plants. *Ann. Rev. Ecol. Evol. Syst.* 29, 319–343. doi: 10.1146/annurev.ecolsys.29.1.319

Schardl, C. L., Leuchtmann, A., and Spiering, M. J. (2004). Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* 55, 315–340. doi: 10.1146/annurev.arplant.55.031903.141735

Slaughter, L. C. (2012). *Soil Microbial Community Response to Climate Change: Reults From a Temperate Kentucky Pasture*. Theses and Dissertations, Plant and Soil Sciences, University of Kentucky. Paper 8. Available online at: http://uknowledge.uky.edu/pss_etds/8

Spiers, D. E., Evans, T. J., and Rottinghaus, G. E. (2005). "Interaction between thermal stress and fescue toxicosis: animal models," in *Neotyphodium in Cool-Season Grasses*, eds C. A. Roberts, C. P. West, and D. E. Spiers (Ames, IA: Blackwell Publishing), 243–270.

Spyreas, G., Gibson, D. J., and Middleton, B. A. (2001). Effects of endophyte infection in tall fescue (*Festuca arundinacea*: Poaceae) on community diversity. *Int. J. Plant Sci.* 162, 1237–1245. doi: 10.1086/323274

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). Board-invited review: St. Anthony's fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci.* 89, 1603–1626. doi: 10.2527/jas.2010-3478

Stuedemann, J. A., and Hoveland, C. S. (1988). Fescue endophyte: history and impact on animal agriculture. *J. Prod. Agric.* 1, 39–44. doi: 10.2134/jpa1988.0039

Weber, D. A. (2014). *Grassland Sustainability in Kentucky: Case Studies Quantifying the Effects of Climate Change on Slug Herbivory in Pastures and Different Home Lawn Types on Turf Greenhouse Gas Emissions*. Theses and Dissertations, Plant and Soil Sciences, University of Kentucky. Paper 39. Available online at: http://uknowledge.uky.edu/pss_etds/39

West, C. P. (2007). "Plant influences on endophyte expression," in *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses*, eds A. J. Popay and E. R. Thom (Christchurch: New Zealand Grassland Association (no. 13)), 117–121.

West, C. P., Izekor, E., Turner, K. E., and Elmi, A. A. (1993). Endophyte effects on growth and persistence of tall fescue along a water-supply gradient. *Agron. J.* 85, 264–270. doi: 10.2134/agronj1993.00021962008500020019x

White, J. Jr. (1987). Widespread distribution of endophytes in the *Poaceae*. *Plant Dis.* 71, 340–342. doi: 10.1094/PD-71-0340

Worchel, E., Giauque, H., and Kivlin, S. (2013). Fungal symbionts alter plant drought response. *Microb. Ecol.* 65, 671–678. doi: 10.1007/s00248-012-0151-6

Yates, S. G., and Powell, R. G. (1988). Analysis of ergopeptine alkaloids in endophyte infected tall fescue. *J. Agr. Food Chem.* 36, 337-340. doi: 10.1021/jf00080a023

Young, C. A., Hume, D. E., and McCulley, R. L. (2013). Forages and pastures symposium: fungal endophytes of tall fescue and perennial ryegrass: pasture friend or foe? *J. Anim. Sci.* 91, 2379–2394. doi: 10.2527/jas.2012-5951

Yu, J., Chen, L., Xu, M., and Huang, B. (2012). Effected of elevated CO_2 on physiological responses of tall fescue to elevated temperature, drought stress, and the combined stresses. *Crop Sci.* 52, 1848–1858. doi: 10.2135/cropsci2012.01.0030

Yurkonis, K. A., Shukla, K., Holdenried, J., Hager, H. A., Bolton, K. A., Klironomos, J. N., et al. (2014). Endophytes inconsistently affect plant communities across *Schedonorus arundinaceus* hosts. *Plant Ecol.* 215, 389–398. doi: 10.1007/s11258-014-0309-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2014; accepted: 23 September 2014; published online: 21 October 2014.
*Citation: McCulley RL, Bush LP, Carlisle AE, Ji H and Nelson JA (2014) Warming reduces tall fescue abundance but stimulates toxic alkaloid concentrations in transition zone pastures of the U.S.. Front. Chem. **2**:88. doi: 10.3389/fchem.2014.00088*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 24 November 2014
doi: 10.3389/fchem.2014.00107

Variation in the expression of ergot alkaloids between individual tillers of perennial ryegrass

Wade J. Mace*, Kristy L. Lunn, Navjot Kaur and Catherine M. Lloyd-West

Grasslands Research Centre, AgResearch Limited, Palmerston North, New Zealand

Edited by:
James Levi Klotz, United States Department of Agriculture - Agricultural Research Service, USA

Reviewed by:
Jiaoyang Jiang, University of Wisconsin-Madison, USA
Daniel Panaccione, West Virginia University, USA

****Correspondence:***
Wade J. Mace, Grasslands Research Centre, AgResearch Limited, Private Bag 11008, Palmerston North 4442, New Zealand
e-mail: wade.mace@agresearch.co.nz

Epichloë fungal endophytes of cool season grasses are well-known to produce a range of alkaloids of benefit to the host. Some of these compounds are advantageous to agriculture due to qualities that promote pasture persistence (e.g., the loline class of alkaloids confer insect protection) while others are detrimental to the well-being of grazing livestock. The ergot alkaloids (e.g., ergovaline), produced in ryegrass and tall fescue associations, causes poor animal health in farming regions in many countries around the world and further study is required to improve our knowledge on this class of compounds. Here we present the application of a quantitative LC-MS/MS (liquid chromatography coupled to mass spectrometry) method measuring eight ergot alkaloids (chanoclavine, agroclavine, elymoclavine, lysergol, lysergic acid, ergine, lysergyl-alanine, ergovaline) produced by endophyte infected grasses, to monitor levels in individual tillers from multiple plants of a single cultivar of perennial ryegrass (*Lolium perenne* cv. "Grasslands Samson") infected with a common toxic endophyte strain (*Epichloë festucae* var. *lolii*). Monitoring the expression in individual tillers allows an estimation of the variability within a plant (between tillers) as well as between plants. The study showed that there is significant variation in the concentration of the ergot alkaloids between tillers of a single plant, at or exceeding the level of variation observed between individual plants of a population. This result emphasizes the fundamental importance of robust experimental design and sampling procedures when alkaloid expression assessment is required and these need to be rigorously tailored to the hypothesis being tested.

Keywords: ergot alkaloids, quantitation, *Lolium perenne*, endophyte, liquid chromatography-mass spectroscopy

INTRODUCTION

Fungal endophytes of cool season grasses, the *Epichloë* "endophytes," are well-known for their production of bioactive alkaloids and the benefits these compounds confer to their plant hosts (Bush et al., 1997; Kuldau and Bacon, 2008). Aside from those alkaloids that protect the plant from insect herbivory (Breen, 1994), these endophytes also produce alkaloid toxins active against mammalian herbivores (Schmidt and Osborn, 1993) such as the indole diterpene, lolitrem B and the ergot alkaloid, ergovaline. In an agricultural context these toxins are undesirable due to their detrimental health effects to grazing animals and subsequent profitability of the farming enterprise (Hoveland, 1993).

Artificially created perennial ryegrass/endophyte associations, or novel associations, are a key factor in New Zealand's agribusiness success (Johnson et al., 2013). This is due to the ability to create novel associations that are absent of the detrimental endophyte toxins (most importantly lolitrem B) and the associated animal ill-health while still possessing advantageous traits like insect deterrent compounds (such as peramine). Similarly, tall fescue/endophyte associations, which lack the production of ergovaline, are available in the USA (Bouton et al., 2002). But the dominance of the natural tall fescue/endophyte associations, which produces ergovaline, still has a significant impact on animal health in North American agriculture. Hence there has been (and continues to be) significant research into the effects of the ergot alkaloids (predominantly ergovaline) on animal health (Schmidt and Osborn, 1993), physiological response, mode of action, management practices that can mitigate animal health impacts, seasonal expression (Rogers et al., 2011), and distribution *in planta* (Spiering et al., 2002).

While ergovaline has been the main focus, it is important to consider the other ergot alkaloids that are expressed with ergovaline in the various perennial ryegrass and tall fescue endophyte associations. **Figure 1** shows the a range of the ergot alkaloids that can be detected in extracts of cool season grasses infected with *Epichloë* endophytes. Ergine and lysergyl-alanine are likely byproducts of the lysergyl peptide lactam intermediate (Panaccione et al., 2003), and as shown by the results of the current study, can comprise a significant proportion of the ergot alkaloids expression in perennial ryegrass/endophyte associations.

Research has been undertaken into the distribution of endophyte alkaloids between different plant structures. This has shown that there is a decreasing gradient of ergovaline concentration from the base to the tip of the tiller. However, many previous studies investigated population averages due to multiple samples from individual plants needing to be pooled to supply sufficient material for quantitative analyses. Similarly, research has

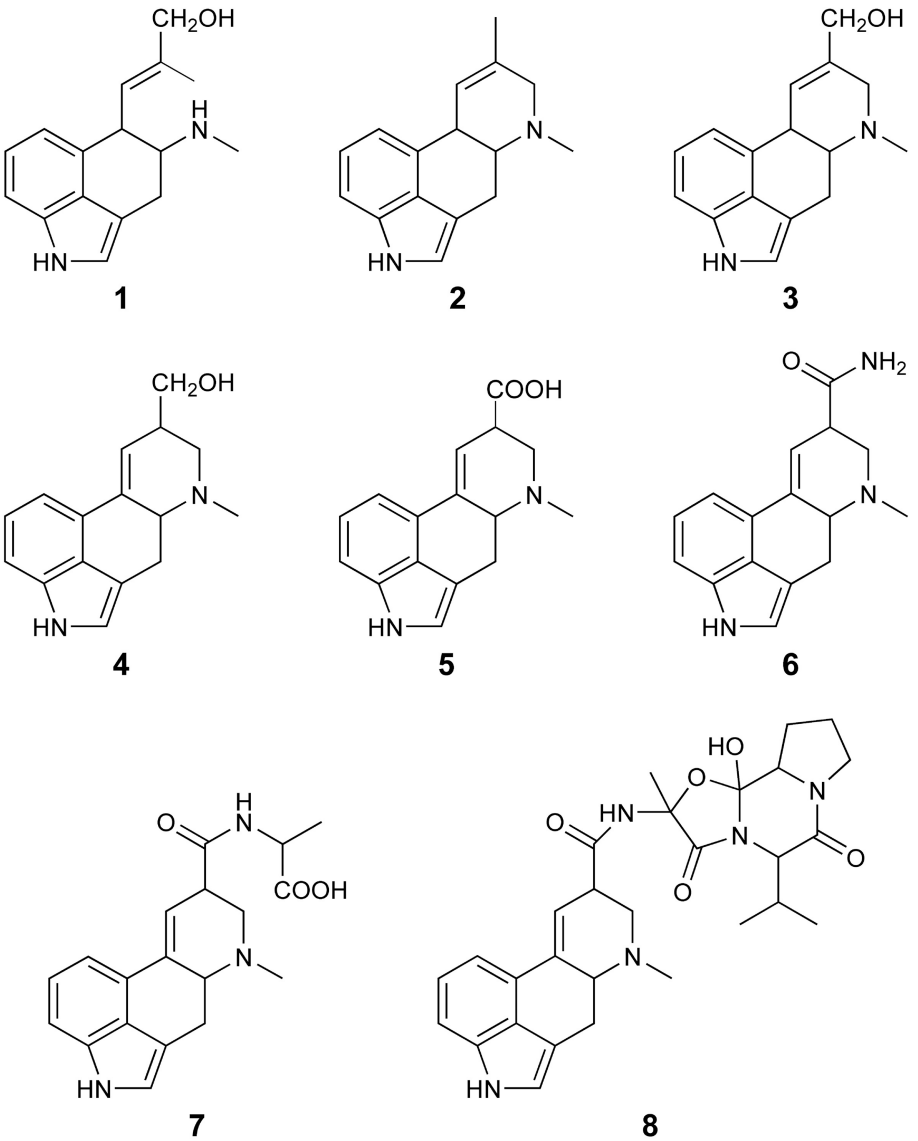


FIGURE 1 | Ergot alkaloids that can be detected in cool season grasses infected with *Epicloë* endophytes; chanoclavine (1), agroclavine (2), elymoclavine (3), lysergol (4), lysergic acid (5), ergine (6), lysergyl-alanine (7), and ergovaline (8).

been undertaken investigating the difference in expression levels between newly emerged leaves and older more mature plant structures, but again this research required the pooling of samples (Spiering et al., 2002, 2005). It has also been shown (Welty et al., 1994) that there are significant differences in expression between plants within a population with the expression difference a heritable trait (Easton et al., 2002). This factor has been exploited in agricultural systems to select for populations with higher or lower ergovaline expression (Adcock et al., 1997; Pennell et al., 2010).

To date no research has been undertaken on individual tillers looking at variation in expression within a single plant. If such variation exists, then a sampling regime for selecting high or low expressing populations would be critical, as would the sampling protocol for assessing any individual plant expression. The aim of the current research was to investigate the range of ergovaline (and selected ergot alkaloids) expression between individual tillers within a plant, and compare this to the range of expression observed between plants in a given population.

MATERIALS AND METHODS

PLANT MATERIAL

Seedlings of perennial ryegrass cv. Samson, infected with a common toxic strain of *Epichloë festucae* var. *lolii* [formally known as *Neotyphodium lolii* (Leuchtmann et al., 2014)] were raised in a glasshouse and their endophyte status assessed after 4 weeks from sowing seed (Simpson et al., 2012). Endophyte-free plants were discarded and endophyte-positive plants (25 individuals)

were transferred to poly-bags (P3/4) with fresh potting mix. Plants were maintained in the glasshouse for approximately 14 months. Plant maintenance included routine watering and application of additional slow release fertilizer as required. Periodic trimming of plants with scissors to 20 mm above ground level to stimulate alkaloid production was conducted with a final trim to ground level conducted in February 2011 (late summer). 10 weeks after the final trim, six tillers were randomly selected from each plant and harvested to ground level, with the basal 40 mm of the tiller frozen, freeze-dried, and weighed. Individual tillers were milled using 2 × 3 mm stainless steel beads (5 m/s, 30 s) in 2 mL plastic vials (FastPrep FP120, Savant Instruments Inc., Farmingdale, NY, USA).

Table 1 | Summary statistics for the ergot alkaloid expression of the plants in the sample population; mean plant expression (mg/kg), minimum and maximum plant expression (mg/kg), coefficient of variation for plant expression.

Alkaloid	Chanoclavine	Ergovaline	Ergine	Lysergyl-alanine
Mean	0.38	5.5	5.81	1.2
Min	0.18	0.65	0.14	0.47
Max	0.56	13.88	12.65	1.98
CV (%)	28.4	53.2	71.3	33.4

SAMPLE EXTRACTION

Tillers were extracted using a modification of previous methods (Mol et al., 2008; Rasmussen et al., 2012). Briefly; milled tiller samples were extracted with 500 μL of 75% methanol (containing ergotamine (0.54 μg/mL) as an internal standard) for 1 h in the dark. Samples were centrifuged (5000 g^1, 5 min) and the supernatant transferred to 2 mL amber HPLC vials via a 0.45 μm syringe filter (PVDF).

ERGOT ALKALOID ANALYSIS

Samples were analyzed according to Rasmussen et al. (2012), using a Thermo TSQ triple-quadrupole mass spectrometer equipped with an Accela 1250 HPLC system. Chromatography was achieved using a Kinetex XB-C18 column (100 × 2.1 mm, 2.6 μ, Phenomenex). The method allows the quantification of chanoclavine (1), agroclavine (2), elymoclavine (3), lysergol (4), lysergic acid (5), ergine (6), lysergyl-alanine (7), ergovaline (8), and epimers thereof. Peak integration was conducted using LCQuan 2.7 (Thermo Fisher Scientific Inc., San Jose, CA, USA) with AgResearch in-house software used to determine alkaloid concentrations from peak areas and calculated standard curves.

Table 2 | Mean concentrations (mg/kg) (and coefficients of variation) of ergot alkaloids detected in tillers of individual plants of the population.

Plant	Chanoclavine		Ergovaline		Ergine		Lysergyl-alanine	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
01	0.43	17.7	5.4	71.9	7.7	52.6	1.4	65.2
02	0.42	34.8	8.1	29.5	0.1	155.1	0.8	37.4
03	0.50	10.0	7.6	14.9	10.3	8.3	1.9	14.3
04	0.37	22.4	13.9	9.8	1.3	35.1	1.0	21.0
05	0.20	28.8	2.5	21.6	6.2	12.1	0.7	13.6
06	0.25	66.8	0.6	90.8	7.1	64.6	1.0	72.4
07	0.49	34.8	5.5	26.3	9.0	12.5	1.6	17.0
08	0.29	20.9	6.6	40.9	4.2	22.4	1.2	37.8
09	0.36	35.1	11.2	30.5	10.7	26.7	1.6	15.1
10	0.27	58.6	3.7	42.2	2.7	60.0	0.7	50.8
11	0.44	25.5	5.5	29.5	12.6	14.3	2.0	14.5
12	0.20	59.6	1.7	26.9	8.3	43.1	1.1	48.2
13	0.28	19.0	5.2	15.6	10.0	32.3	0.8	32.6
14	0.23	49.2	4.9	35.1	0.5	55.8	1.0	34.1
15	0.56	39.8	10.2	30.0	0.2	117.0	1.5	24.2
16	0.50	48.0	3.2	39.3	6.2	35.5	1.2	45.4
17	0.30	17.3	5.4	31.7	0.2	121.2	0.8	41.6
18	0.38	31.8	1.9	53.6	3.6	46.1	0.7	30.3
19	0.18	38.6	6.1	31.9	ND	–	0.5	46.2
20	0.39	25.9	4.7	27.4	8.2	11.1	0.9	18.3
21	0.43	19.2	5.2	22.1	ND	–	1.1	25.9
22	0.34	26.7	5.4	22.4	9.6	26.7	1.6	29.0
23	0.45	21.6	4.6	35.1	9.1	17.4	1.4	34.5
24	0.50	12.9	4.2	33.0	7.0	20.6	1.4	22.4
25	0.43	37.6	4.4	34.5	5.9	25.5	1.5	51.6

ND, Not Detected.

DATA AND STATISTICAL ANALYSIS

A weighted average of the individual tiller alkaloid concentrations was used to calculate a plant concentration for the purposes of comparing variation between plants. The formula $x_{plant} = \sum_{i=1}^{n} w_i x_i / \sum_{i=1}^{n} w_i$ describes the calculation, where x_i = concentration of analyte x in tiller i, w_i = weight of tiller i, and x_{plant} = calculated concentration of analyte x in the plant.

Investigation of the proportions of variance between plant and within plant was preformed via a fully nested ANOVA of the alkaloid expression from individual tillers. A power analysis for determining the number of tillers that would be required to be harvested per plant was undertaken using a One-Way ANOVA model. Statistical analyses were conducted using Minitab® 16 (Minitab Pty Ltd, Sydney, Australia).

RESULTS

ERGOT ALKALOID DETECTION AND QUANTITATION

Not all alkaloids from the ergot alkaloid biosynthetic pathway were detected above the limit of quantitation; only chanoclavine, ergovaline, ergine, and lysergyl-alanine exceeded the limit of quantification (0.1 mg/kg *in planta*). Therefore, only these ergot alkaloids were used to evaluate intra- and inter-plant variation in alkaloid expression.

VARIATION IN ERGOT ALKALOID EXPRESSION

Variation between plants

There was a high level of variation in the expression of the detected alkaloids between plants in the population. Chanoclavine showed the least change in expression level (3 fold range between plants) compared to ergine which showed the highest with a greater than 25 fold range in expression. This was reflected in the coefficients of variation, where ergine showed the highest (71.3%) and chanoclavine the lowest (28.4%) (**Table 1**).

Variation within plants

Within the plants (between tillers of the same plant) there were also observations of a high level of variation in alkaloid expression. Coefficients of variation for any plant/alkaloid combination ranged from 8.3% to 90.8%. **Table 2** shows the mean concentration and coefficient of variation for each plant/alkaloid combination.

To investigate the proportions of variance between plant and within plant, a fully nested ANOVA was undertaken, summarized in **Table 3**. For ergovaline and ergine, the main contributor of variance was variation in expression between plants. Chanoclavine showed the opposite pattern, with the main contributor being variation in expression within a plant. Lysergyl-alanine showed a more balanced distribution of variance.

For chanoclavine, as the concentration increased within a plant, the variance remained relatively constant. The average variance of the lowest five plants was similar to that of the highest five plants. This can be seen in the consistency of the size of the confidence intervals of the means of each plant (**Figure 2**). This compares to ergovaline, where the variance is greater for the higher expressing plants than for the lower expressing plants (**Figure 3**).

Table 3 | Percentage of variance component attributed to between plant or within plant variation for each ergot alkaloid.

Alkaloid	Variance attributed to	
	Between plant (%)	Within plant (%)
Chanoclavine	38.7	61.3
Ergovaline	70.9	29.1
Ergine	79.6	20.4
Lysergyl-alanine	43.5	56.5

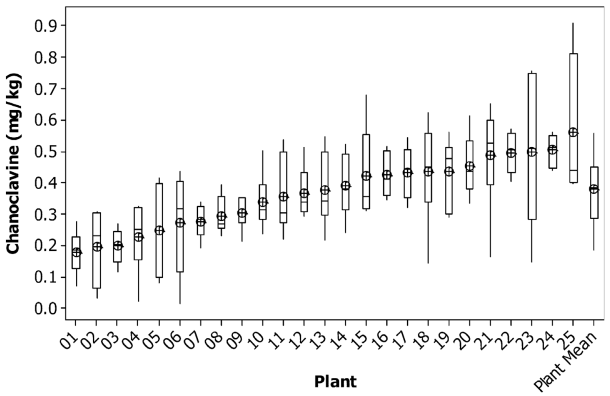


FIGURE 2 | Box plot of chanoclavine concentration for individual tillers of each plant with mean tiller concentration (open circles) and boxes showing interquartile range. Plants are ranked from lowest to highest mean tiller concentration. Also shown are the corresponding parameters for the weighted mean of each plant.

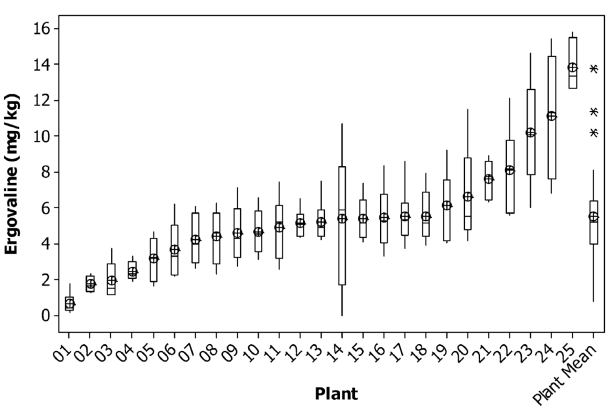


FIGURE 3 | Box plot of ergovaline concentration for individual tillers of each plant with mean tiller concentration (open circles) and boxes showing interquartile range. Plants are ranked from lowest to highest mean tiller concentration. Also shown are the corresponding parameters for the weighted mean of each plant. Outliers are indicated by an asterisk.

Using the data from the current study, power tables have been generated to determine the number of tillers that would be required to be harvested per plant to be confident in selecting the highest or lowest expressing chanoclavine or ergovaline plants of a

Table 4 | Power table indicating the number of tillers required to select a population of plants with indicated difference in mean chanoclavine expression (mg/kg) from the population mean.

Number of tillers	Plants in selection				
	5	10	15	20	25
2	0.82	0.85	0.88	0.91	0.94
4	0.43	0.48	0.51	0.54	0.56
6	0.33	0.37	0.40	0.42	0.44
8	0.28	0.31	0.34	0.36	0.37
10	0.24	0.28	0.30	0.32	0.33
12	0.22	0.25	0.27	0.29	0.30

Calculated for a 5% level of significance and a power of 0.8 using the standard deviation of the study population (0.15).

Table 5 | Power table indicating the number of tillers required to select a population of plants with indicated difference in mean ergovaline expression (mg/kg) from the population mean.

Tillers harvested	Plants in selection				
	5	10	15	20	25
2	4.2	4.4	4.5	4.7	4.8
4	2.2	2.5	2.6	2.8	2.9
6	1.7	1.9	2.1	2.2	2.3
8	1.4	1.6	1.7	1.8	1.9
10	1.3	1.4	1.5	1.6	1.7
12	1.1	1.3	1.4	1.5	1.5

Calculated for a 5% level of significance and a power of 0.8 using the standard deviation of plants with the lowest 20% expression within the study population (0.77).

Table 6 | Power table indicating the number of tillers required to select a population of plants with indicated difference in mean ergovaline expression (mg/kg) from the population mean.

Number of tillers	Plants in selection				
	5	10	15	20	25
2	12.5	12.8	13.3	13.8	14.2
4	6.5	7.2	7.8	8.2	8.5
6	5.0	5.6	6.0	6.4	6.7
8	4.2	4.8	5.1	5.4	5.7
10	3.7	4.2	4.5	4.8	5.0
12	3.3	3.8	4.1	4.3	4.5

Calculated for a 5% level of significance and a power of 0.8 using the standard deviation of plants with the highest 20% expression within the study population (2.27).

population (**Tables 4**, **5**, **6**). From these power tables we can determine that it is not possible to select a set of low or high expressing chanoclavine individuals with 12 or fewer tillers, as the population mean is 0.37 ppm, with the lowest (0.18 ppm) and highest (0.56 ppm) expressing plants expressing within 0.2 ppm of the population mean. However, for ergovaline, it is possible to make selections within the population using relatively few tillers. Due to the smaller standard deviation at the lower concentration levels, it is possible that a sample of four tillers per plant will allow selection of a sub-set of plants with a mean 3 ppm lower than the population mean (equivalent to the bottom 20% of plants in the test population). To do the same selection for a sub-set of plants with a mean equivalent to the top 20% of plants in the test population (4.7 ppm greater than the population mean) would require sampling of more tillers (due to the higher standard deviation), but could still be achieved with 12 tillers per plant for a selection of 25 plants.

DISCUSSION

It has long been understood that there is significant variation in endophyte alkaloid expression between plants in a population (Easton et al., 2002). It is generally accepted that this is driven by the host genetics, and is a heritable response that can be selected for in a plant breeding program. What is not clearly understood is how much variation in endophyte alkaloid expression there is between tillers within a plant. This can have implications to plant breeding (through accuracy of plant selections) and in experimental design when investigating endophyte alkaloid expression, where it is necessary to ensure sufficient sample sizes to generate data that is statistically robust.

The results of this study have shown that it is important to not only have an understanding of the variation in the expression of endophyte alkaloids between plants in a population, but also the variation within the plants of the population. Unless the whole plant is being sampled (which is not always possible depending on the experimental design) the variation within the plants can have an impact of the quality of the data generated and the final conclusions of the study. Hence the variability within plants should be considered as part of the experimental design process.

ACKNOWLEDGMENTS

The authors would like to thank Stuart Card and Siva Ganesh (AgResearch Ltd) for reviewing the manuscript and providing useful discussion.

REFERENCES

Adcock, R. A., Hill, N. S., Bouton, J. H., Boerma, H. R., and Ware, G. O. (1997). Symbiont regulation and reducing ergot alkaloid concentration by breeding endophyte-infected tall fescue. *J. Chem. Ecol.* 23, 691–704. doi: 10.1023/B:JOEC.0000006404.33191.60

Bouton, J. H., Latch, G. C. M., Hill, N. S., Hoveland, C. S., McCann, M. A., Watson, R. H., et al. (2002). Reinfection of tall fescue cultivars with non-ergot alkaloid–producing endophytes. *Agron. J.* 94, 567–574. doi: 10.2134/agronj2002.5670

Breen, J. P. (1994). Acremonium endophyte interactions with enhanced plant resistance to insects. *Annu. Rev. Entomol.* 39, 401–423. doi: 10.1146/annurev.en.39.010194.002153

Bush, L. P., Wilkinson, H. H., and Schardl, C. L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol.* 114, 1–7.

Easton, H. S., Latch, G. C. M., Tapper, B. A., and Ball, O. J. P. (2002). Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. *Crop Sci.* 42, 51–57. doi: 10.2135/cropsci2002.0051

Hoveland, C. S. (1993). Importance and economic significance of the Acremonium endophytes to performance of animals and grass plant. *Agric. Ecosyst. Environ.* 44, 3–12. doi: 10.1016/0167-8809(93)90036-O

Johnson, L., de Bonth, A. M., Briggs, L., Caradus, J., Finch, S., Fleetwood, D., et al. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Divers.* 60, 171–188. doi: 10.1007/s13225-013-0239-4

Kuldau, G., and Bacon, C. (2008). Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. *Biol. Control* 46, 57–71. doi: 10.1016/j.biocontrol.2008.01.023

Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. (2014). Nomenclatural realignment of Neotyphodium species with genus Epichloë. *Mycologia* 106, 202–215. doi: 10.3852/13-251

Mol, H. G. J., Plaza-Bolaños, P., Zomer, P., de Rijk, T. C., Stolker, A. A. M., and Mulder, P. P. J. (2008). Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes. *Anal. Chem.* 80, 9450–9459. doi: 10.1021/ac801557f

Panaccione, D. G., Tapper, B. A., Lane, G. A., Davies, E., and Fraser, K. (2003). Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. *J. Agric. Food Chem.* 51, 6429–6437. doi: 10.1021/jf0346859

Pennell, C., Rolston, M., De Bonth, A., Simpson, W., and Hume, D. (2010). Development of a bird-deterrent fungal endophyte in turf tall fescue. *N. Z. J. Agric. Res.* 53, 145–150. doi: 10.1080/00288231003777681

Rasmussen, S., Lane, G. A., Mace, W., Parsons, A. J., Fraser, K., and Xue, H. (2012). The use of genomics and metabolomics methods to quantify fungal endosymbionts and alkaloids in grasses. *Methods Mol. Biol.* 860, 213–226. doi: 10.1007/978-1-61779-594-7_14

Rogers, W. M., Roberts, C. A., Andrae, J. G., Davis, D. K., Rottinghaus, G. E., Hill, N. S., et al. (2011). Seasonal fluctuation of ergovaline and total ergot alkaloid concentrations in tall fescue regrowth. *Crop Sci.* 51, 1291–1296. doi: 10.2135/cropsci2010.07.0402

Schmidt, S. P., and Osborn, T. G. (1993). Effects of endophyte-infected tall fescue on animal performance. *Agric. Ecosyst. Environ.* 44, 233–262. doi: 10.1016/0167-8809(93)90049-U

Simpson, W. R., Schmid, J., Singh, J., Faville, M. J., and Johnson, R. D. (2012). A morphological change in the fungal symbiont *Neotyphodium lolii* induces dwarfing in its host plant *Lolium perenne. Fungal Biol.* 116, 234–240. doi: 10.1016/j.funbio.2011.11.006

Spiering, M. J., Davies, E., Tapper, B. A., Schmid, J., and Lane, G. A. (2002). Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. *J. Agric. Food Chem.* 50, 5856–5862. doi: 10.1021/jf025602b

Spiering, M. J., Lane, G. A., Christensen, M. J., and Schmid, J. (2005). Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in *Lolium perenne* plants. *Phytochemistry* 66, 195–202. doi: 10.1016/j.phytochem.2004.11.021

Welty, R. E., Craig, A. M., and Azevedo, M. D. (1994). Variability of ergovaline in seeds and straw and endophyte infection in seeds among endophyte-infected genotypes of tall fescue. *Plant Dis.* 78, 845–849. doi: 10.1094/PD-78-0845

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 September 2014; accepted: 05 November 2014; published online: 24 November 2014.

Citation: Mace WJ, Lunn KL, Kaur N and Lloyd-West CM (2014) Variation in the expression of ergot alkaloids between individual tillers of perennial ryegrass. Front. Chem. ***2****:107. doi: 10.3389/fchem.2014.00107*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 08 September 2014
doi: 10.3389/fchem.2014.00076

Ergovaline stability in tall fescue based on sample handling and storage methods

Krista Lea[1], Lori Smith[2], Cynthia Gaskill[2], Robert Coleman[3] and S. Ray Smith[1]*

[1] *Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA*
[2] *Veterinary Diagnostic Laboratory, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA*
[3] *Department of Animal and Food Science, University of Kentucky, Lexington, KY, USA*

Edited by:
Darrin Smith, Eastern Kentucky University, USA

Reviewed by:
Alexander Shekhtman, University at Albany, State University of New York, USA
Zhongping Tan, University of Colorado Boulder, USA

***Correspondence:**
S. Ray Smith, Department of Plant and Soil Sciences, University of Kentucky, N-222E Ag. Science Center North, Lexington, KY 40546, USA
e-mail: raysmith1@uky.edu

Ergovaline is an ergot alkaloid produced by the endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams) found in tall fescue [*Schedonorus arundinacea* (Schreb.) Dumort.] and blamed for a multitude of livestock disorders. Ergovaline is known to be unstable and affected by many variables. The objective of this study was to determine the effect of sample handling and storage on the stability of ergovaline in tall fescue samples. Fresh tall fescue was collected from a horse farm in central Kentucky at three harvest dates and transported on ice to the University of Kentucky Veterinary Diagnostic Laboratory. Plant material was frozen in liquid nitrogen, milled and mixed before being allocated into different sub-samples. Three sub-samples were assigned to each of 14 sample handling or storage treatments. Sample handling included increased heat and UV light to simulate transportation in a vehicle and on ice in a cooler per standard transportation recommendations. Storage conditions included storage at 22°C, 5°C, and −20°C for up to 28 days. Each sub-sample was then analyzed for ergovaline concentration using HPLC with fluorescence detection and this experiment was repeated for each harvest date. Sub-samples exposed to UV light and heat lost a significant fraction of ergovaline in 2 h, while sub-samples stored on ice in a cooler showed no change in ergovaline in 2 h. All sub-samples stored at 22°C, 5°C, and −20°C lost a significant fraction of ergovaline in the first 24 h of storage. There was little change in ergovaline in the freezer (−20°C) after the first 24 h up to 28 days of storage but intermittent losses were observed at 22°C and 5°C. To obtain results that most closely represent levels in the field, all samples should be transported on ice to the laboratory immediately after harvest for same day analysis. If immediate testing is not possible, samples should be stored at −20°C until analysis.

Keywords: tall fescue, *Neotyphodium coenophialum*, ergot alkaloids and ergovaline, transportation and storage, HPLC with fluorescence detection

INTRODUCTION

Tall fescue is a cool season perennial grass native to Europe and well adapted to much of the United States, particularly in the southeast where it covers an estimated 35 million acres of livestock pasture, hay fields and roadways (Ball et al., 2007). Most tall fescue plants in the southeastern US are known to be infected with an endophyte, *Neotyphodium coenophialum* (Morgan-Jones and Gams) that has formed a mutualistic relationship with the plant. The endophyte lives in the intercellular spaces of the plant (Bacon and Siegel, 1988) and produces many compounds. Some of these compounds benefit the plant and result in increased drought and disease tolerance (Arachevaleta et al., 1989; Gwinn and Gavin, 1992). However, the endophyte also produces a range of alkaloids toxic to foraging animals.

Ergovaline, a strong vasoconstrictor, is the major ergopeptine alkaloid produced by the endophyte and is believed to be the primary cause of fescue toxicosis in livestock (Lyons et al., 1986; Belesky et al., 1988; Klotz et al., 2007). Extensive research has suggested that ingestion of endophyte infected tall fescue plants by cattle results in increased core body temperature (Aldrich et al., 1993), decreased average daily gain (Hopkins and Alison, 2006), rough hair coats (McClanahan et al., 2008), and decreased weaning weights (Peters et al., 1992) that result in an annual loss of $600 million in the US beef industry (Hoveland, 1993). Pregnant horses often experience prolonged gestation (Monroe et al., 1988), difficulty foaling (Putnam et al., 1991), low milk production (Kosanke et al., 1987), and decreased breeding efficiency (Brendemuehl et al., 1994). Due to the economic impacts of endophyte infected tall fescue on livestock, monitoring ergovaline in tall fescue from livestock pastures is becoming common for research and farm situations.

Ergovaline in solution is known to be unstable due to isomerization, hydrolysis and sensitivity to light, air, acids, and bases (Garner et al., 1993). However, very little information is known about the effects of sample handling. The objectives of this research are to evaluate the stability of ergovaline

in tall fescue material stored over time under various conditions and to establish ideal sample handling and transportation recommendations.

MATERIALS AND METHODS

All samples were gathered from a single pasture on a horse farm located near Lexington, KY on the following dates: 1 May 2012, 21 August 2012, and 11 June 2013. The pasture was ∼0.4 ha in size and contained primarily cool season grasses including tall fescue, Kentucky bluegrass (*Poa pretensis* L.), and orchardgrass (*Dactylis glomerata* L.). The pasture was seeded with orchardgrass and Kentucky bluegrass in the fall of 2011 to increase vegetative cover. Due to the recent overseeding and an excessive distance from the barn, this paddock was ungrazed by horses throughout the entire sampling period, but was routinely mowed to ∼12.5 cm.

The pasture had been tested several times for tall fescue endophyte infection from 2009 through 2013, and the infection rates ranged from 80 to 100%. Endophyte infection levels were established by collecting ∼20 tillers from 10 locations throughout the field for endophyte testing at the University of Kentucky Regulatory Services Laboratory (immunoblot endophyte test kits, Agrinostics, Athens, GA).

Ergovaline quantitation had also been performed periodically on tall fescue found in this pasture and was observed to consistently contain more than 500 ppb ergovaline during the growing season from 2008 to 2012.

HARVEST AND SAMPLING FOR ERGOVALINE ANALYSIS

On day 0 of each harvest period, ∼2 kg of fresh tall fescue was harvested using a rice knife at 5–7.5 cm above the soil surface. Plant material was placed on ice and transported immediately to the laboratory (less than 1 h). Once at the laboratory, the sample was cut into 2.5–7.5 cm lengths, frozen using liquid nitrogen and milled while frozen. Milling was performed using a Stein Mill (Steinlite Corporation, Atchison, KS). Milled material was then mixed by hand and separated into 42 individual sub-samples, each ∼5 g. Sub-samples were placed into 120 ml whirl-pak bags and immediately placed into their assigned treatments with three sub-samples assigned to each treatment. Average time from harvest to treatment application was 2.5–4 h and samples were kept chilled or frozen the entire time.

TREATMENTS

Treatments were designed to simulate real world situations (**Table 1**). The Control treatment was placed in the freezer (−20°C) for 2 h before sample preparation began. The Ice treatment was designed to simulate a sample that had been collected by a producer or county agent and transported to the laboratory on ice. This was accomplished by placing the appropriate sub-samples in a cooler on ice for 2 h. Similarly, the Light + Heat treatment was designed to simulate the sample transported inside a vehicle under hot and sunny weather conditions. These sub-samples were exposed to 38 ± 1.5°C and UV light by placing them under full spectrum bulbs (three GE Residential Ecolux 40W bulbs, three Hatch Lighting F40T12/D/DX 40W bulbs and one 60 watt incandescent bulb) for 2 h. The entire apparatus was covered with vinyl and aluminum foil to contain heat. Storage treatments included storage at ambient temperature (22°C), refrigerator (5°C), and freezer (−20°C) temperatures for varied amounts of time. These various storage locations and durations were meant to simulate storage at a testing laboratory. On the day of analysis, samples were removed from their treatment locations and set on the lab bench for less than 10 min before sample preparation began.

Table 1 | List of treatments for sample handling and storage.

Treatment abbreviation	Treatment	Time of treatment	Temperature (°C)
C	Control	2 h	−20
I	Ice	2 h	1
LH	Light + Heat	2 h	38.5
A1	Ambient temp	1 Day	22
A2	Ambient temp	2 Days	22
A3	Ambient temp	3 Days	22
R1	Refrigerator	1 Day	5
R3	Refrigerator	3 Days	5
R6	Refrigerator	6 Days	5
F1	Freezer	1 Day	−20
F7	Freezer	7 Days	−20
F14	Freezer	14 Days	−20
F21	Freezer	21 Days	−20
F28	Freezer	28 Days	−20

QUANTITATION BY HPLC AND FLUORESCENCE DETECTION

The analytical method for this experiment was as adapted from Spiering et al. (2002) at the University of Kentucky Veterinary Diagnostic Laboratory for research and diagnostic purposes to assess total ergovaline (ergovaline + ergovalinine) content in tall fescue.

Ergovaline and ergovalinine are stereoisomers that are often present in solution. Degree of epimerization from ergovaline to ergovalinine depends on many variables including type of extraction solvent, amount of light and heat to which the solution is exposed and the amount of time the ergot alkaloid has been dissolved in solution (Smith and Shappell, 2002). Ergotamine and its epimer, ergotaminine, are susceptible to the same variables.

Chemicals and reagents

Ergotamine tartrate was purchased in solid, purified form (>97%) from Sigma-Aldrich (St. Louis, MO, USA). Ergovaline tartrate was obtained as a custom synthetic product (>93%) from F. T. Smith (Pharmaceutical Sciences, Auburn University, Auburn, AL, USA). Ammonium acetate, methanol, acetronitrile, and 2-propanol (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). D,L—lactic acid (85%) was purchased from Acros Organics (New Jersey, USA). Purified water was prepared using a Mega-Pure MP-6A distillation system and an EASYpure II RF water conditioner (Barnstead, Dubuque, IA, USA).

For each sub-sample, 1.00 ± 0.25 g of plant material was placed in a 15 ml disposable conical centrifuge tube and the exact mass was recorded; 4950 μL of internal extraction solution [50%

aqueous 2-propanol/1% (v/v) lactic acid] was added, as well as 50 µL 10 µM ergotamine. Ergotamine was used as an internal standard because it is structurally and chemically similar to ergovaline, therefore losses of ergotamine through the sample preparation process are assumed to be similar to ergovaline losses. The sub-sample was then vortexed for ∼30 s to completely wet the plant material with extraction solvent. All samples analyzed concurrently were then placed on a mixer/rotator [Multi Mixer and Rotator (UNICO MTR22), United Products & Instruments, Inc.], covered with aluminum foil to minimize exposure to light and rotated for 1 h. Samples were centrifuged for 12 min at 3000 rpm. The supernatant was removed, passed through a PVDF syringe filter (0.45 µm, 25 mm diameter) to remove particulates and dispensed into silanized, amber 2-mL autosampler vials.

For each day of analysis, four standard solutions with increasing ergovaline concentrations ranging from 0 to 270 ppb and a reagent blank were prepared and analyzed. The standard solutions all contained 58.2 ppb ergotamine. The reagent blank was prepared using an empty 15 mL centrifuge tube, adding all solutions as if the tube contained a sample and treated it as a sample throughout the analysis.

Moisture content

A separate ∼1 g portion of plant material from each sub-sample was weighed, dried for 24 h at 100°C in a forced-air drying oven (Isotemp Oven, Thermo Fisher Scientific, Waltham, MA, USA) and weighed again to determine moisture content of the original sample. Moisture content of each sub-sample was determined on the date of analysis to correct for the moisture contribution to the total subsample mass.

Chromatography and fluorescence analysis

Reverse-phase chromatographic separation of ergovaline, ergotamine and their respective epimers from fescue matrix components was achieved using a U-3000 dual pump high pressure liquid chromatography (HPLC) system equipped with a RF-2000 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase was delivered at a constant flow rate of 1.0 mL/min. The instrument was equipped with an Acclaim 120 C18 HPLC Analytical Column (3 µm particles, 120 Å; 4.6 mm ID × 100 mm, Thermo Fisher Scientific, Waltham, MA, USA), preceded by a Security Guard Column Cartridge System (C18; 4 mm × 3.0 mm; Phenomenex, Torrance, CA, USA). The column compartment was set and maintained at 30°C throughout the analysis. Binary mobile phases consisted of 75% (v/v) 0.1 M ammonium acetate in acetonitrile (mobile phase A) and 25% (v/v) 0.1 M ammonium acetate in acetonitrile (mobile phase B), each of which was degassed by helium sparging for ∼10 min prior to use. Sample extracts and standard solutions (20 µL) were injected into the initial gradient conditions of 95% mobile phase A/5% mobile phase B. Immediately following injection the mobile phase B was increased at a linear rate to 20% over the next 20 min, then further increased to 50% over the next 15 min and finally increased to 70% over the next 5 min. The gradient profile was then held at 30% mobile phase A/70% mobile phase B for 7 min to wash the analytical column of any residual nonpolar matrix components. At 47 min after injection, the initial gradient conditions were resumed and the column was re-equilibrated in preparation for the next injection. Fluorescence detection parameters included excitation and emission wavelengths set to 310 and 410 nm, respectively, and each sample required 52 min to run. The autosampler tray was covered throughout analysis to prevent light exposure.

Calibration curves were produced by plotting the instrument response against the ergovaline concentration of each standard solution. For this method, the instrument response was defined as the peak area ratio of the total ergovaline detected (e.g., the sum of the peak areas for ergovaline and ergovalinine) to the total ergotamine detected (e.g., the sum of ergotamine and its epimer). A linear regression was fit to the standard data and, using the resulting trendline equation, the total ergovaline concentration was calculated for each fescue sample. The final result was corrected for moisture content and the reported values are presented on a dry matter basis. The term "ergovaline" in all results will refer to ergovaline + ergovalinine.

STATISTICS

All results were analyzed in SAS 9.3 using the general linear models and least-squares means procedures (PROC GLM and lsmeans). A significant difference in ergovaline concentrations was observed across harvests, therefore treatments were compared within the same harvest only and considered significant at $P < 0.05$.

RESULTS

For the May 2012 harvest, no difference in ergovaline concentration was observed between the Ice treatment and the Control (**Figure 1**); Samples exposed to light + heat for 2 h experienced significant losses in ergovaline. Samples stored at 22°C, 5°C, and −20°C for 24 h contained less ergovaline compared to the control (22, 27, and 17%, respectively), (**Table 2**). Losses

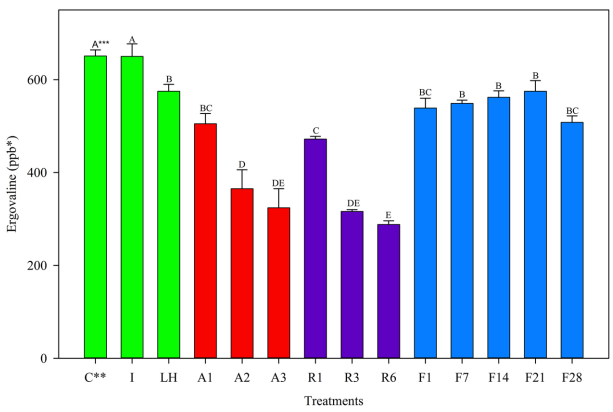


FIGURE 1 | Ergovaline concentration of all tall fescue sub-samples in various storage conditions from May 2012 harvest. *Parts per billion. **Treatment abbreviations are as follows: C, Control; I, Ice; LH, Light + Heat; A1, Ambient Day 1; A2, Ambient Day 2; A3, Ambient Day 3; R1, Refrigerator Day 1; R3, Refrigerator Day 3; R6, Refrigerator Day 6; F1, Freezer Day 1; F7, Freezer Day 7; F14, Freezer Day 14; F21, Freezer Day 21; and F28, Freezer Day 28. ***Matching letters indicate no statistical difference at a significance level of $P < 0.05$.

Table 2 | Ergovaline concentrations after 24 h of storage at various temperatures for three harvests.

	Ergovaline (ppb*)					
	May 2012		August 2012		June 2013	
Control	651	A†	1606	A	524	A
Ambient (22°C)	505	B	638	B	394	B
Refrigerator (5°C)	472	B	648	B	386	B
Freezer (−20°C)	539	B	693	B	423	B

*Parts per billion.

†Matching letters within a column indicate no statistical difference ($p < 0.05$) within a single harvest.

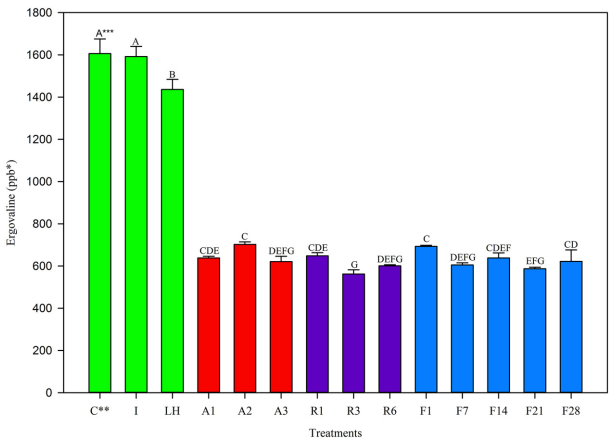


FIGURE 2 | Ergovaline concentration of all tall fescue sub-samples in various storage conditions from August 2012 harvest. *Parts per billion. **Treatment abbreviations are as follows: C, Control; I, Ice; LH, Light + Heat; A1, Ambient Day 1; A2, Ambient Day 2; A3, Ambient Day 3; R1, Refrigerator Day 1; R3, Refrigerator Day 3; R6, Refrigerator Day 6; F1, Freezer Day 1; F7, Freezer Day 7; F14, Freezer Day 14; F21, Freezer Day 21; and F28, Freezer Day 28. ***Matching letters indicate no statistical difference at a significance level of $P < 0.05$.

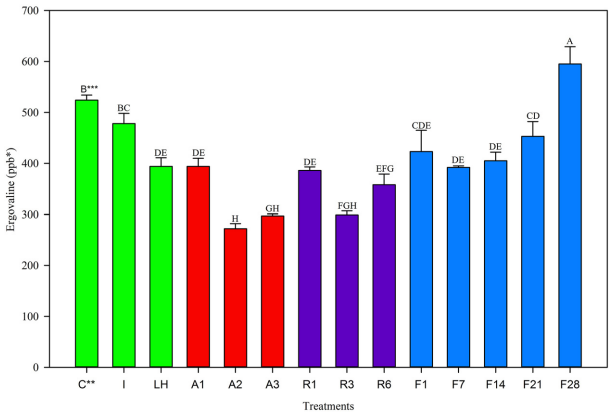


FIGURE 3 | Ergovaline concentration of all tall fescue sub-samples in various storage conditions from June 2013 harvest. *Parts per billion. **Treatment abbreviations are as follows: C, Control; I, Ice; LH, Light + Heat; A1, Ambient Day 1; A2, Ambient Day 2; A3, Ambient Day 3; R1, Refrigerator Day 1; R3, Refrigerator Day 3; R6, Refrigerator Day 6; F1, Freezer Day 1; F7, Freezer Day 7; F14, Freezer Day 14; F21, Freezer Day 21; and F28, Freezer Day 28. ***Matching letters indicate no statistical difference at a significance level of $P < 0.05$.

experienced in the first 24 h were not different from one another, regardless of storage temperature. Ergovaline concentrations did not vary throughout 28 days of −20°C storage in May 2012.

In the August 2012 harvest, similar observations were made to May 2012. The Control and Ice treatment showed equivalent ergovaline concentration (**Figure 2**) while the light + heat lost significant ergovaline. Storage losses in the first 24 h (**Table 2**) were significant from the control, and not different from one another (60% at 22°C and 5°C, 57% at −20°C). Little variation in ergovaline concentration was observed over 28 days of −20°C storage.

Trends in the June 2013 harvest were similar to the two previous harvests. The Control and Ice treatment were not different from one another (**Figure 3**), however the light+heat was reduced. Twenty-four hour storage losses were significantly different than the Control but not different from one another and were 25% at 22°C, 26% at 5°C, and 19% at −20°C (**Table 2**). No change in ergovaline concentration was observed in samples stored at −20°C for 21 days. Interestingly, ergovaline concentration was higher at day 28; laboratory procedures were carefully reviewed to insure that this anomaly was not related to sample preparation or calculation errors.

DISCUSSION

These results indicated that ergovaline in fresh plant material that had been frozen and milled was not consistently stable. In all three harvests, there was no significant loss of ergovaline in the first 2 h when the sub-samples were kept on ice in a cooler, compared to the control. However exposure to heat and UV light resulted in a significant loss after 2 h for all three harvests. This suggested that during transportation, sample handling is an important factor. Samples for research and diagnostics should be transported on ice in a cooler to the testing laboratory as soon as possible.

Ergovaline concentrations were reduced in all material in the first 24 h of storage at all storage temperatures. For each harvest, losses observed at −20°C were not different from losses observed at 5°C or 22°C. This suggests that in the first 24 h, ergovaline is not stable in harvested material. Few laboratories have the capabilities to analyze tall fescue samples the same day as harvest, therefore it is likely that ergovaline levels in most research and diagnostic samples are reported lower than what is in the field at the time of harvest.

Twenty-four hour losses observed in May and June harvests when stored at −20°C were similar in magnitude (17 and 19%, respectively) compared to the August harvest (57%). It is possible that ergovaline stability in the plant is dependent on the time of year. However, the initial concentrations of the control treatments for the May and June harvests (651 ppb in May 2012, 524 ppb in June 2013) were also more similar to one another than the initial concentration for the control treatment in August 2012 (1606 ppb). This suggests that the instability of

ergovaline may be related to the concentration of ergovaline in the plant material at harvest: material with higher concentrations may show a greater loss in the first 24 h than material with lower concentration. Norman et al. (2007) also observed higher percent loss of ergovaline in hay bales with higher initial concentrations than those with lower concentrations at harvest.

After a significant decrease in ergovaline concentration in the first 24 h of storage, ergovaline concentrations changed very little over 28 days of storage at −20°C in all three harvests. This suggests that samples can be stored for up to a month without further degradation. If steps are taken to reduce or account for losses in the first 24 h, then laboratories will be able to store samples for up to at least 1 month before analysis and achieve accurate test results.

It is unclear what effect milling may have on ergovaline before storage. Allocation of whole tillers to treatment groups was attempted, but large differences in ergovaline concentrations (up to 34%) were observed between groups before any storage treatment was applied. This is similar to what Mace and Baker (2012) reported, observing 68% variability in ergovaline concentration between plants and 32% variability between tillers within a plant. It is unclear if milling had any effect on the concentrations throughout this study, but milling was conducted before the application of the storage treatments to ensure homogenous mixing of the plant material. Controlled studies of the effect of milling would be valuable in the future.

CONCLUSION

In conclusion, sample handling and storage conditions have a significant impact on the ergovaline concentrations within the tall fescue plant material. To obtain the most accurate analysis, all samples should be transported on ice to the laboratory immediately after harvest for same day analysis. If immediate testing is not possible, samples should be stored at −20°C until analysis.

Based on the results of this research, ergovaline measurements may be significantly lower after 24 h of storage, regardless of storage temperature. Most testing facilities do not have the capacity to guarantee testing the same day as harvest. Therefore researchers, extension personnel, veterinarians and producers should assume that testing results are significantly lower than actual levels in pasture.

ACKNOWLEDGMENT

The authors would like to thank Lakland Farm for their cooperation in this study and the USDA ARS FAPRU unit located at the University of Kentucky for funding this research through the Specific Cooperative Agreement.

REFERENCES

Aldrich, C. G., Paterson, J. A., Tate, J. L., and Kerley, M. S. (1993). The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *J. Anim. Sci.* 71, 164–170.

Arachevaleta, M., Bacon, C. W., Hoveland, C. S., and Radcliffe, D. E. (1989). Effect of the tall fescue endophyte on plant response to environmental stress. *Agron. J.* 81, 83–90. doi: 10.2134/agronj1989.00021962008100010015x

Bacon, C. W., and Siegel, M. R. (1988). Endophyte parasitism of tall fescue. *J. Prod. Agric.* 1, 45–55. doi: 10.2134/jpa1988.0045

Ball, D. M., Hoveland, C. S., and Lacefield, G. D. (2007). "Cool season grasses," in *Southern Forages*, eds D. M. Ball, C. S. Hoveland, and G. D. Lacefield (Norcross, GA: International Plant Nutrition Institute), 40–49.

Belesky, D. P., Stuedemann, J. A., Plattner, R. D., and Wilkinson, S.R. (1988). Ergopeptine alkaloids in grazed tall fescue. *Agron. J.* 80, 209–212. doi: 10.2134/agronj1988.00021962008000020014x

Brendemuehl, J. P., Boosinger, T. R., Pugh, D. G., and Shelby, R. A. (1994). Influence of endophyte-infected tall fescue on cyclicity, pregnancy rate and early embryonic loss in the mare. *Theriogenology* 42, 489–500. doi: 10.1016/0093-691X(94)90687-E

Garner, G. B., Rottinghaus, G. E., Cornell, C. N., and Testereci, H. (1993). Chemistry of compounds associated with endophyte/grass interaction: ergovaline- and ergopeptine-related alkaloids. *Agric. Ecosyst. Environ.* 44, 65–80. doi: 10.1016/0167-8809(93)90039-R

Gwinn, K. D., and Gavin, A. M. (1992). Relationship between endohyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. *Am. Phytopathol. Soc.* 76, 911–914.

Hopkins, A. A., and Alison, M. W. (2006). Stand persistence and animal performance for tall fescue endophyte combinations in the south central USA. *Agron. J.* 98, 1221–1226. doi: 10.2134/agronj2006.0007

Hoveland, C. S. (1993). Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. *Agric. Ecosyst. Environ.* 44, 3–12. doi: 10.1016/0167-8809(93)90036-O

Klotz, J. L., Bush, L., Smith, D. L., Shafer, W. D., Smith, L. L., Arrington, B. C., et al. (2007). Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassey. *J. Anim. Sci.* 85, 2330–2336. doi: 10.2527/jas.2006-803

Kosanke, J. L., Loch, W. E., Worthy, K., and Ellersieck, M. R. (1987). "Effect of toxic tall fescue on plasma prolactin and progesterone in pregnant pony mares," in *Proceedings of the Tenth Equine Nutrition and Physiology Symposium* (Fort Collins, CO), 663–668.

Lyons, P. C., Plattner, R. D., and Bacon, C. W. (1986). Occurrance of peptide and clavine ergot alkaloids in tall fescue grass. *Science* 232, 487–489. doi: 10.1126/science.3008328

Mace, W. J., and Baker, K. L. (2012). "Inter-tiller vs. inter-plant alkaloid variation: a comparison of total alkaloid profile," in *8th International Symposium on Fungal Endopyte of Grasses* (Lanzhou), 94–96.

McClanahan, L. K., Aiken, G. E., and Dougherty, C. T. (2008). Influence of rough hair coats and steroid implants on the performance and physiology of steers grazing endophyte-infected tall fescue in the summer. *Prof. Anim. Sci.* 24, 269–276.

Monroe, J. L., Cross, D. L., Hudson, L. W., Hendricks, D. M., Kennedy, S. W., and Bridges, W. C. (1988). Effect of selenium and endophyte-contaminated fescue on performance and reproduction in mares. *Equine Vet. Sci.* 8, 148–153. doi: 10.1016/S0737-0806(88)80038-8

Norman, R. C., Coblentz, W. K., Hubbell, D. S. III., Ogden, R. K., Coffey, K. P., Caldwell, J. D., et al. (2007). Effects of storage conditions on the forage quality characteristics and ergovaline content of endophyte-infected tall fescue hays. *Crop Sci.* 47, 1635–1646. doi: 10.2135/cropsci2006.10.0656

Peters, C. W., Grigsby, K. N., Aldrich, C. G., Paterson, J. A., Lipsey, R. J., Kerley, M. S., et al. (1992). Performance, forage utilization and ergovaline consumption by beef cows grazing endohyte fungus-infected tall fescue, endophyte fungus-free tall fescue or orchardgrass pastures. *J. Anim. Sci.* 70, 1550–1561.

Putnam, M. R., Bransby, D. I., Schumacher, J., Boosinger, T. R., Bush, L., Shelby, R. A., et al. (1991). Effects of the fungal endophyte *Acremonium coenphialum* in fescue on pregnant mares and foal viability. *Am. J. Vet. Res.* 52, 2071–2074.

Smith, D. J., and Shappell, N. W. (2002). Technical note: epimerization of ergopeptine alkaloids in organic and aqueous solvents. *J. Anim. Sci.* 80, 1616–1622.

Spiering, M. J., Davies, E., Tapper, B. A., Schmid, J., and Lane, G. A. (2002). Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. *J. Agric. Food Chem.* 50, 5856–5862. doi: 10.1021/jf025602b

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 June 2014; paper pending published: 24 July 2014; accepted: 18 August 2014; published online: 08 September 2014.
*Citation: Lea K, Smith L, Gaskill C, Coleman R and Smith SR (2014) Ergovaline stability in tall fescue based on sample handling and storage methods. Front. Chem. **2**:76. doi: 10.3389/fchem.2014.00076*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 11 December 2014
doi: 10.3389/fchem.2014.00110

Tall fescue seed extraction and partial purification of ergot alkaloids

Huihua Ji[1], F. Fannin[2], J. Klotz[3] and Lowell Bush[2]*

[1] Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY, USA
[2] Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA
[3] Forage Animal Production Research Unit, Agricultural Research Services, United States Department of Agriculture, Lexington, Kentucky, USA

Edited by:
Darrin Smith, Eastern Kentucky University, USA

Reviewed by:
John Wade, Florey Institute of Neuroscience and Mental Health, Australia
Alexander Shekhtman, University at Albany, State University of New York, USA
Darrin Smith, Eastern Kentucky University, USA

****Correspondence:***
Lowell Bush, Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546-0236, USA
e-mail: lowell.bush@uky.edu

Many substances in the tall fescue/endophyte association (*Schedonorus arundinaceus*/*Epichloë coenophiala*) have biological activity. Of these compounds only the ergot alkaloids are known to have significant mammalian toxicity and the predominant ergot alkaloids are ergovaline and ergovalinine. Because synthetically produced ergovaline is difficult to obtain, we developed a seed extraction and partial purification protocol for ergovaline/ergovalinine that provided a biologically active product. Tall fescue seed was ground and packed into several different sized columns for liquid extraction. Smaller particle size and increased extraction time increased efficiency of extraction. Our largest column was a 114 × 52 × 61 cm (W × L × D) stainless steel tub. Approximately 150 kg of seed could be extracted in this tub. The extraction was done with 80% ethanol. When the solvent front migrated to bottom of the column, flow was stopped and seed was allowed to steep for at least 48 h. Light was excluded from the solvent from the beginning of this step to the end of the purification process. Following elution, ethanol was removed from the eluate by evaporation at room temperature and the resulting syrup was freeze-dried. About 80% recovery of alkaloids was achieved with 18-fold increase in concentration of ergovaline. Initial purification of the dried product was accomplished by extracting with hexane/water (6:1, v/v). The aqueous fraction was extracted with chloroform, the aqueous layer discarded, after which the chloroform was removed with a resulting 20-fold increase of ergovaline. About 65% of the ergovaline was recovered from the chloroform residue for an overall recovery of 50%. The resultant partially purified ergovaline had biological activities in *in vivo* and *in vitro* bovine bioassays that approximate that of synthetic ergovaline.

Keywords: ergovaline, ergovalinine, alkaloid extraction, ergopeptine alkaloids, epimerization

INTRODUCTION

There have been many name changes for both tall fescue and the endophyte in recent years, but *Schedonorus arundinaceus and Epichloë coenophiala* are the most generally accepted at present. The biologically active substances include the pyrrolizidine and ergot alkaloids plus peramine. Of these compounds only the ergot alkaloids have significant mammalian toxicity and the predominant, 84–97%, of the ergot alkaloids are ergovaline and ergovalinine (Lyons et al., 1986). These two ergopeptine alkaloids are isomers and in equilibrium depending upon the environment in which they are contained (Smith and Shappell, 2002). The cyclo-tripeptide of these two alkaloids is the amino group of alanine attached to the ergolene ring plus valine and proline. Concentration of alkaloid accumulation in the plant is dependent upon the growing conditions for the association. Generally, higher nitrogen fertilization, clipping frequency and cooler temperatures will increase accumulation of ergovaline in vegetative tissues (Bush and Fannin, 2009). Ergovaline has been shown to be a vasoconstrictor of the bovine lateral saphenous vein (Klotz et al., 2007) and much more potent than other alkaloids present in the grass/endophyte association with constriction induced at 10^{-7} M (see Strickland et al., 2009 for more details). A significant observation is that low amounts of ergovaline are required to elicit a response in many bioassays but sufficient pure ergovaline has not been available to conduct *in vivo* assays. Ergovaline is the more biologically active of the two isomers and neither are easily chemically synthesized. Chemical standards for analytical assays or for bioassays are very difficult to obtain. Ergovaline may be quantified in extracts from endophyte infected grasses by HPLC with fluorescent detection or by HPLC with a mass spectrometer detector. Both of these provide high sensitivity and selectivity in the determination. For analytical purposes solvent extractions from grass material are carried out with the pH adjusted low (e.g., with organic acids) or high (e.g., with NaOH or ammonia) to ensure good solubility (Spiering et al., 2002). Earlier reports had described extraction solvents including mixtures of methanol/ethyl acetate; methylene chloride/ammonium hydroxide, mixtures of organic acids, methanol and methanol/water (see Garner et al., 1993). Rottinghaus et al. (1990) found that a 1:1 methanol:water mixture has the greatest extraction of

ergovaline compared to just methanol or water. Because of the other alkaloids often present in tall fescue forage, Spiering et al. (2002) developed a protocol for microanalytical extraction using 2-propanol-lactic acid as extraction solvent to do one extraction for ergovaline and peramine. This protocol was on a micro-scale and allowed analysis of individual plant parts of one plant. However, it was not conducive to large scale extraction and subsequent purification. Presently, routine extraction for analytical purposes use 80% methanol based on the procedure of Yates and Powell (1988). Extraction of tall fescue seed with lactic acid on a 5 kg scale has been accomplished by Moubarak et al. (1993) and yielding a few mg of ergovaline. They concentrated the alkaloid in the lactic acid solution onto Bio-Beads followed by removal with methanol, reducing the methanol and separating the ergovaline on two different HPLC columns. Based on HPLC analysis they obtained egovaline of about 95% purity with very little ergovalinine present. Absence of ergovalinine is significant in testing the activity of ergovaline in bioassays.

Our objective was to isolate and partially purify ergovaline/ergovalinine from up to 150 kg tall fescue seed infected with the endophyte. In the remainder of this report we will use ergovaline to mean both isomers, unless otherwise indicated.

MATERIALS AND METHODS

Seed contain the greatest accumulation of ergovaline of any tall fescue tissue (Rottinghaus et al., 1991) and we designed several experiments to most efficiently extract the alkaloid. Over time we have used several different batches of seed for these studies, but all had ergovaline levels above 5 mg kg^{-1}. Solvent, seed particle size and time used for extraction had significant impact on efficiency of extraction.

We chose 80% aqueous ethanol to do the extractions because it was less expensive to purchase, easier to discard than methanol and the extraction efficiency was similar to methanol. For these extraction studies, seeds were intact or powdered to pass different screen sizes (0.5, 1, 2 mm) and extracted with 80% aqueous ethanol. Materials were extracted for 2, 24, 48, or 150 h. Because of the long extraction time for our large batches the ergovaline/ergovalinine ratio was monitored and decisions were made to minimize the amount of isomerization to ergovalinine.

For our large scale extractions tall fescue seed that tested high in ergovaline content were ground to pass a 1 mm sieve. Ground seed were packed carefully into a 114 × 52 × 61 cm tub column (**Figure 1**). There is a gasket around the rim of the tub that seals the top lid to the tub for the introduction of N_2 to aid in the removal of the extraction solvent. The screw jacks on the top are visible to hold the lid in place while the system is under pressure from the N_2. The jacks may be used to compress the seed to remove the solvent but we found that using N_2 was more efficient. On the left end is the exit for the solvent into a tube covered with foil that drains into a large surface area container for the removal of the ethanol from the extract. Packing was done to provide a uniform column substrate as possible. This was done by adding about 25 kg at a time, leveling and lightly packing. Care in packing the column was done to insure that the solvent would migrate uniformly to the bottom of the column. This column holds 150 kg

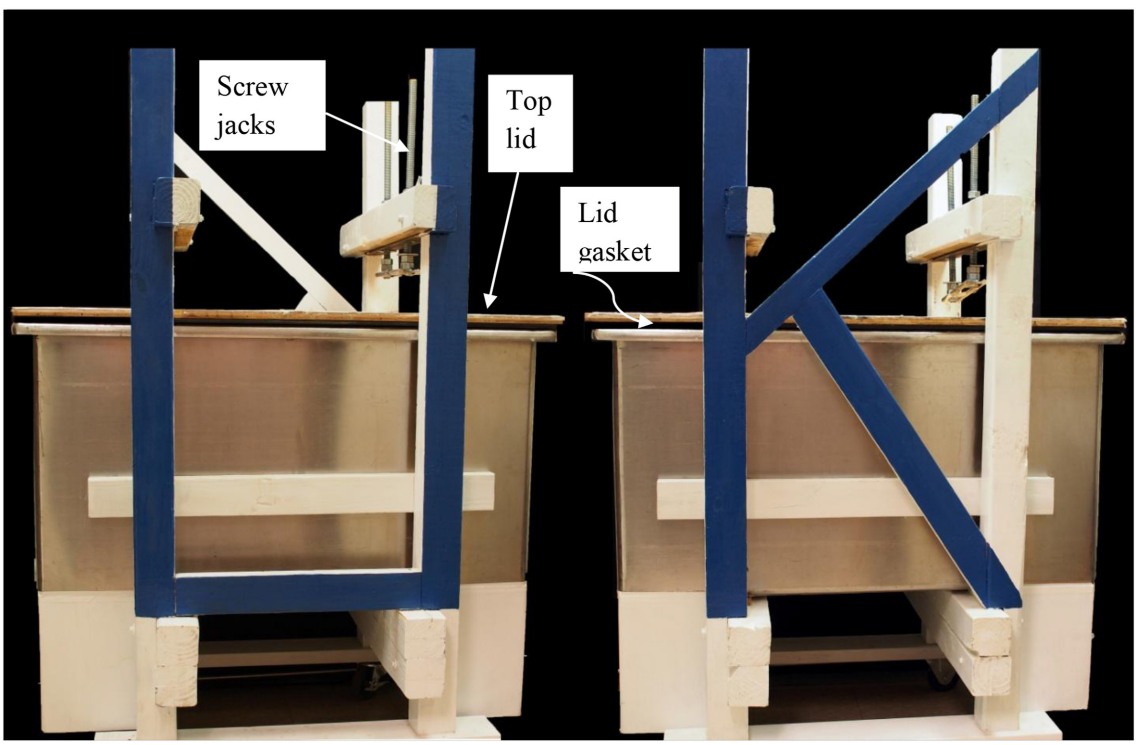


FIGURE 1 | Front and rear view of tub extractor that holds 150 kg powdered seed.

dry powder seed. The bottom of the column was filled with glass marbles above the outlet for solvent. The glass marbles were covered with a metal screen and non-dyed denim filter to keep seed residue from entering the eluate. The seedbed was compressed slightly by 19 mm thick polyethylene plate. Extraction was done with 80% ethanol and to fill the void volume approximately 160 L were added over 18–20 h period. At this time the solvent had reached the bottom of the column and the flow was stopped and seed steeped for 48 h. The tub column was then sealed and compressed N_2 was introduced onto the top of the tub column to aid in removal of the extracting solvent. The eluate was drained into a large surface area tub and the ethanol removed by a fan blowing on the surface. From the point of emergence from the column and throughout the remaining processing the material was kept in the dark. After elution was complete, approximately 80 L of 80% ethanol was added over an 8 h period. Then the flow was stopped and seed steeped again for 48 h. Elution continued as previously described. After most of the ethanol was removed from the extractor and the drying tub, the resulting extract was freeze-dried to remove remaining ethanol and water and then stored at −20°C.

Purification of the 80% ethanol extract was attempted with many different solvents and combinations. The best protocol in our investigations was one of making a water slurry of the dried 80% ethanol extract followed by partitioning with hexane (discarding the hexane), then partitioning the aqueous fraction with $CHCl_3$ (discarding the aqueous fraction) and removal of the $CHCl_3$ under vacuum to yield a product with greatly increased ergovaline concentration. All steps were done in brown bottles to limit epimerization.

Purified extracts from the large scale extractions were used in experiments to measure the biological activity in bovine tissues compared to chemically pure ergovaline (Foote et al., 2012, 2013, 2014). Summaries of experimental protocols are provided in the Bioassay section to facilitate explanation of the results.

Routine assay for the alkaloids in each experiment followed a HPLC/FLD (Florescence detector) procedure to quantify ergovaline and ergovalinine concentrations, as developed by Yates and Powell (1988) and modified as in Aiken et al. (2009). Ergovaline and ergovalinine were identified by excitation at 310 and detection at 420 nm with identity confirmed by LC/MS/MS. The *m/z* 223 from parent ion *m/z* 534 was monitored as well the product ion of *m/z* 534 (Lehner et al., 2004).

RESULTS

Our routine protocol, mentioned above, extracts 500 mg with 10 mL extracting solution while shaking for 2 h. Whole seed were extracted for 48 h with shaking and the powdered seed were extracted via our routine procedure. The smaller the particle size the greater amount of ergovaline that was extracted and the ergovaline/ergovalinine ratio did not change significantly (**Table 1**). Ergovaline/ergovalinine ratio ranged from 64/36 to 61/39. Even extracting whole seed for 48 h only removed about 5% of the alkaloid. The assumption that all the ergovaline was extracted in the first step of the ground seed is not valid but is about 84% efficient for the finest particle size and exhaustive extraction. Because grinding large amounts of tall fescue seed through a 0.5 mm screen is not reasonable (time-wise) for large-scale extractions (kg amounts), we used the 1 mm particle size for our large-scale extractions.

The interaction between particle size and time of extraction demonstrated that increased shaking extraction time, regardless of the particle size increased ergovaline extraction (**Table 2**). Even at the 0.5 mm particle size the increased time from 2 to 48 h increased ergovaline extracted into the ethanol. The increased efficiency of extraction for the 2, 1, and 0.5 mm particle size from 2 to 150 h was 251, 61, and 19%, respectively.

From 150 kg of seed, we obtained 8.3 kg of dried extract. This extraction resulted in an 80% recovery of ergovaline and an 18-fold increase in concentration. Initial seed and the resulting freeze-dried extract are depicted in **Figure 2**. Because we had to use the longer times of extraction for our large batch extractions, we measured the epimerization that may occur between ergovaline and ergovalinine in the solvents to be used. Chemically pure ergovaline dissolved in 80% methanol had no measureable epimerization immediately after being dissolved. However, by 22 and 47 h significant epimerization occurred, 16 and 26 %, respectively (data not shown). Similarly, seed extract solubilized in 80% ethanol (**Table 2**) or acetonitrile (data not shown) also had significant epimerization, about 40% conversion and was very consistent across treatments during extraction and measurement.

Table 1 | Seed particle size and extraction efficiency with 80% aqueous ethanol.

Sample identification	Ergovaline		Ergovalinine		Total extracted
	μg g^{-1}	stdev	μg g^{-1}	stdev	(%)
Whole seed[1]	0.30	0.04	0.47	0.06	5
GROUND THROUGH					
2 mm screen	2.24	0.12	1.42	0.08	34
1 mm screen	3.79	0.14	2.26	0.08	60
0.5 mm screen	5.19	0.13	3.32	0.20	84

[1] *Whole seed were extracted for 48 h, other sizes were extracted for 2 h.*

Table 2 | Extraction time, particle size interaction for efficient extraction with 80% ethanol.

Particle size	Extraction time	Ergovaline		Ergovalinine	
mm	h	μg g^{-1}	stdev	μg g^{-1}	stdev
2	2	2.24	0.12	1.42	0.08
2	24	4.27	0.06	2.83	0.13
2	48	5.00	0.37	3.26	0.21
2	150	5.30	0.16	3.36	0.07
1	2	3.79	0.14	2.26	0.08
1	24	4.92	0.16	3.12	0.15
1	48	5.50	0.01	3.49	0.04
1	150	5.94	0.07	3.82	0.08
0.5	2	5.19	0.13	3.32	0.20
0.5	24	5.80	0.05	3.78	0.14
0.5	48	6.10	0.07	3.96	0.01
0.5	150	6.19	0.02	3.91	0.12

FIGURE 2 | Seed and initial dried extract.

To obtain the best efficiency of recovering ergovaline from the large scale dried extract we tried several solvents, solvent sequences and solvent combinations to get efficient recovery and further purification. Any approaches that utilized water resulted in high recovery of lysergic acid and isolysergic acid but little ergovaline recovery. A water extract followed by $CHCl_3$ removed much of the ergovaline and two $CHCl_3$ extracts removed most of the ergovaline (the first 4 rows) were done in this sequence on one sample and results summarized (**Table 3**). Chloroform without some water wetting of the dried extract removed much smaller portion of the ergovaline. Acidic and basic solutions with organic solvents plus hexane and methyl-tert butyl ether were also tried for extraction of ergovaline from the initial dried extract, but were less effective than $CHCl_3$. However, initially extracting with hexane from a water slurry of the freeze dried extract to remove lipophilic substances greatly increased the purification of the $CHCl_3$ extract from the slurry following the hexane extraction. The hexane fraction did contain 10–15% of the ergovaline (bottom **Table 3**) and this could be back extracted with 80% methanol to improve overall efficiency, if needed. The $CHCl_3$ fraction was filtered and $CHCl_3$ removed under vacuum at 25°C in the dark. Dried residue was solubilized in 80% aqueous methanol.

Based on the above results we selected the following protocol for the second step in purification. Three hundred mL of water was added to 600 g of the dried extract and mixed to a smooth slurry to remove all "crystalline" pieces. Slurry was added to a 4 L brown bottle and 1800 mL of hexane was added. The mixture was vigorously shaken for 10 min and the hexane and aqueous layers were allowed to separate. This could take up to 2 h. Hexane removed many of the lipids and the alkaloids remained in the aqueous fraction. The hexane layer was decanted and the aqueous fraction was extracted with hexane two more times. The aqueous fraction was extracted with 1800 mL chloroform 3-times and the chloroform fractions combined and removed in a rotary evaporator. Residue was stored at −20° C for use. Ergovaline concentration increased from the seed about 350- to 400-fold (**Figures 3A–C**). Chromatogram in **Figure 3A** is from initial seed extract with ergovaline (EV) and ergovalinine (EVI). The initial crude 80% ethanol extract alkaloid content is illustrated in **Figure 3B**. This chromatogram is a 100-fold dilution of the extract for comparison with **Figure 3A**. Ergovaline present in the $CHCl_3$ of the water/hexane/$CHCl_3$ cleanup had no lysergic acids and the solution for the chromatogram had been diluted 600-fold (**Figure 3C**, **Table 3**). Lysergic acid and loline alkaloids were not detected in the final purified extract (not shown on the chromatograms). Fragment ion spectrum of ergovaline and of the partially purified ergovaline *m/z* 534 fragment are indicative of ergovaline (**Figure 4**). Further purification may be done with HPLC separation and collection of ergovaline and ergovalinine. The HPLC purification of the water/hexane/$CHCl_3$ extract is illustrated in **Figure 3D**. The ergovalinine is most likely from epimerization that occurred during the chromatography or processing. A final purification by HPLC was reported earlier by Moubarak et al. (1993).

BIOASSAY OF PURIFIED EXTRACT

Purified extracts were used in experiments to measure the biological activity in bovine vasculature. An experiment was conducted to determine if substances extracted from endophyte infected tall fescue other than ergovaline were responsible for vasculature responses *in vitro* (Foote et al., 2012). Lateral saphenous veins from cattle were tested in a multi-myograph with different alkaloid treatments. Alkaloid treatments were (1) chemically pure ergovaline (EV), (2) endophyte infected seed extract (E+EXT); (3) a mixture of alkaloids (ALK) that mimicked those found in the E+EXT, and (4) an extract from endophyte free seed extract. An extract was generated using endophyte containing seed described above and a second similar extract was generated from endophyte-free seed. The partially purified residue

Table 3 | Purification attempts from initial seed extract.

Extraction solvent	Lysergic acid	Isolysergic acid	Ergovaline		Ergovalinine		Lysergic acid/isolysergic acid	EV/EVI
	Recovery			stdev	Recovery	stdev		
	%	%	%		%		Epimer portion	Epimer portion
1st water	62	51	9		12		56/44	63/37
2nd water	5	4	1		1		55/45	56/44
1st $CHCl_3$	1	5	63	5	67	8	9/91	64/36
2nd $CHCl_3$	0	2	16	2	15	1	0/100	64/36
only $CHCl_3$	ND	ND	37		45			58/42
H_2O/HEXANE/$CHCl_3$								
Hexane fraction	6	6	15	1	12	2	51/49	71/29
$CHCl_3$ fraction	ND	ND	68	6	65	3		61/39
80% methanol	100	100	100		100		51/49	62/38

Measures of lysergic acid and ergovaline recoveries at different steps of the purification. ND, not detected.

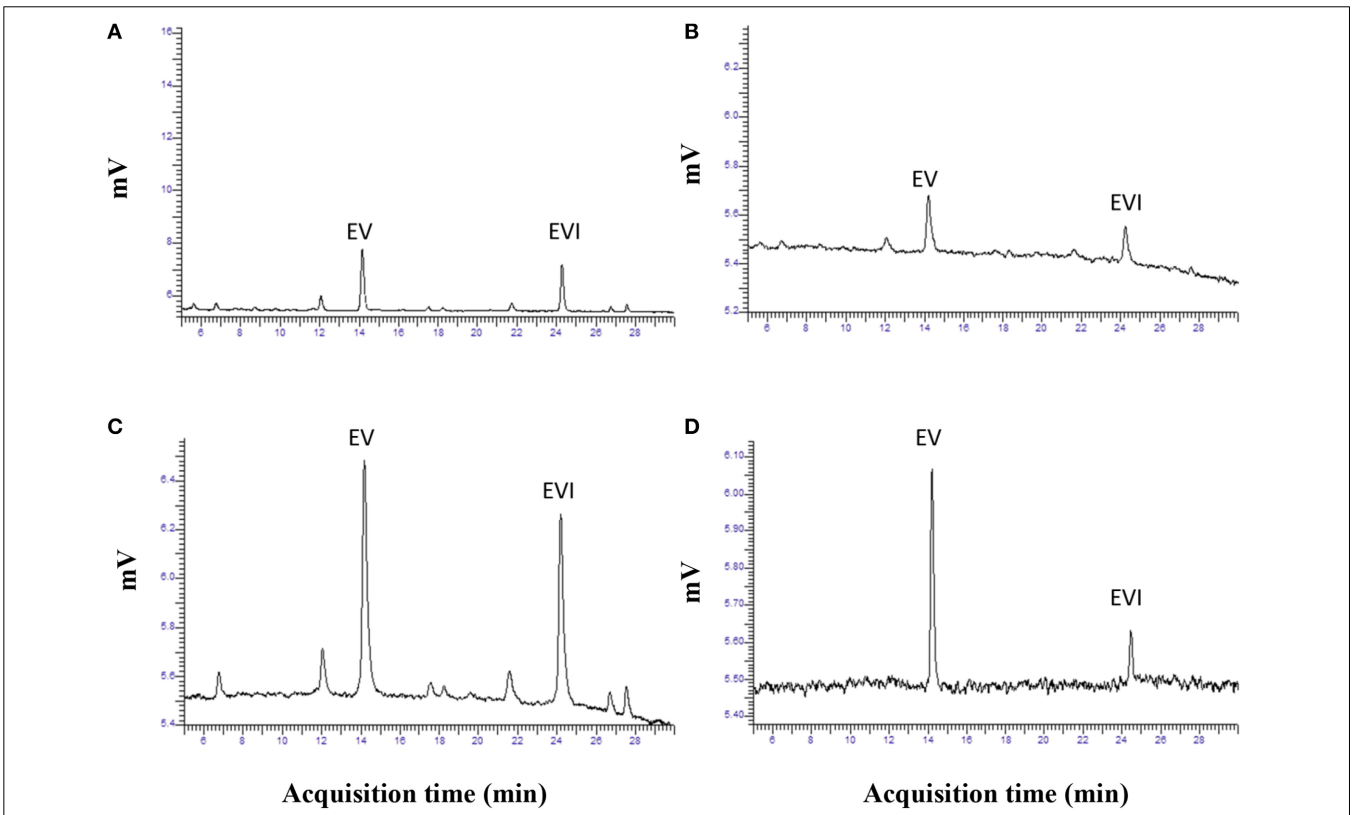


FIGURE 3 | Chromatograms of ergovaline from initial seed to a partially purified material. Panel **(A)** is chromatogram of routine analysis of powdered seed (EV, ergovaline; EVI, ergovalinine). Panel **(B)** is a chromatogram from dried material following 80% ethanol extraction. Panel **(C)** is chromatogram of dried material following the water/hexane/$CHCl_3$ purification. Panel **(D)** is a chromatogram of HPLC purification of ergovaline from **(C)**.

from each extract was solublized in 50 mL of 80% methanol. The endophyte-free extract was diluted in the same manner as for the extract from the endophyte-infected seed. The amount of each extract and chemical alkaloid treatment added to myograph cells were determined by amount of ergovaline required meet the ergovaline concentration along the X-axis (**Figure 5**). Ergovaline/ergovalinine ratio of the alkaloid extract was 60/40. A mixed alkaloid treatment was prepared by dissolving ergovaline, ergotamine, α-ergocryptine, ergocristine D-lysergic acid hydrate, and ergonovine in 80% methanol. The alkaloid concentrations of this mixed alkaloid treatment were based on alkaloids quantified in the endophyte infected seed extract, including ergovaline. Both the E+EXT and ALK mixture induced similar contractile response as ergovaline. Lack of a significant response

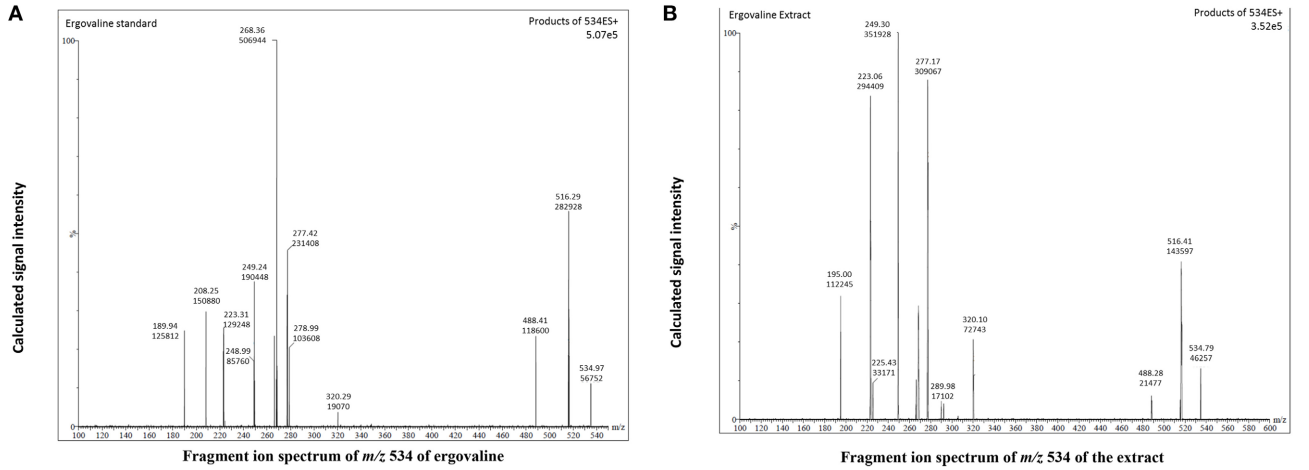


FIGURE 4 | Fragment ion spectrum of *m/z* 534 fragment of chemical standard ergovaline and the partially purified extract from panel 3D. (A) is chemical standard for ergovaline, **(B)** is the ergovaline extract.

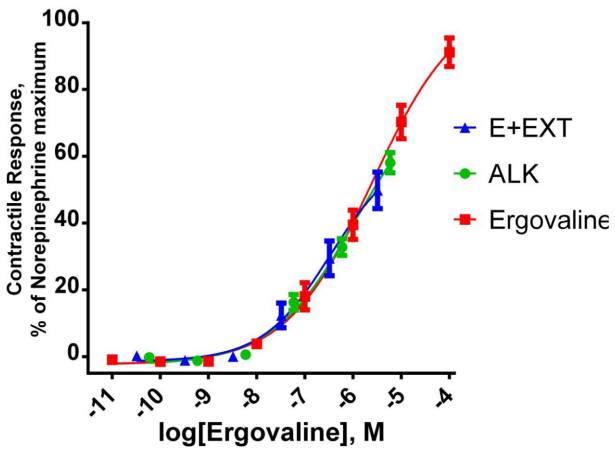


FIGURE 5 | Contractile response of bovine lateral saphenous vein. Response of bovine lateral saphenous vein to ergovaline, an endophyte-infected tall fescue seed extract (E+EXT) standardized to ergovaline concentration, and a mixture of ergot alkaloids (ALK) that reflects the alkaloid profile of E+EXT. Regression lines represent the fitting of data to a sigmoidal concentration response curve. The ALK treatment was prepared by dissolving ergovaline, ergotamine, α-ergocryptine, ergocristine D-lysergic acid hydrate, and ergonovine in 80% methanol. The alkaloid concentrations of this ALK treatment were based on alkaloids quantified in the E+EXT. E+EXT was prepared as described above for purification of the crude extract from the tub extractor. All treatment solutions were based on the ergovaline concentration. The other alkaloids in the E+EXT and the ALK mimic solution were in the 10^{-8} to 10^{-10} M. Figure taken from Foote et al. (2012).

from the endophyte-free seed extract (data not shown) and the fact that the response from the E+EXT and ALK were similar to the chemically pure ergovaline response (**Figure 5**) suggests that ergovaline is the only substance in the endophyte-infected tall fescue extract that is causing the contractile response in this bioassay, and perhaps in any of the affected vasculature. The seed extract also decreased *in vivo* reticuloruminal epithelial blood flow about 50% and volatile fatty acid absorption (acetate, propionate and butyrate) from the washed reticulorumen (Foote et al., 2013). Acute exposure of *in vitro* bovine rumen epithelium to the extract had no effect on acetate or butyrate flux (μmol/cm^2 h) across the epithelium (Foote et al., 2014).

DISCUSSION

Data in **Tables 2**, **3** indicate that chemically pure ergovaline does not epimerize as rapidly as seed extracts that contain many other substances in addition to ergovaline in our studies. Our data are not exactly comparable as the pure ergovaline that was is in 80% methanol and the seed extracts are in 80% ethanol. Smith and Shappell (2002) reported that in methanol, ergovaline had about 20 and 45% epimerization in 22 and 47 h, respectively. In water, they reported less, about 15 and 30%, epimerization over the same time periods. Both their studies were done at 37°C which would enhance the rate of epimerization. The water and lower temperature (∼23°C) in our study probably resulted in the lesser rate of epimerization measured. We did not measure the rate of epimerization of ergovaline in 80% aqueous ethanol but it was stable at about 60/40 ergovaline/ergovalinine over the time periods of our studies (**Tables 2**, **3**). Product ion fragments of *m/z* 534 of the partially purified extract (**Figure 4B**) are indicative of ergovaline and agree with previous mass spectrum published (Lehner et al., 2004) and the current product of purification is biologically active. In different bioassays the partially purified extract resulted in similar biological activity based on the amount of ergovaline in the extract compared to chemical ergovaline.

CONCLUSIONS

Solvent, seed particle size, and time used for extraction had significant impact on efficiency of extraction. Overall this is a protocol for extraction of large amount of high ergovaline plant tissue that yields dried extracts with enhanced levels of ergovaline (350- to 400-fold increase) that are biologically active. Activity is equal to ergovaline alone in selected bioassays. Additional purification was achieved with HPLC separation of ergovaline and ergovaline. This

further purification of ergovaline will be useful in specific cellular bioassays and for analytical purposes.

AUTHOR CONTRIBUTIONS

All authors were involved in aspects of data acquisition, analysis, and interpretation. All authors contributed to writing the manuscript, approved the final version, and are accountable for the data presented and interpretation therein.

ACKNOWLEDGMENTS

This work was supported by a cooperative agreement with Lowell Bush and the USDA-ARS Forage Animal Production Research Unit (3049025370) and the University of Kentucky's College of Agriculture, Food, and the Environment.

REFERENCES

Aiken, G. E., Strickland, J. R., Looper, M. L., Bush, L. P., and Schrick, F. N. (2009). Hemodynamics are altered in caudal artery of beef heifers fed different ergot alkaloid concentrations. *J. Anim. Sci.* 87, 2142–2150. doi: 10.2527/jas.2008-1562

Bush, L. P., and Fannin, F. F. (2009). "Alkaloids," in *Tall Fescue for the Twenty-First Century. Agronomy Monograph 53*, eds H. A. Fribourg, D. B. Hannaway, and C. P. West (Madison WI: American Society of Agronomy, Inc.), 229–249.

Foote, A. P., Harmon, D. L., Brown, K. R., Strickland, J. R, McLeod, K. R., Bush, L. P., et al. (2012). Constriction of bovine vasculature by endophyte-infected tall fescue seed extract is similar to pure ergovaline. *J. Anim. Sci.* 90, 1603–1609. doi: 10.2527/jas.2011-4513

Foote, A. P., Kristensen, N. B., Klotz, J. L., Kim, D. H., Koontz, A. F., McLeod, K. R., et al. (2013). Ergot alkaloids from endophyte-infected tall fescue decrease reticuloruminal epithelial blood flow and volatile fatty acid absorption from the washed reticulorumen. *J. Anim. Sci*. 91, 5366–5378. doi: 10.2527/jas.2013-6517

Foote, A. P., Penner, G. B., Walpole, M. E., Klotz, J. L., Brown, K. R., Bush, L. P., et al. (2014). Acute exposure to ergot alkaloids from endophyte-infected tall fescue does not alter absorptive or barrier function of the isolated ruminal epithelium. *Animal* 8, 1106–1112. doi: 10.1017/S1751731114001141

Garner, G. B., Rottinghaus, G. E., Cornell, C. N., and Testereci, H. (1993). Chemistry of compounds associated with endophyte/grass interactions: ergovaline-, and ergopeptine-related alkaloids. *Agric. Ecosyst. Environ*. 44, 65–80. doi: 10.1016/0167-8809(93)90039-R

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Arrington, B. C., et al. (2007). Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 85, 2330–2336. doi: 10.2527/jas.2006-803

Lehner, A. F., Craig, M., Fannin, N., Bush, L., and Tobin, T. (2004). Fragmentation patterns of selected ergot alkaloids by electrospray ionization tandem quadrupole mass spectrometry. *J. Mass Spectrom.* 39, 1275–1286. doi: 10.1002/jms.678

Lyons, P. C., Plattner, R. D., and Bacon, C. W. (1986). Occurrence of peptide and clavine ergot alkaloids in tall fescue. *Science* 232, 487–489. doi: 10.1126/science.3008328

Moubarak, A. S., Piper, E. L., West, C. P., and Johnson, Z. B. (1993). Interaction of purified ergovaline from endophyte-infected tall fescue with synaptosomal ATPase enzyme system. *J. Agric. Food Chem*. 41, 407–409. doi: 10.1021/jf00027a012

Rottinghaus, G. E., Garner, G. B., and Cornell, C. N. (1990). "Liquid chromatographic determination of ergovaline in endophyte-infested tall fescue," in *Proceedings of International Symposium on Acremonium/Grass Interactions, New Orleans, LA, USA*, eds S. S. Quisenberry and R. E. Joost (Baton Rouge, LA: Louisiana Agriculture Experiment Station), 93–96.

Rottinghaus, G. E., Garner, G. B., Cornell, C. N., and Ellis, J. L. (1991). HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades and seed heads. *J. Agric. Food Chem.* 39, 112–115. doi: 10.1021/jf00001a022

Spiering, M. J., Davies, E., Tapper, B. A., Schmid, J., and Lane, G. A. (2002). Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. *J. Agric. Food Chem.* 50, 5856–5862. doi: 10.1021/jf025602b

Strickland, J. R., Aiken, G. E., Spiers, D. E., Fletcher, L. R., and Oliver, J. W. (2009). "Physiological basis of fescue toxicosis," in *Tall Fescue for the Twenty-First Century. Agronomy Monograph 53*, eds H. A. Fribourg, D. B. Hannaway and C. P. West (Madison, WI: American Society Agron), 203–227.

Smith, D. J., and Shappell, N. W. (2002). Technical note: epimerization of ergopeptine alkaloids in organ and aqueous solvents. *J. Anim. Sci.* 80, 1616–1622.

Yates, S. G., and Powell, R. G. (1988). Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. *J. Agric. Food Chem*. 36, 337–340. doi: 10.1021/jf00080a023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 July 2014; accepted: 21 November 2014; published online: 11 December 2014.

*Citation: Ji H, Fannin F, Klotz J and Bush L (2014) Tall fescue seed extraction and partial purification of ergot alkaloids. Front. Chem. **2**:110. doi: 10.3389/fchem.2014.00110*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 04 November 2014
doi: 10.3389/fchem.2014.00095

Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal?

Carolyn A. Young*[*‡], Nikki D. Charlton[‡], Johanna E. Takach, Ginger A. Swoboda, Michael A. Trammell, David V. Huhman and Andrew A. Hopkins[†]

The Samuel Roberts Noble Foundation, Forage Improvement Division, Ardmore, OK, USA

Edited by:
James Levi Klotz, United States Department of Agriculture - Agricultural Research Service, USA

Reviewed by:
Joana Costa, Center for Neuroscience and Cellular Biology, Portugal
Charles Wilson Bacon, United States Department of Agriculture, USA

****Correspondence:***
Carolyn A. Young, The Samuel Roberts Noble Foundation, Forage Improvement Division, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA
e-mail: cayoung@noble.org

[†]Present address:
Andrew A. Hopkins, Dow AgroSciences Inc., York, NE, USA

[‡]These authors have contributed equally to this work.

Tall fescue (*Lolium arundinaceum*) is a valuable and broadly adapted forage grass that occupies approximately 14 million hectares across the United States. A native to Europe, tall fescue was likely introduced into the US around the late 1800's. Much of the success of tall fescue can be attributed to *Epichloë coenophiala* (formerly *Neotyphodium coenophialum*) a seed borne symbiont that aids in host persistence. *Epichloë* species are capable of producing a range of alkaloids (ergot alkaloids, indole-diterpenes, lolines, and peramine) that provide protection to the plant host from herbivory. Unfortunately, most tall fescue within the US, commonly referred to as "Kentucky-31" (KY31), harbors the endophyte *E. coenophiala* that causes toxicity to grazing livestock due to the production of ergot alkaloids. Molecular analyses of tall fescue endophytes have identified four independent associations, representing tall fescue with *E. coenophiala*, *Epichloë* sp. FaTG-2, *Epichloë* sp. FaTG-3, or *Epichloë* sp. FaTG-4. Each of these *Epichloë* species can be further distinguished based on genetic variation that equates to differences in the alkaloid gene loci. Tall fescue samples were evaluated using markers to simple sequence repeats (SSRs) and alkaloid biosynthesis genes to determine endophyte strain variation present within continental US. Samples represented seed and tillers from the Suiter farm (Menifee County, KY), which is considered the originating site of KY31, as well as plant samples collected from 14 states, breeder's seed and plant introduction lines (National Plant Germplasm System, NPGS). This study revealed two prominent *E. coenophiala* genotypes based on presence of alkaloid biosynthesis genes and SSR markers and provides insight into endophyte variation within continental US across historical and current tall fescue samples.

Keywords: *Neotyphodium coenophialum*, fescue toxicosis, ergot alkaloids, Kentucky-31 (KY31), endophyte diversity

INTRODUCTION

HISTORY OF KY31 TALL FESCUE AND DISCOVERY OF ENDOPHYTE

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh. syn *Festuca arundinaceae* Shreb.] was introduced into the United States from Europe in the 1800's and is considered an important cool season perennial forage crop (Hoveland, 2009). Tall fescue is widely adapted to the eastern United States spanning 14 million hectares (35 million acres) with the fescue belt considered the major region of adaptation and use (**Figure 1**) (Ball et al., 1993). A timeline representing significant research events of tall fescue is shown in **Figure 1** and outlined below.

One of the most well known tall fescue cultivars, "Kentucky-31" (KY31), was collected in 1931 by Dr. E. N. Fergus (University of Kentucky) on a farm owned by William Suiter (Menifee County, KY) (Fergus and Buckner, 1972). KY31 gained wide acceptance as a grass with excellent agronomic attributes under difficult growth conditions, such as drought and poor soils. The KY31 ecotype was released in 1942, but was not officially registered as a cultivar until 1972 (Fergus and Buckner, 1972). Subsequently, the persistence and success of KY31 was attributed to the presence of the systemic fungal endophyte, *Epichloë coenophiala*. It was estimated that 90% of all tall fescue pastures in the US are endophyte infected (Siegel et al., 1985). The fitness benefits the endophyte provides include drought tolerance, improved competitive ability (Arachevaleta et al., 1989; West et al., 1993; Malinowski and Belesky, 2000), as well as protection from herbivores through the production of bioactive alkaloids (Clay et al., 1985; Bacon et al., 1986).

Unfortunately, although KY31 was known as a persistent cultivar, reports of poor animal performance when grazing this forage began in the 1940s (Cunningham, 1949; Jacobson et al., 1963). Animals that grazed on tall fescue suffered maladies such as fescue foot, fat necrosis, and fescue toxicosis (Bush et al., 1979). Cattle experiencing fescue toxicosis can exhibit rough hair coats, heat stress (wallowing in mud), elevated rectal temperatures, vasoconstriction, suppressed appetite, reduced prolactin levels, poor growth (lower average daily gains), and a reduction in calving rates (Hoveland et al., 1983; Hemken et al., 1984;

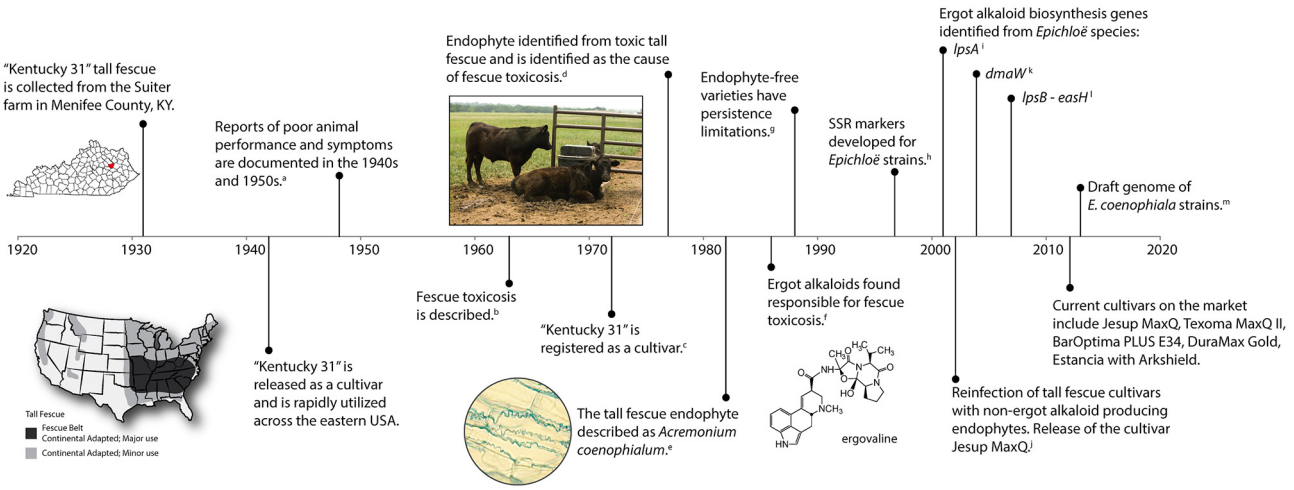


FIGURE 1 | A timeline of significant events associated with endophyte-infected tall fescue. Citations include a, Cunningham, 1948; b, Jacobson et al., 1963; c, Fergus and Buckner, 1972; d, Bacon et al., 1977; e, Morgan-Jones and Gams, 1982; f, Lyons et al., 1986; g, Pedersen et al., 1990; h, Groppe et al., 1995; Moon et al., 1999; i, Panaccione et al., 2001; j, Bouton et al., 2002; k, Wang et al., 2004; l, Fleetwood et al., 2007; m, Schardl et al., 2013b.

Stuedemann and Hoveland, 1988; Roberts and Andrae, 2004; Caldwell et al., 2013). Symptoms of fescue foot resembled those observed from ergot alkaloid toxicity seen with *Claviceps purpurea* on rye suggesting that an ergot alkaloid might be responsible for toxicity (Yates, 1971). However, although the syndrome was first described in 1963 (Jacobson et al., 1963) it took another decade before an endophyte was suggested as the causal agent. In 1977, a fungal endophyte was identified in toxic tall fescue as the likely culprit of these symptoms (Bacon et al., 1977), which was later confirmed by the production of ergot alkaloids by the fungus (Porter et al., 1979; Lyons et al., 1986; Bacon, 1988). The endophyte grows systemically through the upper plant parts and is maternally inherited in the seed (Siegel et al., 1984; Schardl, 2001).

TALL FESCUE ENDOPHYTE DESCRIPTION

Initially, Bacon et al. (1977) identified the tall fescue endophytes as *E. typhina*, which was later renamed *Acremonium coenophialum* to acknowledge the anomorphic state of *Epichloë* species (Morgan-Jones and Gams, 1982). Christensen and Latch (1991) described variation among isolates of *A. coenophialum* from tall fescue, and in 1993 the taxonomy of these endophytes was described (Christensen et al., 1993). The genus *Acremonium* was reclassified using phylogenetic analyses and *A. coenophialum* was renamed *Neotyphodium coenophialum* (Glenn et al., 1996). Finally, under the nomenclatural rule changes for fungi *Neotyphodium* is now included in the genus *Epichloë* resulting in the change to *E. coenophiala* (Leuchtmann et al., 2014). *E. coenophiala* is considered an asexual hybrid, phylogenetically described as a triparental hybrid with inferred ancestral progenitors from *E. festucae*, *E. typhina* subsp. *poae*, and the *Lolium*-associated endophyte (LAE) (Tsai et al., 1994; Moon et al., 2004).

E. coenophiala, like many *Epichloë* species, is capable of producing a variety of bioactive secondary metabolites. The four described classes of alkaloids produced by *Epichloë* species are ergot alkaloids, indole-diterpenes, lolines, and peramine (Siegel et al., 1990). Ergot alkaloids (e.g., ergovaline) and the indole-diterpene, lolitrem B, have been shown to have anti-mammalian activity causing fescue toxicosis (Bacon et al., 1977) and ryegrass staggers (Fletcher and Harvey, 1981), respectively. Peramine is considered an insect feeding deterrent (Johnson et al., 1985; Rowan and Latch, 1994) and the lolines have been documented for their potent insecticidal activity (Bush et al., 1997). *E. coenophiala* as a species complex can produce all four classes of alkaloids (**Table 1**). However, the most commonly produced alkaloids are peramine, lolines, and ergovaline (Christensen et al., 1993; Leuchtmann et al., 2000; Schardl et al., 2013b).

Considerable research has been conducted to understand the biosynthesis of these bioactive compounds including identification and characterization of the gene products required for the biosynthesis of each alkaloid class (Panaccione et al., 2001; Wang et al., 2004; Spiering et al., 2005, 2008; Tanaka et al., 2005; Young et al., 2006, 2009; Fleetwood et al., 2007; Saikia et al., 2012; Pan et al., 2014). This has been supported with genome sequences, including draft genome sequences of three *E. coenophiala* strains (Schardl et al., 2013a,b). This research has provided an understanding of why *Epichloë* species can have diverse alkaloid profiles and provided the sequence to develop markers for mating type and key alkaloid biosynthesis genes to genetically evaluate endophyte diversity *in planta* (Charlton et al., 2012, 2014; Takach et al., 2012; Takach and Young, 2014).

To date, tall fescue is known to form associations with four taxonomic groups, *E. coenophiala*, *Epichloë* sp. FaTG-2, *Epichloë* sp. FaTG-3, and *Epichloë* sp. FaTG-4 that vary based on ploidy (either 2x or 3x) and progenitors (**Table 1**). Initially tall fescue endophytes were distinguished by morphology and isozyme analysis to establish taxonomic groupings, and variation was also seen with the production of peramine, ergovaline, lolitrem B, and lolines (Christensen et al., 1993). Phylogenetic analyses were able

Table 1 | *Epichloë* species and genotype variation associated with endophytes of tall fescue.

Endophyte species	Endophyte genotype[a]	Ploidy	Progenitors[b]	Minimum marker set to distinguish species genotypes					Predicted chemotype class[d]
				Mating type	Ergot alkaloid *EAS*	Indole-diterpene *IDT/LTM*	Loline *LOL*	Peramine *PER*[c]	
E. coenophiala	Profile 1	3x	Efe (II) × LAE (Vb) × Ety (Ib)	AAA	*dmaW, lpsB*		*lolC, lolA*	*perA*-A2	*EAS, LOL, PER*
E. coenophiala	Profile 2	3x	Efe (II) × LAE (Vb) × Ety (Ib)	AAA	*dmaW, lpsB*	*idtQ*	*lolC, lolA*	*perA*-A2	*EAS, LOL, PER*
E. coenophiala	Profile 3	3x	Efe (II) × LAE (Vb) × Ety (Ib)	AAA	*dmaW, lpsB*	*idtG, idtQ*	*lolC, lolA*	*perA*-A2	*EAS, IDT, LOL, PER*
E. coenophiala	Profile 4	3x	Efe (II) × LAE (Vb) × Ety (Ib)	AAA		*idtG, idtQ*	*lolC, lolA*	*perA*-A2	*IDT, LOL, PER*
Epichloë sp. FaTG-2	Profile 1	2x	Efe (II) × LAE (Vb)	BB	*dmaW, lpsB*	*idtG, idtQ, ltmJ*		*perA*-A2, Δ*perA*-A2	*EAS, LTM, PER*
Epichloë sp. FaTG-2	Profile 2	2x	Efe (II) × LAE (Vb)	AB	*dmaW, lpsB*	*idtG, idtQ*		perA-A2, ΔperA-A2	*EAS, IDT, PER*
Epichloë sp. FaTG-2	Profile 3	2x	Efe (II) × LAE (Vb)	AB	*dmaW, lpsB*	*idtG, idtQ, ltmJ*		*perA*-A2, Δ*perA*-A2	*EAS, LTM, PER*
Epichloë sp. FaTG-3	Profile 1	2x	LAE (Vb) × Ety (Ia)	AA		*idtG, idtQ*	*lolC, lolA*	perA-A2, ΔperA-A2	*IDT, LOL, PER*
Epichloë sp. FaTG-3	Profile 2	2x	LAE (Vb) × Ety (Ia)	AA			*lolC, lolA*	perA-A2, ΔperA-A2	*LOL, PER*
Epichloë sp. FaTG-4	Profile 1	2x	LAE (Vb) × Ety (Ia)	AB	*dmaW, lpsB*	*idtG*		perA-A2	*EAS, PER*
Epichloë sp. FaTG-4	Profile 2	2x	LAE (Vb) × Ety (Ia)	AB	*dmaW, lpsB*	*idtG, idtQ*		perA-A2	*EAS, IDT, PER*

[a] *Based on designations from Takach and Young (2014) and draft genome sequences of FaTG-2 isolates NFe45079 and NFe45115.*

[b] *Efe (II), E. festucae (mating population II); LAE, Lolium associated endophyte (mating population Vb); Ety (Ia), E. typhina (mating population 1a); Ety (Ib), E. typhina (mating population 1b); Mating population as designated from Leuchtmann et al. (2014).*

[c] *The perA-A2 marker is designed to the second adenylation domain. Some isolates have a deletion in this domain as represented by ΔperA-A2 (Takach et al., 2012).*

[d] *Predicted chemotype class represents the class of genes that are found in the genome and do not always represent a functionally active locus. EAS, ergot alkaloids; IDT, indole diterpenes; LTM, lolitrem B; LOL, lolines; PER, peramine.*

to define the relationships of each taxonomic group to distinguish the ancestral progenitors of these hybrid species (Schardl et al., 1991, 2013b; Moon et al., 2004). The most studied of these species is *E. coenophiala*, the endophyte first identified in KY31.

Isozyme analyses of *E. coenophiala* isolates from within the US indicated that very little variation existed within this species (Leuchtmann and Clay, 1990). Although isozyme analysis can reflect endophyte diversity, this analysis requires pure cultures and thus is limited by the number of samples per tall fescue line that can be screened. Genetic analysis can now be performed directly with endophyte infected plant material using high throughput systems (Takach and Young, 2014). Markers have recently been used to determine the genetic diversity between tall fescue endophyte isolates and also evaluate their potential for alkaloid production (Ekanayake et al., 2012; Takach et al., 2012; Takach and Young, 2014). In fact, variation of mating type and alkaloid genes determined by PCR could be enough to allow placement of tall fescue endophytes into distinct genotype groups associated with each *Epichloë* species (**Table 1**) (Takach and Young, 2014). At least four unique *E. coenophiala* genotypes are easily distinguishable among tall fescue sourced originally from Europe and the Mediterranean basin (Ekanayake et al., 2012; Takach and Young, 2014).

Literature surrounding endophyte-infected tall fescue that causes fescue toxicosis often refers to *E. coenophiala* as the common toxic endophyte. The objective of this study was to compare the endophytes within tall fescue cultivars, varieties and ecotypes from the US using markers to SSRs and alkaloid biosynthesis genes to identify and characterize these endophytes. We have determined endophyte diversity across historical and current tall fescue samples to evaluate the endophyte diversity that may exist across the US.

MATERIALS AND METHODS

BIOLOGICAL MATERIALS

Tall fescue plant material was provided by researchers in Alabama, Arkansas, Georgia, Kentucky, Missouri, Mississippi, New York, Ohio, Oklahoma, Pennsylvania, South Carolina, Tennessee, Texas, and West Virginia (**Table 2**). Plants were maintained in a space plant nursery under rain-fed conditions or in the greenhouse at the Samuel Roberts Noble Foundation, Ardmore, Oklahoma. Each researcher was requested to provide at least 10 independent

Table 2 | Sources of tall fescue plants from US collection.

State	County	Plant designation[a]	No. of plants maintained	No. plants that died	No. of endophyte-infected plants	Endophyte genotypes present
AL	Dallas	Black belt station	21	0	21	2-1, 2-2
AR	Nevada	Prescott	6	0	6	1-1, 2-1
AR	Hempstead	Deanne	6	0	6	2-1
FL, GA, MD, NY		GA-5	10	2	7	1-1, 1-2, 2-1, 2-3
GA	Walker	Walker county	12	0	8	2-1
GA	Wayne	Jesup	9	2	6	2-1
KY	Mennifee	Suiter farm	10	1	9	1-1, 2-1, 2-4
KY	Caldwell	Pennyrile	10	1	4	2-1
MO	Camden	Lake Farm	12	0	9	2-1
MO	Camden	Ford place	8	0	8	2-1
MO	Camden	Tiny's place	4	0	3	2-1
MO	St. Louis	Hencken	6	0	6	2-1
MS	Oktibbeha	Starksville	9	0	9	2-1, 2-4
NY	Allegany	Alfred	5	0	5	2-1
OH	Coshocton	NAEW graze	6	0	6	1-1, 2-1
OH	Coshocton	NAEW hay	6	0	5	2-1
OK	Carter	NFTF 1000	9	0	7	2-1
OK	Woodward	NFTF 1100	9	1	7	2-1
OK	Hughes	Calvin	2	0	1	2-1
PA	Huntingdon	Soder	1	0	1	1-?
PA	Huntingdon	Soder (Petersburg)	3	0	3	3-1
PA	Centre	Everhart	1	0	1	2-3
PA	Centre	JRE state college	5	1	4	2-1, 2-2
SC	Anderson	NFTF 1491	10	0	5	2-1
TN	Henderson	Lexington	4	0	4	2-1
TN	Henderson	Natchez trace	7	0	6	1-1, 2-1
TX	Fannin	Fannin	4	0	4	2-1
TX	Fannin	NFTF 1492	10	3	6	2-1, 2-2
TX	Crosby, Lubbock, Lamb, Briscoe	NFTF 1230	9	0	8	1-1, 2-1
TX	Kerr	NFTF 1480	9	1	8	2-1
WV	Raleigh	Roscoe upper	10	0	8	1-1, 2-1
WV	Raleigh	Roscoe middle	10	1	7	2-1
WV	Raleigh	Roscoe lower	10	3	6	2-1, 2-4
WV	Raleigh	Reba	10	3	5	2-1

[a] *Plant designation refers to landmark or site location or plant breeding line information (NFTF).*

plants from fields known to cause fescue toxicosis or thought to contain the common toxic endophyte. Tall fescue seed stocks (PI lines) were sourced from the National Plant Germplasm System (NPGS). Georgia-5 (GA-5) seed was provided by JH Bouton and other seed stocks were sourced from the Samuel Roberts Noble Foundation tall fescue (NFTF) breeding program and designated NFTF.

DNA ISOLATION AND ENDOPHYTE GENOTYPING

Total DNA from individual seeds or tillers from stock plants were isolated using QIAGEN MagAttract 96 DNA Plant Core Kit (Qiagen Inc., Valencia, CA). Primers specific for *tefA*, tef1-exon1d (5′-GGGTAAGGACGAAAAGACTCA-3′) and tef1-exon5u-1 (5′-CGGCAGCGATAATCAGGATAG-3′) (Craven et al., 2001; Moon et al., 2002) were used to detect the presence of endophyte. A minimum set of key alkaloid genes and two mating type genes were chosen to differentiate the *E. coenophiala* endophytes present in continental tall fescue based on the previous study by Takach and Young (2014). The markers were designed to *mtAC* and *mtBA* mating type genes, *dmaW* and *lpsB* for representatives of the *EAS* locus, *lolC* and *lolA* for representatives of the *LOL* locus, *idtG* and *idtQ* for representatives of the *IDT* locus, and *perA* second adenylation domain (*perA*-A2) for *PER*. Multiplex PCR was performed in a total volume of 25 μL containing 3 μL DNA, 1.0 U GoTaq™ DNA Polymerase (Promega Corp., Madison, WI), 1× Green GoTaq™ Reaction Buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega Corp.), and 1 μM of each primer as described previously (Takach et al., 2012; Charlton et al., 2014; Takach and Young, 2014). The cycling parameters were an initial denaturation step for 1 min at 94 C, 30 cycles of denaturation at

94 C for 15 s, annealing at 56 C for 30 s, extension at 72 C for 45 s, followed by a final synthesis step at 72 C for 10 min.

PCR of the microsatellite B10 and B11 loci (Moon et al., 1999) were used to differentiate endophytes within an *E. coenophiala* profile. For SSR analysis, one primer at each locus was end labeled with a fluorescent phosphoramidite dye. Specifically, primers B10.1 was labeled with 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) and B11.1 was labeled with 2′-chloro-5′-fluoro-7′,8′-benzo-1,4-dichloro-6-carbo xyfluorescein (NED) (Life Technologies, Carlsbad, CA). PCR was performed in a total volume of 10 μL containing diluted DNA (approximately 0.5 ng), 0.75 U Platinum *Taq* DNA Polymerase (Life Technologies), 1× PCR Buffer (-Mg), 1.5 mM $MgCl_2$, 100 nM of each dNTP (Promega Corp.) and 200 nM of each primer. The cycling parameters were an initial denaturation step for 4 min at 94 C, 35 cycles of denaturation at 94 C for 30 s, annealing at 60 C for 30 s, extension at 72 C for 30 s, followed by a final synthesis step at 72 C for 7 min. PCR products (1.5 μL of a 1:10 dilution) were added to 9.9 μL of Hi-Di formamide and 0.1 μL of GeneScan™ 500 LIZ™ size standard (Life Technologies). Samples were denatured at 94 C for 5 min prior to separation on an ABI 3730 DNA Analyzer. Data analysis was performed using Peak Scanner Software v1.0 (Applied Biosystems).

ERGOVALINE ANALYSIS

Pseudostems were collected from greenhouse grown plants, lyophilized and ground into a fine powder and stored at −20°C. Ergovaline concentrations were measured in duplicate using 10 ± 0.10 mg of tissue for each endophyte infected sample. Samples were extracted in 200 μL of methanol containing 0.005 mg/mL dihydroergotamine tartrate salt (Sigma-Aldrich, St. Louis, MO) for 3 h and then centrifuged at 1800 × *g* for 5 min. Each sample was analyzed for the presence of ergovaline using an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA) as described previously (Takach et al., 2012). Seed extracts in which the ergovaline content was previously quantified (A. M. Craig, Endophyte Testing Laboratory, Oregon State University) were used as standards for quantification. Concentrations used to generate the standard curve included 0, 50, 97, 500, 1000, and 2000 ppb.

The linear standard curve was plotted as ergovaline:ergotamine peak area ratio vs. the actual amount of ergovaline. Standards and samples were analyzed in duplicate and their values averaged.

RESULTS AND DISCUSSION

EVALUATION OF HISTORICAL TALL FESCUE ENDOPHYTES FROM THE UNITED STATES

The cultivar KY31 is well known for causing fescue toxicosis and has been distributed over much of the eastern United States (**Figure 1**). KY31 was established from an ecotype collection from the Suiter farm in Menifee County, KY (released as a cultivar in 1943) and source material (PI 531431) from this location was deposited into NPGS in 1991. Other cultivars, developed after KY31, have also contributed to the dissemination of endophyte-infected tall fescue (Pedersen and Sleper, 1988). In particular, Alta (cultivar in 1945) was considered a successful cultivar in northeast US and was likely interbred with KY31 (Asay et al., 1979). Seed from other early tall fescue cultivars such as Alta, Kenmont (cultivar in 1963), Kenwell (cultivar in 1965), Kenhy (cultivar in 1977), and Missouri 96 (cultivar in 1977) (Pedersen and Sleper, 1988) were also included in our study (see **Table 3** for NPGS deposition

Table 3 | Characterization of endophytes from Kentucky 31 tall fescue seed in US based on microsatellite variation.

Seed stock	Year[a]	Seeds tested	%E+ (number)	%E– (number)	%E+ Ecoe profile 1[b] (number)	%E+ Ecoe profile 2[b] (number)
PI 561431 - KY31	1991	46	91% (42)	9% (4)	48% (22)[c]	43% (20)[d]
KY31 commercial seed[e]	2011	48	79% (38)	21% (10)	17% (8)[c]	63% (30)[d]
KY31 - SW Missouri	2008	46	98% (45)	2% (1)	0% (0)	98% (45)[d]
PI 596701 - Missouri 96	1979	24	0	100 (24)	0% (0)	0% (0)
PI 578714 - Kenmont	1963	24	0	100 (24)	0% (0)	0% (0)
PI 574521 - Kenwell	1965	22	0	100 (22)	0% (0)	0% (0)
PI 434051 - Kenhy	1979	24	0	100 (24)	0% (0)	0% (0)
PI 601020 - Johnstone[f]	1983	24	0	100 (24)	0% (0)	0% (0)
PI 578712 - Alta	1962	48	0	100 (48)	0% (0)	0% (0)
Jesup E+	2003	10	100% (10)	0% (0)	0% (0)	100% (10)
NFTF 1000 - PDF E+[g]	1998	36	100% (36)	0% (0)	8% (3)	92% (33)
NFTF 1011 - PDF E+[g]	2007	12	100% (12)	0% (0)	33% (4)	67% (8)
NFTF 1041 - PDF E+[g]	2010	12	100% (12)	0% (0)	0% (0)	100% (12)

[a] *The year the seed was purchased, generated or when it entered NPGS.*

[b] *As determined by markers consistent with E. coenophiala profile 1 and 2 from* ***Table 1****.*

[c] *SSR B10 = 161, 170, 184; B11 = 147, 191.*

[d] *SSR B10 = 152, 161, 178; B11 = 171, 195.*

[e] *The KY31 commercial seed Tri-Star Seed Co., Inc. located in Spring Hill, KS was purchased from the Tractor Supply Company, Ardmore, Oklahoma, in July 2011.*

[f] *Johnstone was released as an endophyte free cultivar (Buckner et al., 1983).*

[g] *NFTF 1011 and NFTF 1041 represent selections from the original NFTF 1000 (also known as PDF E+ Hopkins et al., 2011) from Oklahoma.*

dates). Studies that have included some of these early cultivars have subsequently indicated they were infected with a common toxic endophyte (Bacon et al., 1977; Cornell et al., 1982; Siegel et al., 1984; Pedersen and Sleper, 1988). We also evaluated more recent cultivars and germplasm from the NFTF breeding program that contain common toxic endophyte (**Table 3**).

Seed from each cultivar or line were analyzed for endophyte infection and genetic variation (**Table 3**). Unfortunately many of the seed samples sourced from NPGS were endophyte-free or had levels less than 5%. Since endophyte viability can be compromised during storage (Siegel et al., 1985; Rolston and Agee, 2007) there was no guarantee these seeds would represent the endophyte status of the original plant material. Only the three KY31 samples, Jesup E+ and NFTF breeding lines were positive for endophyte presence. In addition, two endophyte genotypes, *E. coenophiala* profile 1 and profile 2 that vary based on presence of *IDT* genes, could be distinguished in four of these seed samples. However, the percentage of each endophyte strain varied in each seed lot. Four of the tested seed lines, KY31 (from Missouri), Jesup E+ and NFTF 1041 may represent an *E. coenophiala* profile 2 monoculture, or contain a low incidence of *E. coenophiala* profile 1 as the number of seeds tested for some lines were low (**Table 3**).

Lines that were selected from NFTF 1000-PDF (NFTF 1011 selected from PDF for vigorous growth, high forage yield and digestibility, and NFTF 1041 selected from PDF for high digestibility by marker assisted selection) showed different ratios of each *E. coenophiala* profile (**Table 3**). This may show the influence an endophyte strain, which is maintained in the maternal line, can have on selectable traits such as persistence and vigor if the endophyte provides a host advantage. As such, it would be interesting to evaluate the host genetic shifts under selection, with and without endophytes, while also following selection of different endophyte strains.

ENDOPHYTE ANALYSIS FROM DEVELOPMENT OF THE CULTIVAR GEORGIA-5

Analysis of endophyte variation within a population provides an opportunity to evaluate material incorporated through a tall fescue breeding pipeline and eventually released for commercial production (**Figure 2**). The GA-5 cultivar was developed as a synthetic endophyte-infected cultivar with superior forage yield and persistence in the Southern Coastal Plains that had potential to replace KY31 (Bouton et al., 1993b). The cultivar was established from five clones and was shown to be 75% endophyte infected (Bouton et al., 1993b). We evaluated seed from the original five clones (each clone having originated from a different location) using markers to SSRs and alkaloid biosynthesis genes to determine the initial infection rates of each clone and identify which *E. coenophiala* profiles were present. The endophyte status of the originating lines varied from 32 to 100% infection, and the endophyte profiles were consistent within the seed sample from each clone. Three independent endophyte genotypes (based on SSRs) were identified within the clones (**Figure 2**). Seed from synthetic 1 established in 1980 was also tested for endophyte infection and identification, and all three endophyte genotypes were represented within this sample with an overall endophyte infection level of 79% (**Figure 2**). In 1993, GA-5 was registered as a cultivar (Bouton et al., 1993b) and subsequently released commercially in 1996. When we evaluated a seed stock from the commercial line the overall infection level was 69% and two of the three expected endophyte SSR profiles were identified within the sample. However, an additional endophyte genotype (B10 = 152, 161, 178 and B11 = 171, 195) was present in 5% of the seed sample (**Figure 2**) that has likely arisen from contamination later in production. The level of endophyte free seed increased from Syn 1 (21%) to Syn 6 (31%) and may indicate that production favored this part of the population. Unfortunately we were unable to detect the endophyte genotype profile 2 with B10 = 161, 173, 178 and B11 = 171, 195, which may be due to the number of seeds that were tested.

ENDOPHYTE DIVERSITY FROM US TALL FESCUE COLLECTIONS

To further examine tall fescue endophyte diversity within the US, collections of tall fescue plants from 14 eastern states were evaluated for endophyte presence. In total, 246 tall fescue plants were screened using markers for SSRs B10 and B11, and the minimum set of alkaloid biosynthesis genes to distinguish the different *E. coenophiala* genetic profiles. Of the 246 plants initially screened, 204 (83%) were endophyte-infected. All of the endophyte-infected samples could be amplified with primer sets to *mtAC*, *perA*, and the *LOL* and *EAS* markers, and samples only varied with the presence of *IDT* markers. Three *E. coenophiala* genetic profiles were identified (*E. coenophiala* profiles 1, 2, and 3; **Table 4**).

Further analysis using the B10 and B11 SSR markers showed additional variation within the genetic profiles. There were two unique SSR profiles for genotype 1, four SSR profiles for genotype 2 and one SSR profile for genotype 3. *E. coenophiala* profile 1 is consistent with the same pattern of the KY31 endophyte *E. coenophiala* strain e19 (Schardl et al., 1991; Takach and Young, 2014), while *E. coenophiala* profile 2 is more highly represented in the samples.

The two most common SSR marker patterns were also identified in the KY31 seed (**Table 3**). Interestingly, of the nine plants that were collected from the original Suiter farm (**Table 2**), one plant showed a third unique SSR profile in common with endophytes found in West Virginia and Mississippi. Only one set of plants from Pennsylvania produced *E. coenophiala* genetic profile 3, but unfortunately knowledge about the tall fescue planted at this site was unavailable. This Pennsylvania endophyte-infected tall fescue is the only set of plants that were likely to produce indole-diterpenes.

Plants with unique *E. coenophiala* profiles were selected from the nursery and maintained in the greenhouse. Ergovaline was analyzed from pseudostems of 25 plants representing the seven unique *E. coenophiala* profiles. Each of the plant-endophyte associations produced ergovaline (**Table 5**), indicating that the endophyte in these lines would all be considered a common toxic endophyte. The ergovaline levels generated under greenhouse conditions were also extremely high, well above the threshold of 400–750 ppb required for toxicity in livestock (Hovermale and Craig, 2001). Although the levels tested from these plants are high, it may simply reflect they were well maintained plants subjected to regular fertilization, which is known to result

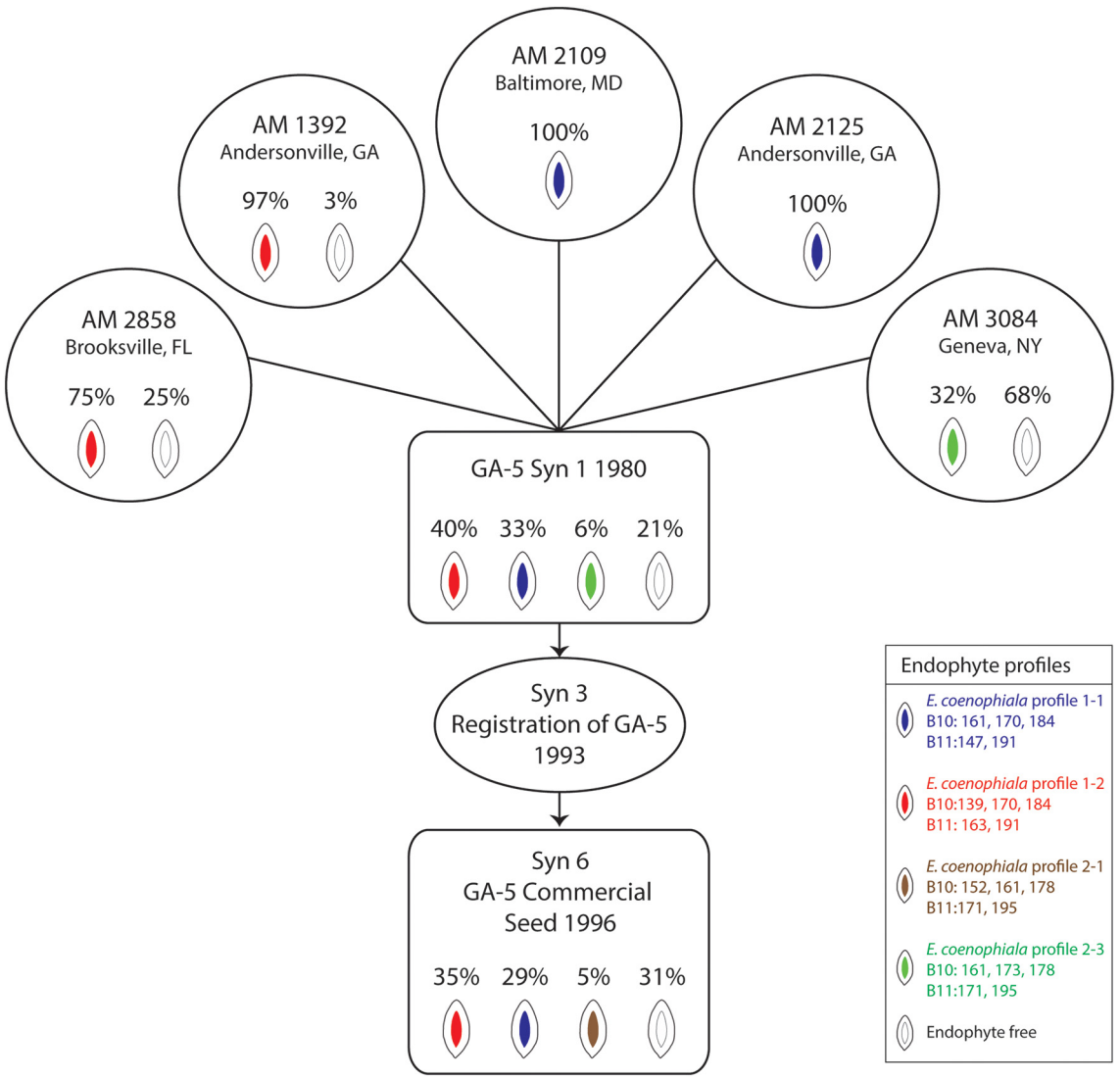


FIGURE 2 | *Epichloë coenophiala* profiles and infection frequencies of seed used in the development of the "Georgia 5" tall fescue cultivar. The five clones consisted of AM 1392, AM 2109, AM 2125, AM 2858, and AM 3084. The synthetic 3 generation represents the breeders seed increase from the five clones (Bouton et al., 1993b).

in higher levels of ergovaline production (Rottinghaus et al., 1991).

This study provides a snapshot of common toxic endophyte-infected tall fescue across the eastern US. The endophyte genotypes we identified were consistent with samples from Europe where tall fescue was originally sourced (Takach and Young, 2014). Endophyte genotype diversity was present at the origin of KY31 but it appears *E. coenophiala* genotype 2 is the dominant endophyte genotype across the US. This endophyte was also prevalent in other breeding populations such as Jesup E+ and NFTF 1000. Given the sample numbers we have selected from each location, it is still possible that other endophyte genotypes are present at low frequencies. Although KY31 is believed to be the predominant source of tall fescue in the US, other sources were developed and planted at various times and locations, and it is possible that during this process one endophyte genotype provided a selectable advantage. Indeed, if we look at NFTF 1011, a selection from the NFTF 1000 line (**Table 3**), we see a shift in endophyte genotype that may be due to specific selection pressures.

NEW CULTIVARS WITH SELECTED ENDOPHYTES

To overcome fescue toxicosis, researchers initially removed the endophyte from toxic tall fescue. Unfortunately, studies that evaluated the endophyte role on tall fescue performance found endophyte-infected lines had better persistence and greater yield than endophyte-free lines (Pedersen et al., 1990; Bouton et al., 1993a, 2002; West et al., 1993; Gunter and Beck, 2004). As expected, when production of ergot alkaloids was low or not present, livestock also had better overall performance and

Table 4 | Characterization of endophytes from tall fescue plants in US based on alkaloid profiles and microsatellite variation.

Endophyte genotype	B10 allele sizes (bp)	B11 allele sizes (bp)	*PER*[a]	*EAS*[a]		*LOL*[a]		*IDT*[a]		No. of plants	States
			perA-A2	*dmaW*	*lpsB*	*lolC*	*lolA*	*idtG*	*ltmQ*		
E. coenophiala profile 1-1	161, 170, 184	147, 191	+	+	+	+	+			11	AR, KY, OH, TN, TX, WV
E. coenophiala profile 1-2	139, 170, 184	163, 191	+	+	+	+	+			2	GA
E. coenophiala profile 2-1	152, 161, 178	171, 195	+	+	+	+	+		+	172	AL, AR, GA, KY, MO, MS, NY, OH, OK, PA, SC, TN, TX, WV
E. coenophiala profile 2-2	152, 161, 178	183, 195	+	+	+	+	+		+	3	AL, TX
E. coenophiala profile 2-3	161, 173, 178	171, 195	+	+	+	+	+		+	3	GA (NY)[b], PA
E. coenophiala profile 2-4	161, 173, 178	171, 210	+	+	+	+	+		+	4	KY, MS, WV
E. coenophiala profile 3-1	161, 170, 178	155, 163	+	+	+	+	+	+	+	3	PA

[a] *The + indicates that a PCR fragment was detected with primers designed to the gene.*

[b] *Identified out of GA-5 and represents the clone from NY (see* ***Figure 2****).*

Table 5 | Ergovaline concentrations of tall fescue infected with different *Epichloë coenophiala* genotypes.

Endophyte genotype[a]	B10 allele sizes (bp)	B11 allele sizes (bp)	No. of plants tested	States represented	Range of [Ergovaline] (ppm)
E. coenophiala profile 1-1	161, 170, 184	147, 191	5	GA, KY, TN, TX, WV	2.2–7.1
E. coenophiala profile 1-2	139, 170, 184	163, 191	1	GA	4.1
E. coenophiala profile 2-1	152, 161, 178	171, 195	11	AL, GA, KY, MO, MS, PA, TN, TX, WV	1.7–7.8
E. coenophiala profile 2-2	152, 161, 178	183, 195	2	AL, TX	1.2–9.3
E. coenophiala profile 2-3	161, 173, 178	171, 195	3	GA (NY)[b], PA	2.4–5.5
E. coenophiala profile 2-4	161, 173, 178	171, 210	2	KY, MS	1.9–8.4
E. coenophiala profile 3-1	161, 170, 178	155, 163	1	PA	4.2

[a] *Based on designations from Takach and Young (2014).*

[b] *Identified from the GA-5 line and represents the clone from NY (see* ***Figure 2****).*

increased average daily gains (Stuedemann and Hoveland, 1988; Gunter and Beck, 2004). An ideal solution to capture both endophyte associated plant persistence and reduced livestock toxicity was to identify an endophyte strain that retained traits for plant persistence, but did not produce the alkaloids toxic to livestock. Analysis of tall fescue across its natural distribution of Europe and the Mediterranean basin has shown diversity of both the endophyte (Christensen et al., 1993; Ekanayake et al., 2012; Takach and Young, 2014) and its plant host (Hand et al., 2012); subsequently this diversity has been exploited to establish selected endophyte-infected tall fescue with low mammalian toxicity (reviewed in Bouton, 2009; Johnson et al., 2013; Young et al., 2013). Typically these endophytes lack many or all of the genes at the *EAS* locus required for ergot alkaloid biosynthesis (e.g., *E. coenophiala* profile 4, **Table 1**) (Takach and Young, 2014), although some endophytes have been selected for lower ergot alkaloid production.

Initial success with selected endophytes was observed when the endophyte strain AR542 (known commercially as MaxQ and MaxP in the US and Australia, respectively) was inoculated into Jesup and GA-5 (Bouton et al., 2002). Agronomic evaluations indicated selected endophyte-tall fescue associations provided the benefits of endophyte infection (stand persistence) with animal performance similar to endophyte-free tall fescue (Bouton et al., 2002; Parish et al., 2003; Gunter and Beck, 2004). Jesup MaxQ (Pennington Seed, Inc.) was the first commercial tall fescue cultivar to be released and used by farmers containing a selected endophyte. Additional endophyte-infected tall fescue lines have since been established and evaluated for both plant and animal performance (Roberts and Andrae, 2004; Hopkins et al., 2010; Parish et al., 2013; Beck et al., 2014). One of the most recently released cultivars, Texoma MaxQ II, is the result of breeding for plant persistence with an ecotypic selection that was subsequently inoculated with the endophyte strain AR584 (MaxQ II) that does not cause livestock toxicity (Hopkins et al., 2010, 2011). Current commercially available cultivars of selected endophyte-tall fescue associations include: Jesup MaxQ (Pennington Seed, Inc.), Texoma MaxQ (Pennington Seed Inc.), BarOptima Plus E34 (Barenbrug), Duramax Gold (DLF International Seeds), and Estancia with ArkShield (MountainView Seeds).

CONCLUSION

Endophytes have clearly affected the success of tall fescue within the US, from the prevalence of the common toxic endophyte through to advancing cultivars with selected endophytes. Many farmers have learned to manage the effects of fescue toxicosis through pasture management techniques, but now there are also options for eliminating toxicity by pasture replacement. The

availability of elite tall fescue lines infected with selected endophytes allows farmers to provide nutritious, non-toxic feed for their livestock without fear of toxic repercussions. Climate change will likely increase the dependency of tall fescue monocultures to rely upon endophytes to provide drought tolerance and protection from insect pests. As more selected endophyte-infected cultivars enter the marketplace to replace tall fescue pastures containing common toxic endophytes, we will be able to examine the constancy of these symbiotic associations over time. Utilization of molecular markers will provide effective methods to identify endophyte strains within tall fescue cultivars, varieties and ecotypes, and help distinguish endophyte friend from foe.

ACKNOWLEDGMENTS

We would like to acknowledge Joe Bouton for providing seed and Simon Abel, Sherrita Bright, and Kenny Word for technical support. We thank Lark Trammell (Forage Analysis Core Facility), the Genomic Core Facility and the Greenhouse Core Facility at The Samuel Roberts Noble Foundation for technical support. The authors thank the following people who provided plants from the listed states: Don Ball (AL), Rob Kallenbach (MO), David Belesky (WV), Gerald Atkinson (KY), Gary Lacefield (KY), Norman Edwards (GA), James Hopkins (MO), Howard Skinner (PA and NY), David Barker (OH), James Rogers (OK and SC), Joseph Bouton (GA), Jimmy Ray Parish (MS), Steve Green (AR), Paul Beck (AR), and Jack Chapman (OK). The Samuel Roberts Noble Foundation provided financial support for this research.

REFERENCES

Arachevaleta, M., Bacon, C. W., Hoveland, C. S., and Radcliffe, D. E. (1989). Effect of the tall fescue endophyte on plant response to environmental stress. *Agron. J.* 81, 83–90. doi: 10.2134/agronj1989.00021962008100010015x

Asay, K., Frakes, R. V., and Buckner, R. C. (1979). "Breeding and cultivars," in *Tall Fescue*, eds R. Buckner and L. Bush (Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America), 111–139.

Bacon, C., Porter, J., Robbins, J., and Luttrell, E. (1977). *Epichloë typhina* from toxic tall fescue grasses. *Appl. Environ. Microbiol.* 34, 576–581.

Bacon, C. W. (1988). Procedure for isolating the endophyte from tall fescue and screening isolates for ergot alkaloids. *Appl. Environ. Microbiol.* 54, 2615–2618.

Bacon, C. W., Lyons, P. C., Porter, J. K., and Robbins, J. D. (1986). Ergot toxicity from endophyte-infected grasses: a review. *Agron. J.* 78, 106–116. doi: 10.2134/agronj1986.00021962007800010023x

Ball, D. M., Pederson, J., and Lacefield, G. D. (1993). The tall-fescue endophyte. *Am. Sci.* 81, 370–379.

Beck, P., Stewart, C., Gray, H., Gadberry, M., Gunter, S., Young, C., et al. (2014). Using tall fescue in a complementary grazing program for Spring calving beef cows in Southern Arkansas. *Prof. Anim. Sci.* 30, 423–431.

Bouton, J. (2009). "Deployment of novel endophytes in the tall fescue commercial seed trade," in *Tall Fescue for the Twenty-First Century, Agronomy Monograph 53*, eds H. Fribourg, D. Hannaway, and C. West (Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America), 367–375.

Bouton, J., Gates, R., Belesky, D., and Owsley, M. (1993a). Yield and persistence of tall fescue in the southeastern coastal plain after removal of its endophyte. *Agron. J.* 85, 52–55. doi: 10.2134/agronj1993.00021962008500010011x

Bouton, J., Gates, R., Hill, G., Owsley, M., and Wood, D. (1993b). Registration of 'Georgia 5' tall fescue. *Crop Sci.* 33, 1405. doi: 10.2135/cropsci1993.0011183X003300060059x

Bouton, J. H., Latch, G. C., Hill, N. S., Hoveland, C. S., McCann, M. A., Watson, R. H., et al. (2002). Reinfection of tall fescue cultivars with non-ergot alkaloid–producing endophytes. *Agron. J.* 94, 567–574. doi: 10.2134/agronj2002.0567

Buckner, R., Boling, J., Burrus, P., Bush, L., and Hemken, R. (1983). Registration of Johnstone tall fescue (Reg. No. 23). *Crop Sci.* 23, 399–400. doi: 10.2135/cropsci1983.0011183X002300020057x

Bush, L., Boling, J., and Yates, S. (1979). "Animal disorders," in *Tall Fescue*, eds R. C. Buckner and L. P. Bush (Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America), 247–292.

Bush, L. P., Wilkinson, H. H., and Schardl, C. L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol.* 114, 1–7.

Caldwell, J. D., Coffey, K. P., Jennings, J. A., Philipp, D., Young, A. N., Tucker, J. D., Hubbell, D. S. 3rd., et al. (2013). Performance by spring and fall-calving cows grazing with full, limited, or no access to toxic *Neotyphodium coenophialum*-infected tall fescue. *J. Anim. Sci.* 91, 465–476. doi: 10.2527/jas.2011-4603

Charlton, N. D., Craven, K. D., Afkhami, M. E., Hall, B. H., Ghimire, S. R., and Young, C. A. (2014). Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic *Epichloë* species of *Bromus laevipes*. *FEMS Microbiol. Ecol.* 90, 276–289. doi: 10.1111/1574-6941.12393

Charlton, N. D., Craven, K. D., Mittal, S., Hopkins, A. A., and Young, C. A. (2012). *Epichloë canadensis*, a new interspecific epichloid hybrid symbiotic with Canada wildrye (*Elymus canadensis*). *Mycologia* 104, 1187–1199. doi: 10.3852/11-403

Christensen, M., and Latch, G. (1991). Variation among isolates of *Acremonium* endophytes (*A. coenophialum* and possibly *A. typhinum)* from tall fescue *(Festuca arundinacea). Mycol. Res.* 95, 1123–1126. doi: 10.1016/S0953-7562(09)80558-3

Christensen, M., Leuchtmann, A., Rowan, D., and Tapper, B. (1993). Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis)* and perennial ryegrass *(Lolium perenne). Mycol. Res.* 97, 1083–1092. doi: 10.1016/S0953-7562(09)80509-1

Clay, K., Hardy, T. N., and Hammond, A. M. (1985). Fungal endophytes of grasses and their effects on an insect herbivore. *Oecologia* 66, 1–5. doi: 10.1007/BF00378545

Cornell, C., Garner, G., Yates, S., and Bell, S. (1982). Comparative fescue foot potential of fescue varieties. *J. Anim. Sci.* 55, 180–184.

Craven, K. D., Hsiau, P. T. W., Leuchtmann, A., Hollin, W., and Schardl, C. L. (2001). Multigene phylogeny of *Epichloë* species, fungal symbionts of grasses. *Ann. Mo. Bot. Gard.* 88, 14–34. doi: 10.2307/2666129

Cunningham, I. (1948). Tall fescue grass is poison for cattle. *N.Z. J. Agric.* 77, 519.

Cunningham, I. (1949). A note on the cause of tall fescue lameness in cattle. *Aust. Vet. J.* 25, 27–28. doi: 10.1111/j.1751-0813.1949.tb04752.x

Ekanayake, P. N., Hand, M. L., Spangenberg, G. C., Forster, J. W., and Guthridge, K. M. (2012). Genetic diversity and host specificity of fungal endophyte taxa in fescue pasture grasses. *Crop Sci.* 52, 2243–2252. doi: 10.2135/cropsci2011.12.0664

Fergus, E., and Buckner, R. C. (1972). Registration of Kentucky 31 Tall Fescue (Reg. No. 7). *Crop Sci.* 12, 714. doi: 10.2135/cropsci1972.0011183X001200050061x

Fleetwood, D. J., Scott, B., Lane, G. A., Tanaka, A., and Johnson, R. D. (2007). A complex ergovaline gene cluster in *Epichloë* endophytes of grasses. *Appl. Environ. Microbiol.* 73, 2571–2579. doi: 10.1128/AEM.00257-07

Fletcher, L. R., and Harvey, I. C. (1981). An association of a *Lolium* endophyte with ryegrass staggers. *N.Z. Vet. J.* 29, 185–186. doi: 10.1080/00480169.1981.34839

Glenn, A. E., Bacon, C. W., Price, R., and Hanlin, R. T. (1996). Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* 88, 369–383. doi: 10.2307/3760878

Groppe, K., Sanders, I., Wiemken, A., and Boller, T. (1995). A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Appl. Environ. Microbiol.* 61, 3943–3949.

Gunter, S., and Beck, P. (2004). Novel endophyte-infected tall fescue for growing beef cattle. *J. Anim. Sci.* 82, E75–E82.

Hand, M. L., Cogan, N. O., and Forster, J. W. (2012). Molecular characterisation and interpretation of genetic diversity within globally distributed germplasm collections of tall fescue (*Festuca arundinacea* Schreb.) and meadow fescue (*F. pratensis Huds.). Theor. Appl. Genet.* 124, 1127–1137. doi: 10.1007/s00122-011-1774-6

Hemken, R., Jackson, J. Jr., and Boling, J. (1984). Toxic factors in tall fescue. *J. Anim. Sci.* 58, 1011–1016.

Hopkins, A., Young, C., Butler, T., and Bouton, J. (2011). Registration of 'Texoma'MaxQ II tall fescue. *J. Plant Regist.* 5, 14–18. doi: 10.3198/jpr2010.02.0082crc

Hopkins, A., Young, C., Panaccione, D., Simpson, W., Mittal, S., and Bouton, J. (2010). Agronomic performance and lamb health among several tall fescue

novel endophyte combinations in the south-central USA. *Crop Sci.* 50, 1552–1561. doi: 10.2135/cropsci2009.08.0473

Hoveland, C., Schmidt, S., King, C., Odom, J., Clark, E., McGuire, J., et al. (1983). Steer performance and association of *Acremonium coenophialum* fungal endophyte on tall fescue pasture. *Agron. J.* 75, 821–824. doi: 10.2134/agronj1983.00021962007500050021x

Hoveland, C. S. (2009). "Origin and history," in *Tall Fescue for the 21st Century*, eds H. Fribourg, D. Hannaway, and C. West (Madison, WI: Amer Society of Agronomy), 3–10.

Hovermale, J. T., and Craig, A. M. (2001). Correlation of ergovaline and lolitrem B levels in endophyte-infected perennial ryegrass (*Lolium perenne*). *J. Vet. Diagn. Invest.* 13, 323–327. doi: 10.1177/104063870101300407

Jacobson, D., Miller, W., Seath, D., Yates, S., Tookey, H., and Wolff, I. (1963). Nature of fescue toxicity and progress toward identification of the toxic entity. *J. Dairy Sci.* 46, 416–422. doi: 10.3168/jds.S0022-0302(63)89066-9

Johnson, L. J., de Bonth, A. C., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D. J., et al. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Divers.* 60, 171–188. doi: 10.1007/s13225-013-0239-4

Johnson, M. C., Dahlman, D. L., Siegel, M. R., Bush, L. P., Latch, G. C., Potter, D. A., et al. (1985). Insect feeding deterrents in endophyte-infected tall fescue. *Appl. Environ. Microbiol.* 49, 568–571.

Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106, 202–215. doi: 10.3852/106.2.202

Leuchtmann, A., and Clay, K. (1990). Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. *Phytopathology* 80, 1133–1139. doi: 10.1094/Phyto-80-1133

Leuchtmann, A., Schmidt, D., and Bush, L. (2000). Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë/Neotyphodium* endophytes. *J. Chem. Ecol.* 26, 1025–1036. doi: 10.1023/A:1005489032025

Lyons, P. C., Plattner, R. D., and Bacon, C. W. (1986). Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. *Science* 232, 487–489. doi: 10.1126/science.3008328

Malinowski, D. P., and Belesky, D. P. (2000). Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci.* 40, 923–940. doi: 10.2135/cropsci2000.404923x

Moon, C. D., Craven, K. D., Leuchtmann, A., Clement, S. L., and Schardl, C. L. (2004). Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. *Mol. Ecol.* 13, 1455–1467. doi: 10.1111/j.1365-294X.2004.02138.x

Moon, C. D., Miles, C. O., Jarlfors, U., and Schardl, C. L. (2002). The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. *Mycologia* 94, 694–711. doi: 10.2307/3761720

Moon, C. D., Tapper, B. A., and Scott, B. (1999). Identification of *Epichloë* endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Appl. Environ. Microbiol.* 65, 1268–1279.

Morgan-Jones, G., and Gams, W. (1982). Notes on Hyphomycetes. XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe typhina*, new taxa in one of two new sections of *Acremonium*. *Mycotaxon* 15, 311–318.

Pan, J., Bhardwaj, M., Faulkner, J. R., Nagabhyru, P., Charlton, N. D., Higashi, R. M., et al. (2014). Ether bridge formation in loline alkaloid biosynthesis. *Phytochemistry* 98, 60–68. doi: 10.1016/j.phytochem.2013.11.015

Panaccione, D. G., Johnson, R. D., Wang, J., Young, C. A., Damrongkool, P., Scott, B., et al. (2001). Elimination of ergovaline from a grass–*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12820–12825. doi: 10.1073/pnas.221198698

Parish, J. A., McCann, M. A., Watson, R. H., Paiva, N. N., Hoveland, C. S., Parks, A. H., et al. (2003). Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in stocker cattle. *J. Anim. Sci.* 81, 2856–2868.

Parish, J. A., Parish, J. R., Best, T. F., Boland, H. T., and Young, C. A. (2013). Effects of selected endophyte and tall fescue cultivar combinations on steer grazing performance, indicators of fescue toxicosis, feedlot performance, and carcass traits. *J. Anim. Sci.* 91, 342–355. doi: 10.2527/jas.2011-4725

Pedersen, J., Lacefield, G., and Ball, D. (1990). A review of the agronomic characteristics of endophyte-free and endophyte-infected tall fescue. *Appl. Agric. Res.* 5, 188–194.

Pedersen, J., and Sleper, D. (1988). Considerations in breeding endophyte-free tall fescue forage cultivars. *J. Prod. Agric.* 1, 127–132. doi: 10.2134/jpa1988.0127

Porter, J. K., Bacon, C. W., and Robbins, J. D. (1979). Ergosine, ergosinine, and chanoclavine I from *Epichloë typhina*. *J. Agric. Food Chem.* 27, 595–598. doi: 10.1021/jf60223a045

Roberts, C., and Andrae, J. (2004). Tall fescue toxicosis and management. *Crop Manage*. 3. doi: 10.1094/CM-2004-0427-01-MG

Rolston, M., and Agee, C. (2007). "Delivering quality seed to specification-the USA and NZ novel endophyte experience," in *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses. Christchurch, New Zealand. Grassland Research and Practice Series*, eds A. Popay and E. Thom (Dunedin, NZ: NZ Grassl. Assoc. Inc.), 229–231.

Rottinghaus, G. E., Garner, G. B., Cornell, C. N., and Ellis, J. L. (1991). HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. *J. Agric. Food Chem.* 39, 112–115. doi: 10.1021/jf00001a022

Rowan, D., and Latch, G. (1994). "Utilization of endophyte-infected perennial ryegrasses for increased insect resistance," in *Biotechnology of Endophytic Fungi of Grasses*, eds C. W. Bacon and J. F. J. White (Boca Raton, FL: CRC Press), 169–183.

Saikia, S., Takemoto, D., Tapper, B. A., Lane, G. A., Fraser, K., and Scott, B. (2012). Functional analysis of an indole-diterpene gene cluster for lolitrem B biosynthesis in the grass endosymbiont *Epichloë festucae*. *FEBS Lett.* 586, 2563–2569. doi: 10.1016/j.febslet.2012.06.035

Schardl, C. L. (2001). *Epichloë festucae* and related mutualistic symbionts of grasses. *Fungal Genet. Biol.* 33, 69–82. doi: 10.1006/fgbi.2001.1275

Schardl, C. L., Liu, J., White, J. F. Jr., Finkel, R. A., An, Z., and Siegel, M. R. (1991). Molecular phylogenetic relationships of nonpathogenic grass mycosymbionts and clavicipitaceous plant pathogens. *Plant Syst. Evol.* 178, 27–41. doi: 10.1007/BF00937980

Schardl, C. L., Young, C. A., Hesse, U., Amyotte, S. G., Andreeva, K., Calie, P. J., et al. (2013a). Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. *PLoS Genet.* 9:e1003323. doi: 10.1371/journal.pgen.1003323

Schardl, C. L., Young, C. A., Pan, J., Florea, S., Takach, J. E., Panaccione, D. G., et al. (2013b). Currencies of mutualisms: sources of alkaloid genes in vertically transmitted Epichloae. *Toxins* 5, 1064–1088. doi: 10.3390/toxins5061064

Siegel, M., Latch, G., Bush, L., Fannin, F., Rowan, D., Tapper, B., et al. (1990). Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. *J. Chem. Ecol.* 16, 3301–3315. doi: 10.1007/BF00982100

Siegel, M. R., Johnson, M. C., Varney, D., Nesmith, W., Buckner, R., Bush, L. P., et al. (1984). A fungal endophyte in tall fescue: incidence and dissemination. *Phytopathology* 74, 932–937. doi: 10.1094/Phyto-74-932

Siegel, M. R., Latch, G. C. M., and Johnson, M. C. (1985). *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: significance and control. *Plant Dis.* 69, 179–181.

Spiering, M. J., Faulkner, J. R., Zhang, D. X., Machado, C., Grossman, R. B., and Schardl, C. L. (2008). Role of the LolP cytochrome P450 monooxygenase in loline alkaloid biosynthesis. *Fungal Genet. Biol.* 45, 1307–1314. doi: 10.1016/j.fgb.2008.07.001

Spiering, M. J., Moon, C. D., Wilkinson, H. H., and Schardl, C. L. (2005). Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. *Genetics* 169, 1403–1414. doi: 10.1534/genetics.104.035972

Stuedemann, J. A., and Hoveland, C. S. (1988). Fescue endophyte: history and impact on animal agriculture. *J. Prod. Agric.* 1, 39–44. doi: 10.2134/jpa1988.0039

Takach, J. E., Mittal, S., Swoboda, G. A., Bright, S. K., Trammell, M. A., Hopkins, A. A., et al. (2012). Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. *Appl. Environ. Microbiol.* 78, 5501–5510. doi: 10.1128/AEM.01084-12

Takach, J. E., and Young, C. A. (2014). Alkaloid genotype diversity of tall fescue endophytes. *Crop Sci.* 54, 667–678. doi: 10.2135/cropsci2013.06.0423

Tanaka, A., Tapper, B. A., Popay, A., Parker, E. J., and Scott, B. (2005). A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. *Mol. Microbiol.* 57, 1036–1050. doi: 10.1111/j.1365-2958.2005.04747.x

Tsai, H. F., Liu, J. S., Staben, C., Christensen, M. J., Latch, G. C., Siegel, M. R., et al. (1994). Evolutionary diversification of fungal endophytes of tall fescue grass by

hybridization with *Epichloë* species. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2542–2546. doi: 10.1073/pnas.91.7.2542

Wang, J., Machado, C., Panaccione, D. G., Tsai, H. F., and Schardl, C. L. (2004). The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. *Fungal Genet. Biol.* 41, 189–198. doi: 10.1016/j.fgb.2003.10.002

West, C., Izekor, E., Turner, K., and Elmi, A. (1993). Endophyte effects on growth and persistence of tall fescue along a water-supply gradient. *Agron. J.* 85, 264–270. doi: 10.2134/agronj1993.00021962008500020019x

Yates, S. (1971). Toxin-producing fungi from fescue pasture. *Microb. Toxins* 7, 191–206. doi: 10.1016/B978-0-12-046507-1.50012-4

Young, C. A., Felitti, S., Shields, K., Spangenberg, G., Johnson, R. D., Bryan, G. T., et al. (2006). A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. *Fungal Genet. Biol.* 43, 679–693. doi: 10.1016/j.fgb.2006.04.004

Young, C. A., Hume, D. E., and McCulley, R. L. (2013). Forages and pastures symposium: fungal endophytes of tall fescue and perennial ryegrass: pasture friend or foe? *J. Anim. Sci.* 91, 2379–2394. doi: 10.2527/jas.2012-5951

Young, C. A., Tapper, B. A., May, K., Moon, C. D., Schardl, C. L., and Scott, B. (2009). Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltm* gene analysis. *Appl. Environ. Microbiol.* 75, 2200–2211. doi: 10.1128/AEM.00953-08

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 July 2014; accepted: 14 October 2014; published online: 04 November 2014.

Citation: Young CA, Charlton ND, Takach JE, Swoboda GA, Trammell MA, Huhman DV and Hopkins AA (2014) Characterization of Epichloë coenophiala within the US: are all tall fescue endophytes created equal? Front. Chem. ***2****:95. doi: 10.3389/fchem.2014.00095*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 13 October 2014
doi: 10.3389/fchem.2014.00090

Development of a methodology to measure the effect of ergot alkaloids on forestomach motility using real-time wireless telemetry

Amanda M. Egert[1], James L. Klotz[2], Kyle R. McLeod[1] and David L. Harmon[1]*

[1] *Ruminant Nutrition Laboratory, Department of Animal & Food Sciences, University of Kentucky, Lexington, KY, USA*
[2] *Forage-Animal Production Research Unit, Agricultural Research Service, United States Department of Agriculture, Lexington, KY, USA*

Edited by:
Darrin Smith, Eastern Kentucky University, USA

Reviewed by:
Gerald Huntington, North Carolina State University, USA
Lori L. Smith, University of Kentucky Veterinary Diagnostic Lab, USA

***Correspondence:**
David L. Harmon, Ruminant Nutrition Laboratory, Department of Animal and Food Sciences, University of Kentucky, 814 W. P. Garrigus Building, Lexington, KY 40546, USA
e-mail: dharmon@uky.edu

The objectives of these experiments were to characterize rumen motility patterns of cattle fed once daily using a real-time wireless telemetry system, determine when to measure rumen motility with this system, and determine the effect of ruminal dosing of ergot alkaloids on rumen motility. Ruminally cannulated Holstein steers ($n = 8$) were fed a basal diet of alfalfa cubes once daily. Rumen motility was measured by monitoring real-time pressure changes within the rumen using wireless telemetry and pressure transducers. Experiment 1 consisted of three 24-h rumen pressure collections beginning immediately after feeding. Data were recorded, stored, and analyzed using iox2 software and the rhythmic analyzer. All motility variables differed ($P < 0.01$) between hours and thirds (8-h periods) of the day. There were no differences between days for most variables. The variance of the second 8-h period of the day was less than ($P < 0.01$) the first for area and less than the third for amplitude, frequency, duration, and area ($P < 0.05$). These data demonstrated that the second 8-h period of the day was the least variable for many measures of motility and would provide the best opportunity for testing differences in motility due to treatments. In Experiment 2, the steers ($n = 8$) were pair-fed the basal diet of Experiment 1 and dosed with endophyte-free (E−) or endophyte-infected (E+; 0 or 10 μg ergovaline + ergovalinine/kg BW; respectively) tall fescue seed before feeding for 15 d. Rumen motility was measured for 8 h beginning 8 h after feeding for the first 14 d of seed dosing. Blood samples were taken on d 1, 7, and 15, and rumen content samples were taken on d 15. Baseline ($P = 0.06$) and peak ($P = 0.04$) pressure were lower for E+ steers. Water intake tended ($P = 0.10$) to be less for E+ steers the first 8 h period after feeding. The E+ seed treatment at this dosage under thermoneutral conditions did not significantly affect rumen motility, ruminal fill, or dry matter of rumen contents.

Keywords: forestomach, contractions, motility, rumen pressure, telemetry, ergot alkaloids, tall fescue

INTRODUCTION

Numerous factors affect motility of the reticulorumen including diet composition, feed and water intake, environmental temperature, feeding vs. resting activity, volatile fatty acid concentrations, and metabolic conditions, such as hypocalcemia, as well as individual animal variation (Church, 1976). Additionally, many methods have previously been used for measuring forestomach motility, such as electromyography (McLeay and Smith, 2006; Poole et al., 2009), radiotelemetry (Cook et al., 1986), and pressure-sensitive recordings of ruminal gas (Colvin and Daniels, 1965) or fluids (Dado and Allen, 1993). In order to adequately evaluate the effect of a treatment on rumen motility, one must first understand typical rumen motility patterns throughout the entire feeding cycle.

Ergot alkaloids, which are produced by a symbiotic endophyte associated with tall fescue grass (Lyons et al., 1986), cause fescue toxicosis in grazing livestock (Strickland et al., 2011). Fescue toxicosis syndrome can be costly for livestock producers due to decreased average daily gains, feed intake, milk production, and conception rates (Strickland et al., 2011). Westendorf et al. (1993) found that about 93–96% of ergot alkaloids consumed are absorbed or transformed in the gastrointestinal tract. Additionally, it was determined that only 50–60% of the ergot alkaloids administered in the diet are recovered in the abomasum, which means that a large portion (40–50%) of ergot alkaloids in the diet are metabolized or absorbed in the forestomach.

Recent research with ergot alkaloids has suggested that rumen motility may also be altered with endophyte-infected tall fescue consumption. For example, Foote et al. (2013) demonstrated that the DM percentage and dry contents of the rumen on a BW basis were greater for cattle that were ruminally dosed with endophyte-infected (**E+**) tall fescue seed compared to cattle dosed with endophyte-free (**E−**) seed. This finding could indicate a difference in particulate or liquid passage rates. One hypothesis is that reduced passage rate in E+ steers could be a result of decreased rumen motility. Ergot alkaloids, specifically

ergotamine and ergovaline, have been shown to decrease contractions and increase baseline tonus of reticulorumen smooth muscle in sheep when administered intravenously (McLeay and Smith, 2006; Poole et al., 2009). Yet, there has not been research investigating the effect of ergot alkaloids or endophyte-infected tall fescue seed on rumen motility patterns in cattle. Furthermore, data on ruminal or oral dosing of ergot alkaloids and the effect on rumen motility is lacking.

Therefore, the objectives of Experiment 1 were to characterize rumen motility patterns relative to feeding using a pressure transducer and real-time, wireless telemetry system and determine when, relative to feeding, to measure motility. Using the time period as determined in Experiment 1, the objective of Experiment 2 was to investigate the effects of ruminal dosing of endophyte-infected tall fescue seed on rumen motility, rumen dry matter contents, and ruminal fill in cattle.

MATERIALS AND METHODS

The procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Experiments were conducted at the University of Kentucky C. Oran Little Research Center in Woodford County.

EXPERIMENT 1

Animal management

Eight ruminally cannulated Holstein steers ($BW = 321 \pm 11$ kg) were fed alfalfa cubes (% composition on a DM basis: $CP = 16.8$; $ADF = 33.5$; $NDF = 43.1$; $NFC = 29.1$; $TDN = 59$; $NE_m = 5.09$ MJ/kg) at $1.5 \times NE_m$ once daily (0830 h) top-dressed with a trace mineral pre-mix (Kentucky Nutrition Service, Lawrenceburg, KY, USA; $NaCl = 92$–96%; $Fe = 9275$ ppm; $Zn = 5500$ ppm; $Mn = 4790$ ppm; $Cu = 1835$ ppm; $I = 115$ ppm; $Se = 18$ ppm; $Co = 65$ ppm) to meet or exceed nutrient requirements (NRC, 2000). Steers were housed indoors at 22°C in individual 3×3 m stalls and given *ad libitum* access to water.

Telemetry system

A wireless telemetry system (emkaPACK*4G* telemetry system, emka TECHNOLOGIES USA, Falls Church, Virginia) was used to monitor real-time pressure changes in the rumen. The system consisted of 2 receivers, 8 transmitters, and 8 bptVAP modules (pressure transducers). Wireless receivers were mounted securely to the wall outside of the pens in the room with the steers. The receivers were hardwired to a POE+ switch (8-port gigabit GREENnet POE+ switch, TRENDnet, Torrance, CA) that was connected to a laptop. All cable connections were made using E5 ethernet cables. Transducers were connected to their corresponding transmitters using the auxiliary port. During experimentation, the transducer and transmitter were housed in a 1 L cylindrical plastic container with screw-on lid (Gordon Food Service, Wyoming, MI), which replaced the cap in the cannula opening. A stainless steel female luer lock bulkhead adapter inserted into the bottom of the container served as the connection between the transducer and catheter. A female luer lock to 2.4 mm barb adapter connected the transducer to a 5.5 cm piece of silicone tubing (i.d. = 2.4 mm; o.d. = 4.0 mm) attached to the barb of the bulkhead adapter. The transducer was taped to the side of the container to prevent kinks in the tubing.

The catheter was a section of 96.5 cm long Tygon tubing (i.d. = 3.2 mm; o.d. = 6.4 mm) with 8 fused Tygon tubing cuffs and a 22.9 cm latex balloon (Bargain Balloons, Niagara Falls, NY) on one end, which was prefilled with 1 L of water. The cuffs enabled consistent placement of the balloons on the end of the catheter. On the other end of the catheter, a 3-way stopcock was connected by means of a female luer lock to 3.2 mm barb adapter. The catheter was weighted with approximately 300 g anchored approximately 4 cm from the top of the balloon. Balloons, which were replaced before each data collection period, were secured to the catheter using latex castration bands (Ideal Instruments, Neogen Corporation, Lansing, MI) placed over the balloon tongue and clamped tightly onto the catheter by plastic hose clamps (acetyl copolymer; i.d. minimum = 11.4 mm; i.d. maximum = 13 mm; Cole-Palmer Instrument Co., Vernon Hills, IL). Cheesecloth was placed into the container to prevent excessive movement of the transmitter. Upon submerging the water-filled balloon in the ventral sac of the rumen, the attached container was inserted into the cannula opening. A piece of nylon webbing over the lid of the container was secured to the cannula with nylon screws and thumb nuts (1/4–20; Non-Ferrous Fastener Inc., Chino, CA) to keep the container in place.

Signal calibration

Each transmitter and pressure transducer combination was manually calibrated using a sphygmomanometer connected to the "out" port of the transducer. Calibration was performed in the two-points (sampled) mode (20 and 200 mm Hg) of iox2 software (iox 2.9.4.27, emka TECHNOLOGIES USA) before data collection commenced on each day. To verify that calibration was successful, various amounts of pressure were applied with the pressure gage to check that values on the gage matched values in iox2 software (emka TECHNOLOGIES USA).

Data collection and analysis

Data were collected for 24 consecutive hours to capture the entire feeding cycle on three separate instances for each steer. Collection periods began, immediately following feeding, at 0830 and ended at 0830 the following day. Data were recorded and stored using iox2 software (emka TECHNOLOGIES USA) with a sampling rate of 100 pressure readings per second. Smoothing was set to 20 samples (200 ms) to help eliminate some background and movement noise in the signal.

The rhythmic analyzer in iox2 was used simultaneously while data were collected to analyze the raw rumen pressure data, identify ruminal contractions, and calculate the following parameters for each contraction: baseline, peak, amplitude, frequency, time to peak (TTP), relaxation time (RT), and area under the curve. The log and storage cadence was set to event related mode calculating the mean of these parameters for each event. By definition, for data to be considered an event or contraction, the signal must have increased at least 4 mm Hg from baseline (event threshold). Additionally, the contraction must have a slope value for the TTP start threshold of at least 0.500 mm Hg/s.

Individual water intake was also recorded using water meters every 8 h after feeding on collection days.

Statistical analysis

Statistical Analysis Systems software (SAS; SAS Inst. Inc., Cary, NC) was used to calculate duration (TTP + RT) of each contraction. Values for baseline, peak, amplitude, frequency, TTP, RT, duration, and area for each animal were averaged for each hour after data collection began using the Proc Means procedure of SAS 9.3. Hourly means for the above variables were analyzed using a Proc GLIMMIX with repeated measures model of SAS considering animal as a random effect and effect of hour and thirds (consecutive 8-h periods within d, i.e., h 1–8, 9–16, and 17–24) as fixed effects. Tests for differences between days for each variable were conducted using contrasts. The Proc TTEST of SAS was run to determine equality of variances between thirds of the day after feeding for amplitude, frequency, duration, and area.

EXPERIMENT 2

Animals and treatments

The same eight ruminally-cannulated Holstein steers ($BW = 378 \pm 12$ kg) used in Experiment 1 were paired by weight in a randomized complete block design. Within block, one steer was assigned to each treatment: E− ("KY 32"; 0 mg ergovaline + ergovalinine/kg DM) or E+ ("KY 31"; 2.87 mg ergovaline + ergovalinine/kg DM; 0.65 mg ergotamine + ergotaminine/kg DM) tall fescue seed treatment. Tall fescue seed was analyzed for ergovaline isomer and ergotamine isomer concentrations using a HPLC with fluorescence detection procedure modified from Yates and Powell (1988). Steers were pair-fed the basal diet in Experiment 1 once daily (0800 h) starting at 1.5 × NE_m (NRC, 2000). Thus, the E− steers only received the quantity of feed their paired E+ steer consumed the previous day. Tall fescue seed was ground by a grinder mixer (MX125, Gehl, West Bend, WI) to pass through a 3-mm screen. Immediately before feeding, all steers were dosed with 1.45 kg tall fescue seed through the cannula opening for 15 d. The E+ steers received 10 μg ergovaline + ergovalinine/kg BW. Therefore, a combination of E+ and E− seed was used to achieve this dosage level for the E+ treatment animals. Steers were housed indoors at the University of Kentucky C. Oran Little Research Center in Woodford County at 22°C in individual 3 × 3 m stalls. *Ad libitum* access to water was provided.

Telemetry system and signal calibration

Experiment 2 utilized the wireless telemetry system (emka TECHNOLOGIES USA) and signal calibration procedure as described previously.

Data collection and analysis

During the first 14 d of ruminal seed dosing, an 8-h data collection period began 8 h after feeding (1600 h) each day. Data were recorded and stored using iox2 software with a sampling rate of 100 pressure readings per second with smoothing averaging every 20 readings (200 ms). Water intake was recorded using water flow meters immediately after feeding, before collection, and after collection (i.e. every 8 h). The rhythmic analyzer in iox2 was used as it was in Experiment 1 for data analysis.

Blood collection

On d 1, 7, and 15, blood was collected from the jugular vein immediately before seed dosing and feeding. Blood samples were allowed to clot for 24 h at 4°C and centrifuged at 1,500 × g for 25 min (4°C). Serum prolactin concentrations were analyzed by radioimmunoassay procedures of Bernard et al. (1993). The intraassay CV was 10.1% and the interassay CV was 7.4%.

Ruminal evacuation

Complete manual evacuation of the rumen contents was conducted 8 h after feeding on d 15 through the cannula. Ruminal fill was measured for each steer by weighing total rumen contents. Three replicate samples (approximately 100 g each) were taken from the ruminal contents of each steer for DM analysis. The remaining contents were placed back into the rumen immediately after sampling.

Statistical analysis

Calculations were as described for Experiment 1. Values for baseline pressure, peak pressure, amplitude, frequency, TTP, RT, duration, and area under the curve for each animal were averaged over the 8 h period every day using the Proc Means procedure of SAS. Motility variables were analyzed as a randomized block design (RBD) with repeated measures for the effects of seed, day, and the interaction of seed × day. Water intake, DM intake, and ruminal content measures were analyzed as an RBD for the effect of seed. Serum prolactin was analyzed as a randomized block design with repeated measures for fixed effects of seed, day, and the interaction. Probability of Type I error less than 0.05 was considered significant.

RESULTS

EXPERIMENT 1

Mean (± s.e.m.) water intakes for the first (1–8 h), second (9–16 h), and third (17–24 h) 8-h periods of the day were 30.28 ± 2.00, 6.14 ± 0.72, and 0.18 ± 0.04 L, respectively. **Table 1** displays the mean value for each rumen contraction variable measured and the range between animals.

Contraction amplitude was greatest around feeding time (**Figure 1A**). Frequency of ruminal contractions was greatest at feeding time and decreased thereafter until 22 h after feeding (**Figure 1B**). The duration of contractions gradually decreased and then increased slightly 2 h before the next feeding

Table 1 | Mean values and range between animals for rumen contraction variables.

Item, units	Mean[a]	s.e.m.[b]	Range[c]
Baseline, mmHg	22.98	2.68	7.28
Peak, mmHg	30.28	2.78	7.29
Amplitude, mmHg	7.29	0.46	1.07
Frequency, contractions/min	2.87	0.23	0.92
Time to peak, s	4.06	0.42	0.85
Relaxation time, s	5.23	0.50	1.04
Duration, s	9.29	0.73	1.34
Area, mmHg*s	30.47	2.99	6.93

[a] *Mean = overall mean, n = 576.*

[b] *s.e.m. = standard error of the mean, n = 8.*

[c] *Range = range of means among the 8 steers.*

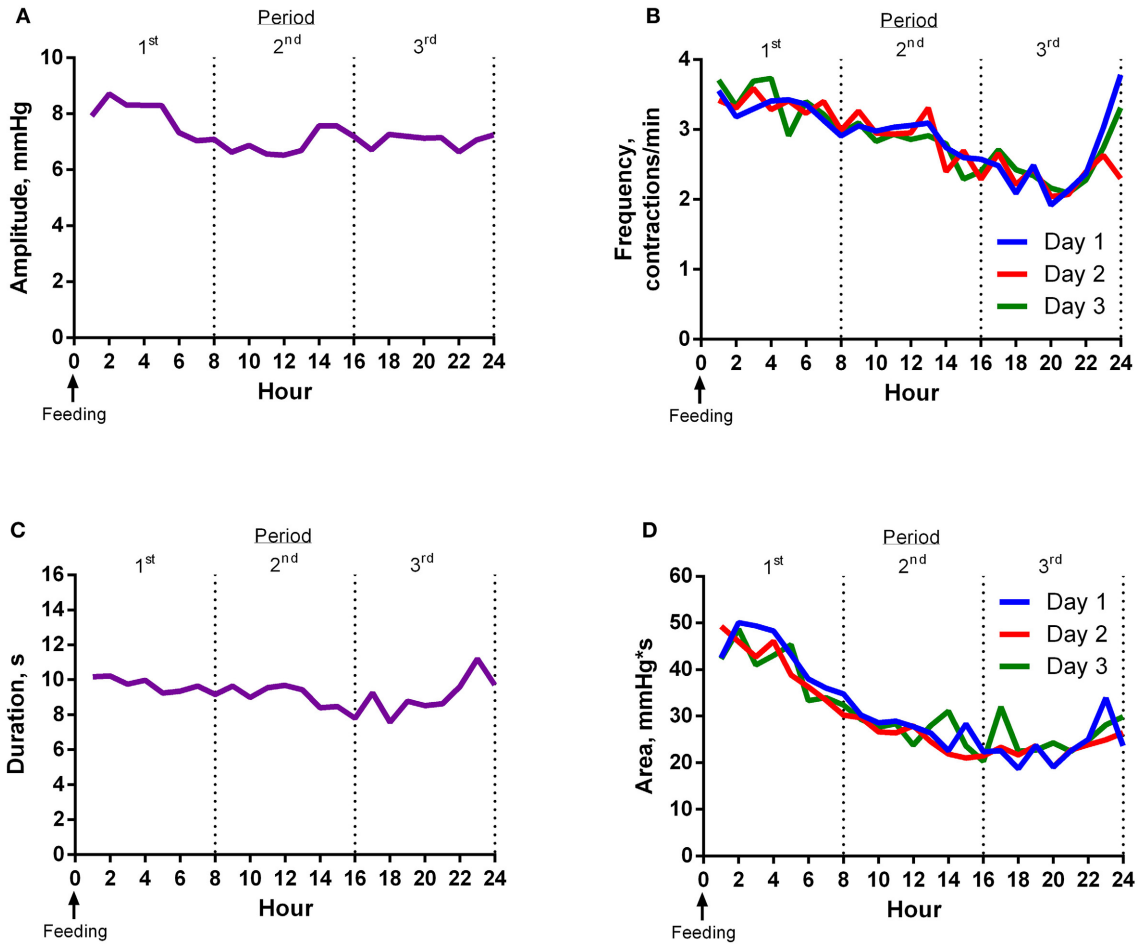


FIGURE 1 | Experiment 1 Results. (A) Mean contraction amplitude of steers ($n = 8$) for each hour relative to feeding. The mean contraction amplitude of the first 8 h period was higher ($P < 0.01$) than the second. The mean contraction amplitude of the second 8 h period was not different ($P = 0.96$) from the third. **(B)** Mean contraction frequency of steers ($n = 8$) for each hour relative to feeding. Mean contraction frequency of day 2 was lower ($P = 0.03$) than day 3. The mean contraction frequency of the first 8 h period was higher ($P < 0.01$) than the second. The mean contraction frequency of the second 8 h period was different ($P < 0.01$) from the third. Additionally, the average of the first and third 8 h periods was not different ($P = 0.35$) from the second. **(C)** Mean contraction duration of steers ($n = 8$) for each hour relative to feeding. The mean contraction duration of the first 8 h period was longer ($P < 0.01$) than the second. The mean contraction duration of the second 8 h period was not different ($P = 0.34$) from the third. **(D)** Mean contraction area of steers ($n = 8$) for each hour relative to feeding. Mean contraction area of day 1 was lower ($P = 0.03$) than day 3. The mean contraction area of the first 8 h period was greater ($P < 0.01$) than the second. The mean contraction area of the second 8 h period tended ($P = 0.07$) to be greater than the third.

(**Figure 1C**). Area under the curve for contractions decreased as time from feeding increased (**Figure 1D**).

All motility variables differed ($P < 0.01$) by hour and period (divided into 3, 8-h periods) of the day. The effect of day was not significant for most variables. However, the mean frequency of day 3 was higher ($P = 0.03$) than day 2, and mean area of day 3 was greater ($P = 0.03$) than day 1. Variance of the second 8 h period of the day was less than ($P < 0.01$) the first and third for area and less than ($P < 0.05$) the third for amplitude, frequency, and duration.

EXPERIMENT 2

Mean water intake tended ($P = 0.10$) to be lower for E+ steers than for E− steers (16.27 ± 5.13 L and 28.86 ± 5.13 L, respectively) before data collection, meaning the 8-h period in between feeding and the start of data collection (**Figure 2**). There were no differences in water intakes between E− and E+ steers during data collection (E−: 6.54 ± 2.82 L; E+: 9.21 ± 2.82 L) or from the end of the data collection to feeding the next day (overnight; E−: 0.37 ± 0.35 L; E+: 1.10 ± 0.35 L).

Table 2 shows the mean results of rumen motility variables between E− and E+ treated steers. Pressure at the peak of the contractions was smaller ($P = 0.04$) for E+ steers. There was also a tendency for baseline pressure to be smaller ($P = 0.06$) in E+ steers. The effect of day was significant ($P < 0.05$) for baseline pressure, peak pressure, and frequency, while tending ($P = 0.10$) to be different for duration. Contraction frequency had a tendency ($P = 0.10$) for a seed × day interaction (**Figure 3**).

Serum prolactin was not different between seed treatments for any of the days (**Figure 4**). There was a large decrease in prolactin

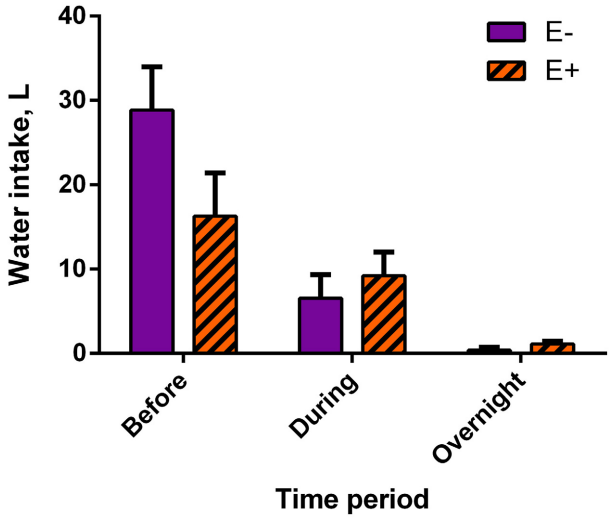


FIGURE 2 | Water intake by time period, relative to data collection, for endophyte-free (E−) and endophyte-infected (E+) tall fescue seed treated steers. Steers in the E+ treatment group received 10 μg ergovaline + ergovalinine/kg BW daily. In the 8 h immediately before data collection commenced, water intake tended ($P = 0.10$) to be greater for E− steers. Water intake was not different between seed treatment groups during data collection ($P = 0.55$) or overnight ($P = 0.23$).

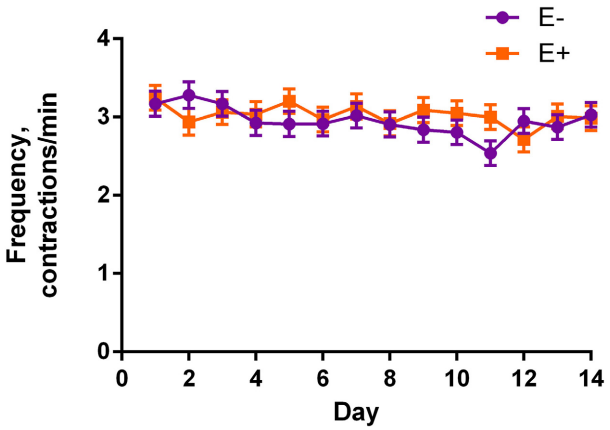


FIGURE 3 | Frequency of contractions for endophyte-free (E−) and endophyte-infected (E+) tall fescue seed treated steers each day of the experiment. Steers in the E+ treatment group received 10 μg ergovaline + ergovalinine/kg BW daily. The effect of day was significant ($P = 0.03$), and there was a tendency ($P = 0.10$) for a seed × day interaction.

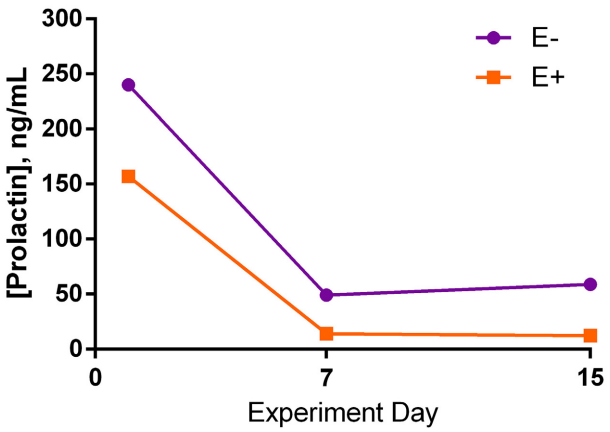


FIGURE 4 | Serum prolactin concentrations (*s.e.m.* = 50.4; *n* = 8) for endophyte-free (E−) and endophyte-infected (E+) seed-treated steers throughout the experiment. Steers in the E+ treatment group received 10 μg ergovaline + ergovalinine/kg BW daily. The effects of seed, day, and the interaction were not significant ($P > 0.05$).

Table 2 | Mean results for rumen motility contraction variables measured for 14 days in E− and E+ tall fescue seed treated steers.

Item	Seed treatment		s.e.m.[c]	*P*-values		
	E−[a]	E+[b]		Seed	Day	Seed × Day
Baseline, mm Hg	29.73	27.11	0.81	0.06	<0.01	0.43
Peak, mm Hg	36.68	34.30	0.65	0.04	<0.01	0.29
Amplitude, mm Hg	6.95	7.20	0.28	0.55	0.46	0.24
Frequency, contractions/min	2.95	3.02	0.12	0.68	0.03	0.10
Time to peak, s	4.29	4.43	0.11	0.43	0.35	0.39
Relaxation time, s	4.98	4.96	0.19	0.90	0.10	0.28
Duration, s	9.29	9.38	0.20	0.50	0.10	0.78
Area, mm Hg*s	28.86	31.55	1.91	0.36	0.13	0.84

[a] *E− = endophyte-free tall fescue seed.*

[b] *E+ = endophyte-infected tall fescue seed.*

[c] *Standard error of the mean; n = 8*

concentration between d 1 and d 7 of the trial for both treatment groups, although it was not significant. Comparison of d 7 and 15 showed relatively similar prolactin concentrations.

Table 3 displays the results of rumen evacuation and rumen content dry matter analysis. Particular consideration has been given to the relative DM intake around the time of evacuation between E− and E+ seed treated steers in an attempt to account for differences in rate of intake, despite the pair feeding situation. Dry matter intakes were not different ($P = 0.71$) between groups throughout the duration of the experiment due to the pair-feeding. Percent DM of ruminal contents, wet contents, and dry contents did not differ ($P > 0.05$) between E− and E+ steers. However, there was a tendency ($P = 0.07$) for the wet contents per 100 kg BW basis to be lower in E+ steers. For the 8 h immediately prior to evacuations on d 15, water consumption was not different ($P = 0.13$) between E− and E+ steers (27.89 ± 5.29 L and 16.66 ± 5.29 L, respectively.

DISCUSSION

EXPERIMENT 1

This experiment was the first recorded adaptation of the emka-PACK*4G* wireless telemetry system for use in cattle. Most studies conducted with this system used canines (Bailey et al., 2011;

Table 3 | DM intakes and ruminal contents measured by rumen evacuations on d 15 and DM analysis.

Item	Seed treatment		s.e.m.[b]	*P*-value
	E−	E+		
INTAKES				
DM[a], kg	9.13	9.12	0.18	0.71
DM 8 h before evacuations, kg	9.84	7.59	1.02	0.22
DM 32 h before evacuations, kg	20.37	17.93	1.10	0.22
RUMINAL CONTENTS				
Percent DM	15.58	16.37	0.55	0.39
Wet contents, kg	66.50	59.68	3.47	0.16
Wet contents, kg/ 100 kg BW	17.58	15.80	0.47	0.07
Dry contents, kg	10.32	9.77	0.55	0.35
Dry contents, kg/ 100 kg BW	2.75	2.58	0.08	0.22
Dry contents, % of intake prior 8 h	105.27	143.59	20.46	0.25
Dry contents, % of intake prior 32 h	50.63	55.60	4.11	0.37

[a] *Mean DM intake for d 1 through d 14 with pair-feeding management.*
[b] *Standard error of the mean; n = 8*

McMahon et al., 2011) or non-human primates (Bruce et al., 2013). With the iox2 software, this system enables the measurement of many other variables beyond contraction amplitude and frequency, which are commonly the only variables reported as measurements of rumen motility (Attebery and Johnson, 1969; Bruce and Huber, 1973; Daniel, 1983; Cook et al., 1986). Additionally, other papers typically show values for these variables for the entire primary or secondary cycle (Froetschel et al., 1986; McSweeney et al., 1989; McLeay and Smith, 2006), whereas with this approach values for each contraction of the ventral sac are obtained. Because of this, it was difficult to find comparisons for some of these variables in published literature. The wireless aspect of this technology enables the animals to move freely and naturally in their environment. Moreover, the procedure for this technology is less invasive than other alternatives for measuring rumen motility, such as electromyography, and enables researchers to obtain a more detailed measurement of ruminal contractions.

Cannulation likely alters rumen motility and some measurements of motility may not be applicable to a non-cannulated animal. Mooney et al. (1971) found that cannulation decreased reticular contraction frequency during rest, but not during feeding or rumination. Also, amplitude of reticular contractions was significantly greater during feeding in intact animals. Frequency and amplitude of ruminal contractions within cannulated cattle also varies between sources. Attebery and Johnson (1969) reported frequencies between 1.74 and 2.23 contractions per min and amplitudes of 7.52–14.86 cm water in fed cows at various temperatures. However, observations were only taken for 30 min on 5 animals. Conversely, Daniel (1983) found an average frequency of the dorsal sac to be 1.13 ± 0.309 contractions per min and average amplitude of 14.7 ± 2.58 mm Hg prior to inducing hypocalcemia in cows. In this study, the mean frequency of ruminal contractions in steers was greater than those previously described, and the mean amplitude was generally lower. Differences could be attributable to the diet composition, method of measurement, time of recording relative to feeding, and length of recording.

Although statistical differences were found between certain days for frequency and area, graphically the days do not appear different. Therefore, these differences may not be physiologically relevant. Baseline and peak pressure displayed the greatest standard errors and ranges of all motility variables measured. This is likely due to the nature of the experiment and animal management as baseline and peak increase when the animal is laying down. Since the animals were allowed to move freely and stand up or lay down at will, the standard error of the mean and ranges for these parameters were more variable. Overall, small standard errors and ranges achieved here for measured parameters suggest that this approach to monitoring rumen motility is repeatable and consistent.

The second 8-h period of the day was the least variable for many measures of motility tested and had a moderate water intake. Therefore, it was concluded that measurements of motility for 9–16 h after feeding provide the best opportunity for testing differences in motility related to treatments because it provided the time when the pressure signal could be most consistently analyzed by the software due to less background noise. Feeding management will affect the values obtained and should be considered when designing experiments.

EXPERIMENT 2

Research on the effects of endophyte-infected tall fescue or ergot alkaloids on rumen motility has been minimal. Previous research has been done via electromyography in sheep utilizing the direct intravenous injection of ergotamine and ergovaline (McLeay and Smith, 2006; Poole et al., 2009). However, there are no published data on the effects in cattle. Additionally, the route of administration could have an impact on the effects. Ergot alkaloids injected directly into the blood stream might cause a greater degree of biological reaction, such as vasoconstriction, than ergot alkaloids consumed orally or given intra-ruminally. As a result, this study attempted to delineate two aspects of information that are lacking: (1) the effect of ergot alkaloids on rumen motility specifically in cattle and (2) the effect on rumen motility when ergot alkaloids are dosed intraruminally (as opposed to intravenously).

Similarities between the ergoline ring of ergot alkaloids and dopamine enables ergot alkaloids to bind D2-dopamine receptors (Berde and Stürmer, 1978; Goldstein et al., 1980; Sibley and Creese, 1983). By binding to and activating these D2 receptors in the anterior pituitary gland, ergot alkaloids can inhibit the secretion of prolactin (Hurley et al., 1980; Schillo et al., 1988; Porter and Thompson, 1992) through second messenger responses (Larson et al., 1995). As a result, reduced serum prolactin concentrations have been used as an indicator of fescue toxicosis, yet do not indicate severity by level of decrease. There are multiple reports of cattle consuming endophyte-infected tall fescue or seed where a depression in serum prolactin concentration was shown (Schillo et al., 1988; Klotz et al., 2012; Koontz et al., 2012; Foote et al., 2013).

In this study, there was a large numerical decrease in serum prolactin concentrations from the first day of seed dosing to mid experiment in both seed treatment groups. The mean prolactin concentration for E+ steers was lower than E− steers throughout the experiment, yet, results were not statistically different. Therefore, prolactin data do not support that these steers were experiencing acute fescue toxicosis. Although it was chosen as an intermediate dosage from published studies showing reduced serum prolactin concentrations in E+ treated steers, the dosage rate of 10 μg ergovaline + ergovalinine/kg BW may have been too small to induce fescue toxicosis under thermoneutral conditions. Kim et al. (2013) administered approximately 8 μg ergovaline + ergovalinine/kg BW, whereas Foote et al. (2013) dosed 15 μg ergovaline + ergovalinine/kg BW. Both of these experiments successfully induced fescue toxicosis and utilized ground endophyte-infected tall fescue seed given intraruminally at thermoneutral temperatures, as was done in this study.

In contrast, other signs of fescue toxicosis were demonstrated. Reductions in DM intake were observed for many E+ steers, and E+ steers routinely consumed their daily ration at a slower rate than E− steers. Similarly, Koontz et al. (2012) showed a greater rate of dry matter intake at thermoneutral conditions for E− steers. Dry matter intake rate could not be controlled in this study with once daily feeding. This may help explain the baseline and peak pressure of E+ steers being lower than E− steers. For instance, if E− steers have consumed all of their feed, they would have likely been lying down more often during data collection, increasing the pressure, compared to the E+ steers, who still had food left to consume from their feed bunks. However, standing and laying behaviors were not monitored.

There was a tendency for a seed × day interaction for frequency of contractions (**Figure 3**). However, this is difficult to discern except that the E− steer contractions were less frequent than E+ steers on d 11.

There was also a tendency for greater water intake by E− steers compared to E+ steers during the first 8-h period following feeding, the period before motility data collection began. This was likely the result of the increased eating rate mentioned above and could have altered subsequent rumen motility (Church, 1976), although no significant differences were found. Aldrich et al. (1993) also reported water intake of steers was not changed with the consumption of tall fescue seed. Overall, the values obtained for rumen motility variables measured in this experiment agree with Experiment 1 and provide more support to the consistency of this approach.

Studies have demonstrated that cyclical contractions of reticuloruminal smooth muscle of sheep can be reduced or inhibited with the intravenous injection of ergot alkaloids (McLeay and Smith, 2006; Poole et al., 2009). This could potentially relate to a decreased passage rate of particulate or liquid matter, which could account for a reduction in intake as is commonly seen with ruminants experiencing fescue toxicosis. Unlike Foote et al. (2013), no differences were found in dry matter percentage of ruminal contents or dry contents (kg/100 kg BW) between E+ and E− steers in this study. The lack of effect on ruminal dry matter contents is likely a result of the E+ seed treatment not effectively inducing fescue toxicosis. Additionally, differences may be due to the time, relative to feeding, that rumen evacuations were conducted and rumen content samples were collected; Foote et al. (2013) gathered rumen content samples before feeding, whereas this study utilized rumen content samples collected 8 h after feeding.

Research has shown that duration of reticular contractions instead of frequency may have a larger influence on passage rate of ruminal fluid and particulate matter (Okine et al., 1989). The same theory could be applied to duration and frequency of ruminal contractions. However, seed treatment did not affect duration of contractions in this experiment.

CONCLUSIONS

The emkaPACK*4G*wireless telemetry system can be used as an accurate, effective, and non-invasive tool to measure rumen motility and obtain detailed measurements of ruminal contractions in ruminally cannulated animals. Endophyte-infected tall fescue seed treatment at a dosage of 10 μg ergovaline + ergovalinine/kg BW under thermoneutral conditions for 14 days (which failed to induce acute fescue toxicosis) did not significantly alter rumen motility, ruminal fill, or dry matter of rumen contents. Therefore, it remains unclear as to whether ergot alkaloids or endophyte-infected tall fescue dosed intraruminally decreases rumen motility. Future experiments should focus on the interactions of ergot alkaloid dosage, ambient temperature, intake and feeding behavior, and rumen motility.

ACKNOWLEDGMENTS

The authors acknowledge Lauren Clark of the University of Kentucky C. Oran Little Research Center Beef Unit for her hard work in helping conduct these experiments and Edward Roualdes for statistical consulting for Experiment 1.

REFERENCES

Aldrich, C. G., Paterson, J. A., Tate, J. L., and Kerley, M. S. (1993). The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *J. Anim. Sci.* 71, 164–170.

Attebery, J. T., and Johnson, H. D. (1969). Effects of environmental temperature, controlled feeding, and fasting on rumen motility. *J. Anim. Sci.* 29, 734–737.

Bailey, E., Grant, C., Moors, J., Wang, J.-L., Napoléoni, J.-G., Ewart, L., et al. (2011). "Simultaneous assessment of blood pressure and respiratory function in dogs: validation of a non-invasive telemetry method," in *Poster Presented by AstraZeneca at the 11th Annual Meeting of the Safety Pharmacology Society* (Innsbruck).

Berde, B., and Stürmer, E. (1978). "Introduction to the pharmacology of ergot alkaloids and related compounds as a basis of their therapeutic applications," in *Ergot Alkaloids and Related Compounds*. eds B. Berde and H. O. Schild (Berlin; Heidelberg; New York, NY: Springer-Verlag), 1–28.

Bernard, J. K., Chestnut, A. B., Erickson, B. H., and Kelly, F. M. (1993). Effects of prepartum consumption of endophyte-infested tall fescue on serum prolactin and subsequent milk production of Holstein cows. *J. Dairy Sci.* 76, 1928–1933.

Bruce, L. A., and Huber, T. L. (1973). Inhibitory effect of acid in the intestine on rumen motility in sheep. *J. Anim. Sci.* 37, 164–168.

Bruce, K. D., Mega, W., Overheim, K., Cirz, R., Cass, R., Valderas, M., et al. (2013). "Telemetry used to determine efficacy of intravenous Plazomicin against inhaled tularemia in Cynomolgus Macaques (CM)," in *Abstract #120. Poster Presented by Lovelace at the 13th Annual Meeting of the Safety Pharmacology Society* (Rotterdam).

Church, D. C. (1976). *Digestive Physiology and Nutrition of Ruminants*. Vol. 1. Digestive Physiology, 2nd Edn. Portland, OR: D. C. Church.

Colvin, H. W. Jr., and Daniels, L. B. (1965). Rumen motility as influences by physical form of oat hay. *J. Dairy Sci.* 48, 935–941.

Cook, W. O., Richard, J. L., Osweiler, G. D., and Trampel, D. W. (1986). Clinical and pathological changes in acute bovine aflatoxicosis: rumen motility and tissue and fluid concentrations of aflatoxins B_1 and M_1. *Am. J. Vet. Res*. 47, 1817–1825.

Dado, R. G., and Allen, M. S. (1993). Continuous computer acquisition of feed and water intakes, chewing, reticular motility, and ruminal pH of cattle. *J. Dairy Sci.* 76, 1589–1600.

Daniel, R. C. W. (1983). Motility of the rumen and abomasum during hypocalcemia. *Can. J. Comp. Med*. 47, 276–280.

Foote, A. P., Kristensen, N. B., Klotz, J. L., Kim, D. H., Koontz, A. F., McLeod, K. R., et al. (2013). Ergot alkaloids from endophyte-infected tall fescue decrease reticuloruminal epithelial blood flow and volatile fatty acid absorption from the washed reticulorumen. *J. Anim. Sci.* 91, 5366–5378. doi: 10.2527/jas.2013-6517

Froetschel, M. A., Croom, W. J. Jr., Hagler, W. M. Jr., Argenzio, R., Liacos, J., and Broquist, H. P. (1986). Effects of slaframine on ruminant digestive function: ruminal motility in sheep and cattle. *J. Anim. Sci.* 63, 1502–1508.

Goldstein, M., Lieberman, A., Lew, J. Y., Asano, T., Rosenfeld, M. R., and Makman, M. H. (1980). Interaction of pergolide with central dopaminergic receptors. *Proc. Natl. Acad. Sci. U.S.A*. 77, 3725–3728.

Hurley, W. L., Convey, E. M., Leung, K., Edgerton, L. A., and Hemken, R. W. (1980). Bovine prolactin, TSH, T4 and T3 concentrations as affected by tall fescue summer toxicosis and temperature. *J. Anim. Sci.* 51, 374–379.

Kim, D. H., Klotz, J. L., and Harmon, D. L. (2013). Effect of endophyte-infected tall fescue seed on ruminal metabolism and physiology in angus steers. *J. Anim. Sci*. 91(E Suppl. 2), 522.

Klotz, J. L., Brown, K. R., Xue, Y., Matthews, J. C., Boling, J. A., Burris, W. R., et al. (2012). Alterations in serotonin receptor-induced contractility of bovine lateral saphenous vein in cattle grazing endophyte-infected tall fescue. *J. Anim. Sci.* 90, 682–693. doi: 10.2527/jas.2011-4323

Koontz, A. F., Bush, L. P., Klotz, J. L., McLeod, K. R., Schrick, F. N., and Harmon, D. L. (2012). Evaluation of a ruminally dosed tall fescue seed extract as a model for fescue toxicosis in steers. *J. Anim. Sci.* 90, 914–921. doi: 10.2527/jas.2011-4292

Larson, B. T., Samford, M. D., Camden, J. M., Piper, E. L., Kerley, M. S., Paterson, J. A., et al. (1995). Ergovaline binding and activation of D2 dopamine receptors in GH4ZR7 cells. *J. Anim. Sci.* 73, 1396–1400.

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

McLeay, L., and Smith, B. (2006). Effects of ergotamine and ergovaline on the electromyographic activity of smooth muscle of the reticulum and rumen of sheep. *Am. J. Vet. Res*. 67, 707–714. doi: 10.2460/ajvr.67.4.707

McMahon, M., Ward, G., Milliken, P., Aylott, M., and Patel, B. (2011). "Assessment of non-invasive telemetric blood pressure and electrocardiogram measurement in conscious dogs," in *Poster Presented by GlaxoSmithKline at the Society of Toxicology Meeting* (Washington, DC).

McSweeney, C. S., Kennedy, P. M., and John, A. (1989). Reticulo-ruminal motility in cattle (*Bos indicus*) and water buffaloes (*Bubalus bubalis*) fed a low quality roughage diet. *Comp. Biochem. Physiol. A Comp Physiol.* 94A, 635–638. doi: 10.1016/0300-9629(89)90607-5

Mooney, G. A., Radcloff, H. D., and Cupal, J. J. (1971). Effects of fistulation on rumen motility. *J. Anim. Sci.* 33, 261 (Abstr.).

Okine, E. K., Mathison, G. W., and Hardin, R. T. (1989). Effects of changes in frequency of reticular contractions on fluid and particulate passage rates in cattle. *J. Anim. Sci.* 67, 3388–3396.

Poole, D., Littler, R., Smith, B., and Mcleay, L. (2009). Effects and mechanisms of action of the ergopeptides ergotamine and ergovaline and the effects of peramine on reticulum motility of sheep. *Am. J. Vet. Res.* 70, 270–276. doi: 10.2460/ajvr.70.2.270

Porter, J. K., and Thompson, F. N. Jr. (1992). Effects of fescue toxicosis on reproduction in livestock. *J. Anim. Sci.* 70, 1594–1603.

Schillo, K. K., Leshin, L. S., Boling, J. A., and Gay, N. (1988). Effects of endophyte-infected fescue on concentrations of prolactin in blood sera and the anterior pituitary and concentrations of dopamine and dopamine metabolites in brains of steers. *J. Anim. Sci.* 66, 713–718.

Sibley, D. R., and Creese, I. (1983). Interactions of ergot alkaloids with anterior pituitary D2 dopamine receptors. *Mol. Pharmacol.* 23, 585–593.

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). St. Anthony's Fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci*. 89, 1603–1626. doi: 10.2527/jas.2010-3478

Westendorf, M. L., Mitchell, G. E. Jr., Tucker, R. E., Bush, L. P., Petroski, R. J., and Powell, R. G. (1993). *In vitro* and *in vivo* ruminal and physiological responses to endophyte-infected tall fescue. *J. Dairy Sci.* 76, 555–563. doi: 10.3168/jds.S0022-0302(93)77375-0

Yates, S. G., and Powell, R. G. (1988). Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. *J. Agric. Food Chem*. 36, 337–340.

Conflict of Interest Statement: The reviewer Lori L. Smith declares that, despite being affiliated with the same institution as authors Amanda M. Egert, Kyle R. McLeod and David L. Harmon, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 July 2014; accepted: 25 September 2014; published online: 13 October 2014.

Citation: Egert AM, Klotz JL, McLeod KR and Harmon DL (2014) Development of a methodology to measure the effect of ergot alkaloids on forestomach motility using real-time wireless telemetry. Front. Chem. ***2****:90. doi: 10.3389/fchem.2014.00090*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 21 August 2014
doi: 10.3389/fchem.2014.00068

Exposure to ergot alkaloids during gestation reduces fetal growth in sheep

Susan K. Duckett [1]*, John G. Andrae [2] and Scott L. Pratt [1]

[1] Animal and Veterinary Sciences Department, Clemson University, Clemson, SC, USA
[2] School of Agricultural Forest and Environmental Sciences, Clemson University, Clemson, SC, USA

Edited by:
James Levi Klotz, United States Department of Agriculture - Agricultural Research Service, USA

Reviewed by:
Christian W. Gruber, Medical University of Vienna, Austria
Alexander Shekhtman, University at Albany, State University of New York, USA
Lester Ronald Fletcher, AgResearch NZ, New Zealand

***Correspondence:**
Susan K. Duckett, Animal and Veterinary Sciences Department, Clemson University, 145 Poole Ag. Center, Clemson, SC, USA
e-mail: sducket@clemson.edu

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh; *Schedonorus phoenix* (Scop.) Holub] is the primary cool season perennial grass in the eastern U.S. Most tall fescue contains an endophyte (*Neotyphodium coenophialum*), which produces ergot alkaloids that cause vasoconstriction and could restrict blood flow to the fetus in pregnant animals. The objective of this study was to examine fetal growth during maternal exposure to ergot alkaloids during gestation. Pregnant ewes ($n = 16$) were randomly assigned to one of two dietary treatments: (1) endophyte-infected (*N. coenophialum*) tall fescue seed (E+; 0.8 ug of ergovaline /g diet DM) and (2) endophyte-free tall fescue seed (E−; 0.0 ug of ergovaline/g diet DM). Birth weight of lambs was reduced by 37% for E+ compared to E−. Organ and muscle weights were also lighter for E+ than E−. Exposure to ergot alkaloids *in utero* reduces fetal growth and muscle development.

Keywords: sheep, ergot alkaloids, fetal growth, muscle development

INTRODUCTION

Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh; *Schedonorus phoenix* (Scop.) Holub] is the primary cool season perennial grass utilized in the eastern U.S. occupying more than 14 million ha (Stuedemann and Hoveland, 1988). The majority of tall fescue contains an endophyte (*Neotyphodium coenophialum*), which produces ergot alkaloids (i.e., ergovaline, ergovalinine, lysergic acid etc.). The endophyte is beneficial to the plant and improves establishment, persistence, and drought tolerance (Stuedemann and Hoveland, 1988); however, ingestion of the ergot alkaloids by grazing livestock results in fescue toxicosis which reduces animal growth (Hoveland, 1993) and reproductive performance (Peters et al., 1992). Ergot alkaloids contain a tetracyclic ergoline ring and are structurally similar to biogenic amines, serotonin, dopamine, norepinephrine, and epinephrine (Berde, 1980; Weber, 1980; Strickland et al., 2011). Ergot alkaloids bind to receptors for the biogenic amines and elicit decreased serum prolactin concentrations and vasoconstriction (Klotz et al., 2006, 2012; Aiken et al., 2009). Dyer (1993) found that ergovaline induced contraction of bovine uterine and umbilical cord arteries via $5HT_{2A}$ serotonergic receptors, which could restrict blood flow to the fetus. Gestating ewes in the Southeastern US would generally be exposed to endophyte-infected tall fescue throughout the gestation period. Little research exists on how ergot alkaloid exposure in gestating ewes impacts fetal growth and development. The objective of this study was to assess how exposure to ergot alkaloids during gestation (d 35 to parturition) of ewes altered fetal growth and development.

MATERIALS AND METHODS

All animal experimental procedures were reviewed and approved by the Clemson University Institutional Animal Care and Use Committee (AUP-2011-053).

Southdown ewes ($n = 20$; BW = 70 kg; BCS = 4) were mated to a single ram that was fitted with a marking harness. Ewes were checked twice daily and crayon marks from the ram's harness were denoted to estimate breeding date. Ewes were confirmed pregnant via transrectal ultrasonography on d 35 of gestation. Ewes confirmed pregnant ($n = 16$) were randomly assigned to one of two dietary treatments: (1) endophyte-infected (*N. coenophialum*) tall fescue seed (E+; 0.8 μg of ergovaline + ergovalinine/g diet DM) and (2) endophyte-free tall fescue seed (E−; 0.0 μg of ergovaline + ergovalinine/g diet DM). Endophyte-infected and endophyte-free tall fescue seed (E+ *cv.* Defiance, and E− *cv.* Fawn, turf-type tall fescue seed, Seed Research of Oregon, Tangent, OR) was first analyzed for ergovaline and ergovalinine levels according to Aiken et al. (2009) and then diets formulated to provide the targeted levels of ergovaline/ergovalinine in the diet. Fescue seed was delivered daily in a total mixed ration (**Table 1**) formulated to meet NRC requirements for pregnant ewes from d 35 to parturition.

Blood samples were collected from the ewes via jugular venipuncture into tubes on d 30, 50, and 130 of gestation. Samples were allowed to clot for 30 min at room temperature and then at 4°C overnight. Serum was obtained by centrifuging at 1000 × *g* for 15 min at 4°C and stored frozen at −20°C. Prolactin (PRL) concentrations were measured using RIA according to the procedures of Bernard et al. (1993).

Table 1 | Composition of the total mixed ration containing endophyte-infected tall fescue seed fed to the ewes during gestation.

Ingredient	% of ration, DM
Tall fescue seed	38.5
Cottonseed hulls	15.4
Molasses	8.6
Corn grain, cracked	18.9
Soybean hulls	11.4
Limestone	0.2
Soybean meal	2.8
NUTRIENT COMPOSITION, DM BASIS	
Crude protein	11%
TDN	60%

At parturition, a male lamb (E+ = 8; E− = 8) was removed from each ewe carrying twins. If two male lambs were born to the same ewe, the firstborn male lamb was removed from the dam. Male lambs were given a fixed amount of artificial colostrum (Lamb's Choice Total, The Saskatoon Colostrum Co., 3 oz. reconstituted dried bovine colostrum) and harvested within 12 h of birth. The attending veterinarian euthanized lambs with an overdose of pentobarbital. Live weight was collected for each lamb and then the lamb was exsanguinated. The hide, head, feet, and tail were removed and weight of the carcass obtained. Weights were collected on all organs and total digestive tract. From the left side of each carcass, individual muscles [longissimus thoracis (LT), gluteus medius, semimembranosus, semitendinosus, biceps femoris, and quadriceps femoris] were collected and weighed. Samples of the longissimus and semitendinosus muscles were immersed in optimal cutting temperature solution, frozen in liquid nitrogen, and stored at −80°C. for subsequent fiber typing. Adipose depots (subcutaneous fat, kidney fat, mesenteric fat) were also collected and weighed. No appreciable subcutaneous fat depots were present in any of the lambs. From the right side of each carcass, all muscle and fat were removed, weighed and ground for total body proximate composition.

PROXIMATE COMPOSITION

For proximate analysis, total muscle and fat samples from the right side of each lamb carcass were chopped (Blixer®3 Series D, Robot Coupe Inc., Ridgeland, MS) to reduce particle size and subset removed for determination of moisture content. The remaining samples were frozen at −20°C, lyophilized (VirTis, SP Scientific, Warminster, PA), ground (Blixer®3), and stored at −20°C. Duplicate samples were analyzed for nitrogen content by the combustion method using a Leco FP-2000 N analyzer (Leco Corp., St. Joseph, MI) and multiplied by 6.25 to determine CP content. Moisture content was determined by weight loss after drying at 100°C for 24 h. Total ash content was determined by ashing at 600°C for 8 h. Total fat content was determined in duplicate using Ankom XT-15 Extractor (Ankom Technology, Macedon, NY) and hexane as solvent.

FATTY ACID COMPOSITION

Freeze dried total muscle and fat samples from the right side of each lamb carcass were transmethylated according to the method of Park and Goins (1994). Fatty acid methyl esters (FAME) were analyzed using an Agilent 6850 (Agilent, San Fernando, CA) gas chromatograph equipped with an Agilent 7673A (Hewlett-Packard, San Fernando, CA) automatic sampler. Separations were accomplished using a 100-m SP2560 (Supelco, Bellefonte, PA) capillary column (0.25 mm i.d. and 0.20 μm film thickness). Column oven temperature increased from 150 to 160°C at 1°C per min, from 160 to 167°C at 0.2°C per min, from 167 to 225°C at 1.5°C per min, and then held at 225°C for 16 min. The injector and detector were maintained at 250°C. Sample injection volume was 1 μL. Hydrogen was the carrier gas at a flow rate of 1 mL per min. Samples were run twice with a split ratio of 100:1 for trans C18:1 and long-chain fatty acids, and again at split ratio of 10:1 for conjugated linoleic acid (CLA) and omega-3 fatty acids. Individual fatty acids were identified by comparison of retention times with standards (Sigma, St. Louis, MO; Supelco, Bellefonte, PA; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating an internal standard, methyl tricosanoic (C23:0) acid, into each sample during methylation and expressed as a weight percentage of total fatty acids.

IMMUNOFLUORESCENCE IMAGE ANALYSIS

Longissimus and semitendinosus samples were immersed in optimal cutting temperature solution, frozen in liquid nitrogen, and stored at −80°C. Muscle samples were cryosectioned and fiber typed using antibodies for myosin heavy chain (MHC)-fast (AbCam, My-32) and MHC-slow (Hybridoma Bank, BA-F8). The number and cross-sectional area of primary and secondary myofibers were counted on 10 different sections for each lamb, and a ratio of secondary to primary myofibers is reported. The cross-sectional area was measured using IMT iSolution Lite (version 9.4, IMT i-Solutions Inc., Vancouver, BC, Canada).

STATISTICAL ANALYSES

Prolactin data were analyzed in a completely randomized design using MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with treatment, time, and two-way interaction in the model. Gestation length data was also measured using the MIXED procedure with treatment in the model. Ewe was the experimental unit for both analyses. For all lamb data, data were analyzed in a completely randomized design using MIXED procedure with treatment in the model and lamb as experimental unit. Least square means were generated and separated using the PDIFF option of SAS. Significance was determined at ($P < 0.05$).

RESULTS AND DISCUSSION

The interaction between day and treatment was significant ($P < 0.001$) for serum PRL levels (**Figure 1**). On d 30 of gestation (5 d prior to the initiation of dietary treatments), serum PRL levels did not differ between E+ and E− ewes. At d 50, serum PRL levels in E+ ewes decreased ($P < 0.01$) from pre-treatment levels (d 30) and were lower ($P < 0.01$) than E− levels. In E− ewes, serum PRL levels at d 50 were similar to the values at pre-treatment (d 30) and higher ($P < 0.01$) than E+ ewe values. At d 130, serum PRL levels increased ($P < 0.05$) in both E+ and E− ewes compared to d 50 levels; however, PRL levels were higher ($P < 0.01$) for E− than E+. The reduction in serum PRL concentration with exposure to

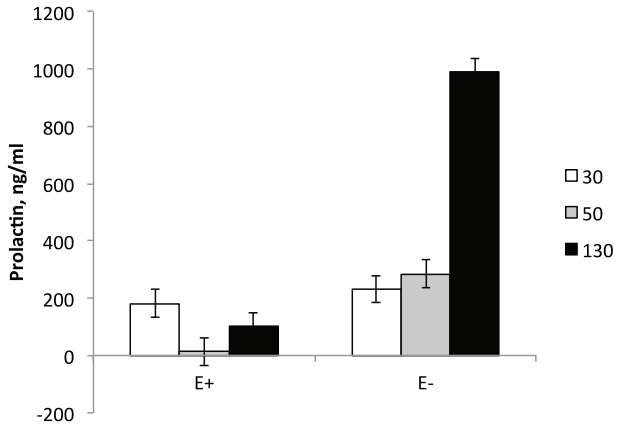


FIGURE 1 | Serum prolactin levels in ewes at d 30, 50, and 130 of gestation. Feeding of tall fescue seed was initiated on d 35 and continued through parturition. Treatment × time interaction was significant ($P < 0.0001$).

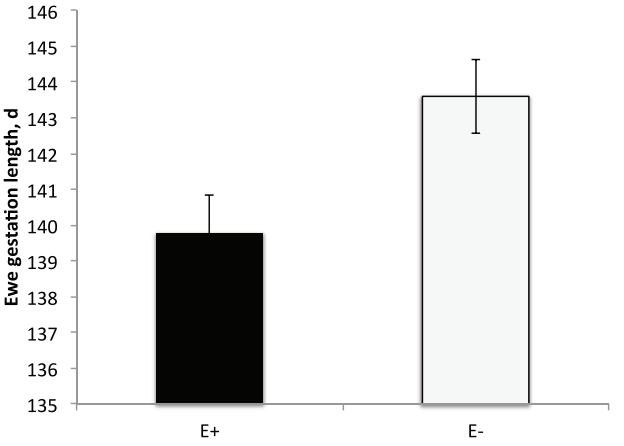


FIGURE 2 | Gestation length (d) of ewes fed tall fescue seed containing endophyte (E+) vs. endophyte-free (E−). Treatment was significant ($P = 0.02$).

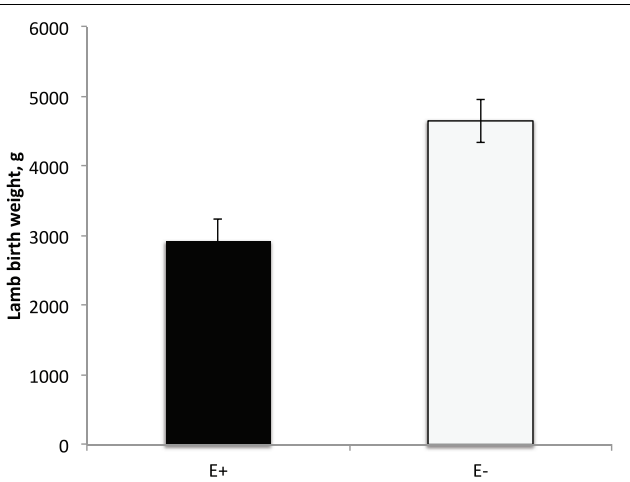


FIGURE 3 | Lamb birth weight from ewes fed tall fescue seed with endophytes (E+) vs. endophyte-free (E−) during gestation. Treatment was significant ($P = 0.001$).

ergot alkaloids via grazing endophyte-infected tall fescue pastures or consumption of endophyte-infected tall fescue seed is a classical response observed in sheep (Elsasser and Bolt, 1987; Emile et al., 2000; Parish et al., 2003), cattle (Emile et al., 2000; Watson et al., 2004; Koontz et al., 2012; Stowe et al., 2013), and horses (McCann et al., 1992). It has been documented in multiple species that as parturition approaches maternal serum PRL concentration increases (Chamley et al., 1973; Bryant and Chamley, 1976; Forsyth, 1986) and this increase is hypothesized to be important for maternal lipid metabolism, mammary growth, and milk production and secretion (Hooley et al., 1978; Banchero et al., 2006; Mabjeesh et al., 2013). The levels of PRL reported here for the E− group are consistent with previous reports; however, the drastically lower levels observed at d 130 for E+ could indicate post-partum issues with ewe metabolism and mammary growth, which would negatively impact postnatal lamb growth.

E+ ewes had approximately 4 d shorter ($P < 0.05$) gestation length than E− controls (**Figure 2**). Similarly, others have reported shorter gestation lengths in ewes with placental insufficiency (Chen et al., 2010) and cows that were nutrient restricted from d 32–83 of gestation (Long et al., 2010). In contrast, horses grazing endophyte-infected tall fescue during gestation have increased gestation lengths (Putnam et al., 1991). Lamb birth weight was reduced ($P < 0.01$) by 37% for E+ compared to E− lambs (**Figure 3**). Watson et al. (2004) observed a 15% reduction in calf birth weight from cows grazing toxic vs. non-toxic fescue during gestation. These reductions in fetal growth with ergot alkaloid feeding are similar to those reported for high ambient temperature exposure throughout pregnancy, which produces the most severe intrauterine growth restriction (IUGR; Bell et al., 1987, 1989; Thureen et al., 1992; Anthony et al., 2003; Arroyo et al., 2006). In sheep, umbilical blood flow increases throughout pregnancy in order to keep pace with fetal growth during the last half of gestation (Reynolds et al., 1986; Reynolds and Ferrell, 1987; Molina et al., 1991). Fetal growth restriction is highly correlated with reduced uteroplacental growth and development (Reynolds and Redmer, 1995, 2001). Experimental conditions like overnutrition, nutrient restriction, hyperthermia, or high altitude that retard fetal growth also reduce uterine and umbilical blood flows (Reynolds et al., 2006). Because adequate blood flow is essential for normal fetal growth, conditions that restrict fetal and placental growth are associated with reduced rates of placental blood flow and nutrient uptakes by the fetus (Reynolds and Redmer, 1995). Since ergot alkaloids cause vasoconstriction in uterine and umbilical blood flow (Dyer, 1993), these effects would induce fetal growth restriction similar to maternal hyperthermia or nutrient deprivation.

Organ weights (heart, lung, kidneys, spleen, thymus, liver, and pancreas) were also smaller ($P < 0.05$) for E+ than E− (**Table 2**) except for the pancreas ($P = 0.52$). Total muscle weight from the right side of each carcass was lighter ($P = 0.0093$) for E+ than

Table 2 | Effect of feeding tall fescue seed with endophyte (E+) vs. endophyte-free (E−) to ewes during gestation (d 35 to parturition) on lamb organ, muscle and adipose tissue weights.

	E+	E−	SEM	*P*-Level
n	8	8		
ORGANS, g				
Heart	22.3	35.1	2.58	0.0035
Lungs	65.4	112.3	8.76	0.0019
Kidneys	16.7	24.0	1.64	0.0067
Spleen	4.7	9.2	1.03	0.0081
Thymus	4.9	11.2	2.00	0.04
Liver	71.6	112.5	10.2	0.017
Pancreas	0.84	1.3	0.050	0.52
Total viscera	234.4	311.0	29.0	0.082
MUSCLES, g				
Longissimus	37.4	63.2	5.06	0.0029
Gluteus medius	11.5	17.6	1.73	0.02
Semitendinosus	8.5	13.6	1.64	0.04
Semimembranosus	24.3	43.4	4.32	0.007
Quadriceps femoris	24.9	41.8	3.42	0.007
Biceps femoris	17.2	29.7	3.30	0.02
Total muscle	313.5	510.7	46.27	0.0093
ADIPOSE, g				
Mesenteric fat	4.6	5.2	0.58	0.51
Kidney fat	12.5	19.8	2.28	0.04

Table 3 | Effect of feeding tall fescue seed with endophyte (E+) vs. endophyte-free (E−) to ewes during gestation (d 35 to parturition) on lamb organ, muscle and adipose tissue weights as a percentage of body weight.

	E+	E−	SEM	P-Level
n	8	8		
ORGANS, %				
Heart	0.76	0.76	0.04	0.96
Lungs	2.2	2.4	0.15	0.42
Kidneys	0.57	0.52	0.03	0.32
Spleen	0.15	0.19	0.02	0.07
Thymus	0.14	0.23	0.04	0.10
Liver	2.3	2.4	0.10	0.40
Pancreas	0.24	0.027	0.009	0.81
Total viscera	8.0	6.7	0.45	0.06
MUSCLES, %				
Longissimus	2.6	2.7	0.13	0.57
Gluteus medius	0.78	0.75	0.04	0.64
Semitendinosus	0.59	0.57	0.07	0.89
Semimembranosus	1.6	1.8	0.14	0.28
Quadriceps femoris	1.7	1.8	0.06	0.23
Biceps femoris	1.1	1.2	0.11	0.48
Total muscle	21.2	21.7	0.95	0.72
ADIPOSE, %				
Mesenteric fat	0.14	0.11	0.01	0.08
Kidney fat	0.42	0.42	0.05	0.98

Table 4 | Proximate composition of total muscle mass from one side of each lamb carcass from ewes fed tall fescue seed with endophyte (E+) vs. endophyte-free (E−) during gestation (d 35 to parturition).

	E+	E−	SEM	*P*-Level
Moisture, %	79.05	78.64	0.09	0.01
Crude protein, %	17.09	18.74	0.69	0.05
Total Lipid, %	2.44	2.50	0.20	0.83
Ash, %	2.12	2.17	0.77	0.78
FATTY ACIDS, %				
C14:0	0.90	1.01	0.07	0.28
C16:0	19.67	20.81	0.84	0.35
C16:1 cis-9	2.14	2.03	0.14	0.59
C17:0	0.36	0.39	0.03	0.33
C18:0	13.13	14.44	0.52	0.10
C18:1 cis-9	49.95	49.54	0.72	0.69
C18:1 cis-11	3.03	2.99	0.12	0.83
C18:2 cis-9,12	0.65	0.51	0.09	0.26
C18:3 cis-9,12,15	0.28	0.26	0.05	0.77
C20:4 cis-5,8,11,14	1.89	0.61	0.32	0.01
C20:5 cis-5,8,11,14,17	0.40	0.18	0.07	0.04
C22:5 cis-7,10,13,16,19	0.31	0.50	0.16	0.42
C22:6 cis-4,7,10,13,16,19	0.26	0.20	0.05	0.44
Saturated	33.70	36.26	1.17	0.14
Monounsaturated	52.09	51.57	0.77	0.64
Polyunsaturated, *n*-6	2.54	1.12	0.37	0.02
Polyunsaturated, *n*-3	1.25	1.16	0.22	0.76
Ratio of *n*-6:*n*-3	1.96	1.07	0.20	0.01
Total fatty acids, g/100g LT	1.76	1.81	0.19	0.85

E−. Individual muscle weights for LT, semitendinosus, semimembranosus, biceps femoris, quadriceps femoris, and gluteus medius were heavier ($P < 0.05$) for E− than E+. Kidney fat amounts were lower ($P < 0.05$) for E+ than E−. Thymus and spleen mass tended ($P < 0.10$) to be smaller for E+ than E− even when adjusted for body or carcass weight. All other organs and muscle weights did not differ ($P > 0.05$) when expressed on a weight basis (**Table 3**). Total viscera weight (weight of the esophagus, rumen, intestines excluding organs) tended to be greater ($P < 0.10$) for E+ than E− when expressed on a body weight or hot carcass weight basis.

The proximate and fatty acid composition of the total muscle mass from the right side of each lamb carcass is shown in **Table 4**. Moisture content was higher ($P < 0.01$) and crude protein content was lower ($P = 0.05$) in total muscle from E+ than E−. Total lipid and ash content of the muscle did not differ between treatments. Stearic (C18:0) acid concentrations of the total muscle tended to be lower ($P = 0.10$) for E+ than E−. Arachidonic (C20:4) and eicosapentaenoic (C20:5) acid concentrations were higher ($P < 0.05$) in total muscle of E+ than E−. Other fatty acid concentrations were not altered by dietary treatment. Total *n*-6 polyunsaturated fatty acid (PUFA) and the ratio of *n*-6 to *n*-3 PUFA were higher ($P < 0.05$) in the muscle of E+ than E−. Total fatty acid content of the muscle did not differ, which indicates that PUFA fatty acid accumulation in muscle was greater with

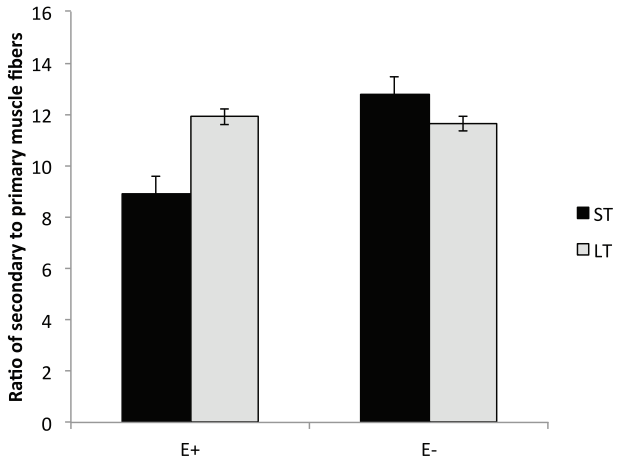


FIGURE 4 | Secondary to primary muscle fiber ratio in the longissimus thoracis (LT) and semitendinosus (ST) muscles in lambs from dams fed tall fescue seed containing endophyte (E+) vs. endophyte-free (E−) during gestation. Treatment was significant ($P = 0.002$) for ST muscles but non-significant for LT ($P = 0.57$).

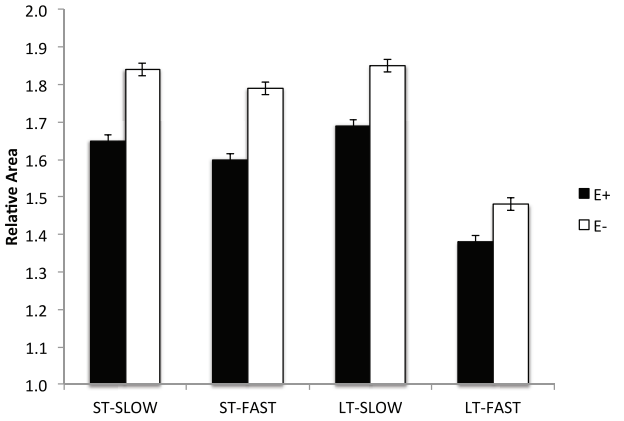


FIGURE 5 | Cross sectional area of muscle fibers (slow- and fast-MHC) in the longissimus thoracis (LT) and semitendinosus (ST) muscles in lambs from dams fed tall fescue seed containing endophyte (E+) vs. endophyte-free (E−) during gestation. Treatment was significant ($P < 0.001$) for both LT and ST muscles.

E+ exposure. Realini et al. (2005) reported that finishing steers on endophyte-infected vs. endophyte-free tall fescue increased stearic acid and lowered monounsaturated fatty acid concentrations with no change in PUFA. Ailhaud et al. (2008) found that increased levels of *n*-6 PUFA and a high ratio of *n*-6 to *n*-3 PUFA during fetal development in rats stimulated adipogenesis to alter hypertrophy and hyperplasia of adipocytes during postnatal growth. These alterations in fatty acid composition at birth could impact adipogenesis and subsequent adipose tissue deposition.

Lambs exposed to ergot alkaloids *in utero* had a lower ($P < 0.05$) secondary to primary muscle fiber ratio in the semitendinosus muscle compared to E− (**Figure 4**). The ratio of secondary to primary muscle fiber did not differ in the LT. Early prenatal muscle fiber growth is due to hyperplasia of muscle fibers and fiber number is set before birth. Research indicates that muscle fiber hyperplasia is complete by about 70 d of gestation in the pig (Swatland, 1973), 180 d in the cow (Albrecht et al., 2013), and 105 d in the sheep (Du et al., 2010). Intrauterine growth restriction of the fetus during the second trimester of gestation reduces the formation of secondary muscle fibers. The ratio of secondary to primary muscle fibers is reduced with intrauterine crowding in pigs (i.e., runt pig, Aberle, 1984; Pardo et al., 2013) and maternal under-nutrition from d 28 to 78 in sheep (Zhu et al., 2004). Cross-sectional area was also reduced ($P < 0.05$) in slow and fast-MHC myofibers of the LT and ST muscles in E+ compared to E− (**Figure 5**). Because postnatal muscle growth is predominately through hypertrophy of existing muscle fibers, a reduction in secondary fiber number also impacts postnatal muscle growth. Pigs that are runts at birth have less total carcass muscle mass and altered adipose tissue cellularity when finished to slaughter weights (Powell and Aberle, 1981). Underwood et al. (2010) found that mid to late nutrient restriction of gestating cows altered growth, adipose, and meat tenderness in the offspring. Long et al. (2012) also reported changes in adipocyte size and carcass parameters in beef offspring from cows with early to mid-gestation undernutrition. Thus, ingestion of ergot alkaloids by ewes during critical time periods of gestation alters fetal muscle growth and development that may have lasting impact on postnatal muscle growth, carcass composition, and palatability throughout the offspring's lifetime.

These results show that fetal growth is restricted in ewes fed endophyte-infected tall fescue seed to simulate fescue toxicosis syndrome during gestation (d 35 to parturition). This reduction in lamb birth weight with ergot alkaloid exposure is similar to lambs exposed *in utero* to high ambient temperatures, which is the most severe IUGR. Exposure *in utero* to ergot alkaloids altered skeletal muscle formation by reducing the ratio of secondary to primary myofibers, myofiber hypertrophy *in utero*, and protein content of muscles. Due to the number of ruminant animals that graze endophyte-infected tall fescue during gestation, additional research is needed to determine mechanisms by which ergot alkaloids reduce fetal growth and the critical time periods of exposure in order to mitigate its effects on fetal growth.

ACKNOWLEDGMENTS

This research project was supported in part by USDA-NIFA-2010-38942-20745. Appreciation is expressed to M. C. Miller and T. A. Burns for assistance with animal care and sample collection, and N. Korn for myofiber image acquisition. Technical contribution no. 6263 of the Clemson University Experiment Station.

REFERENCES

Aberle, E. D. (1984). Myofiber development in skeletal muscles of newborn runt and normal weight. *J. Anim. Sci.* 59, 1651–1656.

Aiken, G. E., Strickland, J. R., Looper, M. L., Bush, L. P., and Schrick, F. N. (2009). Hemodynamics are altered in the caudal artery of beef heifers fed different ergot alkaloid concentrations. *J. Anim. Sci.* 87, 2142–2150. doi: 10.2527/jas.2008-1562

Ailhaud, G., Guesnet, P., and Cunnane, S. C. (2008). An emerging risk factor for obesity: does disequilibrium of polyunsaturated fatty acid metabolism contribute to excessive adipose tissue development? *Brit. J. Nutr.* 100, 461–470. doi: 10.1017/S0007114508911569

Albrecht, E., Lembcke, C., Wegner, J., and Maak, S. (2013). Prenatal muscle fiber development and bundle structure in beef and dairy cattle. *J. Anim. Sci.* 91, 3666–3673. doi: 10.2527/jas.2013-6258

Anthony, R. V., Scheaffer, A. N., Wright, C. D., and Regnault, T. R. H. (2003). Ruminant models of prenatal growth restriction. *Reprod. Suppl.* 61, 183–194.

Arroyo, J. A., Anthony, R. V., Parker, T. A., and Galan, H. L. (2006). Differential expression of placental and vascular endothelial nitric oxide synthase in an ovine model of fetal growth restriction. *Am. J. Obest. Gynecol.* 195, 771–777. doi: 10.1016/j.ajog.2006.06.018

Banchero, G. E., Perez Clariget, R., Bencini, R., Lindsay, D. R., Milton, J. T. B., and Martin, G. B. (2006). Endocrine and metabolic factors involved in the effect of nutrition on the production of colostrum in female sheep. *Reprod. Nutr. Dev.* 46, 447–460. doi: 10.1051/rnd:2006024

Bell, A. W., McBride, B. W., Slepetis, R., Early, R. J., and Currie, W. B. (1989). Chronic heat stress and prenatal development in sheep: I. Conceptus growth and maternal plasma hormones and metabolites. *J. Anim. Sci.* 67, 3289–3299.

Bell, A. W., Wilkening, R. B., and Meschia, G. (1987). Some aspects of placental function in chronically heat-stressed ewes. *J. Dev. Physiol.* 9, 17–29.

Berde, B. (1980). "Ergot compounds: a synopsis," in *Ergot Compounds and Brain Function: Nueroendocrine and Neuropsychiatric Aspects*, eds M. Goldstein, A. Lieberman, D. B. Calne, and M. O. Thorner (New York, NY: Raven Press), 4–23.

Bernard, J. K., Chestnut, A. B., Erickson, B. H., and Kelly, F. M. (1993). Effects of prepartum consumption of endophyte-infected tall fescue on serum prolactin and subsequent milk production of Holstein cows. *J. Dairy Sci.* 76, 1928–1933. doi: 10.3168/jds.S0022-0302(93)77526-8

Bryant, G. D., and Chamley, W. A. (1976). Plasma relaxin and prolactin immunoactivities in pregnancy and at parturition in the ewe. *J. Reprod. Fert.* 48, 201–204. doi: 10.1530/jrf.0.0480201

Chamley, W. A., Buckmaster, J. M., Cerini, M. E., Cumming, I. A., Goding, J. R., Obst, J. M., et al. (1973). Changes in the levels of progesterone, corticosteroids, estrone, estradiol-17β, luteinizing hormone and prolactin tin the peripheral plasma of the ewe during late pregnancy and at parturition. *Biol. Reprod.* 9, 30–35.

Chen, X., Fahy, A. L., Green, A. S., Anderson, M. J., Rhoades, R. P., and Limesand, S. W. (2010). β2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. *J. Physiol.* 588 (Pt 18), 3539–3549. doi: 10.1113/jphysiol.2010.192310

Du, M., Tong, J., Zhao, J., Underwood, K. R., Zhu, M., Ford, S. P., et al. (2010). Fetal programming of skeletal muscle development in the ruminant animals. *J. Anim. Sci.* 88, E51–E60. doi: 10.2527/jas.2009-2311

Dyer, D. C. (1993). Evidence that ergovaline acts on serotonin receptors. *Life Sci.* 53, PL223–PL228. doi: 10.1016/0024-3205(93)90555-H

Elsasser, T. H., and Bolt, D. J. (1987). Dopaminergic-like activity in toxic fescue alters prolactin but not growth hormone or thyroid stimulating hormone in ewes. *Domest. Anim. Endocrinol.* 4, 259–269. doi: 10.1016/0739-7240(87)90022-1

Emile, J. C., Bony, S., and Ghesquiere, M. (2000). Influence of consumption of endophyte-infested tall fescue hay of heifers and lambs. *J. Anim. Sci.* 78, 358–364.

Forsyth, I. A. (1986). Variation among species in the endocrine control of mammary growth and function: the roles of prolactin, growth hormone, and placental lactogen. *J. Dairy Sci.* 69, 886–903. doi: 10.3168/jds.S0022-0302(86)80479-9

Hooley, R. D., Campbell, J. J., and Findlay, J. K. (1978). The importance of prolactin for lactation in the ewe. *J. Endocrinol.* 79, 301–310. doi: 10.1677/joe.0.0790301

Hoveland, C. S. (1993). Importance and economic significance of the Acremonium endophytes on performance of animals and grass plants. *Agr. Ecosyst. Environ.* 44, 3–12. doi: 10.1016/0167-8809(93)90036-O

Klotz, J. L., Brown, K. R., Xue, Y., Matthews, J. C., Boling, K. A., Bush, L. P., et al. (2012). Alterations in serotonin receptor-induced contractility of bovine lateral saphenous vein in cattle grazing endophyte-infected tall fescue. *J. Anim. Sci.* 90, 682–693. doi: 10.2527/jas.2011-4323

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Vevoda, A. C., et al. (2006). Assessment of vasoconstrictive potential of D-lysergic acid using an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 84, 3167–3175. doi: 10.2527/jas.2006-038

Koontz, A. F., Bush, L. P., Klotz, J. L., McLeod, K. R., Schrick, F. N., and Harmon, D. L. (2012). Evaluation of a ruminally dosed tall fescue seed extract as a model for fescue toxicosis in steers. *J. Anim. Sci.* 90, 914–921. doi: 10.2527/jas.2011-4292

Long, N. M., Prado-Cooper, M. J., Krehbiel, C. R., DeSilva, U., and Wettemann, R. P. (2010). Effects of nutrient restriction of bovine dams during early gestation on postnatal growth, carcass, and organ characteristics, and gene expression in adipose tissue and muscle. *J. Anim. Sci.* 88, 3251–3261. doi: 10.2527/jas.2009-2512

Long, N. M., Tousley, C. B., Underwood, K. R., Paisley, S. I., Means, W. J., Hess, B. W., et al. (2012). Effects of early- to mid-gestational undernutrition with or without protein supplementation on offspring growth, carcass characteristics, and adipocyte size in beef cattle. *J. Anim. Sci.* 90, 197–206. doi: 10.2527/jas.2011-4237

Mabjeesh, S. J., Sabastian, C., Gal-Garber, O., and Shamay, A. (2013). Effect of photoperiod and heat stress in the third trimester of gestation on milk production and circulating hormones in dairy goats. *J. Dairy Sci.* 96, 189–197. doi: 10.3168/jds.2012-5624

McCann, J. S., Caudle, A. B., Thompson, F. N., Stuedemann, J. A., Heusner, G. L., and Thompson, D. L. Jr. (1992). Influence of endophyte-infected tall fescue on serum prolactin and progesterone in gravid mares. *J. Anim. Sci.* 70, 217–223.

Molina, R. D., Meschia, G., Battaglia, F. C., and Hay, W. W. Jr. (1991). Gestational maturation of placental glucose transfer capacity in sheep. *Am. J. Physiol.* 261, R697–R704.

Pardo, C. E., Berard, J., Kreuzer, M., and Bee, G. (2013). Intrauterine crowding impairs formation and growth of secondary myofibers in pigs. *Animal* 7, 430–438. doi: 10.1017/S1751731112001802

Parish, J. A., McCann, M. A., Watson, R. H., Hoveland, C. S., Hawkins, L. L., Hill, N. S., et al. (2003). Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in sheep. *J. Anim. Sci.* 81, 1316–1322.

Park, P. W., and Goins, R. E. (1994). *In situ* preparation of fatty acid methyl esters for analysis of fatty acid composition in foods. *J. Food Sci.* 59, 1262–1266. doi: 10.1111/j.1365-2621.1994.tb14691.x

Peters, C. W., Grigsby, K. N., Aldrich, C. G., Paterson, J. A., Lipsey, R. J., Kerley, M. S., et al. (1992). Performance, forage utilization, and ergovaline consumption by beef cows grazing endophyte fungus-infected tall fescue, endophyte fungus-free tall fescue, and orchardgrass pastures. *J. Anim. Sci.* 70, 1550–1561.

Powell, S. E., and Aberle, E. D. (1981). Skeletal muscle and adipose tissue cellularity in runt and normal birth weight swine. *J. Anim. Sci.* 52, 748–756.

Putnam, M. R., Brandby, D. I., Schumacher, J., Boosinger, T. R., Bush, L., Shelby, R. A., et al. (1991). Effects of the fungal endophyte Acremonium coenophialum in fescue on pregnant mares and foal viability. *J. Anim. Sci.* 52, 2071–2074.

Realini, C. E., Duckett, S. K., Hill, N. S., Hoveland, C. S., Lyon, B. G., Sackmann, J. R., et al. (2005). Effect of endophyte type on carcass traits, meat quality, and fatty acid composition of beef cattle grazing tall fescue. *J. Anim. Sci.* 83, 430–439.

Reynolds, L. P., Caton, J. S., Redmer, D. A., Grazul-Bilska, A. T., Vonnahme, K. A., Borowicz, P. P., et al. (2006). Evidence for altered placental blood flow and vascularity in compromised pregnancies. *J. Physiol.* 572, 51–58. doi: 10.1113/jphysiol.2005.104430

Reynolds, L. P., and Ferrell, C. L. (1987). Transplacental clearance and blood flows of bovine gravid uterus at several stages of gestation. *Am. J. Physiol.* 253, R753–R759.

Reynolds, L. P., Ferrell, C. L., Robertson, D. A., and Ford, S. P. (1986). Metabolism of the gravid uterus, foetus, and uteroplacenta at several stages of gestation in cows. *J. Agric. Sci. (Camb.)* 106, 437–444. doi: 10.1017/S0021859600063309

Reynolds, L. P., and Redmer, D. A. (1995). Utero-placental vascular development and placental function. *J. Anim. Sci.* 73, 1839–1851.

Reynolds, L. P., and Redmer, D. A. (2001). Minireview: angiogenesis in the placenta. *Biol. Reprod.* 64, 1033–1040. doi: 10.1095/biolreprod64.4.1033

Stowe, H. M., Miller, M., Burns, M. G., Calcatera, S. M., Andrae, J. G., Aiken, G. E., et al. (2013). Effects of fescue toxicosis on bull growth, semen characteristics, and breeding soundness evaluation. *J. Anim. Sci.* 91, 3686–3692. doi: 10.2527/jas.2012-6078

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). Board-invited review: St. Anthony's fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci.* 89, 1603–1626. doi: 10.2527/jas.2010-3478

Stuedemann, J. A., and Hoveland, C. S. (1988). Fescue endophyte: history and impact on animal agriculture. *J. Prod. Agric.* 1, 39–44. doi: 10.2134/jpa1988.0039

Swatland, H. J. (1973). Muscle growth in the fetal and neonatal pig. *J. Anim. Sci.* 37, 536–545.

Thureen, P. J., Trembler, K. A., Meschia, G., Makowski, E. L., and Wilkening, R. B. (1992). Placental glucose transport in heat-induced fetal growth retardation. *Am. J. Physiol.* 263, R578–R585.

Underwood, K. R., Tong, J. F., Price, P. L., Roberts, A. J., Grings, E. E., Hess, B. W., et al. (2010). Nutrition during mid to late gestation affects growth, adipose tissue deposition, and tenderness in cross-bred beef steers. *Meat Sci.* 86, 588–593. doi: 10.1016/j.meatsci.2010.04.008

Watson, R. H., McCann, M. A., Parish, J. A., Hoveland, C. S., Thompson, F. N., and Bouton, J. H. (2004). Productivity of cow-calf pairs grazing tall fescue pastures infected with either the wild-type endophyte or a nonergot alkaloid-producing endophyte strain, AR542. *J. Anim. Sci.* 82, 3388–3393.

Weber, H. P. (1980). "Ergot compounds: a synopsis," in *Ergot Compounds and Brain Function: Nueroendocrine and Neuropsychiatric Aspects*, eds M. Goldstein, A. Lieberman, D. B. Calne, and M. O. Thorner (New York, NY: Raven Press), 25–34.

Zhu, M. J., Ford, S. P., Nathanielsz, P. W., and Du, M. (2004). Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. *Biol. Reprod.* 71, 168–173. doi: 10.1095/biolreprod.104.034561

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 June 2014; accepted: 04 August 2014; published online: 21 August 2014.
Citation: Duckett SK, Andrae JG and Pratt SL (2014) Exposure to ergot alkaloids during gestation reduces fetal growth in sheep. Front. Chem. ***2****:68. doi: 10.3389/fchem.2014.00068*
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 12 November 2014
doi: 10.3389/fchem.2014.00096

Physiological responses to known intake of ergot alkaloids by steers at environmental temperatures within or greater than their thermoneutral zone

Joan H. Eisemann*, Gerald B. Huntington, Megan Williamson, Michelle Hanna and Matthew Poore

Department of Animal Science, North Carolina State University, Raleigh, NC, USA

Edited by:
John Wade, Florey Institute of Neuroscience and Mental Health, Australia

Reviewed by:
David L. Harmon, University of Kentucky, USA
Andrew P. Foote, United States Department of Agriculture, USA

****Correspondence:***
Joan H. Eisemann, Department of Animal Science, North Carolina State University, 120 Broughton Drive, Box 7621, Raleigh, NC 27695-7621, USA
e-mail: joan_eisemann@ncsu.edu

Two studies separated effects of dietary ergot alkaloids from effects of feed intake or ambient temperature on respiration rate (RR), heart rate (HR), surface temperature (ST), rectal temperature (RT), blood pressure (BP), serum hormone, and plasma metabolite concentrations in beef steers. The balanced, single reversal design for each experiment used 8 beef steers fed tall fescue seed [2.5 g/kg body weight (BW)] with (E+) or without (E−) ergot alkaloids as part of a 60:40 switchgrass hay: supplement diet. Periods were 35 days with 21 days of preliminary phase and 14 days of feeding fescue seed once daily. Measures of dependent variables were collected on d 20, 25, 29, and 35 of each period at 0730 (before feeding), 1230 and 1530. In Experiment 1 steers weighed 286 kg, gained 0.61 kg BW/day, E+ supplied 2.72 mg ergot alkaloids including 1.60 mg ergovaline per steer daily, and mean minimum and maximum daily ambient temperatures were 23.6 and 32.3°C. In Experiment 2 steers weighed 348 kg, gained 1.03 kg BW/day, E+ supplied 3.06 mg ergot alkaloids including 2.00 mg ergovaline daily, and mean minimum and maximum daily ambient temperatures were 11.9 and 17.4°C. Dry matter intake was not affected by fescue seed treatment ($P < 0.20$) in either experiment. In both experiments, E+ reduced HR ($P < 0.01$) and increased insulin ($P = 0.07$). Systolic BP minus diastolic BP decreased ($P < 0.05$) for E+ in both experiments, due to increased diastolic BP in Experiment 1 ($P < 0.03$) and decreased systolic BP in Experiment 2 ($P < 0.07$). In Experiment 1, above the thermoneutral zone, E+ increased ($P < 0.05$) RR, RT, and left side ST in comparison to E−, but in Experiment 2, within the thermoneutral zone, E+ and E− did not differ ($P < 0.18$). Ergot alkaloids from fescue seed affect the cardiovascular system of steers separately from effects of feed intake or environmental temperature. Ergot alkaloids interact with ambient temperatures above the steers' thermoneutral zone to exacerbate the symptoms of hyperthermic stress.

Keywords: steers, tall fescue, ergot alkaloids, environmental temperature, hemodynamics, metabolism

INTRODUCTION

Consumption of toxic, endophyte-infected, tall fescue results in ingestion and absorption of ergot alkaloids produced by the endophyte, *Neotyphodium coenophialum*, which causes fescue toxicosis in grazing cattle. Ergovaline, the alkaloid produced in greatest concentration, or total ergot alkaloids have been measured in fescue to describe its potential toxicity. Concentrations of ergovaline increased from 250 to 450–500 μg/kg in leaf blades and from 500 to 800–1300 μg/kg in leaf sheaths from April to May. Seed heads contained the greatest concentration of toxins and reached concentrations as high as 5000 μg/kg in June (Rottinghaus et al., 1991). Total ergot alkaloid concentration showed the same seasonal changes as ergovaline (Hill et al., 2000).

Studies with steers or heifers consuming different sources of fescue hay (Hemken et al., 1981) or consuming alkaloids from fescue seed (Burke et al., 2001) at differing ambient temperatures in a factorial experimental design indicate a greater response to diets with endophyte-infected vs. endophyte-free fescue (increased respiration and rectal temperatures (RTs) and decreased voluntary intake) during hyperthermic heat stress compared to ambient temperatures within the animals' thermoneutral zone. Adverse responses of cattle consuming endophyte-infected tall fescue, including lower tolerance to ambient temperatures outside the animals' thermoneutral zone, decreased voluntary intake, weight gain, and milk production have been linked to hemodynamic effects of ergot alkaloids in the tall fescue (Strickland et al., 2011). The alkaloids, particularly ergovaline, ergovalanine, and ergonovine administered i.v. (Oliver et al., 1994; Browning and Leite-Browning, 1997; Browning, 2000) or fed at doses comparable to amounts of alkaloids ingested as endophyte-infected fescue seed (Rhodes et al., 1991; Aiken et al., 2007, 2009), decreased heart rate (HR), increased blood pressure (BP), and caused vasoconstriction in steers or heifers. The responses include decreased skin temperature or increased RT and increased respiration rate (RR) at ambient temperatures greater than the animals' thermoneutral

zone. Usually, ingestion or administration of alkaloids decreased serum or plasma prolactin concentrations. In a thermoneutral environment, the hemodynamic responses appeared to be muted or not detectable.

Efforts to delineate potential interactions between hemodynamic effects of alkaloid and ambient temperatures above the animals' thermoneutral zone have been complicated by concomitant changes in voluntary intake when animals consume endophyte-infected tall fescue (Hemken et al., 1981; Boling et al., 1989; Aiken et al., 2007, 2009). Additionally, close human contact required to obtain physiological measures may itself alter the animals' response and contribute to variation in response to dose levels or duration of experimental protocol (Aiken et al., 2007); therefore, most reported studies describe acute responses over a period of hours or a few days. Some researchers have addressed this potential problem by adapting animals to facilities and conditions prior to experiments, e.g., Browning (2000).

The main objectives of the current experiments were to separate the pharmacological effects of endophyte alkaloids from effects of ambient temperatures above the animals' thermoneutral zone and effects attributable to changes in intake or discomfort due to close human contact.

MATERIALS AND METHODS

Two experiments were conducted under the supervision and approval of the university animal care and use committee. Angus steers from the North Carolina State University Beef Education Unit university farm of known pedigree, age, and background were trained to be led by halter and accustomed to close human contact while eating a hay diet devoid of fescue. Experiment 1 was in June to August, 2011, and Experiment 2 was in October to December, 2012. Each experiment used 8 steers. In Experiment 1 mean ± *SD* steers' age was 247 ± 24 d and in Experiment 2 it was 380 ± 27 d. Steers were housed in individual stalls with a chain attached to their halter. The stalls were 115 × 178 cm, with automatic waterers and rubber mats on concrete floors. The daily protocol was removal of orts (if any), feed supplement at 0830 h, move steers outside for exercise in a common pen for about 1 h, then back to stalls for morning hay feeding by about 1000 h. At 1530 h, the second ration of supplement was fed, followed by the second ration of hay. Lights in the barn were 18 h on: 6 h off each day, with adjustment of on and off times to accommodate the season of the year. Steers were weighed weekly, feed and orts recorded daily. Steers' stall assignment was determined when they were randomly allocated to the treatment protocol.

All steers were fed sliced switchgrass hay (**Table 1**) at daily amounts equal to 10 g/kg BW and a supplement, each divided into AM and PM feedings. The hay was stored in rectangular bales, and was pressed through a Van Dale Bale Processor, Model S600 (J-star industries, Ft. Atkinson WI) with knives spaced 12.5 cm apart.

Each experiment had two, 35-d periods with 21 d of preliminary phase and 14 d of feeding endophyte-infected fescue seed (E+) or endophyte-free fescue seed (E−). During the preliminary phase soybean hulls were added to the supplement instead of fescue seed. During the treatment phase of Experiment 1 steers were fed 2.15 kg supplement DM and 0.62 kg of fescue seed DM daily (**Table 1**), and in Experiment 2 steers were fed 2.62 kg supplement and 0.69 kg fescue seed DM daily (**Table 1**), with the total weight of supplement plus fescue divided equally in the AM and PM feedings. In Experiment 1, E+ supplied 2.72 mg ergot alkaloids including 1.60 mg ergovaline per steer daily and in Experiment 2, E+ supplied 3.06 mg ergot alkaloids including 2.00 mg ergovaline daily. The dose chosen was similar to that of Aldrich et al. (1993) who fed diets containing 285 μg/kg of ergovaline from fescue seed. The goal was to produce physiological changes but maintain similar intake during E− and E+ feeding to avoid confounding effects due to intake changes.

Table 1 | Organic matter (OM), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations, g/kg dry matter, in feedstuffs for the 2 experiments.

Item	OM	CP	NDF	ADF
Soybean hulls	949	164	533	385
Endophyte-infected seed	937	152	440	248
Endophyte-free seed	933	132	389	231
SUPPLEMENT				
Experiment 1	946	191	191	125
Experiment 2	945	206	191	125
SWITCHGRASS HAY				
Experiment 1	959	56	691	393
Experiment 2	960	74	707	390

For Experiment 1 the ration was formulated to meet National Research Council (1996) nutrient requirements for CP, TDN, Ca, and P for a steer weighing 272 kg and gaining 0.6 kg/d and for Experiment 2 the ration was formulated to meet those requirements for a steer weighing 318 kg and gaining 0.6 kg/d. The fescue seed passed through a 1.1 cm screen in a hammer mill (Meadow Mills, North Wilksboro, NC) before feeding to partially disrupt the seed coat. All of the fescue seed was fed in the morning, so the amount of AM supplement fed was reduced accordingly. Steers were assigned at random to receive E− (Southern States Cooperative, Inc., Cloverdale, VA) or E+ (EverGreen Seed, LLC, Fuquay-Varina, NC) seed in a single reversal design, 4 steers fed each type of seed in period 1. The treatments were reversed in period 2. The amount of fescue seed fed was gradually increased and the amount of soybean hulls was gradually decreased during the first 3 d when seed was fed in each period (days 22–24), so d 25 was the first day on full treatment.

After 3 weeks' adaptation to facilities and protocol, steers were fed their assigned diets for 14 d then all steers returned to the adaptation diet. After 21 d, the 14-d treatment period was repeated. As in the first period, the amount of fescue seed fed was gradually increased during the first 3 d when seed was fed, so d 25 was the first day on full treatment. Steers' hair was clipped during adaptation, 5 to 7 d before the first day of feeding fescue seed with electric animal clippers that left 3 mm hair (Aesculap® Econom II, Suhl, Germany). Daily minimum, maximum, and 1200 h barn temperature and relative humidity were measured using a calibrated humidity/thermometer (Fisher Scientific, Pittsburgh, PA). These variables were measured at 3 locations in the barn and an average value was calculated.

Additionally, temperature and humidity at sampling times were recorded.

On d 20, 25, 29, and 35 of each period palmar surface temperature (ST) in the area of the large metacarpal bone of the front legs, plantar ST in the area of the large metatarsal bone of the rear legs, and left side ST were measured by digital infrared thermal imaging. The legs were chosen because of accessibility, cleanliness, and potential relationship to ST changes in response to peripheral constriction. The left side was chosen because of the layout of the animal handling facilities and the ability to consistently measure the same area (Huntington et al., 2012). The digital thermal images were recorded with a Ti45FT (Fluke Corporation, Everett WA) with a 20 mm lens, manual focusing, 30 Hz 160 × 120 pixel focal array, and a vanadium oxide uncooled microbolometer. Emissivity was set at 0.95 and background temperature was set at 20°C. The camera has an accuracy of 2°C in the physiological temperature range, and a sensitivity of <0.1°C. Images were stored in files containing approximately 2.5 Mbyte of information that included date, time, image number, emissivity, background temperature, and visual and infrared images. Software provided by the manufacturer allowed detailed isolation of portions of the image (see **Figure 1**) and presented minimal, maximal, average, and the standard deviation of the pixels in the selected portion. BP and HR were measured with a 16 to 24 cm BP cuff around the tail head connected to a digital monitor (Lifesource® A&D Engineering, Inc., San Jose, CA), RT was measured with a digital thermometer (Becton, Dickenson and Co., Franklin Lakes, NJ), and RR by rib cage movement was measured for 10 s at 0730 (before collection of orts), 1230, and 1500 (before PM feeding). Data were averaged for front legs and averaged for rear legs before statistical analysis. The steers were restrained in a squeeze chute while blood was removed by jugular venipuncture, starting at 1300 h on sampling days.

In each experiment, 2 groups of 4 steers ($n = 8$ total) were staggered by 1 week to allow collection of physiological data within 30 min on each sampling day. Steers were adapted to the procedures by several practice sessions during the preliminary phases of the experiments. Two people were in the barn to collect BP and HR measures, one steer at a time in their stall, steer in standing position. One minute elapsed between deflation of the cuff and the subsequent measure. The instrumental criteria and personal experience were used to assess validity of each BP and HR measure. The steers' demeanor on a given day and time affected the number of BP and HR measures used in statistical evaluation of treatments. Of the 384 measurement episodes within steer, period, day, and time, 6 contained 2 measures, 264 contained 3 measures, 108 contained 4 measures, and 6 contained 5 measures.

In Experiment 1, a fecal grab sample was collected between 1100 and 1300 h for 3 d at the end of each fescue period for analysis of alkanes to calculate DM digestibility.

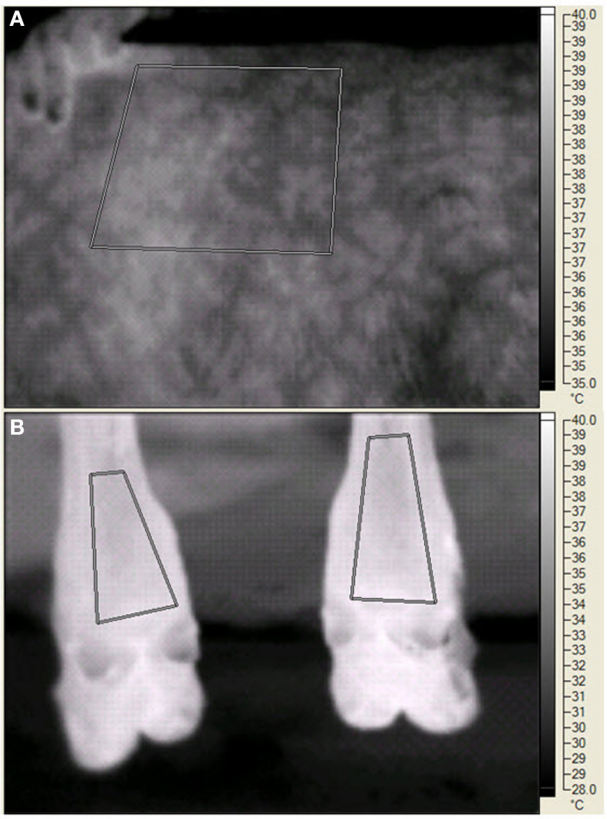


FIGURE 1 | Digital infrared images of the left side (A) and the plantar surface in the area of the large metatarsal bone in the rear legs (B) of a steer during the experiment. The human fingers **(A)** indicate the edge of the scapula. The paralumbar fossa is to the right of the measured area. For both panels, the geometric shapes indicate areas in which temperature data were recorded. The vertical bar on the far right side of each panel indicates the temperature range (°C) and the associated light (warmer) to dark (cooler) gradient within the image.

SAMPLE ANALYSIS

Feed samples were analyzed for nutrient content by a commercial laboratory (North Carolina Department of Agriculture, Raleigh, NC). Concentration of total alkaloids in the fescue seed was analyzed by a commercial laboratory (Agrinostics Ltd. Co., Watkinsville, GA) using an ELISA (Hill and Agee, 1994). Ergovaline concentration in the fescue seed was analyzed by a commercial laboratory (University of Missouri Veterinary Medical Diagnostic Laboratory, Colombia, MO) using HPLC (Rottinghaus et al., 1991, 1993). Serum prolactin (Bernard et al., 1993) and serum insulin (Cartiff et al., 2013) were determined by radioimmunoassay. For prolactin, the intra-assay CV was 6.8% and the inter-assay CV was 7.1%. For insulin, the intra-assay CV was 5.5% and the inter-assay CV was 8.4%. Plasma glucose was analyzed using a glucose oxidase method (Yellow Springs Instruments, Yellow Springs, OH). Concentration of hentriacontane in feed and fecal samples and calculation of DM digestibility in Experiment 1 were determined as described by Chavez et al. (2011).

STATISTICAL ANALYSIS OF DATA

The Mixed procedure of SAS (SAS institute, Cary NC) was used for statistical analysis of data. The model had main effects of treatment, day, time of day, group, period, and all possible interactions of treatment, time and day. Steer, steer × period, steer

× treatment, and steer × time were random effects. Except for measures in Experiment 1 of serum insulin, serum prolactin, and plasma glucose, mean values within steers across times collected on day 20 of each period were used as covariates within periods. The effect of period on baseline values was tested using a model with period, treatment, and period × treatment as the main effect. Data for intake, DM digestibility, plasma glucose, and blood hormones did not have day or time of day in the model.

RESULTS

Feed intake was not affected by treatment in either experiment (**Table 2**) and therefore treatment responses for E+ compared to E− are independent of intake effects. Steers were fed at a slightly restricted intake in both studies to minimize orts and remove confounding effects of intake from the responses. Steers in Experiment 1 had greater orts as a proportion of hay offered and ate slightly less DM as a proportion of BW than steers in Experiment 2. Dry matter digestibility did not differ ($P = 0.76$) for E− and E+ and was 0.584 and 0.590 g/g DM (*SE* 0.014), respectively. Barn temperature, humidity, and calculated temperature-humidity index (THI, Mader et al., 2002) indicate that steers in Experiment 1 were near or above their thermoneutral zone, and steers in Experiment 2 were within their thermoneutral zone during the experiments (**Table 3**). Daily minimums and maximums were within or close to those recorded during sampling times in Experiment 1 (**Figure 2**) and daily minimums were close to those recorded during sampling times in Experiment 2 (**Figure 3**). Maximum values were after sampling times in Experiment 2.

BASELINE DATA

In both experiments, there were few differences ($P < 0.21$) between baseline values collected on d 20 of each period that were used as covariates in the statistical model. In Experiment 1 there were differences ($P < 0.05$) for left side ST standard deviation (0.38 vs. 0.31°C) and trends ($0.05 < P < 0.07$) for systolic pressure (117 vs. 103 mm Hg) and diastolic pressure (52 vs. 47 mm Hg). In Experiment 2 there were differences ($P < 0.05$) for RR (31 vs. 24 breaths/min) and RT (38.2 vs. 38.5°C). In no case was there a period × treatment interaction supporting the fact that there was no carryover between periods for the variables measured.

RESPONSES TO DAYS OF FEEDING FESCUE SEED

In Experiment 1 there was a trend ($P < 0.10$) for increased left side ST, but in Experiment 2 STs decreased ($P < 0.05$) with days of feeding fescue seed (**Table 4**). There were trends ($P < 0.10$) for day × treatment interactions; diastolic BP increased in Experiment 1 for E+ but did not change for E− with days of feeding fescue seed, and there was a decrease in systolic BP in Experiment 2 for E− whereas systolic BP increased for E+ with days of feeding fescue seed. The difference between systolic and diastolic BP decreased for E− but increased for E+ with days of feeding fescue seed in Experiment 2 (**Table 4**). The day × treatment interaction ($P < 0.05$) for RR in Experiment 1 was caused by a greater increase for E+ than E− with days of feeding fescue seed (**Table 4**). The trend ($P < 0.10$) for the

Table 2 | Body weight (BW), dry matter intake (DMI), and orts for steers fed endophyte-free (E−) or endophyte-infected (E+) fescue seed above (Experiment 1) or within (Experiment 2) their thermoneutral zone.

Item	Experiment 1				Experiment 2			
	E−	*E+*	*SE*[a]	*P* =	*E−*	*E+*	*SE*[a]	*P* =
BW, kg	285	288	4	0.24	348	347	10	0.30
DMI, KG/D								
Hay	2.72	2.63	0.1	0.25	3.67	3.68	0.06	0.82
Total	5.44	5.33	0.1	0.18	6.98	6.99	0.06	0.82
Total g/kg BW	19.1	18.5	0.4	0.12	20.2	20.3	0.5	0.40
Orts g/kg hay	174	197	27.5	0.28	60	57	14.2	0.82

[a] *n = 8.*

Table 3 | Barn temperature, relative humidity, and temperature-humidity index (THI) for steers fed endophyte-free (E−) or endophyte-infected (E+) fescue seed above (Experiment 1) or within (Experiment 2) their thermoneutral zone.

Item	Experiment 1				Experiment 2			
	Period 1		Period 2		Period 1		Period 2	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
TEMPERATURE, °C								
Minimum[a]	23.2	1.2	24.0	1.2	11.9	1.9	11.9	2.3
Maximum[a]	32.4	0.8	32.2	1.9	17.4	4.1	17.4	2.8
1200[a]	30.3	1.3	30.0	2.7	15.8	4.0	15.6	2.8
Sampling days[b]								
0730	24.5	1.3	24.3	0.8	12.2	2.1	12.5	1.9
1230	30.9	1.1	29.1	2.8	15.5	3.6	15.4	3.2
1530	30.6	2.0	30.1	1.7	16.2	4.0	16.4	3.5
RELATIVE HUMIDITY, %								
Minimum[a]	46.5	10.7	50.5	10.9	46.6	10.7	53.3	13.3
Maximum[a]	81.3	6.7	86.2	6.9	77.9	7.3	83.1	10.6
1200[a]	58.3	10.2	64.1	14.7	57.3	13.2	64.6	13.9
Sampling days[b]								
0730	74.6	7.7	79.8	8.6	62.9	7.2	64.5	13.7
1230	57.4	10.1	65.1	16.0	54.3	8.8	58.8	11.3
1530	56.5	15.2	57.6	14.5	46.7	7.7	54.2	11.8
THI[c]								
Minimum[a]	69.0	2.0	70.3	1.7	54.7	2.5	54.8	2.9
Maximum[a]	86.9	1.6	87.4	2.7	62.7	6.6	63.0	4.7
1200[a]	79.9	2.2	80.1	2.5	59.7	5.5	59.8	4.2
Sampling days[b]								
0730	73.5	2.4	73.7	1.6	54.7	3.0	55.2	2.7
1230	80.2	2.6	78.9	3.1	59.3	4.8	59.3	4.7
1530	79.5	1.9	79.3	2.0	60.0	4.9	60.8	5.0

[a] *Data from the last 16 d of each period.*

[b] *d 20, 25, 29, and 35 of each period.*

[c] *THI = 0.8Ta + [(0.01 RH × (Ta−14.3)] + 46.3 where Ta = ambient temperature, °C (Mader et al., 2002).*

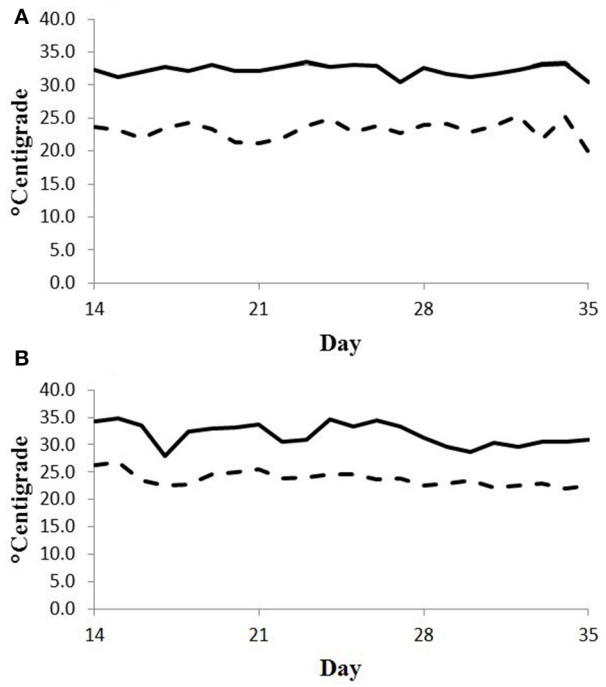


FIGURE 2 | Daily minimum (dashed line) and maximum (solid line) temperature (°C) during days 14 to 35 of period 1 (A) and period 2 (B) in Experiment 1. Day 21 was the first day of seed feeding in each period.

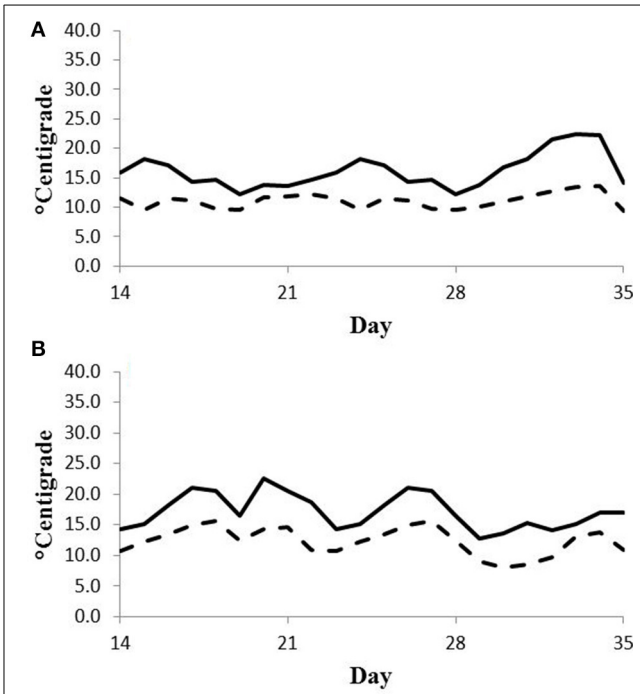


FIGURE 3 | Daily minimum (dashed line) and maximum (solid line) temperature (°C) during days 14–35 of period 1 (A) and period 2 (B) in Experiment 2. Day 21 was the first day of seed feeding in each period.

same interaction in Experiment 2 (**Table 4**) was caused by a lesser decrease in RR for E+ than E− with days of feeding fescue seed. The ST of rear legs tended ($P < 0.10$) to fluctuate more for E+ than E− with days of feeding fescue seed in Experiment 2, but there was no interaction in Experiment 1 (**Table 4**).

The day × treatment interaction of ST difference between front and rear legs ($P < 0.01$) in Experiment 2 reflected similar decreased surface ST of front legs for E− and E+ and of rear legs for E− while ST of rear legs for E+ showed greater fluctuation with days of feeding fescue seed (**Table 4**).

Serum insulin concentration was greater ($P < 0.05$, Experiment 1) or tended ($P = 0.07$, Experiment 2) to be greater for E+ than E− and plasma glucose concentration was greater ($P < 0.01$) for E+ than E− in Experiment 2 (**Table 5**). Serum prolactin concentrations were numerically lower for E+ than E− in both experiments, but variation among steers precluded statistical significance. The average prolactin concentration for E+ was 26% of E− in Experiment 1 and 60% of E− in Experiment 2.

TIME OF DAY AND TREATMENT RESPONSES

Systolic BP decreased ($P < 0.05$, Experiment 1) with time of day (**Table 6**) and tended to decrease ($P < 0.10$, Experiment 2) with E+. Diastolic BP decreased ($P < 0.05$) with time of day in Experiment 2, and was greater for E+ than E− in Experiment 1. In both experiments, systolic – diastolic BP difference was lesser for E+ than E−, with a trend for greater decrease with time of day for E+ than E− in Experiment 1 (time of day × treatment interaction, $P < 0.10$). HR increased with time of day and was lesser for E+ than E− in both experiments (**Table 6**). RR increased ($P < 0.01$) with time of day in both experiments. It was greater ($P < 0.01$) for E+ than for E− in Experiment 1 with a greater increase for E+ than E− with time of day in Experiment 1 (time of day × treatment interaction, $P < 0.05$). In both experiments, RT and STs of legs and the left side increased ($P < 0.05$) with time of day, but there were no time of day × treatment interactions (**Table 6**). In Experiment 1, RT and left side ST were greater ($P < 0.05$) for E+ than E−.

DISCUSSION

Scharf et al. (2011) reported the critical point for ambient temperature to increase core body temperature of growing cattle in feedlots at about 25°C. Hahn (1999) suggested a thermal stress threshold of 25°C for growing cattle fed *ad libitum* which coincided with decreased feed intake and 21°C as the threshold for increased RR. Scharf et al. (2011) observed also that cattle showed nighttime recovery to decrease core body temperature. In both of the current experiments RT, RR, and STs were at their minimum at the 0730 sampling for both E− and E+. This sampling time had ambient temperature and THI that were close to the minimum values. RT, RR, and ST cycled in all steers and an increase in RR, RT, and left side ST due to E+ was observed only when steers were housed above their thermoneutral zone. The increases due to E+ were associated with THI of around 74–80, above 75 which is the point suggested for using thermal stress-limiting measures (Hahn, 1999).

Table 4 | Blood pressure, heart rate (HR), respiration rate (RR), rectal temperature (RT), and surface temperature for steers fed endophyte-free (E−) or endophyte-infected (E+) fescue seed for 14 d above (Experiment 1) or within (Experiment 2) their thermoneutral zone.

Item	Day of feeding fescue seed[a]								
	4		8		14			*P* =	
	E−	*E*+	*E*−	*E*+	*E*−	*E*+	*SE*	Day	Day × Trt[b]
EXPERIMENT 1									
Systolic, mm Hg	104.2	97.7	104.1	102.0	104.8	101.6	2.9	0.63	0.49
Diastolic, mm Hg	48.7	48.0	48.3	54.1	47.5	52.4	1.9	0.26	0.08
S–D[b], mm Hg	54.5	56.0	54.8	49.0	56.2	50.2	2.5	0.81	0.78
HR, beats/min	62	57	62	57	63	58	1	0.17	0.95
RR, breaths/min	31	30	32	34	32	50	1.8	0.01	0.01
RT, °C	38.3	38.3	38.3	38.5	38.3	38.9	0.1	0.01	0.01
Surface Temperature, °C									
Rear legs[c]	33.8	33.9	33.5	34.3	34.1	34.4	0.24	0.11	0.32
Front legs[c]	34.8	35.1	34.9	35.0	35.1	35.4	0.21	0.35	0.82
Front–rear legs	1.07	1.18	1.41	0.73	0.91	0.93	0.21	0.54	0.07
Left side	36.4	36.8	36.6	37.0	36.9	37.4	0.2	0.08	0.99
EXPERIMENT 2									
Systolic, mm Hg	116.1	103.7	114.9	108.3	110.0	106.7	3.2	0.45	0.07
Diastolic, mm Hg	57.1	58.9	56.4	59.7	55.7	56.6	1.9	0.49	0.72
S-D, mm Hg	56.7	47.2	56.1	50.9	52.0	52.4	2.6	0.79	0.02
HR, beats/min	74	66	72	65	72	65	1.5	0.32	0.91
RR, breaths/min	27	26	22	23	22	23	0.6	0.01	0.09
RT, °C	38.3	38.1	38.2	38.1	38.2	38.1	0.1	0.74	0.54
Surface Temperature, °C									
Rear legs[c]	25.1	25.8	23.4	22.6	23.2	23.8	0.48	0.01	0.10
Front legs[c]	28.6	28.7	26.6	26.7	26.6	26.8	0.36	0.01	0.97
Front–rear legs	3.64	2.83	3.30	4.05	3.74	2.94	0.34	0.27	0.01
Left side	31.9	32.4	30.4	31.5	30.6	30.7	0.35	0.01	0.18

[a] *Periods lasted 35 days; fescue seed was fed for 14 days (d22–d35) of each period; n = 8 per treatment.*

[b] *Trt, treatment; S–D, systolic–diastolic blood pressure.*

[c] *Surface temperature of rear legs represents the plantar surface in the area of the large metatarsal bone; surface temperature of front legs represents the palmar surface in the area of the large metacarpal bone.*

Table 5 | Serum insulin, serum prolactin, and plasma glucose concentrations for steers fed endophyte-free (E−) or endophyte-infected (E+) fescue seed above (Experiment 1) or within (Experiment 2) their thermoneutral zone.

Item	Day of feeding fescue seed[a]									
	4		8		14			*P* =		
	E−	*E*+	*E*−	*E*+	*E*−	*E*+	*SE*	Trt[b]	Day	Day × Trt
EXPERIMENT 1										
Prolactin, ng/mL	175	75	160	24	117	21	86	0.37	0.03	0.44
Insulin, uIU/mL	14.1	18.4	16.5	20.9	17.6	23.3	1.7	0.02	0.03	0.88
Glucose, mM	3.53	3.71	3.58	3.70	3.63	3.74	0.02	0.31	0.40	0.76
EXPERIMENT 2[c]										
Prolactin, ng/mL	92	55	54	29	67	44	13.1	0.14	0.30	0.84
Insulin, uIU/mL	19.1	20.5	20.1	20.5	20.6	23.1	1.2	0.07	0.76	0.18
Glucose, mM	4.01	4.28	4.12	4.25	4.07	4.33	0.05	0.01	0.45	0.19

[a] *Periods lasted 35 days; fescue seed was fed for 14 days (d22–d35) of each period.*

[b] *Trt, treatment.*

[c] *Day 20 values were used as a covariate in Experiment 2.*

Table 6 | Blood pressure, heart rate (HR), respiration rate (RR), rectal temperature (RT), and surface temperature at different times of day for steers fed endophyte-free (E−) or endophyte-infected (E+) fescue seed above (Experiment 1) or within (Experiment 2) their thermoneutral zone.

Item	Time of day									
	E−			*E+*				*P =*		
	0730	1230	1530	0730	1230	1530	*SE*	Trt[a]	Time[a]	T × T[a]
EXPERIMENT 1										
Systolic, mm Hg	106.9	106.1	100.0	105.0	97.9	98.5	2.8	0.18	0.03	0.17
Diastolic, mm Hg	49.7	48.1	46.7	51.4	51.3	51.7	1.9	0.03	0.79	0.57
S-D[a], mm Hg	56.2	57.0	52.3	54.5	47.5	47.7	2.41	0.05	0.03	0.08
HR, beats/min	60.2	63.8	63.1	55.1	58.1	58.4	0.96	0.01	0.01	0.84
RR, breaths/min	27	33	35	29	41	44	2	0.01	0.01	0.03
RT, °C	38.1	38.3	38.5	38.2	38.6	38.9	0.1	0.05	0.01	0.16
Surface Temperature, °C										
Rear legs[b]	31.8	34.9	34.7	31.9	35.2	35.4	0.24	0.13	0.01	0.32
Front legs[b]	33.5	35.8	33.5	33.7	35.6	36.0	0.21	0.29	0.01	0.25
Front–rear legs	1.67	0.84	0.87	1.80	0.42	0.63	0.21	0.41	0.01	0.32
Left side	35.2	37.0	37.5	35.8	37.3	38.1	0.19	0.05	0.01	0.83
EXPERIMENT 2										
Systolic, mm Hg	114.2	111.4	110.5	108.2	106.2	104.1	3.1	0.07	0.22	0.86
Diastolic, mm Hg	58.5	55.7	55.0	60.7	56.1	58.4	1.8	0.32	0.04	0.61
S-D, mm Hg	53.7	56.7	54.5	49.9	52.6	48.1	2.4	0.04	0.13	0.73
HR, beats/min	69.6	74.8	73.2	63.3	66.9	66.0	1.5	0.01	0.02	0.74
RR, breaths/min	22	25	25	22	24	24	1	0.98	0.01	0.75
RT, °C	38.0	38.2	38.4	38.0	38.2	38.3	0.1	0.49	0.02	0.78
Surface Temperature, °C										
Rear legs[b]	21.6	25.0	25.1	21.5	25.3	25.3	0.48	0.76	0.01	0.96
Front legs[b]	25.7	28.0	28.1	25.6	28.2	28.4	0.35	0.71	0.01	0.81
Front–rear legs	4.2	3.5	3.0	4.0	2.8	3.0	0.35	0.28	0.01	0.52
Left side	29.6	31.3	32.0	30.0	32.1	32.4	0.35	0.18	0.01	0.58

[a] *Trt, treatment; Time, time of day; T × T, treatment × time of day; S–D, systolic–diastolic blood pressure.*

[b] *Surface temperature of rear legs represents the plantar surface in the area of the large metatarsal bone; surface temperature of front legs represents the palmar surface in the area of the large metacarpal bone.*

Routes of heat flow from the animal to the environment are conduction, convection, and radiation which depend on thermal gradients within the animal and between the animal and the environment; and evaporation which depends on humidity. Skin temperature below 35°C provided a large enough temperature gradient between the body core and the skin to use all 4 routes of heat exchange (Collier et al., 2006). Mechanisms for heat dissipation in response to thermal stress include increased RR, increased peripheral vasodilation, increased skin temperature, and increased sweat rate. Blood flow to the periphery increases to increase heat loss via conduction and convection. Hair coat can reduce heat flow via these two routes. Heat stress increases sweating rate and RR. Evaporation is the major route of heat loss as ambient temperature approaches skin temperature (Hansen, 2004; Scharf et al., 2010). In Experiment 1, steers were housed above their thermoneutral zone, and left side ST was above 35°C which may have reduced the effectiveness of heat transfer from the body core to the skin and resulted in increased RR.

Scharf et al. (2010) observed increased RR, RT, skin temperature, and sweat rate in Angus or Romosinuano steers housed at temperatures above the thermoneutral zone, cycling from 26°C during the night to 36°C during the day, compared to thermoneutral housing (21°C). Skin temperatures were highly correlated with ambient temperatures. Decreased sweat rate was correlated with increased RT during heat stress for Angus cattle (Scharf et al., 2010). STs in both of the current experiments were consistent with a correlation between skin temperature and ambient temperature.

Steers receiving E+ had greater left side ST than those receiving E− when housed above the thermoneutral zone suggesting vasodilation and greater transfer of core body heat to the periphery which should increase heat loss by conduction and convection. The maximum ambient temperature was 32.3°C. Previous studies in which heat stress was constant showed no change in ST due to short-term feeding of E+ (Rhodes et al., 1991; Al-Haidary et al., 2001; Koontz et al., 2012) or decreased ST due to a single injection of ergot alkaloids (Browning and Leite-Browning, 1997;

Browning, 2000). Changes in ST reflect changes in ambient temperatures, hair coat, and peripheral blood flow. We clipped hair in an effort to minimize hair coat effect among steers or in response to treatment. The thermal imaging camera provides data on minimum, maximum, average and standard deviation of ST. Previous work with the same camera showed an inverse correlation between average and the standard deviation of side ST in bulls not exposed to toxic fescue (Huntington et al., 2012), indicating that thermal imaging may detect variation in ST due to thermal patterns created by vasodilation. In the current experiments correlations between mean and standard deviations of ST within experiments (data not shown) were not statistically significant ($P < 0.10$). Steer's hair in our study was not clipped as close to the skin as it was for the Angus bulls in Huntington et al. (2012). ST of front legs was consistently greater than ST of rear legs although the difference in temperature between them declined with time of day. Lack of interactions between days of feeding fescue or time of day with treatment indicates that either front or rear legs could be used to evaluate changes in ST.

RR increased to a greater rate in response to E+ in conditions above the thermoneutral zone in Experiment 1. It is possible that other avenues of heat dissipation were not responding to environmental conditions. Increased RR, sweat rate, and peripheral vasodilation contribute to internal body temperature response to heat stress (Scharf et al., 2010). Decreased skin vaporization was observed in steers housed at 32°C and fed a similar dose of E+ to that fed in the present study compared to steers fed E− (Aldrich et al., 1993). RT was greater for E+ than E− in the current study.

HR decreased due to E+ in cattle housed at both ambient temperatures. Decreased HR should result in decreased BP if other variables that affect pressure are unchanged (Melbin and Detweiler, 1993). Systolic-diastolic pressure difference, pulse pressure, decreased for steers eating E+ in both environments but for different reasons. Under thermoneutral conditions in Experiment 2, there was a trend for decreased systolic BP whereas under conditions above the steers' thermoneutral zone in Experiment 1 diastolic BP increased. Increased diastolic pressure may reflect increased peripheral resistance (Ganong, 1975) due to the known effects of ergot alkaloids from fescue to promote vasoconstriction in some vascular tissues (Oliver et al., 1998; Aiken et al., 2007, 2009; Klotz et al., 2007). These effects may be more pronounced due to regulatory changes in response to thermal stress which promote increased blood flow to the skin than under conditions of basal skin blood flow. At greater doses of alkaloids than that used in the current experiments, vasodilation in response to thermal stress may be more limited due to alkaloid-induced vasoconstriction.

Effects of ergot alkaloids in the fescue seed on decreasing HR, increasing diastolic BP, and RR are consistent with other reports in cattle (Browning and Leite-Browning, 1997; Browning, 2000; Koontz et al., 2012) and sheep (McLeay et al., 2002). However, Rhodes et al. (1991) found no effects of consuming 1.14 mg ergovaline/d on HR, BP or skin temperature of small (88 kg BW) Holstein steers housed at 32°C and restricted to intake equal to 25 g/kg BW. Aiken et al. (2007) observed decreased systolic BP, diastolic BP, and HR in response to ergot alkaloids in beef heifers (375 kg BW), housed below their upper critical temperature and consuming 7.65 mg ergovaline/d. Intake of the heifers tended ($P < 0.15$) to be lesser for those consuming alkaloids (9 kg DM/d) than control heifers (10.7 kg/d). Baseline values for the heifers (measured with a pressure cuff on the tail head) for systolic BP (143 mm Hg), diastolic BP (77 to 86 mm Hg), and HR (106 beats/min) were greater than those measures in steers in the current experiments before (data not shown) or during feeding E− or E+. Aiken et al. (2007) did not describe details of location of heifers during measures, or adaptation to BP procedures. The use of a pressure cuff on the tail head (Browning and Leite-Browning, 1997) in animals accustomed to its use and measured in their usual pens provides credible values for HR and BP similar to those reported for cattle (Rhodes et al., 1991) and sheep (McLeay et al., 2002) with indwelling pressure monitors. Koontz et al. (2012) observed a trend toward short-term increases in diastolic BP in response to toxic fescue seed extract administered to the rumen. The steers in Koontz et al. (2012) were evaluated within and above their upper critical temperature, but feed intake decreased in response to intraruminal dosing of extract from E+ and increased ambient temperature from 22 to 32°C. Our results demonstrate cardiovascular effects from E+ independent of potential effects of DMI at ambient temperatures within or above the animals' thermoneutral zone. Increased serum insulin concentrations in both experiments, and either no change (Experiment 1) or increased plasma glucose concentrations (Experiment 2) in response to E+ indicate changes in homeorhetic control of glucose metabolism which could be linked to insulin resistance and subsequent effects on glucose metabolism when the steers were fed E+.

Ergot alkaloids from fescue seed affect the cardiovascular system of steers separately from effects of feed intake or environmental temperature. Ergot alkaloids interact with ambient temperatures above the steers' thermoneutral zone to exacerbate the symptoms of hyperthermic stress. Based on the data from these studies, there may be insulin-dependent glucose metabolism changes in response to ergot alkaloids.

ACKNOWLEDGMENTS

Research reported in this publication was funded in part by the North Carolina Agricultural Research Service (ARS) and the North Carolina Cattlemen's Association. The authors thank T. Wilson, C. Baum-Lane, and A. Lamb for their excellent assistance.

REFERENCES

Aiken, G. E., Kirch, B. H., Strickland, J. R., Bush, L. P., Looper, M. L., and Schrick, F. N. (2007). Hemodynamic responses of the caudal artery to toxic tall fescue in beef heifers. *J. Anim. Sci.* 85, 2337–2345. doi: 10.2527/jas.2006-821

Aiken, G. E., Strickland, J. R., Looper, M. L., Bush, L. P., and Schrick, F. N. (2009). Hemodynamics are altered in the caudal artery of beef heifers fed different ergot alkaloid concentrations. *J. Anim. Sci.* 87, 2142–2150. doi: 10.2527/jas.2008-1562

Aldrich, C. G., Paterson, J. A., Tate, J. L., and Kerley, M. S. (1993). The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *J. Anim. Sci.* 71, 164–170.

Al-Haidary, A., Spiers, D. E., Rottinghaus, G. E., Garner, G. B., and Ellersieck, M. R. (2001). Thermoregulatory ability of beef heifers following intake of endophyte-infected tall fescue during controlled heat challenge. *J. Anim. Sci.* 79, 1780–1788.

Bernard, J. K., Chestnut, A. B., Erickson, B. H., and Kelly, F. M. (1993). Effects of prepartum consumtion of endophyte-infested tall fescue on serum prolactin

and subsequent milk production of Holstein cows. *J. Dairy Sci.* 76, 1928–1933. doi: 10.3168/jds.S0022-0302(93)77526-8

Boling, J. A., Bunting, L. D., Davenport, G. M., Van der Veen, J. L., Meekings, K. M., Bradley, N. W., et al. (1989). Physiological responses of cattle consuming tall fescue to environmental temperature and supplemental phenothiazine. *J. Anim. Sci.* 67, 2377–2385.

Browning, R. Jr. (2000). Physiological responses of Brahman and Hereford steers to an acute ergotamine challenge. *J. Anim. Sci.* 78, 124–130.

Browning, R. Jr., and Leite-Browning, M. L. (1997). Effect of ergotamine and ergonovine on thermal regulation and cardiovascular function in cattle. *J. Anim. Sci.* 75, 176–181.

Burke, J. M., Spiers, D. E., Kojima, F. N., Perry, B. G., Salfen, A. E., Wood, S. L., et al. (2001). Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. *Biol. Reprod.* 65, 26–268. doi: 10.1095/biolreprod65.1.260

Cartiff, S. E., Fellner, V., and Eisemann, J. H. (2013). Eicosapentaenoic and docosahexaenoic acids increase insulin sensitivity in growing steers. *J. Anim. Sci*. 91, 2332–2342. doi: 10.2527/jas.2012-5694

Chavez, S. J., Burns, J. C., and Huntington, G. B. (2011). Use of plant hydrocarbons as markers to estimate voluntary intake and digestibility in beef steers. *Livest. Sci.* 139, 245–251. doi: 10.1016/j.livsci.2011.01.012

Collier, R. J., Dahl, G. E., and VanBaale, M. J. (2006). Major advances associated with environmental effects on dairy cattle. *J. Dairy Sci.* 89, 1244–1253. doi: 10.3168/jds.S0022-0302(06)72193-2

Ganong, W. F. (1975). *Review of Medical Physiology*. Los Altos, CA: Lange Medical Publications.

Hahn, G. L. (1999). Dynamic responses of cattle to thermal heat loads. *J. Anim. Sci.* 77, 10–20.

Hansen, P. J. (2004). Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim. Reprod. Sci.* 82–83, 349–360. doi: 10.1016/j.anireprosci.2004.04.011

Hemken, R. W., Boling, J. A., Bull, L. S., Hatton, R. H., Buckner, R. C., and Bush, L. P. (1981). Interaction of environmental temperature and antiquality factors on the severity of summer fescue toxicosis. *J. Anim. Sci.* 52, 710–714.

Hill, N. S., and Agee, C. S. (1994). Detection of ergoline alkaloids in endophyte-infected tall fescue by immunoassay. *Crop Sci.* 34, 530–534. doi: 10.2135/cropsci1994.0011183X003400020041x

Hill, N. S., Thompson, F. N., Stuedemann, J. A., Dawe, D. L., and Hiatt, E. E. 3rd. (2000). Urinary alkaloid excretion as a diagnostic tool for fescue toxicosis in cattle. *J. Vet. Diagn. Invest.* 12, 210–217. doi: 10.1177/104063870001200303

Huntington, G., Cassady, J., PAS Gray, K., Poore, M., Whisnant, S., and Hansen, G. (2012). Use of digital infrared thermal imaging to assess feed efficiency in Angus bulls. *Prof. Anim. Sci.* 28, 166–172.

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Arrington, B. C., et al. (2007). Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 85, 2330–2336. doi: 10.2527/jas.2006-803

Koontz, A. F., Bush, L. P., Klotz, J. L., McLeod, K. R., Schrick, F. N., and Harmon, D. L. (2012). Evaluation of a ruminally dosed tall fescue seed extract as a model for fescue toxicosis in steers. *J. Anim. Sci.* 90, 914–921. doi: 10.2527/jas.2011-4292

Mader, T. L., Holt, S. M., Hahn, G. L., Davis, M. S., and Spiers, D. E. (2002). Feeding strategies for managing heat load in feedlot cattle. *J. Anim. Sci.* 80, 2373–2382.

McLeay, L. M., Smith, B. L., and Reynolds, G. W. (2002). Cardiovascular, respiratory, and body temperature responses of sheep to the ergopeptides ergotamine and ergovaline. *Am. J. Vet. Res.* 63, 387–393. doi: 10.2460/ajvr.2002.63.387

Melbin, J., and Detweiler, D. K. (1993). "The cardiovascular system and blood flow," in *Dukes' Physiology of Domestic Animals*, eds M. J. Swenson and W. O. Reece (Ithaca, NY: Cornell University Press), 64–89.

National Research Council. (1996). *Nutrient Requirements of Beef Cattle*, *7th Rev. Edn.* Washington, DC: National Academy Press.

Oliver, J. W., Linnabary, R. D., Abney, L. K., van Manen, K. R., Knoop, R., and Adari, H. S. (1994). Evaluation of a dosing method for studying ergonovine effects in cattle. *Am. J. Vet. Res.* 55, 173–176.

Oliver, J. W., Strickland, J. R., Waller, J. C., Fribourg, H. A., Linnabary, R. D., and Abney, L. K. (1998). Endophyte fungal toxin effect on adrenergic receptors in lateral saphenous veins (cranial branch) of cattle grazing tall fescue. *J. Anim. Sci.* 76, 2853–2856.

Rhodes, M. T., Paterson, J. A., Kerley, M. S., Garner, H. E., and Laughlin, M. H. (1991). Reduced blood flow to peripheral and core body tissues in sheep and cattle induced by endophyte-infected tall fescue. *J. Anim. Sci.* 69, 2033–2043.

Rottinghaus, G. E., Garner, G. B., Cornell, C. N., and Ellis, J. L. (1991). HPLC method for quantitating ergovaline in endohyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades and seed heads. *J. Agric. Food Chem.* 39, 112–115.

Rottinghaus, G. E., Schultz, L. M., Ross, P. F., and Hill, N. S. (1993). An HPLC method for detection of ergot in ground and plelleted feeds. *J. Vet. Diagn. Invest.* 5, 242–247. doi: 10.1177/104063879300500216

Scharf, B., Carroll, J. A., Riley, D. G., Chase, C. C. Jr., Coleman, S. W., Keisler, D. H., et al. (2010). Evaluation of physiological and blood serum differences in heat-tolerant (Romosinuano) and heat-susceptible (Angus) *Bos taurus* cattle during controlled heat challenge. *J. Anim. Sci.* 88, 2321–2336. doi: 10.2527/jas.2009-2551

Scharf, B., Leonard, M. J., Weaber, R. L., Mader, T. L., Hahn, G. L., and Spiers, D. E. (2011). Determinants of bovine thermal response to heat and solar radiation exposures in a field environment. *Int. J. Biometeorol.* 55, 469–480. doi: 10.1007/s00484-010-0360-y

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). Board-invited review: St. Anthony's Fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci.* 89, 1603–1626. doi: 10.2527/jas.2010-3478

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 June 2014; accepted: 15 October 2014; published online: 12 November 2014.

Citation: Eisemann JH, Huntington GB, Williamson M, Hanna M and Poore M (2014) Physiological responses to known intake of ergot alkaloids by steers at environmental temperatures within or greater than their thermoneutral zone. Front. Chem. ***2****:96. doi: 10.3389/fchem.2014.00096*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 20 November 2014
doi: 10.3389/fchem.2014.00101

Vasoconstrictive responses by the carotid and auricular arteries in goats to ergot alkaloid exposure[1]

Glen E. Aiken* and Michael D. Flythe

Forage-Animal Production Research Unit, United States Department of Agriculture Agricultural Research Service, Lexington, KY, USA

Edited by:
John Wade, Florey Institute of Neuroscience and Mental Health, Australia

Reviewed by:
Kenneth Michael Andries, Kentucky State University, USA
Joan M. Burke, United States Department of Agriculture Agricultural Research Service, USA

****Correspondence:***
Glen E. Aiken, Forage-Animal Production Research Unit, United States Department of Agriculture Agricultural Research Service, N220 Ag Science North Bldg., University of Kentucky Campus, Lexington, KY 40546, USA
e-mail: glen.aiken@ars.usda.gov

A fungal endophyte (*Neotyphodium coenophialum*) infects most plants of "Kentucky 31" tall fescue (*Lolium arundinaceum*) and produces ergot alkaloids that cause persistent constriction of the vascular system in grazing livestock. Consequently, animals undergoing this toxicosis cannot regulate core body temperature and are vulnerable to heat and cold stresses. An experiment was conducted to determine if the caudal and auricular arteries in goats (*Capra aegagrus hircus*) vasoconstrict in response to ergot alkaloids. Seven, rumen fistulated goats were fed *ad libitum* orchardgrass (*Dactylis glomeratia*) hay and ruminally infused with endophtye-free seed (E−) for a 7-day adjustment period. Two periods followed with E− and endophyte-infected (E+) seed being randomly assigned to the 2 goat groups in period 1 and then switching treatments between groups in period 2. Infused E+ and E− seed were in equal proportions to the hay such that concentrations of ergovaline and ergovalanine were 0.80 μg per g dry matter for the E+ treatment. Cross-sections of both arteries were imaged using Doppler ultrasonography on days 0, 2, 4, 6, 8, and 12 in period 1 and on days 0, 1, 2, 3, 6, 7, and 9 in period 2. Differences from average baseline areas were used to determine presence or absence of alkaloid-induced vasoconstriction. Carotid arteries initiated constriction on imaging day 2 in both periods, and auricular arteries initiated constriction on imaging day 2 in period 1 and on day 6 in period 2. Luminal areas of the carotid arteries in E+ goats were 46% less than baseline areas in both periods after vasoconstriction occurred, whereas auricular arteries in E+ goats were 52% less than baseline areas in period 1 and 38% in period 2. Both arteries in E+ goats in period 1 relaxed relative to baseline areas by imaging day 2 after they were switched to the E− treatment. Results indicated that goats can vasoconstrict when exposed to ergot alkaloids that could disrupt their thermoregulation.

Keywords: ergot alkaloids, fescue toxicosis, goats, tall fescue, vasoconstriction

INTRODUCTION

Tall fescue is a cool-season, perennial grass that is extensively utilized for grazing and hay production on approximately 15 million hectares in the eastern half of the USA. The grass is persistent and productive under low management, but this benefit is primarily due to alkaloids produced by a *Neotyphodium* endophyte (Hoveland et al., 1983; Strickland et al., 2011) that inhabits the intracellular spaces of most tall fescue plants. Groups of alkaloids (e.g., lolines and peramines) are recognized for providing the plant with tolerances to environmental stresses, such as drought and herbivory (Siegel et al., 1990; Bacon, 1993).

Ergot alkaloids are a class of alkaloids also produced by the endophyte that causes a toxicosis in grazing livestock that reduces both reproductive performance (Porter and Thompson, 1992) and growth rate (Schmidt and Osborn, 1993; Strickland et al., 1993; Paterson et al., 1995). Ergot alkaloids bind biogenic amine receptors in the vasculature to induce persistent vasoconstriction (Oliver, 2005). Constricted blood flow to peripheral tissues reduces the animal's ability to regulate body temperature and, therefore, be vulnerable to heat and cold stress (Aldrich et al., 1993). Ergovaline is an ergopeptine ergot alkaloid that was demonstrated to have the highest vasoconstrictive potency of the ergot alkaloids (Klotz et al., 2008) and also is of highest concentration, ranging from 84 to 97% of the ergopeptine fraction (Lyons et al., 1986).

Browning (2012) reported reduced growth rates of Boer, Kiko, and Spanish goats *(Capra aegagrus hircus)* fed diets containing 1.16 ppm ergovaline as compared to a diet free of ergot alkaloids. However, alterations in the physiology of goats grazing toxic endophyte-infected tall fescue have not been investigated (Ditsch and Aiken, 2009), but is needed as the goat industry expands into the transition zone and goats are exposed to toxic ergot alkaloids. Vasoconstrictive responses to ergot alkaloids have been reported in cattle (Rhodes et al., 1991; Aiken et al., 2009b), sheep (Aiken et al., 2011), and horses (McDowell et al., 2013), but not in goats. Therefore, a pen study was conducted using Color Doppler ultrasonography to compare luminal areas of carotid and auricular arteries in wether goats that were

[1]Mention of trade names or commercial products in the article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

fed either endophyte-infected (E+) or endophyte-free (E−) tall fescue seed.

MATERIALS AND METHODS

The experiment was conducted in indoor pens under a controlled ambient temperature of approximately 20°C. All animal research followed procedures approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol number 2013-1152).

Seven Spanish wether goats that were ruminally fistulated (age = yearling; body weight = 34.3 ± 1.7 kg) were randomly assigned to 4 goats in one pen group and 3 in the other group to compare luminal areas of the carotid and auricular arteries between diets containing either E+ or E− tall fescue seed. A 7-d adjustment period was conducted to measure DM intake of commercially chopped orchardgrass (*Dactylis glomerata* L.) hay. Two experimental periods followed the adjustment period as a crossover design, with the group of 3 goats receiving the E+ seed in the first period and with the treatments being switched between the two groups at the beginning of period 2. Rumens were infused daily at an average of 143 and 142 g of E− or E+ seed, respectively, during period 1 and 135 and 170 g of E− or E+ seed, respectively, during period 2. Duration of period 1 was 14 days and for period 2 was 13 days.

The cultivar "Defiance" was used for the E+ treatment and the cultivar "Kentucky 32" was used for the E− treatment. Ergovaline plus its epimer, ergovalanine were analyzed for both cultivars using procedures of Yates and Powell (1998) and modified as described by Carter et al. (2010).

Hay was fed *ad libitum* and seed that was previously course ground using a Wiley Mill with a 5.0 mm screen was infused into each rumen using a funnel. Feeding of hay and treatment with seed was done at 1500 h each day. During the experimental periods the previous day's intake for each pen were used to estimate intake per goat and the ratio of fed chopped hay to rumenally placed seed for providing a daily diet concentration of 0.8 μg of ergovaline and ergovalanine, per gram of dry matter. Ergovaline plus ergovalanine concentrations in E+ and E− seed contained 2.71 and 0 μg per gm DM, respectively, which resulted in seed averaging 14.5% of the diet dry matter for the E+ and E− treatments.

Color Doppler ultrasound images of the cross-sections of the right carotid (**Figure 1**) and auricular (**Figure 2**) arteries were collected using a Classic Medical TeraVet 3000 Ultrasound Unit (Classic Universal Ultrasound, Tequesta, FL) with a 12L5-VET (12 MHz) linear array transducer. Baseline measures were collected for each goat during the adjustment period on days 3, 5, and 7. Images were collected during period 1 on days 1, 2, 4, 6, 8, and 14, and were collected during period 2 on days 1, 2, 3, 4, 7, 8, and 10. Each imaging session was started at approximately 1100 h and was completed within 30 to 40 min. The goats were handled frequently prior to the start of the study to reduce excitability when being imaged. Individual goats were removed from their pens and their heads were stabilized by gently holding their horns without being placed in a chute. Each goat was clipped at the start of the experiment using surgical clippers under the left ear cross-sectional images were collected for each artery using a frequency of 5.0 MHz and a pulse repetitive frequency that ranged between

FIGURE 1 | Color Doppler ultrasonic image of the cross-section of the right carotid artery in a wether goat on an orchardgrass diet with no ruminal infusion of endophyte-infected or endophyte-free tall fescue seed. Color delineates blood flow in the lumen of the artery.

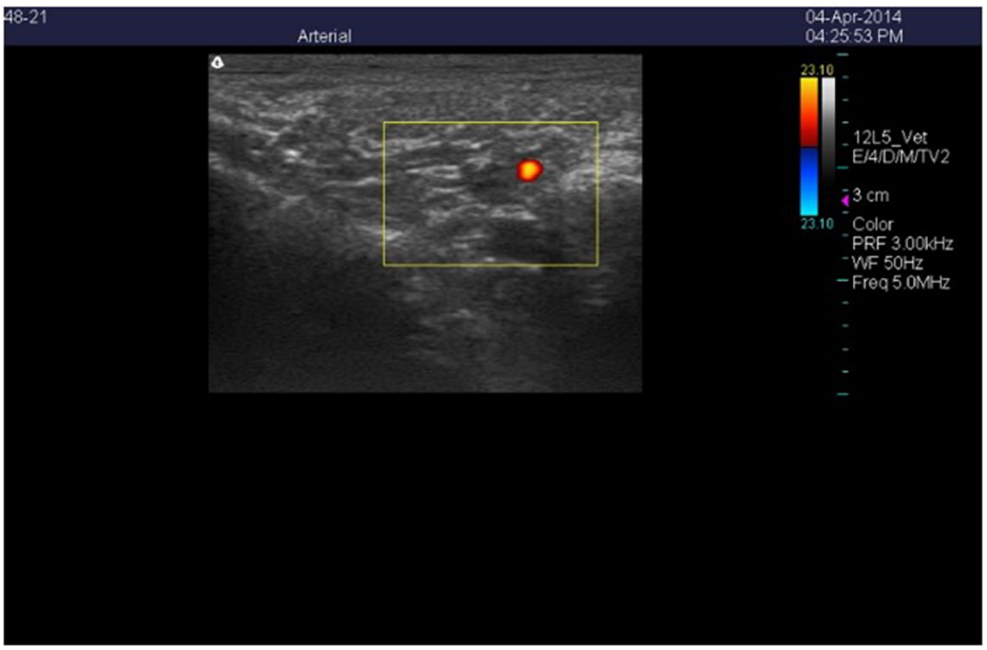


FIGURE 2 | Color Doppler ultrasonic image of the cross-section of the right auricular artery in a wether goat on an orchardgrass diet with no ruminal infusion of endophyte-infected or endophyte-free tall fescue seed. Color delineates blood flow in the lumen of the artery.

2.5 and 3.0 kHz. Scan depth was set at 4 cm for the carotid arteries and 3 cm for the auricular artery. Following freezing of an individual scan, frames stored in the cine memory of the unit were searched to store the image exhibiting the maximum flow signal and assumed to be at peak systolic phase. The flow signal was traced to estimate lumen area (Aiken et al., 2009a).

Caudal and auricular artery luminal areas were analyzed using mixed models of SAS as repeated measures with the heterogeneous autoregressive covariance structure for responses of carotid arteries in periods 1 and 2 and auricular arteries in period 2 and with the autoregressive covariance structure for the auricular arteries in period 1 (Littell et al., 1996). The analysis used individual animals as the experimental unit. Treatment (E+ vs. E−), imaging day, and the interaction between treatment and imaging day were analyzed as fixed effects for both experiments. Measures for the adjustment period were averaged and used as the baseline measure (image day 0) for period 1 and those for period 1 were averaged for each treatment and used as the baseline measure for period 2. In the presence of a significant ($P < 0.05$) treatment x imaging day interaction, differences in least square means between baseline measures and imaging days and differences between treatments at each imaging day were determined using the PDIFF option of SAS.

RESULTS

Mean dry matter intake during the adjustment period was 0.97 kg/goat. During period 1, it was 0.80 and 0.81 kg/goat for E− and E+ treatments, respectively, and during period 2 it was 0.76 and 0.97 kg/goat, respectively. Consumption of ergovaline and ergovalanine averaged approximately 1.1×10^{-5} μg per kg body weight.

PERIOD 1

Mean luminal areas of the carotid arteries in E+ goats were less ($P < 0.05$) than those in E− goats, and there was no treatment × imaging day interaction ($P = 0.805$; **Figure 3A**). Mean luminal area for goats on the E− treatment during the period was 9.4 ± 0.9 mm^2 and for those on the E+ treatment it was 5.8 ± 1.1 mm^2.

Different from carotid arteries, there was a treatment × imaging day interaction ($P < 0.001$) on luminal areas of the auricular arteries (**Figure 3B**). Luminal areas of auricular arteries for the E− treatment were not different ($P > 0.12$) from the mean baseline value, whereas those for the E+ treatment differed from mean baseline areas for all imaging days on and after day 2. Auricular arteries in E+ goats showed vasoconstriction ($P < 0.05$) as compared to E− goats on the imaging sessions for days 2, 4, and 12, even though after the second imaging day lumen areas in E+ goats were never greater than 2.3 mm^2 and in E− goats they were never less than 2.5 mm^2.

PERIOD 2

There was a treatment × imaging day interaction ($P < 0.001$) on carotid arteries (**Figure 4A**). Goats that were switched to E+ exposure after being on the E− treatment in period 1 showed less luminal areas than the baseline measure on and after imaging day 3. Luminal areas for the E+ treatment were less ($P < 0.05$) than those for the E− for imaging days 2, 3, 6, and 7, whereas there was a tendency ($P < 0.10$) for a difference on the day 9 imaging when the standard errors for both treatment groups were highest.

A treatment × imaging day interaction also was detected ($P < 0.01$) for luminal areas of auricular arteries (**Figure 4B**). Auricular arteries in goats switched to E− from the E+ treatment

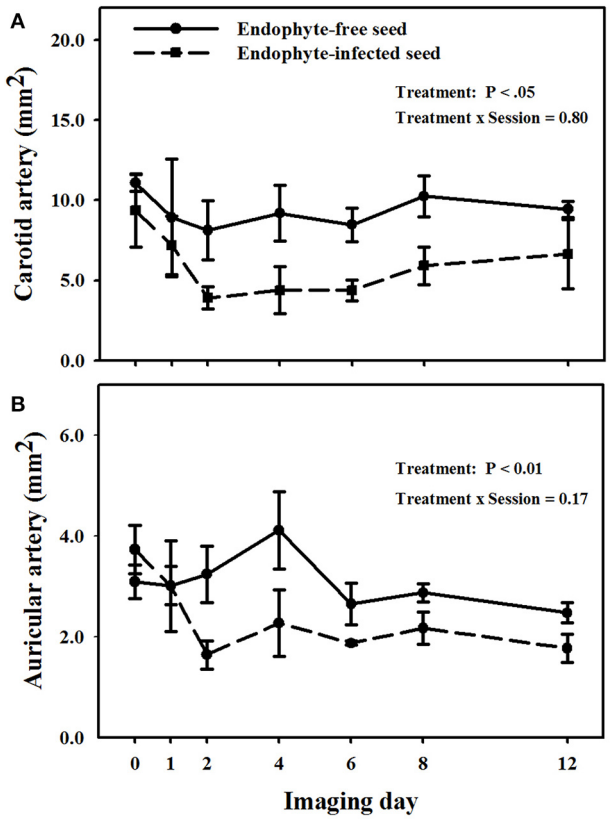


FIGURE 3 | Trends over image days during period 1 in luminal area of the right (A) carotid and (B) auricular arteries in weither goats that were ruminally infused with either endophyte-infected or endophyte-free tall fescue seed. The day 0 is the average of luminal areas during the adjustment period. Standard errors of the mean were calculated using the conservative formula.

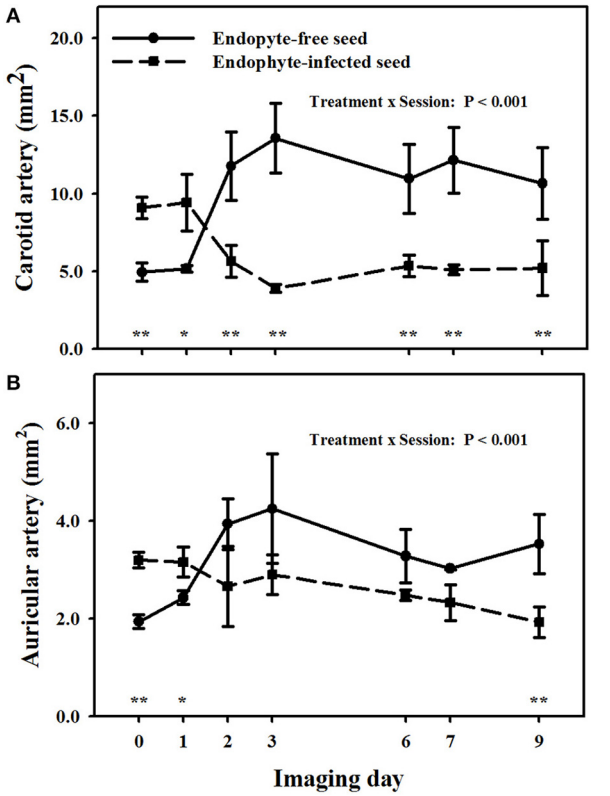


FIGURE 4 | Trends over image days during period 2 in luminal area of the right (A) carotid and (B) auricular arteries in weither goats that were ruminally infused with either endophyte-infected or endophyte-free tall fescue seed. The day 0 is the average of luminal areas during period 1 before treatments were switched. Standard errors of the mean were calculated using the conservative formula. Differences between treatments for imaging days and denoted by $^{*}P < 0.10$ and $^{**}P < 0.05$.

in period 1 were relaxed from the baseline measure on and after day 2. Those in goats switched to E+ in period 2 had a tendency ($P < 0.10$) for vasoconstriction as compared to baseline measures on day 2, and exhibited significant ($P < 0.05$) vasoconstriction on and after day 3. Luminal areas in the E+ goats tended ($P < 0.10$) to be less ($P \leq 0.05$) than for the E− goats day 1, but exhibited significant ($P < 0.05$) vasoconstriction on and after day 2 ($P < 0.05$).

DISCUSSION

Blood flow resistance under normal conditions is regulated by vasoconstriction and vasorelaxation/vasodialation of arteries and veins that is controlled by the autonomic nervous system. Blood flow volume is proportional to the fourth power of luminal radius, which results in large decreases in blood flow volume occurring with small decreases in luminal area (Carter, 2000). Therefore, responses of luminal areas of blood vessel cross-sections provide a direct measure of vasoconstriction or vasorelaxation that has a direct bearing on blood flow volume.

Although ergovaline is the ergopeptine of highest concentration and has the highest potency, other ergot alkaloids were assumed to be in the seed that could have additive effects on vasoconstriction (Klotz et al., 2010). Klotz et al. (2007) used an *in vitro* procedure with lateral saphenous veins biopsied from endophyte-naïve heifers to determine that ergovaline elicited a vasoconstrictive response at a concentration 1×10^{-8} M and had a maximum contractility of 69.9% relative to norepinephrine contractility. From an earlier experiment, Klotz et al. (2006) reported a concentration greater than 1×10^{-4} M was necessary for lysergic acid to induce vasoconstriction with a maximum contractility of 15.6% relative to norepinephrine contractility. Klotz et al. (2008) later reported that ergonovine, ergocryptine, ergocristine, and ergocornine induced a contractile response at similar concentrations (1×10^{-7} M) to ergovaline, but the greatest maximum contractility intensity relative to norepinephrine was achieved by ergonovine (68.5%), and maximum contractility intensities were similar between ergocryptine (45.5%), ergocristine (42.9%), and ergocornine (57.2%). Although ergovaline has demonstrated to be the most potent vasoconstrictor, Klotz et al. (2009) surmised that ergopeptines likely have additive effects on intensity of vascular contraction, thus indicating all are likely contributors to fescue toxicosis.

Overall, luminal areas tended to be more variable in E− goats than in E+ goats. This was clearly indicated by the responses of carotid arteries in both periods and the auricular arteries in period 2 which had Akaike's Information Criterion values in statistical analyses that favored the heterogeneous autoregressive covariance structure, which accounts for heterogeneity of treatment variances (Littell et al., 1996). Luminal areas of caudal arteries in cattle also have been observed to be more variable with E− than E+ diets (Aiken et al., 2007, 2009a). Alkaloid-induced vasoconstriction apparently reduces vascular responsiveness to environmental stimuli.

The goats used in the study were not endophyte-naïve. Prior to placement in the indoor pens they resided in a mixed grass pasture that had moderate percentages of endophyte-infected tall fescue; however, they had been on a non-toxic hay diet in the pens for 38 days prior to the 7 day adjustment period when their rumens were daily infused with E− seed. It was assumed that most of the ergot alkaloids had cleared from the vasculature prior to initiating the E+ diet, which was indicated by the alkaloid-induced vasoconstriction that occurred in both arteries during period 1. Overall luminal areas of carotid and auricular arteries were less ($P < 0.05$) than baseline measures taken during the adjustment periods by day 2.

Day-to-day fluctuations in luminal areas in both arteries in E+ goats that were parallel with those in E− goats are indicative of these goats not being saturated with ergot alkaloids and still having an ability to make vascular adjustments. Using an *in vitro* model with biopsied saphenous veins from endophyte-naïve heifers, Klotz et al. (2009) reported increased smooth muscle contractility with increasing repetitive additions of 1×10^{-7} M concentrations of ergovaline. It was further shown that ergovaline bioaccumulated in the saphenous veins with the repeated exposures and washings. Therefore, this indicates that ergovaline can bioaccumulate in the bovine vasculature through their affinity to biogenic amine receptors.

Switching treatments between the two goat groups in period 2 allowed for an evaluation of possible recovery of the vascular system to previous ergot alkaloid exposure. Detection of relaxation by the carotid and auricular arteries starting on image day 2 indicated that the goats were not saturated with ergot alkaloids by completion of feeding E+ seed during period 1. Aiken et al. (2011) reported luminal areas of auricular arteries in ewe lambs to linearly increase over time after they were switched to E− perennial ryegrass pasture after 19 days of grazing E+ pasture.

Vasoconstrictive responses in period 2, as detected by differences with baseline measures during period 1, were not as immediate as in period 1, with significant differences from baseline measures from period 1 not being observed until image day 3 for the carotid artery and day 6 for the auricular arteries. Treatment differences on individual imaging days cannot be interpreted because of confounding between relaxation of the arteries in goats that were switched from E+ to E− diets and vasoconstriction in those that were switched from E− to E+ diets. Nonetheless, the objectives of period 2 were to evaluate differences in luminal areas of the arteries between period 2 images and baselines means for detecting artery relaxation in E− goats and alkaloid-induced vasoconstriction in E+ goats.

CONCLUSION

Results of this experiment indicated the vasculature of goats can vasoconstrict when exposed to ergot alkaloids. Alkaloid-induced vasoconstriction of the carotid and auricular arteries was mediated within 1 to 2 days after being exposed to ergot alkaloids during period 1, and 2 to 3 days during period 2. Luminal areas of carotid and auricular artereries in E+ goats during period 1 showed rapid increases after they were switched to the E− in period 2, with significant relaxation being accomplished in 2 days for both arteries. The 12-day exposure to ergot alkaloids was short and likely was not long enough for vascular systems in the goats to be saturated with alkaloids. This experiment provides the first documentation of ergot alkaloid-induced vasoconstriction in goats.

REFERENCES

Aiken, G. E., Kirch, B. H., Strickland, J. R., Bush, L. P., Looper, M. L., and Schrick, F. N. (2007). Hemodynamic responses of the caudal artery to toxic tall fescue in beef heifers. *J. Anim. Sci.* 85, 2337–2345. doi: 10.2527/jas.2006-821

Aiken, G. E., Klotz, J. L., Kirch, B. H., Strickland, J. R., and Boykin, D. L. (2009a). Comparison of two tracing methods with ultrasonography to determine lumen area of the caudal artery in beef cattle. *J. Anim. Sci.* 87, 371–374. doi: 10.2527/jas.2008-1087

Aiken, G. E., Strickland, J. R., Looper, M. L., Bush, L. P., and Schrick, F. N. (2009b). Hemodynamics are altered in the caudal artery of beef heifers fed different ergot alkaloid concentrations. *J. Anim. Sci.* 87, 2142–2150. doi: 10.2527/jas.2008-1562

Aiken, G. E., Sutherland, B. L., and Fletcher, L. R. (2011). Haemodynamics of lambs grazing perennial ryegrass (*Lolium perenne* L.) either infected with AR6 novel, wild-type endophyte, or endophyte-free. *N. Z. Vet. J.* 59, 179–184. doi: 10.1080/00480169.2011.579247

Aldrich, C. G., Paterson, J. A., Tate, J. L., and Kerley, M. S. (1993). The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *J. Anim. Sci.* 71, 164–170.

Bacon, C. W. (1993). Abiotic stress tolerance (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue. *Agric. Ecosyst. Environ.* 44, 123–141. doi: 10.1016/0167-8809(93)90042-N

Browning, R. Jr. (2012). Effect of wild-type tall fescue endophyte on growth rate and feed consumption in nulliparous meat goat does. *Small Rum. Res.* 105, 29–32. doi: 10.1016/j.smallrumres.2011.11.025

Carter, J. M., Aiken, G. E., Dougherty, C. T., and Schrick, F. N. (2010). Steer responses to feeding soybean hulls and steroid hormone implantation on toxic tall fescue pasture. *J. Anim. Sci.* 88, 3759–3766. doi: 10.2527/jas.2009-2536

Carter, S. A. (2000). "Hemodynamic considerations in peripheral vascular and cerebrovascular disease," in *Introduction to Vascular Ultrasonography, 4th Edn.*, ed W. J. Zwiebel (Philadelphia, PA: WB Saunders, Co), 3–16.

Ditsch, D. D., and Aiken, G. E. (2009). Endophyte infected tall fescue and small ruminant production: do we have a problem? *Forages Grazinglands* FG-2009-1104-02-RS. doi: 10.1094/FG-2009-1104-02-RS

Hoveland, C. S., Schmidt, S. P., King, C. C. Jr., Odom, J. W., Clark, E. M., McGuire, J. A., et al. (1983). Steer performance and *Acremonium coenophialum* fungal endophyte on tall fescue pasture. *Agron. J.* 75, 821–824. doi: 10.2134/agronj1983.00021962007500050021x

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Arrington, B. C., et al. (2007). Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 85, 2330–2336. doi: 10.2527/jas.2006-803

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Vevoda, A. C., et al. (2006). Assessment of vasoconstrictive potential of D-lysergic acid using an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 84, 3167–3175. doi: 10.2527/jas.2006-038

Klotz, J. L., Kirch, B. H., Aiken, G. E., Bush, L. P., and Strickland, J. R. (2008). Effects of selected combinations of tall fescue alkaloids on the vasoconstricitive capacity

of fescue-naïve bovine lateral saphenous veins. *J. Anim. Sci*. 86, 1021–1028. doi: 10.2527/jas.2007-0576

Klotz, J. L., Kirch, B. H., Aiken, G. E., Bush, L. P., and Strickland, J. R. (2009). Bioaccumulation of ergovaline in bovine lateral saphenous vein *in vitro*. *J. Anim. Sci*. 87, 2437–2447. doi: 10.2527/jas.2008-1692

Klotz, J. L., Kirch, B. H., Aiken, G. E., Bush, L. P., and Stickland, J. R. (2010). Contractile response of fescue naive bovine lateral saphenous veins to increasing concentrations of tall fescue alkaloids. *J. Anim. Sci.* 88, 408–415. doi: 10.2527/jas.2009-2243

Littell, R. C., Milliken, G. A., Stroup, W. W., and Wolfinger, R. D. (1996). *SAS System for Mixed Models*. Cary, NC: SAS Inst., Inc.

Lyons, P. C., Plattner, R. D., and Bacon, C. W. (1986). Occurrence of peptide and clavinet ergot alkaloids in tall fescue grass. *Science* 232, 487–489. doi: 10.1126/science.3008328

McDowell, K. J., Moore, E. S., Parks, A. G., Bush, L. P., Horohov, D. W., and Lawrence, L. M. (2013). Vasoconstriction in horses caused by endophyte-infected tall fescue seed is detected with Doppler ultrasonography. *J. Anim. Sci*. 91, 1677–1684. doi: 10.2527/jas.2012-5852

Oliver, J. (2005). "Pathophysilogic response to endophyte toxins," in *Neotyphodium in Cool-Season Grasses*, eds C. A. Roberts, C. P. West, and D. E. Spiers (Ames, IA: Blackwell Publishing), 291–304.

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

Porter, J. K., and Thompson, F. N. Jr. (1992). Effects of toxicosis on reproduction in livestock. *J. Anim. Sci*. 70, 1594–1603.

Rhodes, M. T., Paterson, J. A., Kerley, M. S., Garner, H. E., and Laughlin, M. H. (1991). Reduced blood flow to peripheral and core body temperatures in sheep and cattle induced by endophyte-infected tall fescue. *J. Anim. Sci*. 69, 2033–2043.

Schmidt, S. P., and Osborn, T. G. (1993). Effects of endophyte-infected tall fescue on animal performance. *Agric. Ecosyst. Environ.* 44, 233–262.

Siegel, M. R., Latch, G. C. M., Bush, L. P., Fannin, F. F., Rowan, D. D., Tapper, B. A., et al. (1990). Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. *J. Chem. Ecol.* 16, 3301–3315.

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr. (2011). Board invited review: St. Anthony's fire in livestock: causes, mechanisms, and potential solution. *J. Anim. Sci*. 89, 1603–1626. doi: 10.2527/jas.2010-3478

Strickland, J. R., Oliver, J. W., and Cross, D. L. (1993). Fescue toxicosis and its impact on animal agriculture. *Vet. Hum. Toxicol*. 35, 454–464.

Yates, S. G., and Powell, R. G. (1998). Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. *J. Agric. Food Chem*. 36, 337–340. doi: 10.1021/jf00080a023

Conflict of Interest Statement: The Reviewer Joan M. Burke declares that, despite being affiliated to the same institution as authors Glen E. Aiken and Michael D. Flythe, the review process was handled objectively and not conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 July 2014; accepted: 27 October 2014; published online: 20 November 2014.

Citation: Aiken GE and Flythe MD (2014) Vasoconstrictive responses by the carotid and auricular arteries in goats to ergot alkaloid exposure. Front. Chem. ***2****:101. doi: 10.3389/fchem.2014.00101*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in Chemistry

ORIGINAL RESEARCH
published: 11 March 2015
doi: 10.3389/fchem.2015.00016

Relationships among ergot alkaloids, cytochrome P450 activity, and beef steer growth

Charles F. Rosenkrans Jr. and Nicholas S. Ezell*

Department of Animal Science, University of Arkansas, Fayetteville, AR, USA

Determining a grazing animal's susceptibility to ergot alkaloids has been a research topic for decades. Our objective was to determine if the Promega™ P450-Glo assay could be used to indirectly detect ergot alkaloids or their metabolites in urine of steers. The first experiment validated the effects of ergot alkaloids [0, 20, and 40 μM of ergotamine (ET), dihydroergotamine (DHET), and ergonovine (EN)] on human CYP3A4 using the P450-Glo assay (Promega™ V9800). With this assay, luminescence is directly proportional to CYP450 activity. Relative inhibition of *in vitro* cytochrome P450 activity was affected ($P < 0.001$) by an interaction between alkaloids and concentration. That interaction resulted in no concentration effect of EN, but within ET and DHET 20 and 40 μM concentrations inhibited CYP450 activity when compared with controls. In experiment 2, urine was collected from Angus-sired crossbred steers ($n = 39$; 216 ± 2.6 days of age; 203 ± 1.7 kg) after grazing tall fescue pastures for 105 days. Non-diluted urine was added to the Promega™ P450-Glo assay, and observed inhibition (3.7 % ± 2.7 of control). Urine content of total ergot alkaloids (331.1 ng/mg of creatinine ± 325.7) was determined using enzyme linked immunosorbent assay. Urine inhibition of CYP450 activity and total alkaloids were correlated ($r = -0.31$; $P < 0.05$). Steers were genotyped at CYP450 single nucleotide polymorphism, C994G. Steer genotype affected ($P < 0.03$) inhibition of CYP450 activity by urine; heterozygous steers had the least amount of CYP450 inhibition suggesting that genotyping cattle may be a method of identifying animals that are susceptible to ergot alkaloids. Although, additional research is needed, we demonstrate that the Promega™ P450-Glo assay is sensitive to ergot alkaloids and urine from steers grazing tall fescue. With some refinement the P450-Glo assay has potential as a tool for screening cattle for their exposure to fescue toxins.

Keywords: fescue toxicosis, predictive biology, cattle

OPEN ACCESS

Edited by:
James Levi Klotz, United States Department of Agriculture – Agricultural Research Service, USA

Reviewed by:
Nick M. Filipov, University of Georgia, USA
Raja S. Settivari, The Dow Chemical Company, USA

****Correspondence:***
Charles F. Rosenkrans Jr., Department of Animal Science, 1-University of Arkansas, B-107E AFLS Building, Fayetteville, AR 72701, USA
crosenkr@uark.edu

Specialty section:
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry

Received: *08 July 2014*
Accepted: *23 February 2015*
Published: *11 March 2015*

Citation:
Rosenkrans CF Jr. and Ezell NS (2015) Relationships among ergot alkaloids, cytochrome P450 activity, and beef steer growth. Front. Chem. 3:16. doi: 10.3389/fchem.2015.00016

Introduction

Tall fescue [*Lolium arundinaceum* (Schreb.) S. J. Darbyshire] infected with the endophytic fungus (*Neotyphodium coenophialum*) is responsible for the production of ergot alkaloids which have a significant economic impact on beef cattle. Consumption of those mycotoxins is known to lower average daily gain, negatively impact reproductive traits, decrease parasite resistance, and reduce heat tolerance, all of which drive up the cost of production (for review see Strickland et al., 2011). Ergot alkaloids have an interesting relationship with animal cytochrome P450 (CYP) enzymes; CYP have been shown

to metabolize ergot alkaloids via hydroxylation, but CYP enzyme activity is inhibited by ergot alkaloids (Althaus et al., 2000; Settivari et al., 2006). Furthermore, CYP is a key enzyme in the biosynthetic pathway for ergot alkaloids (Haarmann et al., 2006).

Current methods for detecting ergot alkaloid concentrations in bodily fluids are limited. Initially, chromatographic methods had relatively high detection limits (Yates and Powell, 1988; Rottinghaus et al., 1991; Moubarak et al., 1996); however, more recent advances in chromatographic and detection methods have vastly improved the sensitivities of the assays (Lehner et al., 2005; Smith et al., 2009; Foote et al., 2012; Wang et al., 2013). Enzyme linked immunosorbent assay is a faster method for detection of ergot alkaloids and has been used to screen livestock exposure to fescue toxins (Shelby and Kelley, 1990; Hill and Agee, 1994). Recently, we demonstrated that the Promega™ P450-Glo assay could be used to assess ergot alkaloid content of tall fescue extracts (Moubarak et al., 2012). The Glo-assay is relatively easy with a quick protocol which makes it a convenient tool for biologically-relevant indirect alkaloid detection. Our objectives were to validate our previous findings related to ergot alkaloid inhibition of CYP450 activity, and determine if urine collected from steers grazing tall fescue inhibited CYP450 activity *in vitro*.

Materials and Methods

Experiment 1

CYP450 Analysis

Ergotamine (ET), dihydroergotamine (DHET), and ergonovine (EN) were dissolved in methanol (100%) resulting in final assay concentrations of 0, 20, and 40 μM. Enzyme activity for CYP450 was assayed using the Promega™ P450-Glo assay (product # V9920; Cali et al., 2009). Briefly, sample (12.5 μL) was added in triplicate to a 96-well plate, followed by CYP3A4 solution (12.5 μL), and incubated at 37°C for 10 min. Reaction was initiated by addition of NADPH regeneration system (25 μL) and plate was incubated 30 min at 37°C. Luciferin Detection Reagent (50 μL) was added and plate was incubated at 20°C for 20 min. Luminescence was recorded with a luminometer (Perkin-Elmer, Victor 1420 multilabel counter) using no filters and an integration time of 1 s/well.

Statistical Analysis

Luminescence data for each alkaloid were analyzed independently using mixed procedures. Main effects were replicate, alkaloid, and concentration; response variable was CYP450 activity as indicated by luminescence. If *F*-tests were significant ($P < 0.05$), multiple *t*-tests were used to separate means. Results are presented as a percent of CYP450 activity with zero alkaloid present.

Experiment 2

Steer Grazing

The University of Arkansas Animal Care and Use Committee approved all animal procedures (protocol # 04024). Healthy, fall-born Angus-sired crossbreed steers ($n = 39$; 203 ± 27 kg; 216 ± 2.6 days of age) were weaned, vaccinated, and treated with anthelmintic in May. Fourteen days after weaning, steers were randomly assigned to a pasture of either Kentucky 31 (K31; four pastures; $n = 20$ steers) or HiMag4 (HM4; four pastures; $n = 19$ steers) for a summer grazing trial. Pastures and tall fescue cultivars were as previously described (Nihsen et al., 2004). During the post-weaning grazing trial (105 days) steers received a daily supplement (1.8 kg/day; 80% ground corn: 20% soybean meal; 12% CP). During the grazing period steers had free access to water and minerals. Steers remained healthy throughout the trial and were not treated with pharmaceuticals.

Sample Collection

At weaning, approximately 10 mL of blood was collected from each steer, stored at 5°C until centrifuged at 1200 × *g* for 30 min, serum was decanted and stored at −20°C until hormone [cortisol, prolactin, and insulin-like growth factor 1 (IGF-1)] concentrations were determined by validated radioimmunoassay. At day 105, each animal was weighed and a urine sample collected. Urine samples were stored at -20°C until assayed. Total alkaloids were determined by ELISA (Agrinostics Ltd. Co., Watkinsville, GA). Inhibition of CYP450 was determined using the assay (Promega™ P450-Glo) described above using 12.5 μL of non-diluted urine in each reaction test.

Cytochrome P4503A28 Genotyping

Steers were genotyped at CYP3A28 single nucleotide polymorphism (SNP), C994G, using methods previously described (Sales et al., 2012). Briefly, genomic DNA was isolated from the buffy coat of EDTA treated whole blood samples (QIAGEN Inc., Valencia, CA). Diluted DNA (20 ng/μL) served as the template for polymerase chain reaction with specific primers [forward (5′-CAACAACATGAATCAGCCAGA-3′) and reverse (5′-CCTACATTCCTGTGTGTGCAA-3′)]. The 565-base pair amplicon was within the coding sequence of bovine *CYP3A28* [National Center for Biotechnology Information (NCBI) gi1769423; (Natsuhori et al., 1997)]. Purified amplification products (QIAGEN Inc., Valencia, CA) were restriction enzyme digested with *Alu* I (New England BioLabs, Beverly, MA). Steer genotypes at SNP site C994G were homozygous cytosine, homozygous guanine, and heterozygous.

Statistical Analysis

Results of the CYP450 enzyme assay were reported in luminescence (arbitrary units). Pearson correlation coefficients were determined between dependent variables. Data were analyzed using mixed procedures with pasture as the experimental unit, genotype within pasture as random term, fescue cultivar as repeated, and creatinine as a covariate. If *F*-test for main or interactive effects were significant ($P < 0.05$) then means were separated using multiple *t*-tests.

Results

Figure 1 validates our previous work (Moubarak et al., 2012) that ET, and DHET inhibit CYP450 activity as detected by the Promega™ P450-Glo assay. In contrast, CYP450 activity was numerically stimulated when 20 and 40 μM of EN where added

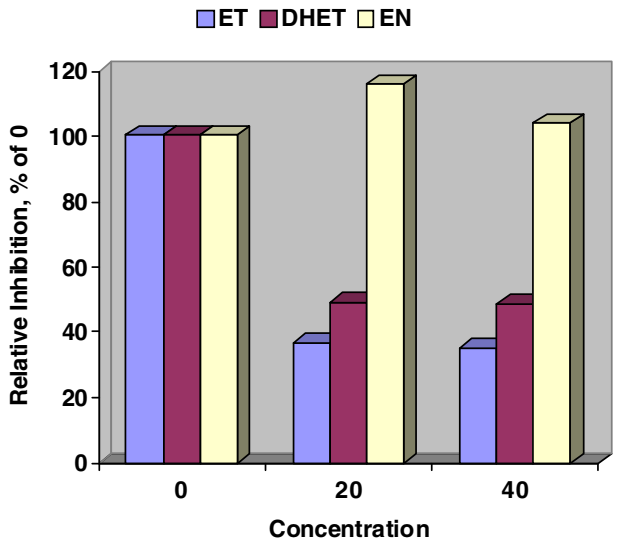


FIGURE 1 | Relative inhibition of *in vitro* cytochrome P450 activity by ergotamine (ET), dihydroergotamine (DHET), and ergonovine (EN). Interaction between alkaloid and concentration (μM) affected ($SE = 4.67$; $P < 0.001$) P450-Glo assay luminescence. Concentration of EN did not affect relative inhibition. Within ET and DHET 20 and 40 μM concentrations did not differ.

to the Glo assay. Our lowest test concentration, 20 μM, resulted in a steep inhibition of CYP450 by ET and DHET suggesting that the sensitivity of assay was exceeded.

Steer weight gain (0.53 ± 0.14 kg/d) was typical for summer grazing of tall fescue with light grain supplementation. **Table 1** presents the Pearson correlation coefficients among body weight, gain, circulating concentrations of cortisol, prolactin, and IGF-1, urine inhibition of cytochrome P450 activity, and urine alkaloid concentrations. Cortisol concentrations at weaning were not correlated with body weight or gain. In contrast, prolactin concentrations and prolactin:cortisol were correlated ($r > 0.30$; $P < 0.05$) with body weight and ADG. Although steer body weights were correlated with IGF-1, ADG was not correlated to IGF-1. Inhibition of CYP450 activity by urine was correlated ($r = -0.50$; $P < 0.05$) with total ergot alkaloid concentration. Inhibition of CYP450 activity by urine also tended to be correlated ($r = -0.26$; $P < 0.10$) with ADG.

Figure 2 displays the effects of C994G genotype on inhibition of CYP450 activity by urine. Steers that were heterozygous at C994G had more luminescence than steers that were either CC or GG. Luminescence was directly proportional to CYP450 activity; therefore, our work suggests that CG steers excreted urine that was less inhibitory to CYP450 activity. Tall fescue cultivar did not affect ($P > 0.1$) urine inhibition of CYP450.

Discussion

Determining the bioavailability of ergot alkaloids in the biological fluids of animals grazing tall fescue (E+) pastures infested with wild-type *N. coenophialum* is difficult. Previously, ergot alkaloids have been determined in cattle serum via HPLC (Moubarak et al., 1996), and in steer urine and bile (Stuedemann et al., 1998). Our results demonstrate that the Promega™ P450-Glo assay offers another method of indirectly determining ergot alkaloid concentrations in the urine of steers. Our preliminary research using the P450-Glo assay for the detection of animal to animal variation in circulating alkaloid concentrations proved too inconsistent to be useful (data not presented); however, refinement of the methodology may lead to a valid assay. Linearity of inhibition was problematic when testing cattle serum and plasma.

Stuedemann et al. (1998) successfully quantified total ergot alkaloid concentration in steer urine and bile using a validated

TABLE 1 | Correlations of steer weight and gain with serum hormones and urine alkaloids.

Item[a]	Mean ± SE	Weaning weight, kg 203 ± 27	End weight, kg 258 ± 34	ADG, kg 0.53 ± 0.14
Cortisol, ng/mL	36.1 ± 11.6	−0.05	−0.01	0.07
Prolactin, ng/mL	35.9 ± 31.9	0.31*	0.46**	0.51**
Prl:cortisol	1.2 ± 1.6	0.34*	0.41**	0.31*
IGF-1	197.1 ± 46.6	0.48**	0.47**	0.20
Urine inhibition, %	3.7 ± 2.7	0.03	−0.07	−0.26+
Urine alkaloids, ng/mg	331.1 ± 325.7	0.04	−0.02	0.21

[a] *Steers (n = 39) were weaned and grazed mixed tall fescue pastures for 105 days. Serum concentrations of cortisol, prolactin, and insulin-like growth factor 1 were determined at weaning. Urine was collected at day 105 of summer grazing, effects of urine on in vitro CYP450 expressed as percent of control (Urine inhibition), and total ergot alkaloids in urine (Urine alkaloids) expressed as ng of alkaloids per mg of creatinine.*

+P < 0.1.

**P < 0.05.*

***P < 0.01.*

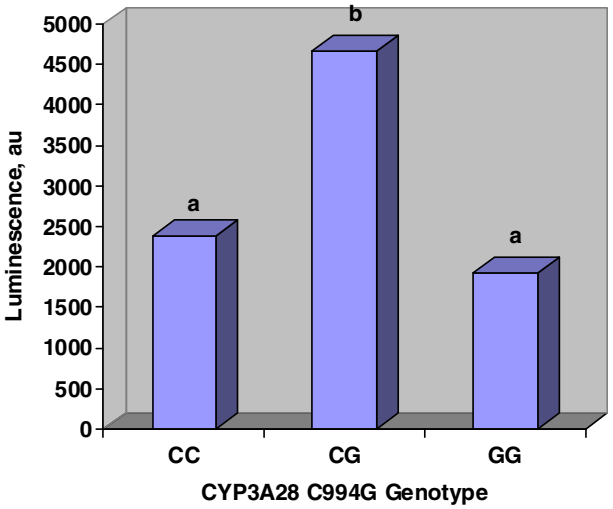


FIGURE 2 | Main effects of CYP3A28 genotype on CYP450 activity *in vitro*. Genotype was determined at C994G mutation. Urine was incubated in P450-Glo assay and luminescence was directly proportional to CYP450 activity. Genotype affected ($SE = 770$; $P < 0.03$) urine inhibition of CYP450 activity. Fescue cultivar did not affect ($P > 0.1$) luminescence.

ELISA. They found that 96% of all ergot alkaloids consumed were excreted through the urine. We analyzed urine from steers in our study using the same ELISA methods and found a strong negative correlation with luminescence from the Promega™ P450-Glo assay, suggesting the P450-Glo assay could indirectly determine animal clearance of ergot alkaloids. The ELISA method has been used primarily when scientists are interested in determining the concentration of total ergot alkaloids, not particular alkaloids (Stuedemann et al., 1998; Hill et al., 2000; Ayers et al., 2009). However, the ELISA assay lacks selectivity as it does not differentiate between ergot alkaloids which have necessitated the use of chromatographic methods to detect specific ergot alkaloids (Schultz et al., 2006; De Lorme et al., 2007; Foote et al., 2012; Wang et al., 2013). Schnitzius et al. (2001), compared HPLC and ELISA detection of ergot alkaloids and found the methods were inconsistent in their identification of alkaloids, concluding that both had their advantages and disadvantages. Antigen (ergot alkaloids) detection is restricted to the specificity of the antibody used in the ELISA test.

The P450-Glo assay also lacks selectivity as any compound that will inhibit CYP450 enzymes will be detected. However, the antibody used for ELISA is specific for the lysergic acid moiety (Hill and Agee, 1994), meaning any precursor molecules present, such as clavines, will not be detected using ELISA, but they would be detected in the P450-Glo assay if they inhibit CYP450. Based on our research, we cannot predict which alkaloid(s) will be associated with animal toxicosis. In fact, it is interesting that one of the more water-soluble ergot alkaloids, ergonovine, did not inhibit the P450-Glo assay yet factors in the urine did inhibit formation of luminescence. Perhaps other water-soluble alkaloids such as clavines and(or) lysergic acid and its derivatives are accumulating in urine, that would be consistent with previous metabolic research (Ayers et al., 2009). Ergopeptine alkaloids (e.g., ergovaline) are not excreted in the urine, so they would not be there to inhibit CYP450 (Schultz et al., 2006; De Lorme et al., 2007; Merrill et al., 2007); however, their metabolites may be in the urine following phase 1 and phase 2 toxin clearance in the liver.

Selecting livestock that are less susceptible to ergot alkaloid poisoning has the potential to improve livestock production where tall fescue is a component of the grazing system. In reality that is what livestock producers have been doing for the last 60 years. If the primary forage is tall fescue for a producer and the livestock do not reproduce then they are culled which results in selection for animals that tolerate the ergot alkaloids. Previously, we demonstrated that mutations in bovine cytochrome *P4503A28* were associated with cattle profitability traits (Sales et al., 2012, 2013). In the current study, steer genotype at SNP C994G was associated with urine inhibition of CYP450 activity. Steers that were CG at C994G excreted urine that was less inhibitory to CYP450 activity. We speculate that heterozygous steers had metabolically altered the alkaloids which resulted in compounds that were not inhibitory of CYP450. Alternatively, CG steers may have deposited alkaloids in fat stores and were not excreted in the urine. Additional research will be required to determine ergot alkaloid deposition, and the dynamics and sensitivity of the P450-Glo-assay to biological fluids from cattle grazing various tall fescue cultivars.

The overall goal of our research is to develop management tools that can increase the sustainability of livestock production. In addition, we are interested in understanding the genetic and physiological mechanisms related to animal productivity and toxicology. Our results suggest that cytochrome P450 can be used as management tool for selecting animals based on their cytochrome *P4503A28* genotype. In addition, we can envision the use of the P450-Glo-assay as a selection assay. We have not tested our hypothesis, but it is plausible that a group of replacement animals could be purposely exposed to a large concentration of ergot alkaloids and urine monitored for inhibition of the P450-Glo-assay. Presumably those animals with less inhibitory urine would be those with more tolerance of ergot alkaloids, but that would need to be tested before being recommended as a selection practice.

References

Althaus, M., Retzow, A., Castell, J. V., Gomez-Lechon, M. J., Amalou, Z., Rose, T., et al. (2000). *In vitro* identification of the cytochrome P450 isoform responsible for the metabolism of alpha-dihydroergocryptine. *Xenobiotica* 30, 1033–1045. doi: 10.1080/00498250010002261

Ayers, A. W., Hill, N. S., Rottinghaus, G. E., Stuedemann, J. A., Thompson, F. N., Purinton, P. T., et al. (2009). Ruminal metabolism and transport of tall fescue ergot alkaloids. *Crop. Sci.* 49, 2309–2316. doi: 10.2135/cropsci2009.01.0018

Cali, J. J., Sobol, M., Ma, D., Uyeda, H. T., and Meisenheimer, P. (2009). *CYP3A4 P450-Glo® Assays with Luciferin-IPA: The Most Sensitive and Selective Bioluminescent CYP3A4 Assay*. Promega Corporation. Available online at: http://www.promega.com/resources/pubhub/cellnotes/cyp3a4-p450-glo-assays-with-luciferin-ipa-the-most-sensitive-and-selective-bioluminescent-cyp3a4/Updated 2009 (Accessed July 15, 2014).

De Lorme, M. J. M., Lodge-Ivey, S. L., and Craig, A. M. (2007). Physiological and digestive effects of *Neotyphodium coenophialum* infected tall fescue fed to lambs. *J. Anim. Sci.* 85, 1199–1206. doi: 10.2527/jas.2005-430

Foote, A. P., Harmon, D. L., Brown, K. R., Strickland, J. R., McLeod, K. R., Bush, L. P., et al. (2012). Constriction of bovine vasculature caused by endophyte-infected tall fescue seed extract is similar to pure ergovaline. *J. Anim. Sci.* 90, 1603–1609. doi: 10.2527/jas.2011-4513

Haarmann, T., Ortel, I., Tudzynski, P., and Keller, U. (2006). Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways. *ChemBioChem* 7, 645–652. doi: 10.1002/cbic.200500487

Hill, N. S., and Agee, C. S. (1994). Detection of ergoline alkaloids in endophyte-infected tall fescue by immunoassay. *Crop Sci.* 34, 530–534. doi: 10.2135/cropsci1994.0011183X003400020041x

Hill, N. S., Thompson, F. N., Stuedemann, J. A., Dawe, D. L., and Hiatt, E. E. III. (2000). Urinary alkaloid excretion as a diagnostic tool for fescue toxicosis in cattle. *J. Vet. Diagn. Invest.* 12, 210–217. doi: 10.1177/104063870001200303

Lehner, A. F., Craig, M., Fannin, N., Bush, L., and Tobin, T. (2005). Electrospray[+] tandem quadrupole mass spectrometry in the elucidation of ergot alkaloids chromatographed by HPLC: screening of grass or forage samples for novel toxic compounds. *J. Mass Spectrom.* 40, 1484–1502. doi: 10.1002/jms.933

Merrill, M. L., Bohnert, D. W., Harmon, D. L., Craig, A. M., and Schrick, F. N. (2007). The ability of a yeast-derived cell wall preparation to minimize the toxic

effects of high-ergot alkaloid tall fescue straw in beef cattle. *J. Anim. Sci.* 85, 2596–2605. doi: 10.2527/jas.2007-0075

Moubarak, A. S., Johnson, Z. B., and Rosenkrans, C. F. Jr. (2012). Liver cytochrome P450 system as affected by endophyte infected tall fescue seed extracts and ergot alkaloids. *Agric. Sci.* 3, 1–4. doi: 10.4236/as.2012.31001

Moubarak, A. S., Piper, E. L., Johnson, Z. B., and Flieger, M. (1996). HPLC method for detection of ergotamine, ergosine, and ergine after intravenous injection of a single dose. *J. Agric. Food Chem.* 44, 146–148. doi: 10.1021/jf950425i

Natsuhori, M., van Raak, M., Ligtenberg, M., Kleij, L., Ten Berge, D., Zweers-Zeilmaker, W. M., et al. (1997). Isolation of a bovine full length cytochrome P450 (CYP3A) cDNA sequence and its functional expression in V79 cells. *Environ. Toxicol. Pharmacol.* 3, 17–24. doi: 10.1016/S1382-6689(96)00133-0

Nihsen, M. E., Piper, E. L., West, C. P., Crawford, R. J. Jr., Denard, T. M., Johnson Z. B., et al. (2004). Growth rate and physiology of steers grazing tall fescue inoculated with novel endophytes. *J. Anim. Sci.* 82, 878–883.

Rottinghaus, G. E., Garner, G. B., Conell, C. N., and Ellis, J. L. (1991). HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. *J. Agric. Food Chem.* 39, 112–115. doi: 10.1021/jf00001a022

Sales, M. A., Larson, M. J., Reiter, S. T., Brown, A. H. Jr., Brown, M. A., Looper, M. L., et al. (2012). Effects of bovine cytochrome P450 single nucleotide polymorphism, forage type, and body condition on production traits in cattle. *J. Anim. Physiol. Nutr.* 96, 545–553. doi: 10.1111/j.1439-0396.2011.01176.x

Sales, M. A., Murphy, K. Y., Reiter, S. T., Brown, A. H. Jr., Brown, M. A., Looper, M. L., et al. (2013). Effects of forage type, body condition and singlenucleotide polymorphisms in the bovine cytochrome P450 regulatory region on cow productivity. *J. Anim. Physiol. Anim. Nutr.* 97, 91–96. doi: 10.1111/j.1439-0396.2011.01246.x

Schnitzius, J. M., Hill, N. S., Thompson, C. S., and Craig, A. M. (2001). Semiquantitative determination of ergot alkaloids in seed, straw, and digesta samples using a competitive enzyme-linked immunosorbent assay. *J. Vet. Diagn. Invest.* 13, 230–237. doi: 10.1177/104063870101300307

Schultz, C. L., Lodge-Ivey, S. L., Bush, L. P., Craig, A. M., and Strickland, J. R. (2006). Effects of initial and extended exposure to an endophyte-infected tall fescue seed diet on faecal and urinary excretion of ergovaline and lysergic acid in mature geldings. *New Zealand Vet. J.* 54, 178–184, doi: 10.1080/00480169.2006.36692

Settivari, R. S., Bhusari, S., Evans, T., Eichen, P. A., Hearne, L. B., Antoniou, E., et al. (2006). Genomic analysis of the impact of fescue toxicosis on hepatic function. *J. Anim. Sci.* 84, 1279–1294.

Shelby, R. A., and Kelley, V. C. (1990). An immunoassay for ergotamine and related alkaloids. *J. Agric. Food Chem.* 38, 1130–1134. doi: 10.1021/jf00094a048

Smith, D., Smith, L., Shafer, W., Klotz, J., and Strickland, J. (2009). Development and validation of an LC-MS method for quantitation of ergot alkaloids in lateral saphenous vein tissue. *J. Agric. Food Chem.* 57, 7213–7220. doi: 10.1021/jf901086q

Strickland, J. R., Looper, M. L., Matthews, J. C., Rosenkrans, C. F. Jr., Flythe, M. D., and Brown, K. R. (2011). Board-invited review: St. Anthony's Fire in livestock: causes, mechanisms, and potential solutions. *J. Anim. Sci.* 89, 1603–1626. doi: 10.2527/jas.2010-3478

Stuedemann, J. A., Hill, N. S., Thompson, F. N., Fayere-Hosken, R. A., Hay, W. P., Dawe, D. L., et al. (1998). Urinary and biliary excretion of ergot alkaloids from steers that grazed endophyte-infected tall fescue. *J. Anim. Sci.* 76, 2146–2154.

Wang, C. C., Fernandez, L. P., and Gomez, M. R. (2013). Sensitive ergotamine determination in pharmaceuticals and biological samples using cloud point preconcentration and spectrofluorimetric detection. *Anal. Chim. Acta* 768, 90–95. doi: 10.1016/j.aca.2013.01.023

Yates, S. G., and Powell, R. G. (1988). Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. *J. Agric. Food Chem.* 36, 337–340. doi: 10.1021/jf00080a023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in CHEMISTRY

PERSPECTIVE ARTICLE
published: 18 February 2015
doi: 10.3389/fchem.2015.00008

Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed

A. Morrie Craig[1]*, James L. Klotz[2] and Jennifer M. Duringer[3]

[1] *Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR, USA*
[2] *Forage-Animal Production Research Unit, United States Department of Agriculture-Agricultural Research Service, Lexington, KY, USA*
[3] *Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, USA*

Edited by:
Darrin Smith, Eastern Kentucky University, USA

Reviewed by:
Youngjoo Kwon, Ewha Womans University, South Korea
Cynthia L. Gaskill, University of Kentucky, USA

****Correspondence:***
A. Morrie Craig, Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, 105 Magruder Hall, Corvallis, OR 97331, USA
e-mail: a.morrie.craig@oregonstate.edu

Ergot-induced disease in humans was known long before Biblical times and has been the root cause for countless human epidemics spanning from the early fourteenth century to the late sixteenth century. In contrast, many of these same ergot alkaloids have been utilized for their medicinal properties to mitigate migraine headaches and have had indications as anti-carcinogens. Although ergot alkaloids have been used for centuries by humans, basic pharmacokinetic data has not been documented for clinical disease in livestock. Consequently, a threshold dose and accurate dose-response data have yet to be established. Throughout the past several years, new detection techniques have emerged to detect these alkaloids at the parts per billion (ppb) level which has allowed for new efforts to be made with respect to determining threshold levels and making accurate clinical diagnoses in affected animals. This perspectives article provides a critical initial step for establishing a uniform interpretation of ergot toxicosis from limited existing data.

Keywords: ergot, ergotism, ergovaline, ergotamine, ergocornine, clinical disease, saphenous vein, cattle

INTRODUCTION

Ergot alkaloids are made as secondary metabolites of fungi. Their production occurs in the sclerotia of several species of the genus *Claviceps*, the most common being *Claviceps purpurea*. These compounds belong to the family of indole alkaloids, one of the ten classes of alkaloids that pharmacologists have defined from plant secondary metabolites. Lysergic acid, a tetracycline ergoline ring system, is a common structure to all of the ergot alkaloids (Krska et al., 2008). The chemical structure of ergotamine, the most commonly found ergot alkaloid and the one responsible for the clinical disease known as ergot, has been documented in a classic text (Matossian, 1989; Bennett and Bentley, 1999; Council for Agricultural Science and Technology, 2003). Other major alkaloids detected in ergot sclerotia include ergocornine, ergocristine, ergosine, and ergonovine, and α-ergocryptine (Krska et al., 2008; Wallwey and Li, 2011; Takach and Young, 2014; also see **Figure 1**, the chromatogram).

Historically, ergot alkaloids have had a large impact on societies. They are likely the cause for the last of the 10 plagues of Egypt; it is believed that many of the oldest sons succumbed following the opening of grain and storage facilities whose contents were contaminated by the *Claviceps* fungi (Marr and Malloy, 1996). Accounts from the Middle Ages describe "St. Anthony's fire," a symptom of intense inflammation attributed to human consumptions of food prepared with ergot-contaminated rye. Furthermore, Marie Antoinette's comment, "Let them eat cake, not bread," most likely surfaced because of ergot contaminated rye bread which was a common food among peasants.

Currently, modern grain cleaning techniques have eliminated ergotism as a human disease, but it remains a significant and devastating disease in the veterinary field primarily affecting sheep, cattle, pigs, and chickens. Clinical signs of ergotism in animals include gangrenous extremities, abortion, convulsions, agalactia, and ataxia (Lorenz and Hoseney, 1979; Richard, 2007; Duringer et al., 2012). Agalactia and the loss of ears, tails, and hooves are common sequelae seen in animals due to vasoconstriction: ergot alkaloids affect the supply of blood to the extremities of the body in addition to acting directly on the central nervous system via the pituitary where they activate the D2 dopamine receptors (Oliver, 2005; Richard, 2012). The tetracyclic ergoline ring of ergot alkaloids is similar to the structure of biogenic amines (Eckert et al., 1978), allowing them to act on the dopaminergic (Larson et al., 1999), serotonergic (Dyer, 1993; Schöning et al., 2001), and α-adrenergic receptors (Oliver et al., 1998; Schöning et al., 2001). Most recently, plant agronomists have shown different varieties within a plant species can portray different "chemotypes" or constitutions different alkaloids (Wallwey and Li, 2011; Panaccione et al., 2012, 2014; Takach and Young, 2014). Sclerotia show significant differences in their total alkaloid content, varying between 0.01 and 0.05% (Krska and Crews, 2008). Patterns that occur in alkaloid production are attributed to an individual strain in a geographic region (Krska and Crews, 2008).

In the United States, there is no regulatory limit defined for ergot alkaloids in grain. In Europe, while there are discussions on establishing legal limits, the European Food Safety Agency (EFSA) at this time has not recommended a limit other than 0.03–3.6 and 0.6 μg/kg b.w. per day for children and adults, respectively (European Food Safety Authority, 2012).

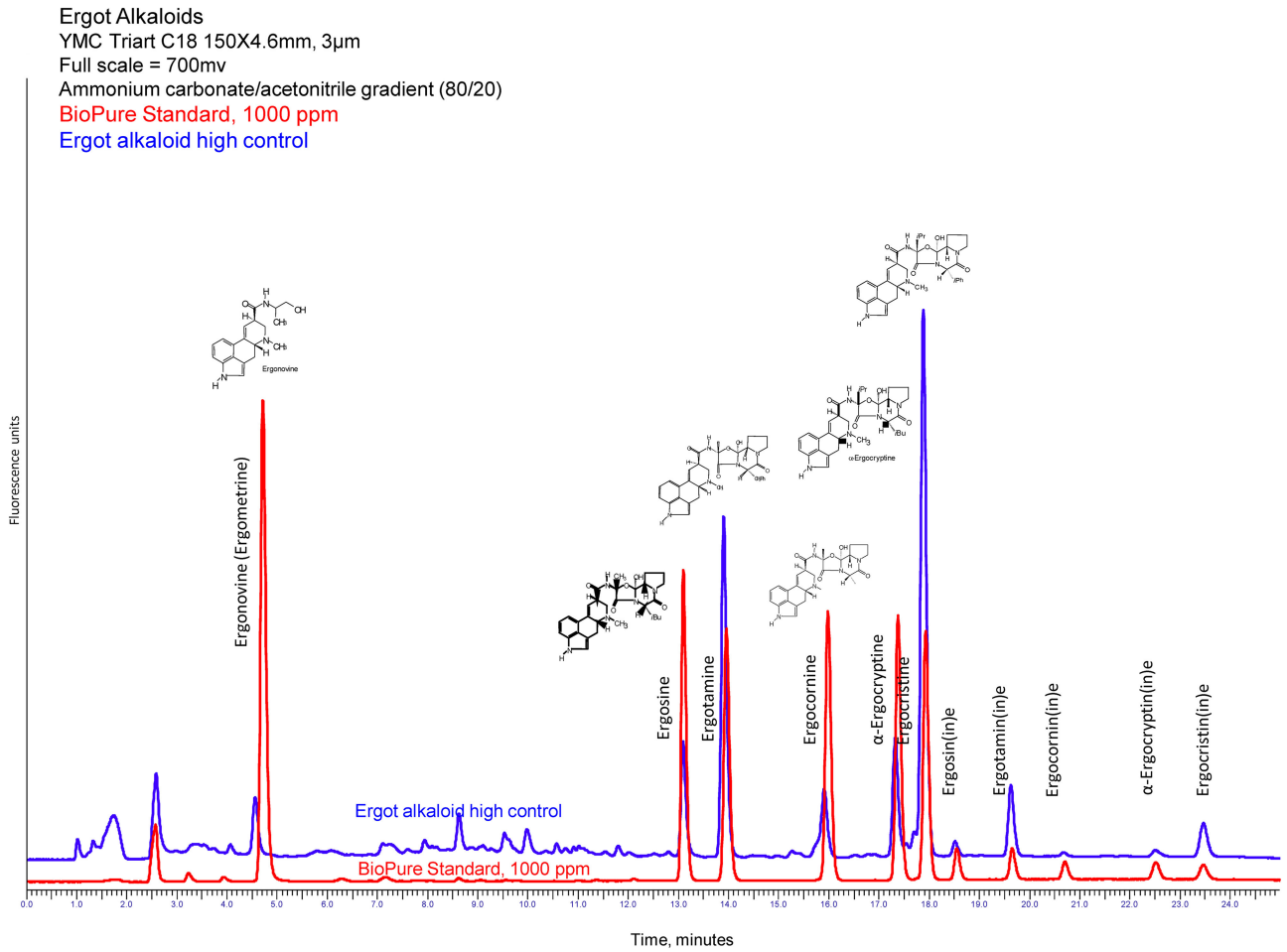


FIGURE 1 | HPLC fluorescence chromatogram of ergot alkaloids, both pure standards and extracted feed sample containing ergot bodies (Craig et al., 2014).

Two types of ergotism have been described: gangrenous (vasoconstrictive) and convulsive (neurological and abortogenic) (da Rocha et al., 2014). Though these two designations exist, the etiology of both is rooted in the vasoconstrictive ability of ergot alkaloids. Associating each alkaloid with a vasoconstrictive measurement (bovine lateral saphenous vein contractile response) has been a key factor in an accurate evaluation of clinical disease and will be discussed in **Table 1**.

The Endophyte Service Laboratory (ESL) (Craig et al., 1994, 2014), along with others (Rottinghaus et al., 1993; Saiga, 1998; Miyazaki, 2004), has conducted studies on threshold levels of alkaloids associated with tall fescue toxicosis. Moreover, these studies have been related to clinical signs as seen in fescue toxicosis (Brendemuehl et al., 1995; Blodgett, 2001; Tor-Agbidye et al., 2001; Marin et al., 2013). In contrast, few studies have been done on ergot-derived alkaloids to establish a threshold in livestock for ergotism. Thus, this article discusses the known correlations between the ergot alkaloids and development of ergotism in clinical cases to serve as a starting point for suggesting threshold levels and metabolism of these toxicants.

METHODS

EXTRACTION OF THE ERGOT ALKALOIDS

A method for extraction of ergot alkaloids from plant material was developed based on previous studies, for subsequent analysis by HPLC-fluorescence (Rottinghaus et al., 1991; Hill et al., 1993; Craig et al., 1994; Duringer et al., 2012). A standard mixture comprised of ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine was purchased from Romer Labs, Inc. (Union, MO, USA). Seed, straw, hay, or feed pellet samples were ground in a Cyclotec 1093 sample mill and passed through a 0.5-mm screen. To each tube, one gram of sample, 11 mL of chloroform and 1 mL of 0.001 M NaOH were added and mixed for 18–24 h in the dark, then centrifuged at 650 × *g*. Five milliliters of supernatant was applied to a 500 mg/6 mL solid phase extraction (SPE) column containing 0.5 g Ergosil® and 0.5 g anhydrous sodium sulfate. The ergot alkaloids were extracted by adding 5 mL eluent to the SPE, followed by a 2 mL 4:1 acetone:chloroform (v/v) wash and elution with 2.5 mL methanol. The eluent was dried under nitrogen at 50°C, then reconstituted in 0.5 mL methanol. Samples were mixed for 10 s,

Table 1 | Concentration at onset of contractile response, half maximal effective concentration or potency (EC50), and the maximal response or efficacy (EMAX) of ergot alkaloids in bovine lateral saphenous veins[a].

Alkaloid	Onset (M)[b]	EC_{50} (M)	E_{MAX} (%)	Ergotamine equivalence level
Ergovaline	1×10^{-8}	$4.0 \times 10^{-6} \pm 1.5 \times 10^{-6}$	104.1 ± 6.0	1
Ergotamine	1×10^{-8}	$4.0 \times 10^{-6} \pm 1.5 \times 10^{-6}$	104.1 ± 6.0	1
Ergonovine	1×10^{-7}	$3.4 \times 10^{-6} \pm 8.8 \times 10^{-7}$	68.5 ± 4.1	0.1
Ergocristine	1×10^{-7}	$5.6 \times 10^{-6} \pm 1.3 \times 10^{-6}$	45.5 ± 4.5	0.1
Ergocornine	1×10^{-7}	$4.0 \times 10^{-5} \pm 2.3 \times 10^{-5}$	57.2 ± 9.9	0.1
α-Ergocryptine	1×10^{-6}	$5.4 \times 10^{-6} \pm 1.2 \times 10^{-6}$	42.9 ± 4.1	0.01
Lysergic acid	1×10^{-5}	$5.5 \times 10^{-5} \pm 2.3 \times 10^{-5}$	22.6 ± 4.1	0.001

[a] *Taken from Klotz et al. (2006, 2007, 2010).*

[b] *Expressed as a percent of norepinephrine maximum, which was a* 1×10^{-4} *M norepinephrine reference addition. Onset is normalized to the closest order of magnitude.*

sonicated for 10 s, and centrifuged at 650 × *g* for 5 min then analyzed via HPLC-fluorescence.

HPLC-FLUORESCENCE ANALYSIS FOR ERGOT ALKALOIDS

Reverse-phase HPLC analysis is coupled with fluorescence detection (excitation and emission wavelengths of 250 nm and 420 nm, respectively) and a gradient run at 0.9 mL/min. Mobile phases of 1 mM ammonium carbonate (A) and ACN (B) were programmed as follows: equilibrate from 0 to 5 min at 75% A, then decrease linearly to 65% A from 5 to 15 min, hold at 65% A from 15 to 20 min, then decrease linearly to 25% A from 20 to 25 min. A Gemini 3.0 μ C18 110Å (Phenomenex, Torrance, CA) column was used in conjunction with a guard column cartridge of similar packing. **Figure 1** shows an example of a HPLC-fluorescence chromatogram for ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine produced using this assay. [Ergonovine and the epimers of the other ergot alkaloids are not retained by the Ergosil® SPE columns (reference Krska et al., 2008) and are not included in reporting the total ergot alkaloid content of the feed sample]. The method performance has a limit of detection (LOD) of 11 ppb for ergosine, ergotamine, and ergocornine, 13 ppb for α-ergocryptine, and 14 ppb for ergocristine. The limit of quantitation (LOQ) is 39 ppb for ergosine and ergotamine, 41 ppb for ergocornine, 49 ppb α-ergocryptine, and 50 ppb for ergocristine. Within day/Day to day variations are 8.5%/6.5% for ergosine and ergotamine, 4.5%/6.9% for ergocornine, 5.8%/7.8% for α-ergocryptine, and 6.8%/10.6% for ergocristine. The recovery for the above-mentioned alkaloids is 92–97%.

WHAT WE KNOW ABOUT VASOCONSTRICTION AND ABORTION FROM ERGOT ALKALOIDS

Using the data presented in Klotz et al. (2006, 2007, 2010, 2011), we have constructed **Table 1**, which summarizes the vascular potency and efficacy of ergovaline (the predominant ergot alkaloid found in tall fescue), ergotamine, ergonovine, ergocristine, ergocornine, ergocryptine, and lysergic acid in relative terms. **Table 1** bridges what is known in tall fescue toxicosis, i.e., vasoconstriction and the extrapolated abortogenic sequelae. It should be noted that the principal column evaluated in determining the onset of vasoconstriction in clinical disease is column two [Onset (M)].

As **Table 1** emphasizes, ergovaline and ergotamine have equal sensitivity for vasoconstriction, whereas ergonovine, ergocristine, and ergocornine are about one-tenth as powerful. Pharmacologically, the half-maximal concentration (EC_{50}) and the onset concentration are measures of a compound's potency (the lower the concentration the more potent the compound); ergotamine and ergovaline are identical in this aspect. The onset of vasoconstriction is reported in concentration (moles/L), and is viewed as the best indicator of toxicosis when evaluating levels in feedstuffs. Interpreting the chromatogram in **Figure 1** in relation to the additive effects of all ergot alkaloids present is performed by utilizing a multiplicative factor in front of various alkaloids; therefore, 10 ergonovine molecules are equally vasoconstrictive to one ergovaline molecule. More recently, Klotz et al. (2013) has demonstrated that the EC_{50} for ergovaline, ergotamine, and ergocornine can be reduced by an animal's prior exposure to ergot alkaloids. This may have an influence on the animal's perceived sensitivity (as reported by the clinician/researcher) and may affect the threshold of an individual animal (Pesqueira et al., 2014). Further, severe winters and wet springs (wet and cold weather patterns in general) have been shown to be more conducive to ergot-related disease (Welty et al., 1994). This strong correlation between weather and ergot disease is easily seen throughout historical events and in our own clinical cases below (**Table 2**).

CORRELATION OF ERGOT TOXINS WITH CLINICAL DISEASE

The alkaloids that cause fescue foot and summer slump syndrome are produced by fungi in pasture grasses. Because of similar vasoconstrictive abilities of ergovaline and ergotamine, we have correlated some of the studies done with fescue with what we have seen from clinical cases with ergot. Field and barn studies of natural fescue foot and herds have been conducted (Oliver, 2005; Marin et al., 2013). Therefore, we are illustrating typical cases seen in feed sample submissions over the last 2–3 years to the ESL in Oregon.

In **Table 2**, column 2 represents the total alkaloids observed, which include a summation of all six ergot alkaloids. Since different alkaloids have different vasoconstrictive onset values, column 3 relates the six alkaloids (**Table 1**) back to "ergotamine

Table 2 | Clinical evaluation of ergot toxicosis in cattle with ergotamine equivalence levels (ppb) in feed on a dry weight basis.

Case number	Total ergot level observed (ppb)[a]	Ergotamine equivalence level (ppb)	Weather conditions	Clinical signs observed
a	473	473	−20°C, Canada, February	Tail loss
b	1500	415	1°C, Oregon, December	Moderate lameness
c	2909	466	−2°C, Idaho, January	Decreased feed intake
d	3555	778	−5°C, E. Oregon, February	Early term abortions, low milk yield
e	5999	626	−5°C, Idaho, January	No feed consumption
f	11,538	1161	−4°C, Canada, April	Sloughing of hooves
g	54,916	3728	1°C, Oregon, January	Early term abortions
h	62,245	10,124	−1°C, Idaho, January	Hooves sloughing completely off

[a] *Includes ergonovine, ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine.*

equivalents." Therefore, ergotamine equivalents are correlated to clinical signs (column 5).

Eight examples of clinical cases associated with feed samples submitted to the ESL by veterinarians suspecting ergot toxicosis are shown to illustrate typical cases that have been seen. The ESL consults with veterinarians and evaluates all aspects of the case to determine if testing of feed samples is warranted. If so, results are often discussed with the client to ensure that proper measures are taken to resolve ergot toxicosis. Five to ten years ago the ESL would observe ergot cases approximately once per month; however, this has progressed to several cases per week and sometimes multiple cases each day in recent years despite maintaining approximately the same number of total samples tested. Our worst toxicosis cases have been in cattle and horses. The increase in cases seen in the Pacific Northwest and throughout the country is likely due to climate change or the lack of field burning that has been phased out throughout the years. The stoppage of burning has greatly reduced the ability to destroy *C. purpurea* by fire.

Evaluation of the cases included in **Table 2** is as follows:

The following cases in cattle involved pellets as nearly 100% of the provided feed source, with the exclusion of case b, which used a grass seed sample. Pellets were usually a combination of grass seed with additional alfalfa or other hay sources mixed in. In cases f and h, cattle had exposure to overgrazed pasture, and it is estimated that little nutrition came from the pasture sources. In both cases, the pellet mixture comprised most of the usable diet.

The first case (a) presents an ergotamine level of nearly 500 parts per billion (ppb). This case originated in February when the colder weather prompted vasoconstriction in the extremities, specifically, the tissue around the tail to become necrotic. No sloughing of ears were observed nor reported in this particular case.

The second case (b) reported 1500 ppb ergot alkaloids and occurred in December as the feed bunks were being cleaned out. There was moderate lameness and some necrosis about the feet. The 1500 ppb consisted of ergotamine and ergocornine, resulting in a lower ergotamine equivalence level (415 ppb) that was similar to case (a).

The third case (c) revealed approximately 2900 ppb ergot alkaloids in pellets which triggered a decreased consumption in feed, as well as a necrotic area around the coronet band of the hoof. This outcome was observed in steers in January. Ergotamine, ergocornine, and ergocryptine were found in the feed. The ergotamine equivalence level (466 ppb) illustrated similarities to that of case (a).

The fourth case (d) presented nearly 3500 ppb ergot alkaloids and took place in February. This case showed greater severity of clinical signs, i.e., term abortions, agalactia, and reduced milk production in the dairy herd. This case had a 778 ppb ergotamine equivalence level.

The fifth case (e) presented itself in January and had a total ergot alkaloid concentration of 6000 ppb. The animals refused to consume any feed, resulting in near starvation. This case was composed of very little ergotamine and mostly other ergot alkaloids, which dramatically brought the ergotamine equivalence level down to 626 ppb.

The sixth case (f) occurred during April, with ergot alkaloid levels measuring almost 12,000 ppb. The steers exhibited sloughing of the hooves, tails, and ears. So many animals sloughed their hooves that approximately 40% of the herd had to be terminated. This case consisted of multiple ergot alkaloids and had an ergotamine equivalence level of approximately 1000 ppb.

The seventh case (g) was the second highest case of total ergot alkaloids (54,916 ppb) that we have observed and occurred in January. Though the weather was fairly warm and dry, there were early term abortions in a high percentage of the cow-calf operation. Approximately 45–47 of the 59 animals that were pregnant had early term abortions over a 2 week period. This case had an approximate 4000 ppb ergotamine equivalence level.

The eighth (h) and final case entailed the observance of cattle during January in the northern region of the United States. This case produced approximately 62,000 ppb of total ergot alkaloids. The clinical consequences of the ergot alkaloids resulted in 3/4 of the cow's hooves to slough off, after which the animals had to be sacrificed. This case consisted of both ergotamine and other ergot alkaloids, and had an ergotamine equivalence level to approximately 10,000 ppb. Although the weather was warm, the high concentration of ergot alkaloid induced enough vasoconstriction to clinically affect the animals.

The bioavailability and pharmacokinetic data of ergot alkaloids in cattle are limited and affected by many different factors. However, using the highest and the lowest dietary ergotamine concentrations reported in **Table 2**, calculations were done to illustrate a theoretical relationship with the ergotamine concentration in a feedstuff and a maximum potential

concentration in blood. A feedstuff containing the lowest concentration of 473 ppb (case a) or 0.473 mg/kg ergotamine fed to a 500 kg cow consuming 2.5% of BW will consume a total of 5.9 mg of ergotamine per day. Assuming that the ergotamine is 100% bioavailable from the feedstuff (not always the case as this is negatively correlated with the maturity of the feedstuff), 100% absorbed (not always the case, as these compounds are likely biotransformed by gut microbes prior to absorption), 100% bioavailable in the blood (assuming negligible bioaccumulation in tissues or initial hepatic detoxification) and total blood volume of 55 mL/kg of BW, a maximum ergotamine concentration ever reaching the blood would be 0.215 μg ergotamine/mL of blood. Although this is far below the EC_{50} reported for ergotamine in **Table 1** of 4×10^{-6} M or an approximate of 2.32 μg of ergotamine/mL in blood, it is much higher than the corresponding onset concentration for ergotamine of 1×10^{-8} M or 0.005 μg /mL. If we substituted the highest reported ergotamine concentration in **Table 2** (case h), the maximum concentration of ergotamine that could ever reach the blood is 4.6 μg /mL. This is twice the concentration of the EC_{50} of ergotamine and could easily explain the severe clinical signs observed.

The data that resulted from these clinical cases illustrate that ergot alkaloids can cause vasoconstriction and deleterious consequences regardless of seasonal conditions (Egert et al., 2014). Severe vasoconstriction will cause the hooves to slough off and/or abortions. However, in cooler or freezing weather, the values for the vasoconstrictiveness of the different ergot alkaloids are not dissimilar from what is found in endophyte-infected tall fescue with the predominant ergot alkaloid ergovaline (Duringer et al., 2012; Foote et al., 2012). This observation should be documented in future scientific studies. As for now, there have been no specific studies looking at individual alkaloids other than the physiometry values documenting vasoconstriction. Furthermore, there have been no studies related to the convulsive aspect of this disease (neurological and abortogenic) and the individual alkaloids. The only clinical evaluation conducted has been by the papers from Klotz et al. (2006, 2007, 2008, 2010). These papers examine the onset of vasoconstriction. Threshold levels have yet to be established for different animal species. When considering the international transportation of feed, ergot levels become an important factor to evaluate in developing Food Safety standards and programs. In particular, feed produced in the European continent and transported to the Middle East are susceptible to *C. purpurea* due to the wet springs and summers that promote fungal growth.

WHAT SHOULD BE DONE IN FUTURE STUDIES?

Though this disease has been known for hundreds of years, only a paltry amount of information is available to accurately diagnose disease at a clinical level (where animal production is affected and animal welfare becomes a concern). The first step in expanding this information should be establishing the threshold level in different species, particularly cattle and horses that could be exposed to ergot alkaloids in their feed. It should be noted that overseas, camels, goats, and sheep are also frequently exposed to these toxic alkaloids. The second step is to establish other bio-indicators of clinical disease other than prolactin levels, weight gain, and some of the more classical animal observances. Lastly, the vasoconstrictive and convulsive effects and their subsequent clinical consequences need to be separated.

ACKNOWLEDGMENT

Special thanks and recognition go out to Avalon McKune and McKenzie Hughes for helping in the progress of this article.

REFERENCES

Bennett, J. W., and Bentley, R. (1999). Pride and prejudice: the story of ergot. *Perspect. Biol. Med.* 42, 333–355. doi: 10.1353/pbm.1999.0026

Blodgett, D. J. (2001). Fescue toxicosis. *Vet. Clin. North Am. Equine Pract.* 17, 567–577.

Brendemuehl, J. P., Boosinger, T. R., Bransby, D. I., and Schumacher, J. (1995). The effect of short term exposure to and removed from the fescue endophyte *Acremonium coenophialum* on pregnant mares and foal viability. *Biol. Reprod. Mono* 1, 61–67.

Council for Agricultural Science and Technology. (2003). *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Ames, IA.

Craig, A. M., Bilich, D., Hovermale, J. T., and Welty, R. E. (1994). Improved extraction and HPLC methods for ergovaline from plant material and rumen fluid. *J. Vet. Diagn. Invest.* 6, 348–352. doi: 10.1177/104063879400600311

Craig, A. M., Blythe, L. L., and Duringer, J. M. (2014). The role of the Oregon state university endophyte service laboratory in diagnosing clinical cases of endophyte toxicosis. *J. Agric. Food Chem.* 62, 7376–7381. doi: 10.1021/jf5027229

da Rocha, M. E., da Chagas Oliveira Freire, F., Feitosa Maia, F. E., Florindo Guesed, M. I., and Rondina, D. (2014). Mycotoxins and their effects on human and animal health. *Food Control* 36, 159–165. doi: 10.1016/j.foodcont.2013.08.021

Duringer, J. M., Murty, L. D., and Craig, A. M. (2012). "Endophyte mycotoxins in animal health," in *Phytchemicals, Plant Growth, and the Environment*, ed D. R. Gang (New York, NY: Springer), 37–58. doi: 10.1007/978-1-4614-4066-6_3

Dyer, D. C. (1993). Evidence that ergovaline acts on serotonin receptors. *Life Sci.* 53, 223–228. doi: 10.1016/0024-3205(93)90555-H

Eckert, H., Kiechel, J. R., Rosenthaler, J., Schmidt, R., and Schreier, E. (1978). "Biopharmaceutical aspects: analytical methods, pharmokinetics metabolism and bioavailability," in *Ergot Alkaloids and Related Compounds*, eds B. Berde and H. O. Schild (Berlin: Springer Verlag), 719–803.

Egert, A. M., Kim, D. H., Schrick, F. N., Harmon, D. L., and Klotz, J. L. (2014). Dietary exposure to ergot alkaloids decreases contractility of bovine mesenteric vasculature. *J. Anim. Sci.* 92, 1768–1779. doi: 10.2527/jas.2013-7141

European Food Safety Authority. (2012). Scientific opinion on ergot alkaloids in food and feed. EFSA panel on contaminants in the food chain (CONTAM). *Eur. Food Saf. Auth. J.* 10, 2798. doi: 10.2903/j.efsa.2012.2798

Foote, A. P., Harmon, D. L., Brown, K. R., Strickland, J. R., McLeod, K. R., Bush, L. P., et al. (2012). Constriction of bovine vasculature caused by endophyte-infected tall fescue seed extract is similar to pure ergovaline. *J. Anim. Sci.* 90, 1603–1609. doi: 10.2527/Jas.2011-4513

Hill, N. S., Rottinghaus, G. E., Agee, C. S., and Schultz, L. M. (1993). Simplified sample preparation for HPLC analysis of ergovaline in tall fescue. *Crop Sci.* 33, 331–333. doi: 10.2135/cropsci1993.0011183X003300020024x

Klotz, J. L., Aiken, G. E., Johnson, J. M., Brown, K. R., Bush, L. P., and Strickland, J. R. (2013). Antagonism of lateral saphenous vein serotonin receptors from steers grazing endophyte-free, wild-type, or novel endophyte-infected tall fescue. *J. Anim. Sci.* 91, 4492–4500. doi: 10.2527/jas.2012-5896

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Arrington, B. C., et al. (2007). Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 85, 2330–2336. doi: 10.2527/jas.2006-803

Klotz, J. L., Bush, L. P., and Strickland, J. R. (2011). A vascular contractility bioassay using bovine right ruminal artery and vein. *J. Anim. Sci.* 89, 1944–1951. doi: 10.2527/jas.2010-3532

Klotz, J. L., Bush, L. P., Smith, D. L., Shafer, W. D., Smith, L. L., Vevoda, A. C., et al. (2006). Assessment of vasoconstrictive potential of D-lysergic acid using an isolated bovine lateral saphenous vein bioassay. *J. Anim. Sci.* 84, 3167–3175. doi: 10.2527/jas.2006-038

Klotz, J. L., Kirch, B. H., Aiken, G. E., Bush, L. P., and Strickland, J. R. (2008). Effects of selected combinations of tall fescue alkaloids on the vasoconstrictive capacity of fescue-naive bovine lateral saphenous veins. *J. Anim. Sci.* 86, 1021–1028. doi: 10.2527/jas.2007-0576

Klotz, J. L., Kirch, B. H., Aiken, G. E., Bush, L. P., and Strickland, J. R. (2010). Contractile response of fescue-naive bovine lateral saphenous veins to increasing concentrations of tall fescue alkaloids. *J. Anim. Sci.* 88, 408–415. doi: 10.2527/jas.2009-2243

Krska, R., and Crews, C. (2008). Significance, chemistry and determination of ergot alkaloids: a review. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess* 25, 722–731. doi: 10.1080/02652030701765756

Krska, R., Stubbings, G., Macarthur, R., and Crews, C. (2008). Simultaneous determination of six major ergot alkaloids and their epimers in cereals and foodstuffs by LC–MS–MS. *Anal. Bioanal. Chem.* 391, 563–576. doi: 10.1007/s00216-008-2036-6

Larson, B. T., Harmon, D. L., Piper, E. L., Griffis, L. M., and Bush, L. P. (1999). Alkaloid binding and activation of D2 dopamine receptors in cell culture. *J. Anim. Sci.* 77, 942–947.

Lorenz, K., and Hoseney, R. C. (1979). Ergot on cereal grains. *CRC Crit. Rev. Food Sci. Nutr.* 11, 311–354. doi: 10.1080/10408397909527267

Marin, S., Ramos, A. J., Cano-Sancho, G., and Sanchis, V. (2013). Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chem. Toxicol.* 60, 218–237. doi: 10.1016/j.fct.2013.07.047

Marr, J. S., and Malloy, C. D. (1996). An epidemiologic analysis of the ten plagues of Egypt. *Caduceus* 12, 7–24.

Matossian, M. A. K. (1989). *Poisons of the Past: Molds, Epidemics, and History*. New Haven, CT: Yale University Press.

Miyazaki, S. (2004). Effects of mycotoxins to livestock: could there be industrial and public health problems in Japan? *Mycotoxins* 54, 113–117. doi: 10.2520/myco.54.113

Oliver, J. W. (2005). "Pathophysiologic response to endophyte toxins," in *Neotyphodium in Cool-season Grasses*, eds C. A. Roberts, C. P. West, and D. E. Spiers (Ames, IA: Blackwell Publishing), 291–304. doi: 10.1002/9780470384916.ch13

Oliver, J. W., Strickland, J. R., Waller, J. C., Fribourg, H. A., Linnabary, R. D., and Abney, L. K. (1998). Endophytic fungal toxin effect on adrenergic receptors in lateral saphenous veins (cranial branch) of cattle grazing tall fescue. *J. Anim. Sci.* 76, 2853–2856.

Panaccione, D. G., Beaulieu, W. T., and Cook, D. (2014). Bioactive alkaloids in vertically transmitted fungal endophytes. *Funct. Ecol.* 28, 299–314. doi: 10.1111/1365-2435.12076

Panaccione, D. G., Ryan, K. L., Schardl, C. L., and Florea, S. (2012). "Analysis and modification of ergot alkaloid profiles in fungi," in *Natural Product Biosynthesis by Microorganisms and Plant, Pt A Methods in Enzymology*, ed D. A. Hopwood (San Diego: Elsevier Academic Press Inc), 267–290. doi: 10.1016/B978-0-12-394290-6.00012-4

Pesqueira, A., Harmon, D. L., Branco, A. F., and Klotz, J. L. (2014). Bovine lateral saphenous veins exposed to ergopeptine alkaloids do not relax. *J. Anim. Sci.* 92, 1213–1218. doi: 10.2527/jas.2013-7142

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses–an overview. *Int. J. Food Microbiol.* 119, 3–10. doi: 10.1016/j.ijfoodmicro.2007.07.019

Richard, J. L. (2012). "Mycotoxins - an overview," in *Guide to Mycotoxins*, ed E. M. Binder (Leicestershire: Anytime Publishing Services), 1–47.

Rottinghaus, G. E., Garner, G. B., Cornell, C. N., and Ellis, J. L. (1991). HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. *J. Agric. Food Chem.* 39, 112–115. doi: 10.1021/jf00001a022

Rottinghaus, G. E., Schultz, L. M., Ross, P. F., and Hill, N. S. (1993). An HPLC method for the detection of ergot in ground and pelleted feeds. *J. Vet. Diagn. Invest.* 5, 242–247. doi: 10.1177/104063879300500216

Saiga, S. (1998). Frequent occurences of cattle poisoning through endophyte-infected hay. *Livest. Tech.* 21–25.

Schöning, C., Flieger, M., and Pertz, H. H. (2001). Complex interaction of ergovaline with 5-HT2A, 5-HT1B/1D, and alpha1 receptors in isolated arteries of rat and guinea pig. *J. Anim. Sci.* 79, 2202–2209.

Takach, J. E., and Young, C. A. (2014). Alkaloid genotype diversity of tall fescue endophytes. *Crop Sci.* 54, 667. doi: 10.2135/cropsci2013.06.0423

Tor-Agbidye, J., Blythe, L. L., and Craig, A. M. (2001). Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. *Vet. Hum. Toxicol.* 43, 140–146.

Wallwey, C., and Li, S.-M. (2011). Ergot alkaloids: structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes. *Nat. Prod. Rep.* 28, 496–510. doi: 10.1039/c0np00060d

Welty, R. E., Craig, A. M., and Azevedo, M. D. (1994). Variability of ergovaline in seeds and straw and endophyte infection in seeds among endophyte-infected genotypes of tall fescue. *Plant Dis.* 78, 845–849. doi: 10.1094/PD-78-0845

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 September 2014; accepted: 22 January 2015; published online: 18 February 2015.

*Citation: Craig AM, Klotz JL and Duringer JM (2015) Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed. Front. Chem. **3**:8. doi: 10.3389/fchem.2015.00008*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers

EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org