EVALUATION OF CORRELATION BETWEEN WITHIN-BARN CURING ENVIRONMENT AND TSNA ACCUMULATION IN DARK AIR-CURED TOBACCO

THESIS

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By

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ABSTRACT

EVALUATION OF CORRELATION BETWEEN WITHIN-BARN CURING ENVIRONMENT AND TSNA ACCUMULATION IN DARK AIR-CURED TOBACCO

Significant variability in cured leaf tobacco-specific nitrosamine (TSNA) content is commonly observed when sampling within dark air-curing barns. This variability may be due to inconsistency in the curing environment within different areas of the barn. A study was initiated in 2012 through support from a CORESTA Study Grant to evaluate if leaf TSNA content is related to microenvironmental conditions in the barn. Seed screened for low conversion of nicotine to nornicotine (sc) and high converter (HC) selections of TR Madole dark tobacco were cured in barns near Princeton and Lexington, Kentucky in 2012 and 2013. Temperature and relative humidity were measured with data loggers placed at 27 locations within each barn for the duration of curing. TSNA content was determined from 20-leaf samples collected from each selection at each of the 27 locations within each barn. There were no significant effects of individual data logger placement in either variety selection on hours above 24°C temperature, hours above 80% relative humidity, or TSNA; therefore, we investigated these data within 3-dimensional aspects of tier, room, and bent within each barn. There were various effects of tier, room, and bent on temperature, relative humidity, and TSNA; but limited significant relationships between temperature, relative humidity, and TSNA.

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Chapter 1: Literature Review

Introduction

Kentucky is the leading state for dark tobacco production with a combined total for both dark fire-cured and dark air-cured types of almost 39 million pounds, seventy percent of this being dark fire-cured tobacco. The average yield of dark air-cured tobacco is 3,024 kg ha^{-1} while the average yield of dark fire-cured tobacco is 3,472 kg ha^{-1}. Dark fire-cured and dark air-cured tobaccos are currently valued at an average of \$5.74 kg^{-1} and \$5.17 kg^{-1}, respectively (USDA NASS, 2013). These tobacco types are primarily used in smokeless products and specialty-type cigars (Miller, 1999). Smokeless product sales have increased in the United States by 65.5 percent between 2005 and 2011, while cigarette consumption has continually decreased (Delnevo et al., 2014).

Challenges within the Tobacco Industry

Tobacco growers face many challenges in tobacco production that can significantly affect production techniques, future planning, and market demands. Effective managerial decisions have to be made since the costs of inputs are always increasing, and fluctuating market prices provide no guarantee of profit. Due to the labor intensive nature of dark tobacco production, availability and costs of labor are major challenges that tobacco growers face throughout the growing season and from year to year. Studies dealing with tobacco production have indicated that it takes 150-200 hours of labor to grow one acre of burley tobacco and 300 hours or more for dark tobacco even with the advances that have come with increased labor efficiency (Snell and Powers, 2013). Access to reliable labor is also a concern to many burley and dark tobacco growers (Snell and Powers, 2013).

Harvesting and Curing of Dark Tobacco

The process of harvesting dark air-cured tobacco is comparable to dark fire-cured tobacco. Dark air-cured tobacco leaves are thick and leathery in texture with a somewhat oily sheen at maturity, and ready to harvest between five and seven weeks after the removal of the terminal bud (Bailey, 2006). The brittle nature of mature dark tobacco necessitates a period of field wilting after cutting but prior to impaling plants on sticks (Bailey et al., 2013). Dark tobacco is stalk harvested, with five to six plants placed on sticks for housing in curing barns.

Uncontrollable weather variables during the growing and curing season are also a major challenge in producing quality air-cured tobacco. Palmer and Pearce (1999) explain that air-curing facilities must take advantage of the ambient curing conditions while minimizing conditions that are detrimental to quality such as rain and wind damage, to achieve the best possible cure.

In recent years, there has been more of a challenge placed on tobacco growers to supply buyers with tobacco that meets certain visual and sensory quality standards. Tobacco that has lower amounts of undesirable chemicals that have been deemed carcinogenic has become another component of quality. This research focuses on Tobacco-Specific Nitrosamines (TSNA), the major carcinogens in tobacco, and the influence of curing conditions on their accumulation in dark air-cured leaf.

Tobacco-Specific Nitrosamines

One of the major carcinogens found in tobacco are TSNAs, which are produced primarily during curing. TSNAs are nitrogenous compounds that are formed only from tobacco alkaloids and are detectable in the tobacco leaf and in the particulate phase of tobacco smoke. There are four major TSNAs: Nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB) (Brunnemann et al. 1983, Fisher et al. 1990, Hecht et al. 1998, Hoffman et al. 1994). The tobacco industry has had a major interest in reducing TSNA content in tobacco products since the early 1990's when a report was published showing that some TSNAs induce malignant tumors in mice, rats, and hamsters (Hoffman and Hecht, 1985., Burton et al., 1989a, Peng, 1990). Since the U.S. Food & Drug Administration gained authority over tobacco products in 2009 (FDA, 2009), the tobacco industry has further emphasized reducing TSNA content to lower the health risk to consumers. TSNA reduction will potentially become more important with pending tobacco regulation from the U.S. Food & Drug Administration (FDA, 2014). A major focus of tobacco research for the past several years has been TSNA reduction in cured leaf by modifying agronomic, curing, processing, and manufacturing practices (W. A. Bailey, personal communication). The formation of TSNAs is influenced by many factors throughout the production process. Accumulation of TSNAs in cured leaf has been inherently variable even within the same tobacco and curing facility. Currently, it is thought that curing conditions, primarily temperature and relative humidity, are the most influential factors in the formation of TSNA.

Factors Influencing Tobacco-Specific Nitrosamine Accumulation

Alkaloids and Nitrosating Agent

Alkaloids are an essential component of leaf quality in commercial tobacco and are important to providing a physiological stimulus that makes the consumption of tobacco products pleasurable (Bush and Crowe, 1989). Bush (1999) made the general conclusion that cultural practices and environmental conditions that improve plant growth will also increase alkaloid formation and accumulation. A commonly accepted mechanism of the formation of tobacco-specific nitrosamines is the nitrosation of naturally occurring alkaloids within the tobacco plant (Peele et al. 2001). Bush et al. (2001) state that the most important of the reactions between alkaloids and nitrosating agents is the reaction between nitrite and the secondary amine alkaloids which occurs during air curing. This reaction is most likely due to microbial activity, since nitrite does not accumulate in the plant. Burton et al. (1989b) concluded that nitrite was formed in significant quantities from nitrate under aerobic conditions.

The amount of specific alkaloid precursor influences the amount of TSNA accumulation. The specific alkaloid precursor that is the most prevalent in burley and dark tobacco is nornicotine, which is converted from nicotine (Jack et al. 2013). Jack et al. (2013) also state that the relative amount of nornicotine depends on the amount of conversion and the absolute amount of nornicotine depends on the amount of nicotine originally present. Use of screened or low converter (LC) seed has had a definite impact on reducing the amount of TSNA content in tobacco. Screened or LC seed reduces the amount of nornicotine, the precursor to nitrosonornicotine (NNN), and is one of the most effective steps in reducing TSNA accumulation (Jack et al. 2011).

Although curing is the overriding factor in TSNA formation, several agronomic factors that occur in the field may also influence TSNA in cured leaf.

Nitrogen Fertility

It has been observed that nitrogen fertility of the soil can influence the accumulation of TSNA in tobacco. The amount of alkaloids and nitrate accumulated in the plant is influenced by the amount of nitrogen fertilizer used (Bush et al. 2001). An experiment conducted using differing rates of nitrogen fertilizers concluded that TSNA accumulation in green leaf samples (fresh tobacco) did not increase, but cured leaf samples had significant increases in TSNA accumulation with the highest rate of applied nitrogen fertilizer (Bokelman and Hempfling, 1999). Other studies investigated nitrate, nicotine, and TSNA accumulation as a result of increased nitrogen fertilization. Tobacco with high nitrogen application had higher nitrate, nicotine, and TSNA accumulation compared to tobacco that received a lower rate of nitrogen fertilizer application (Wahlberg et al, 1999). It also has been reported that increased soil incorporated nitrogen rate or foliar applied nitrogen did not result in significant increases in nitrogenous compounds within the leaf (Ritchey et al., 2014). Bailey (2014) reported that excessive nitrogen applications of 560 to 1,120 kg N ha^{-1} resulted in increased TSNA in two of six dark fire-cured experiments and two of three dark air-cured experiments. Caldwell et al. (2010) conducted a study using differing rates of nitrogen (112 kg ha^{-1},168 kg ha^{-1}, and 224 kg ha^{-1}) and found that reduced nitrogen application resulted in reduced TSNA content in cured leaf but also had a negative impact on yield and quality.

Maturity

Previous literature discussing the relationship between TSNA accumulation and tobacco plant maturity (senescence) is limited due to the innate complexity of this relationship. Nicotine accumulation reaches its maximum content when the tobacco plant reaches maturity (Bush and Crowe, 1989). Burton et al. (1989a) conducted a study to determine how senescence (maturity) influenced the accumulation of TSNA and nitrite using burley tobacco cured at two temperature/relative humidity conditions in curing chambers. This study did show that under normal curing conditions (24°C/70%RH), a rapid increase of TSNA accumulation took place during the first 14 days of air curing but no significant conclusions were drawn that linked maturity to TSNA content.

Formation of Tobacco-Specific Nitrosamines during Curing

It is known that nearly all of the formation of TSNA occurs during curing, specifically during the yellowing to early browning stage (Wiernik et al. 1995), although negligible amounts of TSNA can be found in green leaf (Peele et al. 2001, Bush et al. 2001, Shi et al. 2013). The main genetic trait involved in the formation of TSNA is the propensity of a variety to convert nicotine to nornicotine (Roton et al. 2005).

Under conditions where there are higher concentrations of nitrite, there were also corresponding higher concentrations of TSNA under an environment considered ideal for curing burley tobacco (Burton et al. 1989b). A study conducted by Burton et al. (1992) using dark air cured tobacco found that only a small amount of nitrate was converted to nitrite under normal air-curing conditions and that factors other than nitrate concentration influence nitrite accumulation. Rapid drying or desiccation of the leaf limits the formation of nitrite, which also reduces the formation of TSNA (Roton et al. 2005.)

Levels of TSNA's in the cured leaf have been inherently variable even within the same tobacco and same curing barn (Jack et al. 2013).

Curing environment affects Tobacco-Specific Nitrosamines

The air curing of dark tobacco generally occurs in a period of six to eight weeks which is comparable to burley tobacco (Bailey, 2006). It is known that curing environment may have the most significant impact on TSNA formation. In general, TSNA's are undetectable or at a very low level in fresh leaves before harvest, but are readily measurable after air-curing (Shi et al. 2013.) Temperature, relative humidity, and air flow are the environmental conditions that are believed to be the most important factors that influence the variability of TSNA within curing facilities.

Massey and Smiley (1974) concluded that the more favorable curing conditions in air cured burley tobacco depended on keeping average daily relative humidity between 65 – 70%. However, this humidity range tended to be associated with lighter "buff" colored burley tobacco that the market demanded in the 1960's and 1970's. Today's market generally demands darker cured leaf and so the optimum range for average daily humidity is now 70 to 75%. (R. C. Pearce, personal communication). This optimum relative humidity range would also apply to dark air-cured tobacco.

Relative humidity determines the rate of moisture loss by the tobacco plant (Tso, 1990). Relative humidity and temperature are factors of the curing environment that may affect the variability of TSNA content even within the same curing barn. Traditional air-curing conditions support the idea that high relative humidity during curing result in higher TSNA levels, while drier curing conditions result in tobacco with lower TSNA

and nitrite (Staaf et al. 2005.) Literature suggests that the critical period of TSNA formation during air curing can be defined as when the plant cell membranes break down due to the loss of moisture, therefore causing cell contents to become available to microorganisms (i.e. microbial reduction of nitrate to nitrite) that exist on or in the leaf of tobacco (Staaf et al 2005.) Staaf also concluded that this critical period of cell membrane breakdown can be shortened if this moisture loss from the tobacco leaf is rapid, or lengthened if the environmental conditions favor microbial growth. Curing conditions that promote rapid drying generally result in lower TSNA while curing conditions that promote slower moisture loss or drying generally result in higher TSNA.

Previous literature suggests that during the yellowing stage of dark air-cured tobacco the barn should be held at about 80% relative humidity and only ventilated enough to prevent house burn (Tso, 1990). Curing of mature tobacco at higher temperature and humidity (32°C/83%RH) led to a 400-fold increase in TSNA level (Wiernik et al, 1995.) Burton et al. (1989b) found a positive correlation between nitrite nitrogen and nitrosamines when tobacco is air-cured in a normal environment (24 °C/70% RH). Curing tobacco at higher temperature and humidity (32 °C/ 83% RH) dramatically increased the accumulation of individual nitrosamines and nitrite (Burton et al, 1989a.) Roton et al. (2005) concluded that microbial populations responsible for the formation of nitrite may grow in cured tobacco, and TSNA concentrations may continue to increase after curing if the leaves are kept hanging in the barn under humid conditions after the end of cuing. It is likely that the level of residual nitrite in cured tobacco and temperature play a major role in the reaction (Roton et al, 2005).

This project focuses on the influence of curing environment on TSNA formation, and how changes in microenvironments within the same barn may result changes in TSNA in cured leaf. The intention of this research was to attempt to correlate the variability in curing conditions to the variability in TSNA accumulation. This knowledge could enable growers to make targeted barn repairs or modifications that would stabilize conditions and lower the TSNA content in problem areas of curing barns.

The objectives of this study were:

1.) Evaluate variability in curing conditions within dark air-cured barns.

2.) Attempt to correlate changes in curing conditions with changes in nitrite and TSNA levels of cured leaf within dark air-cured barns.

Chapter 2

Materials and Methods

Research was conducted in 2012 and 2013 at the University of Kentucky Research and Education Center near Princeton KY and at the Kentucky Agricultural Experiment Station Spindletop Farm near Lexington KY to evaluate variability in curing conditions within dark air-cured barns and attempt to correlate changes in curing conditions with changes in nitrite and Tobacco-Specific Nitrosamine (TSNA) content in cured leaf of dark air-cured tobacco. The curing barn used at each location was a three-tiered design with tiers parallel to the length of the barn. Soil types at each location were Crider silt loam (fine-silty, mixed, active Typic Paleudalfs) at Princeton and Bluegrass-

Maury silt loam (fine-silty, mixed, active, mesic Typic Paleudalfs) at Lexington (USDA-NRSC, 2014). Irrigation at the Princeton location was used throughout the growing season in 2012.

TR Madole (TRsc) screened for low nicotine to nornicotine conversion and TR Madole high converter (TRHC), with greater propensity for high conversion of nicotine to nornicotine, were used in this experiment. Approximately 4500 plants (750 sticks of tobacco) were grown at each location, with 2250 plants (375 sticks of tobacco) of each variety. Transplants were grown using current University of Kentucky recommendations (Pearce et al. 2013). Tobacco plants were transplanted to the field in Princeton on May 31, 2012 and June 4, 2013 and in Lexington on June 5, 2012 and May 29, 2013. Field management at each location followed current University of Kentucky recommendations. At Princeton, nitrogen was applied at 336 kg N ha^{-1} with 224 kg N ha^{-1} broadcast prior to transplanting and 112 kg N ha^{-1} sidedressed four weeks after transplanting. Urea (46-0-0) was used as the nitrogen source for broadcast and UAN (32% N liquid) was the nitrogen source used for sidedressing at Princeton. At Lexington, nitrogen was applied at 308 kg N ha^{-1} with 168 kg N ha^{-1} broadcast prior to transplanting and 140 kg N ha^{-1} sidedressed four weeks after transplanting. Urea (46-0-0) was used as the nitrogen source for broadcast and ammonium nitrate (34-0-0) was the nitrogen source used for sidedressing at Lexington. Phosphorus and potassium were applied broadcast prior to transplanting following soil test recommendations at each location. Tobacco was topped at bud-early bloom stage to 16-18 usable leaves. A manual stalk rundown application of fatty alcohol and butralin was used to control suckers. Harvest took place on September 28, 2012 and September 5, 2013 in Princeton and on August 20, 2012 and August 21, 2013 in

Lexington. Both varieties were stalk harvested, allowed to adequately field wilt, and then six plants were placed evenly on each stick. Replicated soil and green leaf samples were taken prior to harvest at each location. Six soil samples were collected from the area in each field where plants were grown (three from the TR area and three from the TR HC area) and analyzed for nitrate. Six green leaf samples (three from TR and three from TR HC) were collected and analyzed for nitrite and TSNA content according to the methods used by Morgan et al. Each green leaf sample contained 20 leaves from the 4th leaf position from 20 different plants.

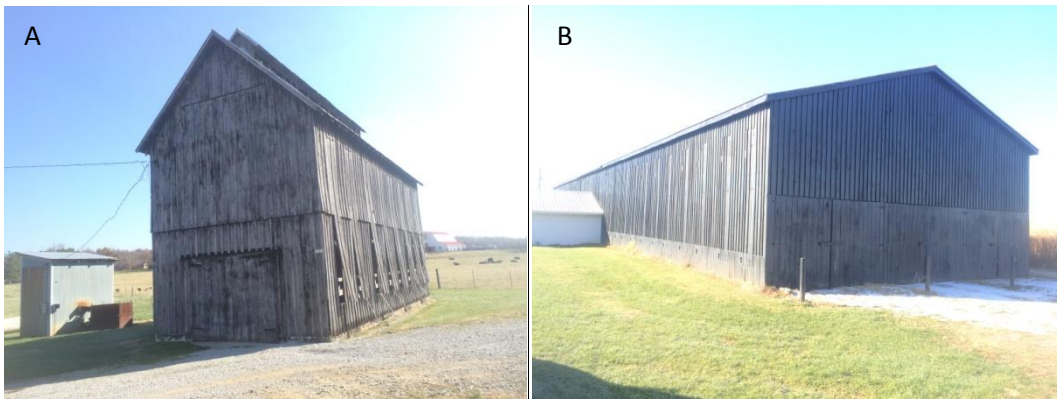


Figure 1.1 Differences in barn dimensions. A) Princeton barn B) Lexington Barn.

27 HOBO® data loggers (Onset Computer Corporation, Bourne, MA) were placed in each curing barn as tobacco was housed. Differences in barn dimensions are shown in Figure 1.1. The Princeton barn was only 5 tiers wide and this study occupied the entire barn whereas in the Lexington barn only the Northeast corner was used and other tobacco was cured to fill the barn. Each barn was a three-tiered design with five rooms used in the experiment as demonstrated in Figure 1.2.

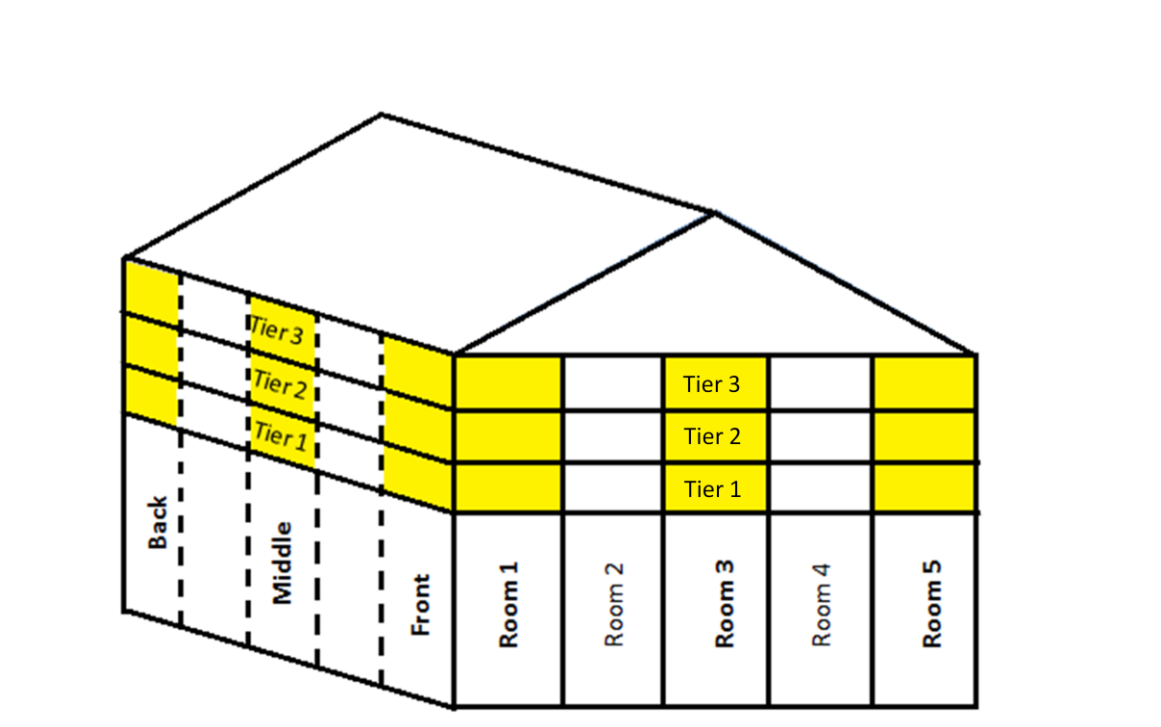


Figure 1.2 Diagram of long-tier orientation barn demonstrating the 3-Dimensional area that was studied.

All data loggers were positioned vertically on each tier at three locations across the width of each barn (left side room 1, center room 3, and right side room 5), and three locations down the length of the barn (front bent, middle bent, and back bent) as represented in Figure 1.2. (3 locations x 9 loggers at each location = 27 data loggers). Figure 1.3 illustrates tobacco housing and meter placement scheme within each room. At the time of tobacco housing, each data logger was launched to collect temperature and relative humidity data every hour for the entire curing season. Ambient temperature and relative humidity data were collected from outside of the barns using a single data logger outside each barn backed up by data from a permanent field weather station nearby.

Housing and Meter Placement in Barns

Room 1	Tier 3 (top)		5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC
	Tier 2 (middle)		5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC
	Tier 1 (bottom)		5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC	5 TRsc	5 TRHC	5 TRsc ★	5 TRHC

★ = Placement of HOBO meter (27 meters per barn).

Figure1.3. Housing scheme showing the placement of data loggers and tobacco varieties in each room of the three sampled rooms within the barns.

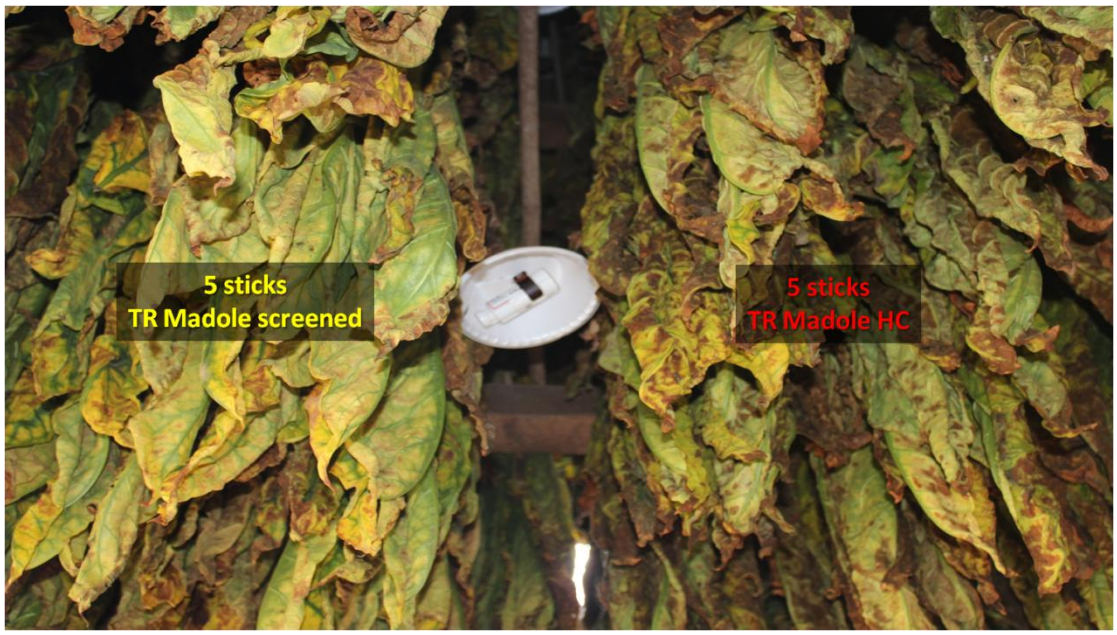


Figure 1.4. Data logger placement within TR Madole screened and TR Madole HC.

Stick spacing used at housing of each barn was approximately 30 cm between sticks. Tobacco was housed in each barn by alternating 5 sticks screened TR Madole followed by 5 sticks TR Madole HC so that 10 sticks will be allocated as a set for each of

the 27 monitoring and sampling locations. Tobacco between each monitoring and sampling location within the barns was also placed with 5 sticks alternations of screened TR Madole and TR Madole HC. Sticks for sampling were tagged and housed in the designated monitoring locations. Each data logger was placed between the 5 sticks of screened TR Madole and the 5 sticks TR Madole HC at each location (Fig. 1.3). Loggers were placed at approximately the same level as the 4th leaf on plants. After curing, all data loggers were taken down with the tobacco and downloaded. Leaf samples were collected from the screened TR Madole and TR Madole HC tobacco on each side of each data logger, totaling 54 leaf samples collected for nitrite and TSNA analysis from each barn (27 samples screened TR Madole and 27 samples TR Madole HC). Each sample consisted of 20 leaves, which were taken from the 4th leaf from the top of 20 different plants in each 5 stick segment. If the 4th leaf was absent, that plant was not included in the sample. Leaves were only collected from the center 4 plants on each stick and were not collected from the outside plants on each stick. Samples were then freeze dried, ground to 1mm, and sent to be analyzed for nitrite and TSNA content.

All leaf samples were analyzed at the University of Kentucky Tobacco Analytical Laboratory located at the Kentucky Tobacco Research and Development Center. The TSNA analysis method followed the method used by Morgan et al. (2004) with use of a Gas Chromatography- Thermal Energy Analyzer (GC-TEA). Nitrate and Nitrite contents were analyzed using the method developed by Crutchfield and Grove (2011) at the University of Kentucky.

Data were analyzed using Statistical Analysis Software (SAS, Cary NC) version 9.3. The experiment was a completely randomized design (CRD). CRD was chosen

because we wanted to determine the environmental effects on placement of data loggers within each barn. The main effects of year, location, data logger placement, tier, bent, and room were treated as qualitative variables. Total TSNA content, number of hours above 80% relative humidity, number of hours above 24°C temperature, and leaf nitrite were treated as quantitative variables. Total TSNA content was determined by the summation of NNN, NAB, NAT, and NNK for each sample. Before analysis, data were checked to determine if the basic assumptions of Analysis of Variance (ANOVA) were met. As expected, variety was significantly different thus varieties were analyzed separately. PROC GLIMMIX was used as the statistical model to develop an ANOVA table and means were separated using least squares (LS)-means procedure at alpha of <0.05. PROC REG was used for regression analysis.

Chapter 3

Results and Discussion

3.1 Weather for Entire Crop Season and Soil Data Overview

There were dramatic weather differences between the 2012 and 2013 production seasons. 2012 was warmer and drier compared to 2013 throughout the growing season for Princeton (Figure 3.1.1). Average monthly temperatures for June through November were relatively close for both years except for July, which was much cooler in 2013. The entire 2013 growing and curing season had more rainfall than 2012, especially earlier in the growing season (June and July). Temperature and rainfall throughout the curing season for both years were similar at Princeton, with 2013 slightly higher.

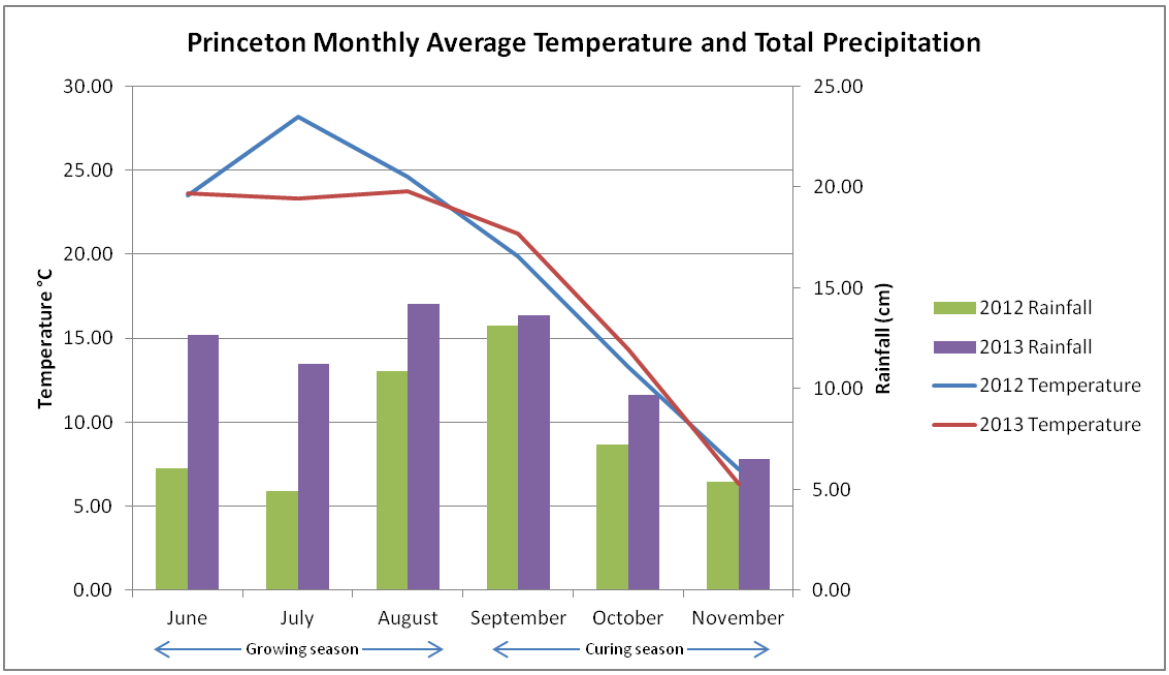


Figure 3.1.1 Princeton monthly average temperature and total monthly rainfall (cm).

The monthly average temperature and total monthly rainfall in Lexington was similar to Princeton for both years with a warmer, drier growing season in 2012, as shown in Figure 3.1.2. Average monthly temperatures were higher for June and July at Lexington in 2012 compared to 2013. In 2012, highest total rainfall was in May, July, and September, while higher total rainfall occurred at Lexington from June through August compared to the other months in 2013. Figures 3.1.1 and 3.1.2 illustrate large differences in rainfall between 2012 and 2013 for June, July, and August at either location, with much higher rainfall occurring during these months in 2013.

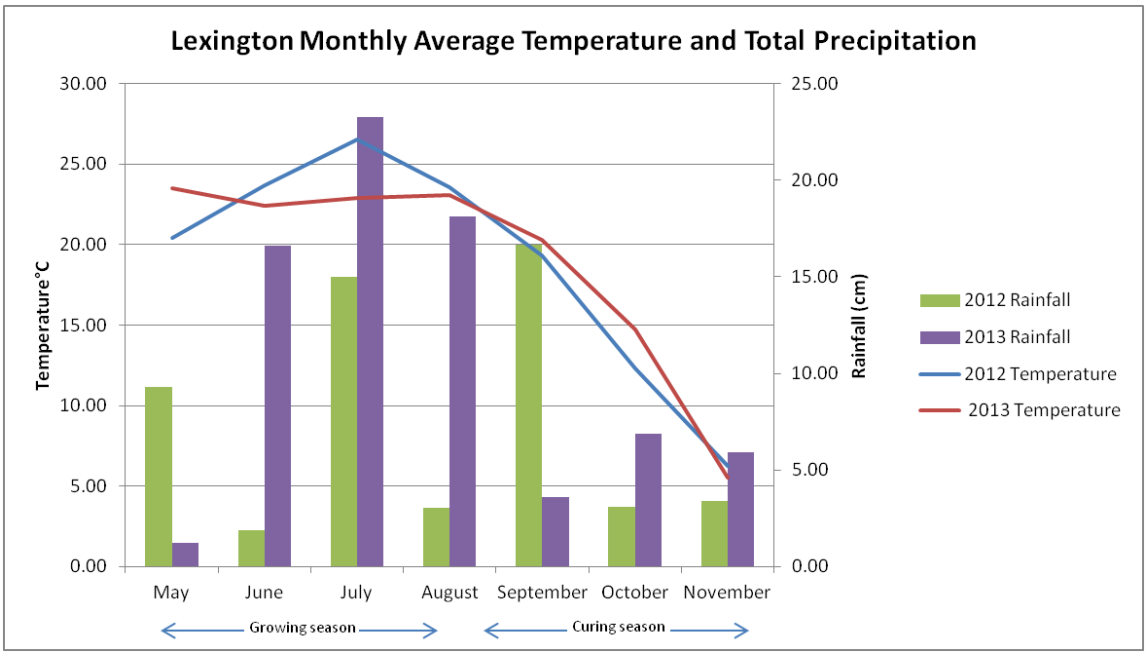
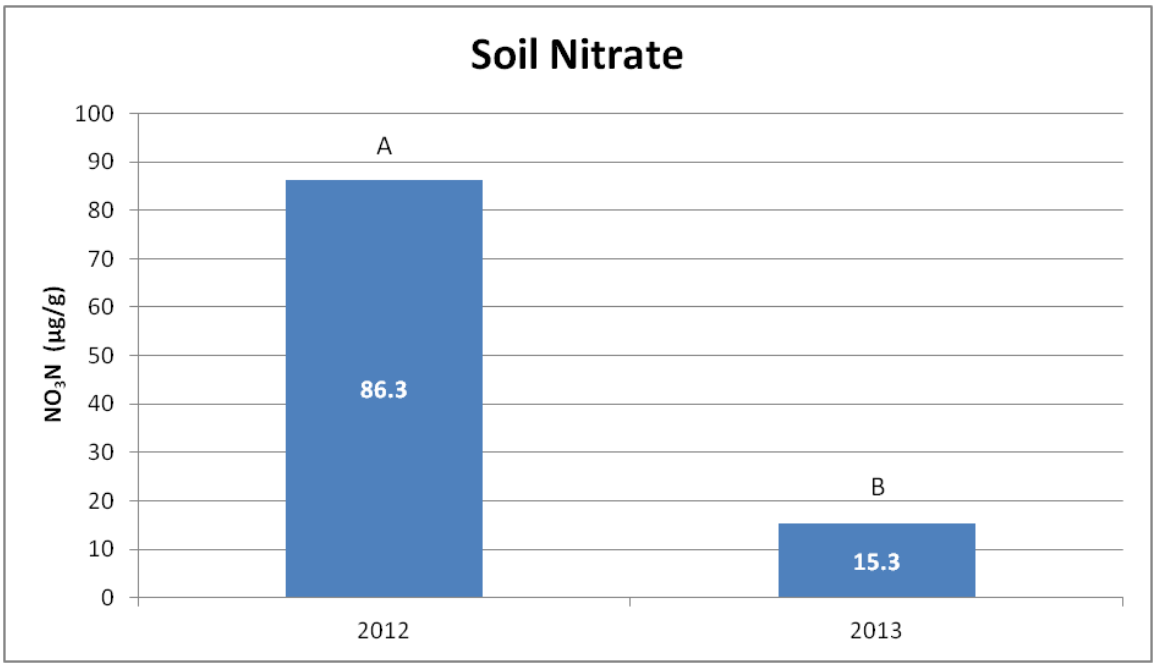


Figure 3.1.2 Lexington monthly average temperature and total monthly rainfall (cm).

Figure 3.1.3 and 3.1.4 show the soil nitrate and ammonium levels from soil samples that were taken on the day of harvest at both locations, in each year from soil where each variety was grown. The ANOVA for soil nitrate (NO_3 N) is shown in Table 3.1.1. There was a significant main effect of year but no other significant main effects or interactions. Soil NO_3 N was much higher in 2012 when compared to 2013, as shown in Figure 3.1.3. This could be explained by the negligible interaction that nitrate has with the negatively charged topsoil, which is why nitrate is very mobile in the soil (Lehmann and Schroth, 2003, Brady and Well, 2008.), and leaches more readily through the soil profile during periods of increased rainfall. The ANOVA for soil ammonium is shown in Table 3.1.2. There was a year by location interaction. There were no significant differences between locations in 2013. Soil ammonium (NH_4N) was significantly higher in Lexington for 2012 at the field site when averaged over variety, as shown in Figure 3.1.4.

Soil Nitrate		
Source of Variation	**F Value**	**Pr>F**
Year	60.72	<.0001
Location	0.02	0.8988
Year*Location	0.59	0.4552
Variety	1.91	0.1857
Year*Variety	1.51	0.2375
Location*Variety	0.21	0.6549
Year*Location*Variety	0.30	0.5919

Table 3.1.1. Analysis of Variance for soil nitrate.

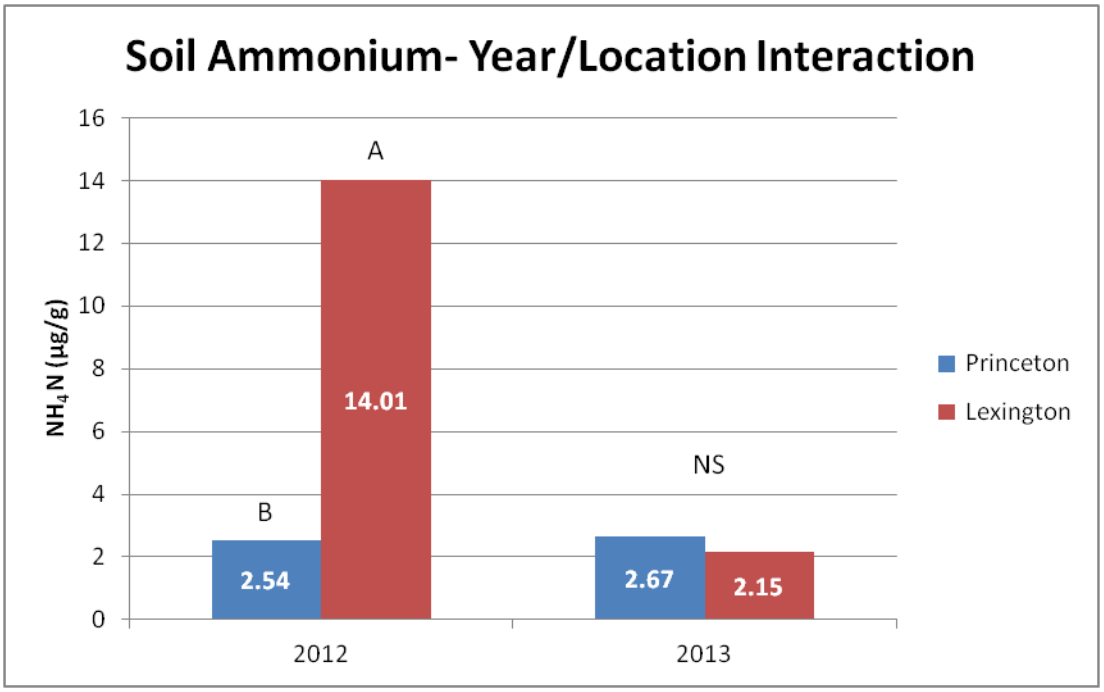


*Means within year and location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.1.3. Main effect of year on soil nitrate (NO_3 N) averaged over location and variety.

Soil Ammonium		
Source of Variation	**F Value**	**Pr>F**
Year	10.24	0.0056
Location	8.93	0.0087
Year*Location	10.69	0.0048
Variety	0.23	0.6352
Year*Variety	0.05	0.8328
Location*Variety	0.15	0.6997
Year*Location*Variety	0.63	0.4385

Table 3.1.2. Analysis of Variance for soil ammonium.



*Means within year and location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05 NS = not significant

Figure 3.1.4. The year by location interaction for soil Ammonium (NH4 N) when averaged over variety.

There were several interactions observed for count of the number of hours above 24°C temperature, count of the number of hours above 80% relative humidity, TR Madole high converter (TRHC) and TR Madole screened (TRsc) total TSNA and leaf nitrite, as shown in Table 3.1.3.

Factor	Temperature	RH	TRHC		TRsc	
			TSNA	Leaf Nitrite	TSNA	Leaf Nitrite
Year	X	X	X	X	X	
Location	X	X			X	
Year*Location	X	X	X	X	X	
Tier	X	X				
Year*Tier	X					
Location*Tier	X	X	X		X	X
Year*Location*Tier	X					
Room	X	X	X	X	X	
Year*Room		X	X	X	X	
Location*Room	X	X			X	
Year*Location*Room				X		
Bent	X	X	X			
Year*Bent	X	X				
Location*Bent	X	X	X			
Year*Location*Bent	X		X			

Table 3.1.3. All significant interactions for within barn curing environment, total TSNA, and leaf nitrite. (X) indicates significance at $P<0.05$.

3.2 Temperature within Curing Barns

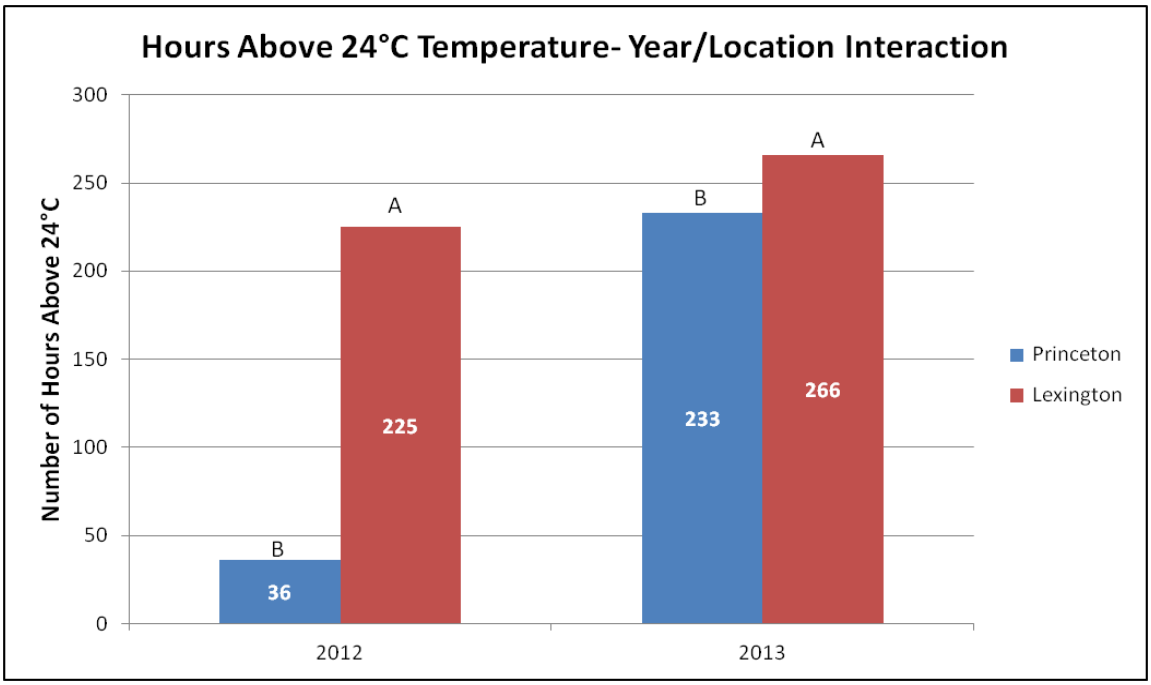
There was no significant effect of individual data logger placement on temperature; therefore, we investigated the 3-dimensional aspects of the tier (bottom, middle, top), room (left, center, right), and bent (front, middle, back) within each barn. All within-barn temperature data are presented as a cumulative count of the number of hours above 24°C for the entire duration of the cure. The proceeding data were analyzed with hours of temperature above 24°C as a dependent variable and year, location, tier, room, and bent as independent variables to determine temperature behavior within barns. There was an overall year by location interaction for temperature data. All temperature data except room data are shown by year and location because there were significant year by location by tier and year by location by bent interactions. Temperature data for room

are shown by location due to significant location by room interaction (Table 3.2.1.). Mean comparisons are shown at the 0.05 level of significance.

Temperature within Barns		
Source of Variation	**F Value**	**Pr>F**
Year	11409.10	<.0001
Location	9900.11	<.0001
Year*Location	4827.80	<.0001
Tier	592.01	<.0001
Year*Tier	140.51	<.0001
Location*Tier	135.43	<.0001
Year*Location*Tier	6.54	0.0018
Room	52.75	<.0001
Year*Room	0.35	0.7043
Location*Room	76.37	<.0001
Year*Location*Room	0.76	0.4682
Bent	51.06	<.0001
Year*Bent	3.88	0.0224
Location*Bent	87.23	<.0001
Year*Location*Bent	20.40	<.0001

Table 3.2.1. Analysis of Variance for Temperature.

The year by location interaction for temperature is shown in Figure 3.2.1. There was a location interaction within each year. In 2012 and 2013, Lexington had more hours above 24°C than Princeton, 225 to 36 and 266 to 233, respectively. Princeton had more hours above 24 C in 2013 compared to 2012, which could be a result of differences in the time of year that curing took place. The 2012 curing season began when tobacco was housed on September 28, 2012 at Princeton compared to August 20, 2012 at Lexington. Housing dates were more similar between locations in 2013.

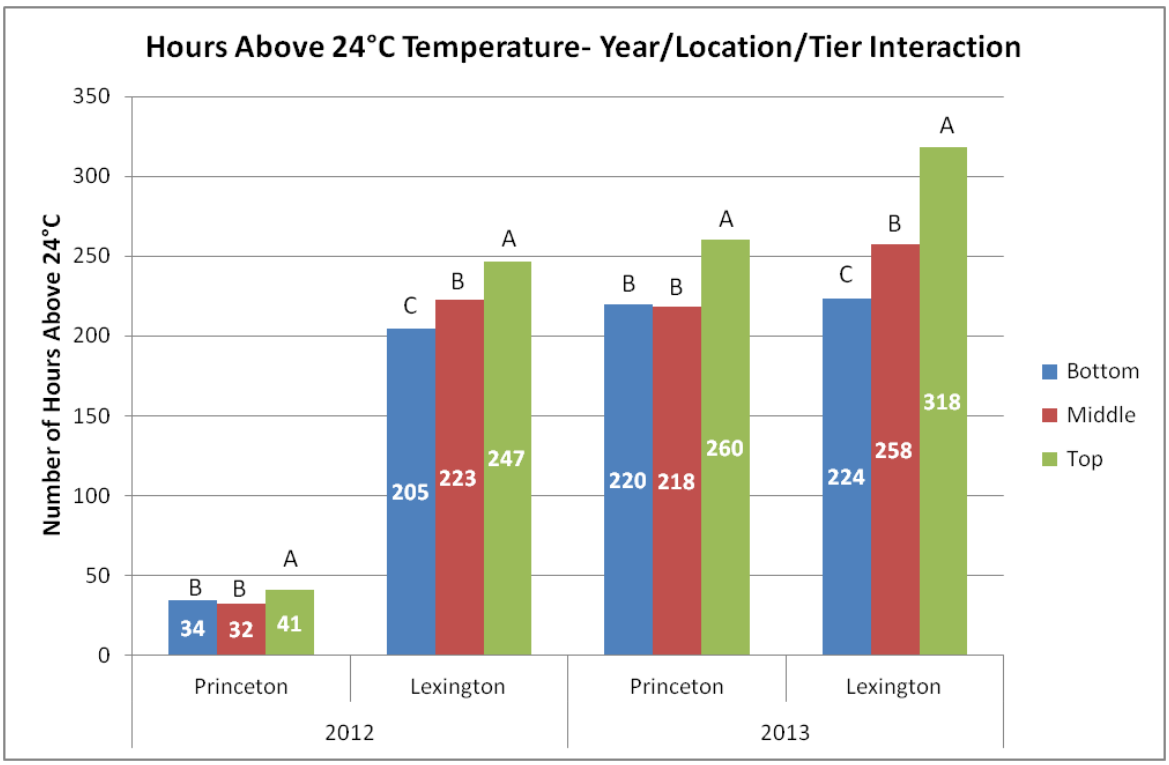


*Means within year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.2.1. Year by location interaction hours above 24°C temperature.

Three-way interactions with temperature

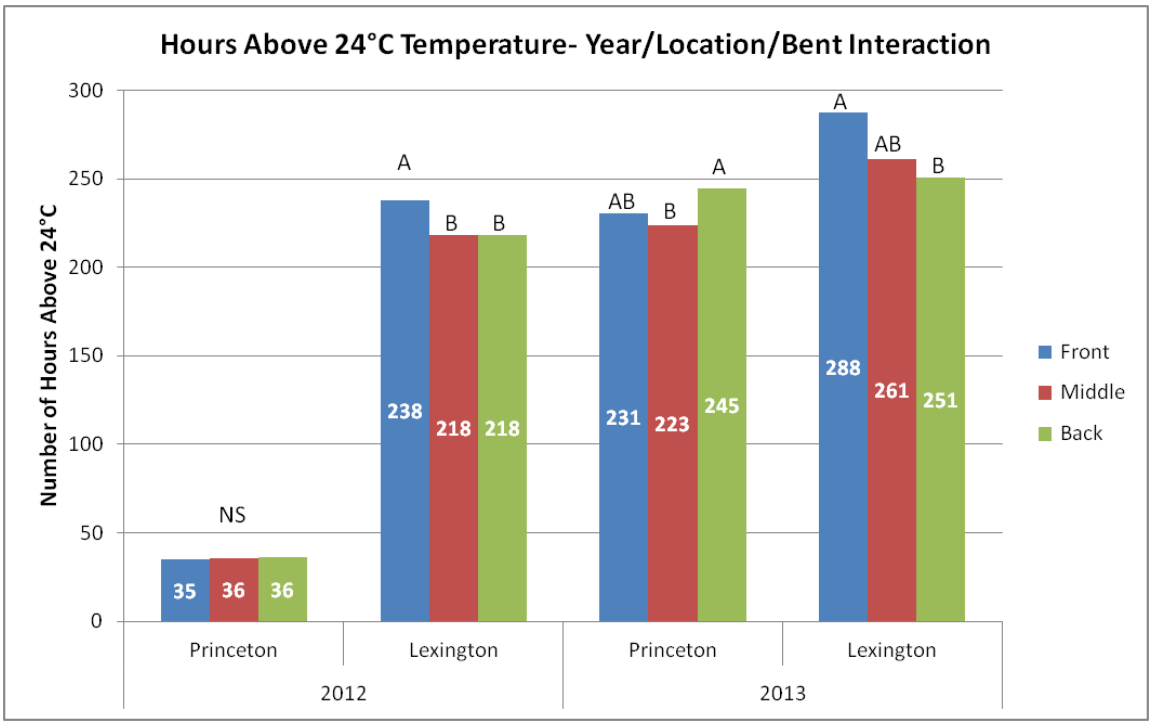
Figure 3.2.2 shows the tier effects on temperature for both locations within each year. It is clear that the top tier of the barn at each location within each year had higher temperature than the middle and the bottom tiers. Temperature was significantly different between all tiers at Lexington each year, with temperature increasing from the bottom tier to the top tier. The bottom and middle tiers had similar temperature at Princeton each year.



*Means within a year and location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.2.2. Year by location by tier interaction hours above 24°C temperature.

The year by location by bent interaction for temperature is shown in Figure 3.2.3. There were no differences in temperature between bents at the Princeton barn in 2012. In 2013 at Princeton, highest temperatures were in the back bent. Lexington followed the same trend for both years with the front bent of the barn having significantly higher hours above 24°C.



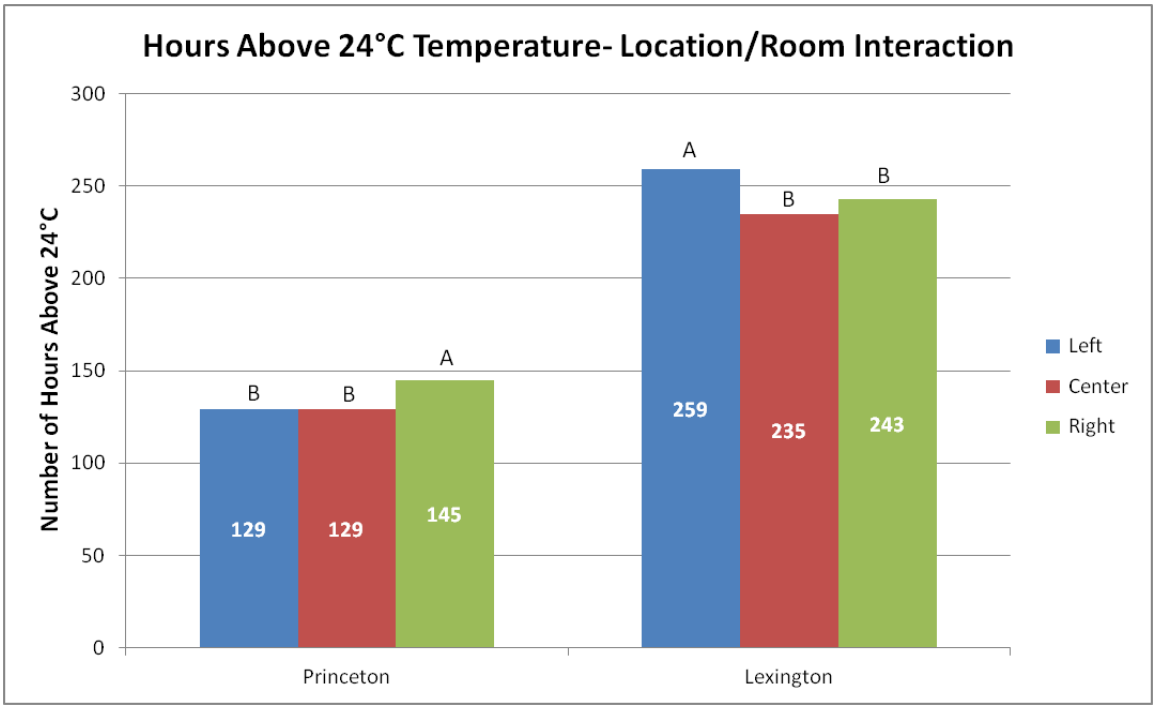
*Means within year and location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05. NS = not significant

Figure 3.2.3. Year by location by bent interaction hours above 24°C temperature.

Location effects on temperature

Figure 3.2.4. shows significant temperature differences between rooms of each barn when averaged over year by location. Differences in effects between barns were to be expected, as these barns were very different in size and around 320 km apart. These barns were different in directional orientation with North-South orientation in Lexington and East-West orientation in Princeton. The barn in Princeton was smaller and only held tobacco from this experiment whereas tobacco from this experiment only occupied the Northeast corner of the Lexington barn. In Lexington, the left room had more hours above 24°C than the center and right rooms. The left room faced the east and was the only room that was adjacent to a wall of the barn. The opposite was observed in Princeton with more hours above 24°C occurring within the right room when compared

to the center and left rooms. The right room at Princeton was on the Southwest side of the barn.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.2.4. Location by room interaction hours above 24°C.

3.3 Relative Humidity during the Curing Season

There was no significant effect of individual data logger placement on relative humidity; therefore, we investigated the 3-dimensional factors of the tier, room, and bent within the barns. All relative humidity data are presented as a cumulative count of the number of hours above 80% relative humidity for the entire duration of the cure. The proceeding data were analyzed with hours of relative humidity greater than 80% as the dependent variable and year, location, tier, room, and bent as independent variables to determine relative humidity behavior within barns. There was an overall year by location interaction for relative humidity data. Within 3-dimensional barn effects, tier data are

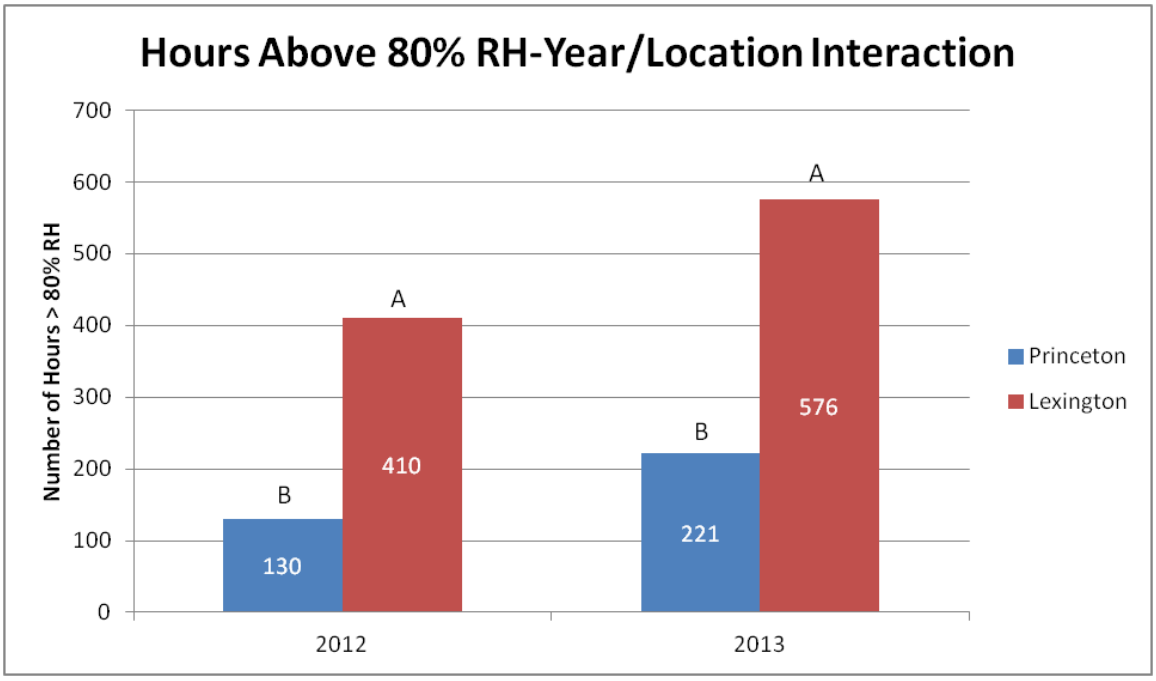
shown by location due to a significant location by tier interaction. Room data are shown by year and location due to significant year by room and location by room interactions. Bent data are shown by year and by location due to significant year by bent and location by bent interactions (Table 3.3.1).

Relative Humidity		
Source of Variation	**F Value**	**Pr>F**
Year	86.19	<.0001
Location	514.54	<.0001
Year*Location	7.89	0.0055
Tier	2.89	0.0580
Year*Tier	0.97	0.3822
Location*Tier	5.83	0.0035
Year*Location*Tier	0.81	0.4463
Room	26.72	<.0001
Year*Room	4.19	0.0167
Location*Room	2.89	0.0581
Year*Location*Room	0.98	0.3777
Bent	14.85	<.0001
Year*Bent	6.75	0.0015
Location*Bent	4.83	0.0090
Year*Location*Bent	0.93	0.3945

Table 3.3.1. Analysis of Variance for relative humidity.

The interaction between year and location for hours above 80% relative humidity is shown in Figure 3.3.1. There was a significant difference between locations within each year. Lexington had significantly higher relative humidity than Princeton each year, with 410 hours compared to 130 hours in 2012, and 576 hours compared to 221 hours in 2013. Each location had more hours above 80% relative humidity in 2013 when compared to 2012, which is likely related to the higher rainfall in October and November

in 2013. The year by location interactions for relative humidity followed the same trend as the year by location interactions for temperature (Figure 3.2.1).

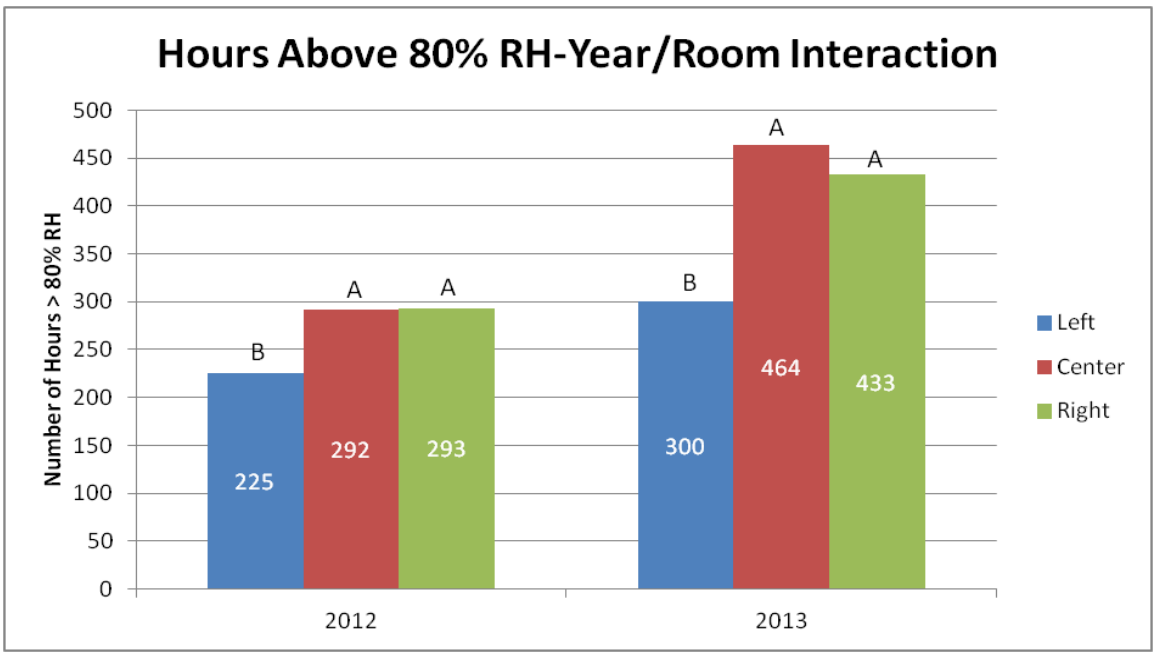


*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.3.1. Year by location interaction hours above 80% relative humidity.

Year effects on relative humidity.

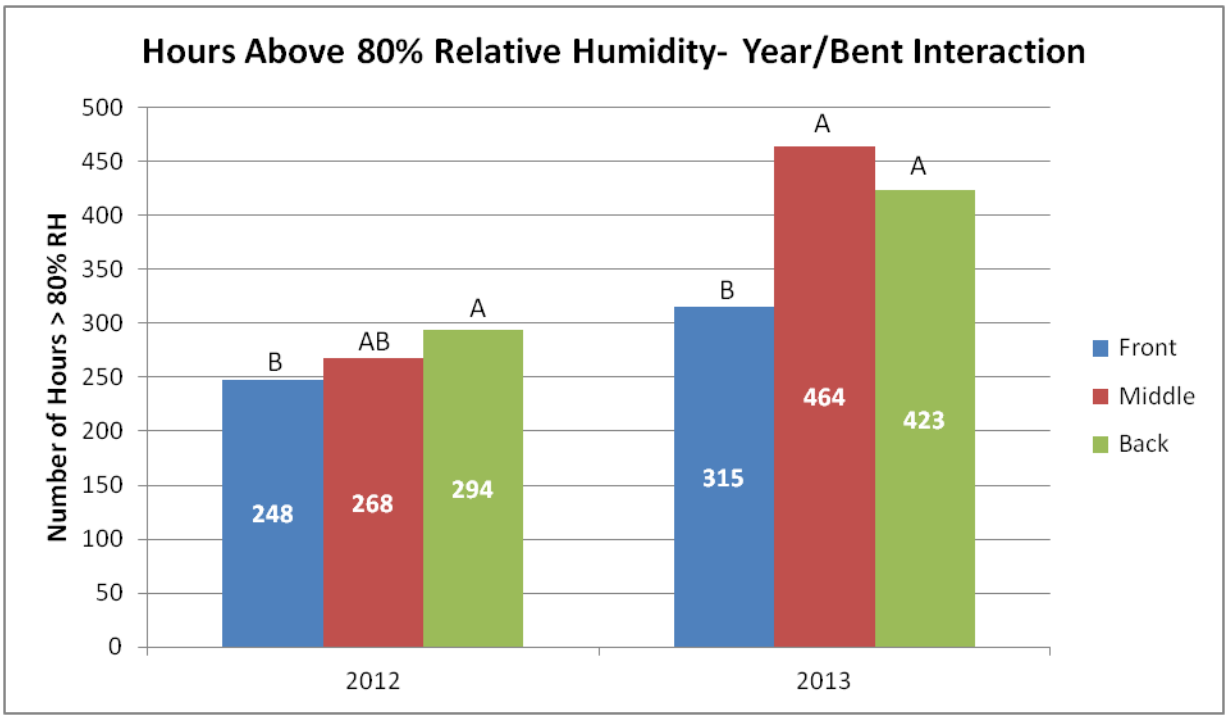
The year by room interaction is shown in Figure 3.3.2. The right and center rooms were significantly higher than the left room when averaged over location in each year.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.3.2. Year by room interaction hours above 80% relative humidity.

The year by bent interaction averaged over location is shown in Figure 3.3.3. In 2012, the back bent was significantly higher than the front bent of the barns with the middle bent being not significantly different from either. In 2013, the middle and back bents were significantly higher than the front bent. The back bents of the barns had significantly more hours above 80% relative humidity than the front bents each year. The back bent at Princeton was on the East end of the barn. The front bent of Lexington was on the wall at the North end of the barn.

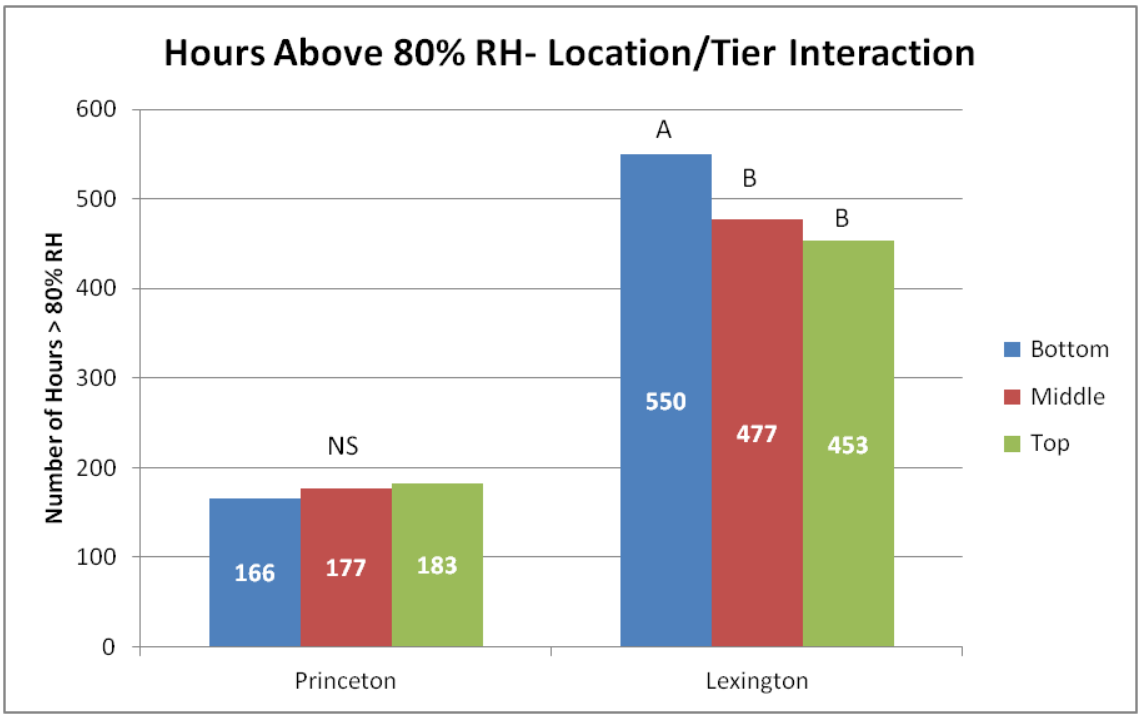


*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.3.3. Hours above 80% relative humidity year by tier interaction.

Location effects on relative humidity.

The location by tier interaction for hours above 80% relative humidity averaged over year is shown in Figure 3.3.4. Numerically, the Princeton barn had higher relative humidity in the top of the barn, but this difference was not significant. The Lexington barn had significantly higher relative humidity in the bottom of the barn when compared to the middle and top. A possible explanation for location differences may be related to differences in the floor of each barn. The Princeton barn has a concrete floor whereas the Lexington barn has a dirt/gravel floor.

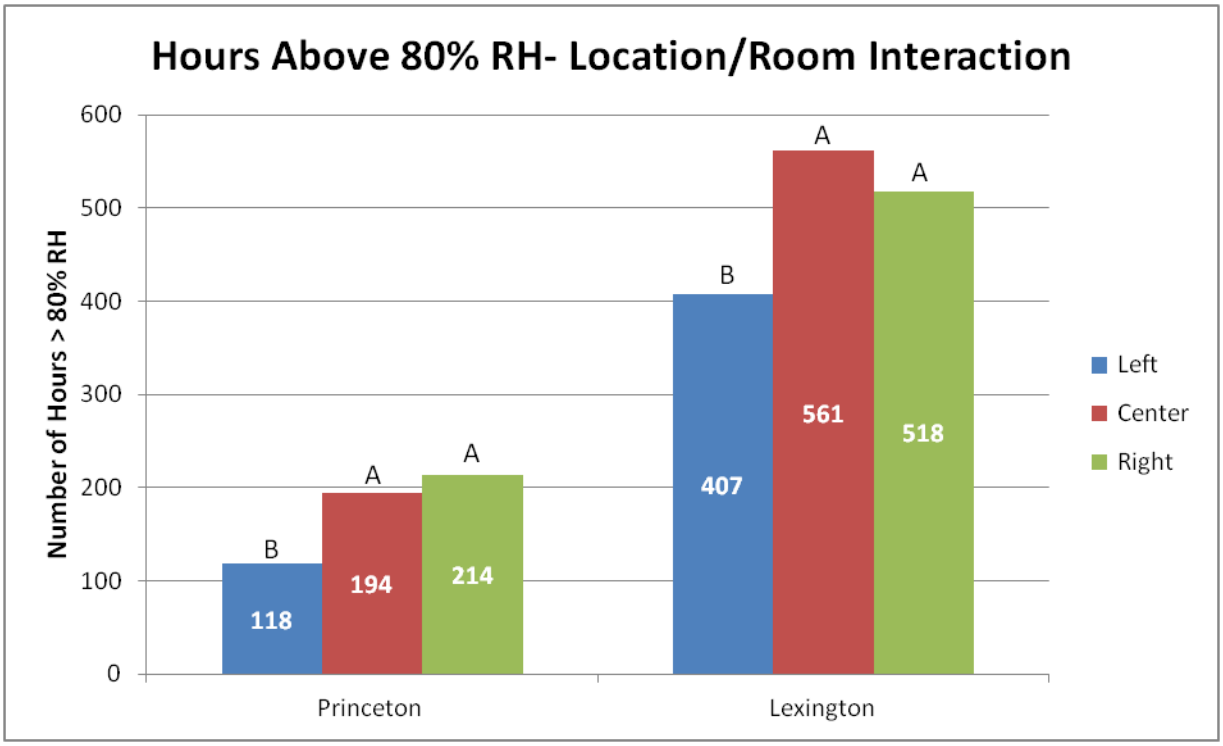


*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05, NS = no significant difference.

Figure 3.3.4. Hours above 80% relative humidity location by tier interaction.

The interaction between location and room for hours above 80% relative humidity is shown in Figure 3.3.5. There was a significant interaction between location and room when averaged over year. Overall, relative humidity was higher in Lexington than in Princeton when averaged over year, although this difference could be related to differences in harvest and housing dates between locations in 2012. Between rooms over years, the center room and right room of each location was significantly higher than the left room of each barn. As previously stated, the barn at Lexington was much larger than the barn at Princeton. The left and right rooms in the Princeton barn were next to the exterior walls whereas only the left room bordered an exterior wall in the Lexington barn. The right room in the Lexington barn was near the center of the barn which could also be

related to the significantly higher number of hours above 80% within the Lexington barn compared to the Princeton barn.

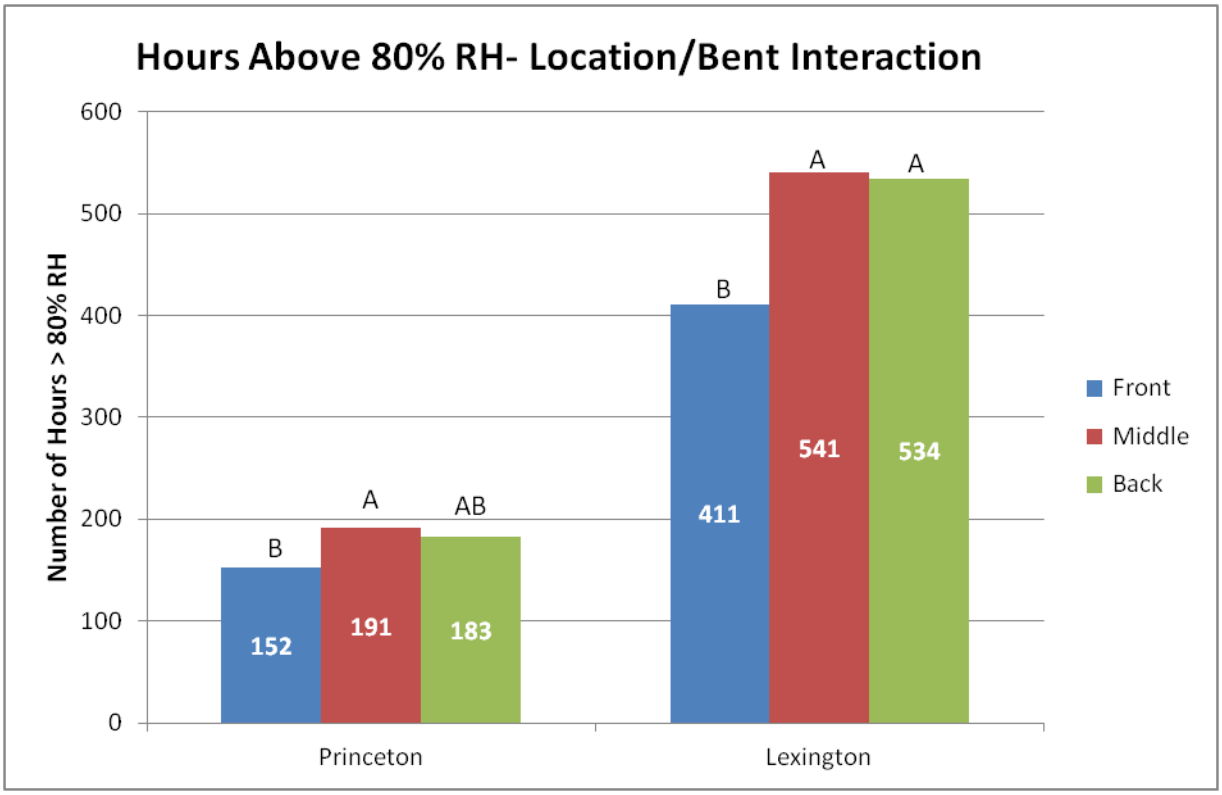


*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.3.5. Hours above 80% relative humidity location by room interaction.

The location by bent interaction when averaged over year is shown in Figure 3.3.6. Each barn location had more hours of relative humidity greater than 80% in the middle bent when comparing to the front bent. The back bent of the Princeton barn was not significantly different from the middle or front bent, however, the back bent was significantly higher than the front in Lexington. The back bent in Princeton was at the east end of the barn and the back bent at Lexington was near the middle of the barn. This could explain why the middle and back bents were significantly higher than the front bent

in Lexington. The front bent was on the North end of the Lexington barn and was the only bent that was exposed to an external wall on two sides.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.3.6. Hours above 80% relative humidity location by bent interaction.

3.4 Tobacco Specific Nitrosamine Content

Tobacco Specific Nitrosamine (TSNA) data are presented as total TSNA, which is the sum of all individual TSNAs (NNN, NAT, NAB, NNK). TR-Madole High Converter (TRHC) data are presented separately from TR-Madole screened (TRsc) because of the significant differences between varieties, as expected. Total TSNA are presented in $\mu g\ g^{-1}$. There was not a significant effect of individual data logger placement on either variety used in this experiment; therefore, we investigated the 3-dimensional aspects of

the tier, room, and bent within each barn as was done with temperature and relative humidity data. The proceeding data were analyzed with TRHC or TRsc as dependent variables and year, location, tier, room, and bent as independent variables to determine how TSNA content varied within barns. Mean comparisons are shown at the 0.05 level of significance. Simple linear regression was also used to model the relationship of TSNA and hours above 24°C, hours above 80% relative humidity, and leaf nitrite.

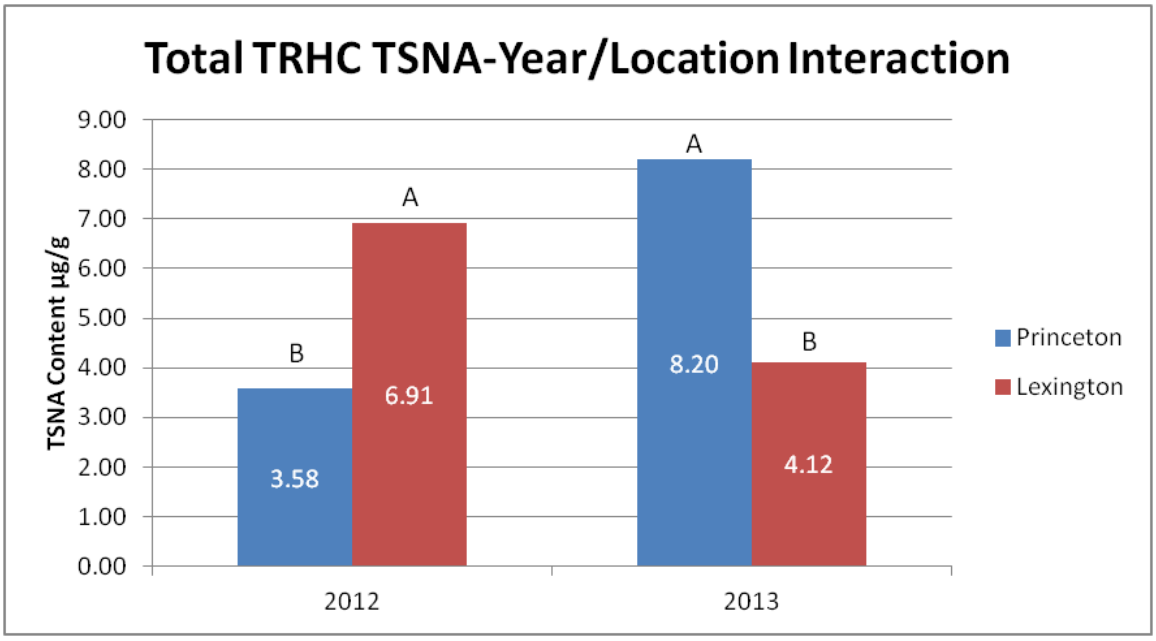
TRHC total TSNA

There was an overall year by location interaction for TRHC total TSNA data. Within 3-dimensional barn effects, tier data are shown by location due to a significant location by tier interaction. Room data are presented by year due to a significant year by room interaction. Bent data are presented by year and by location due to a significant year by location by bent interaction (Table 3.4.1).

TRHC Total TSNA		
Source of Variation	**F Value**	**Pr>F**
Year	9.95	0.0023
Location	1.49	0.2257
Year*Location	173.7	<.0001
Tier	1.56	0.2157
Year*Tier	0.02	0.9757
Location*Tier	9.82	0.0002
Year*Location*Tier	0.69	0.5041
Room	6.66	0.0021
Year*Room	6.52	0.0024
Location*Room	0.39	0.6802
Year*Location*Room	0.04	0.9618
Bent	3.33	0.0408
Year*Bent	2.61	0.0800
Location*Bent	6.04	0.0036
Year*Location*Bent	4.95	0.0094

Table 3.4.1. Analysis of Variance for total TSNA in TRHC.

There was a year by location interaction for TRHC, as presented in Figure 3.4.1. Each location was significantly different within 2012 and 2013. The Lexington barn had approximately twice the TSNA accumulation that the Princeton barn had in 2012. The opposite effect was observed in 2013, with the Princeton barn having approximately twice the TSNA content that the Lexington barn had. The opposing differences in locations within each year is not well explained with the temperature above 24°C or relative humidity greater than 80% data, as hours above 24°C and 80% relative humidity were higher in the Lexington barn each year. There was a positive correlation between TSNA and hours above 80% relative humidity as shown in the linear regression in Figure 3.4.2. Differences in timing of harvest between locations in 2012 may have contributed to this positive correlation. In 2013, this was a negative correlation (Figure 3.4.3) as Lexington had lower TRHC TSNA content but still had higher relative humidity. Before the temperature data were analyzed, our preliminary thoughts were that temperature may have been lower during curing in 2013, possibly overriding the effect of increased relative humidity. However, the temperature data did not support this.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.4.1. Total TRHC TSNA content year by location interaction.

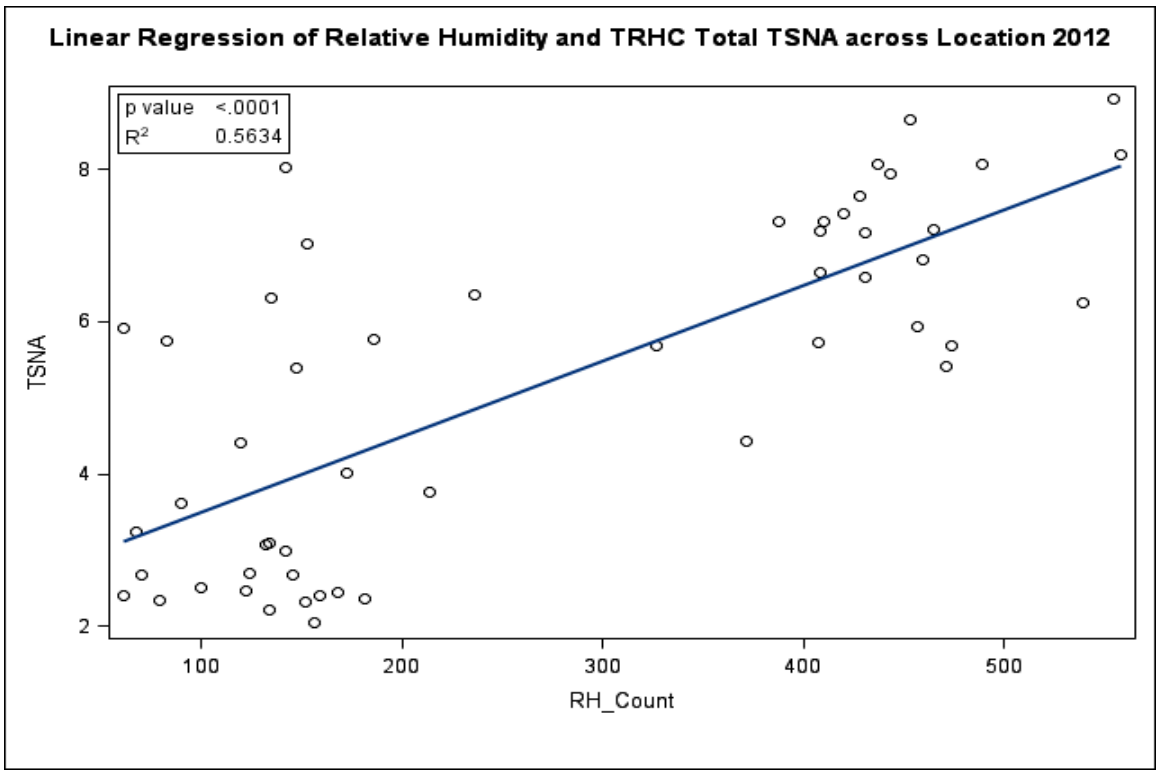


Figure 3.4.2. Simple Linear Regression of total TRHC TSNA and relative humidity within 2012.

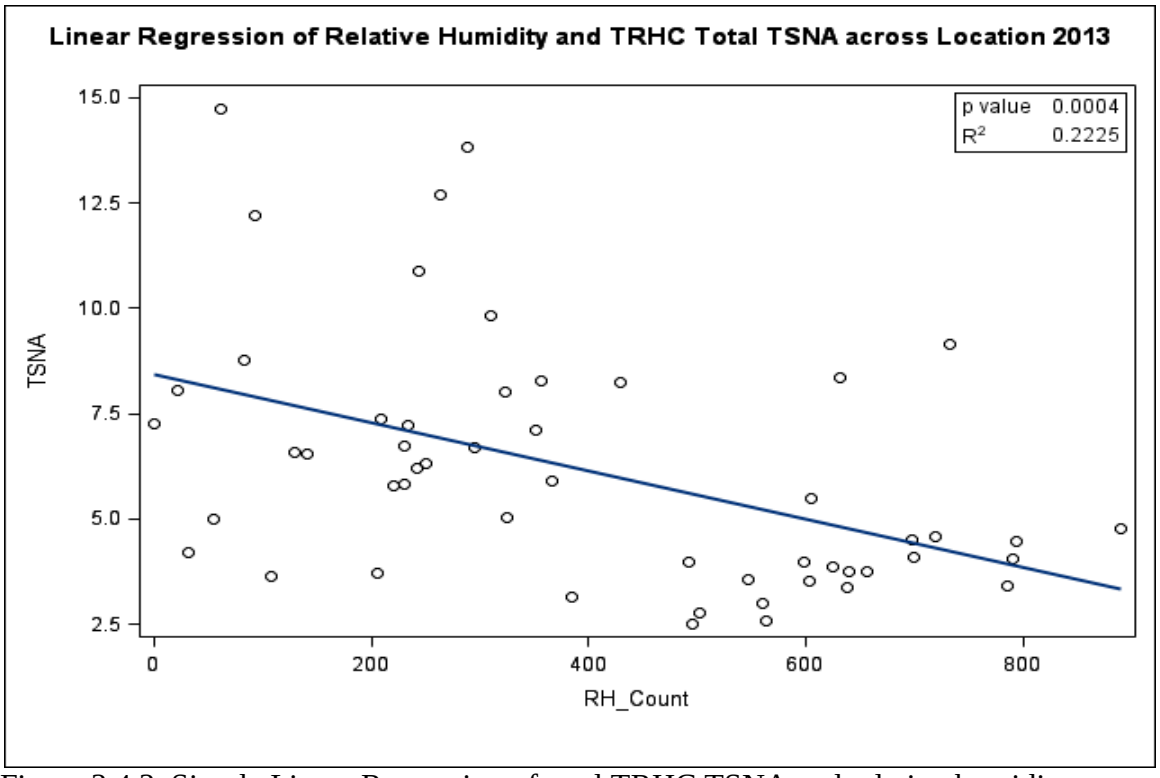
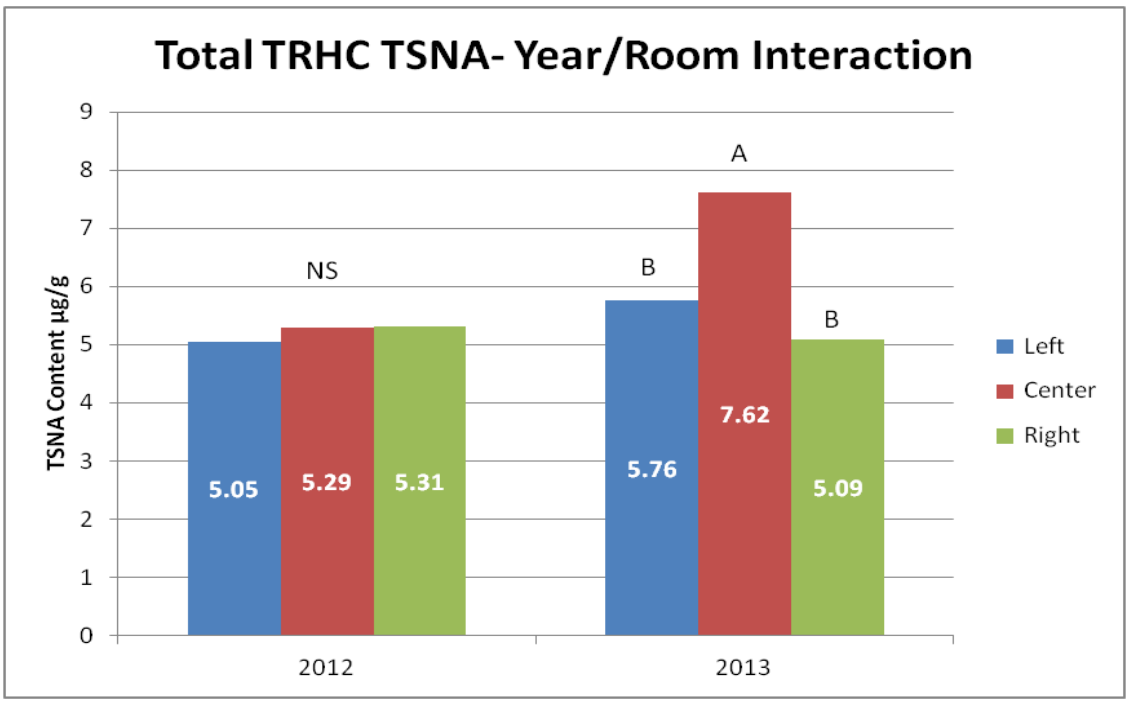


Figure 3.4.3. Simple Linear Regression of total TRHC TSNA and relative humidity within 2013.

Year effects on TRHC Total TSNA content.

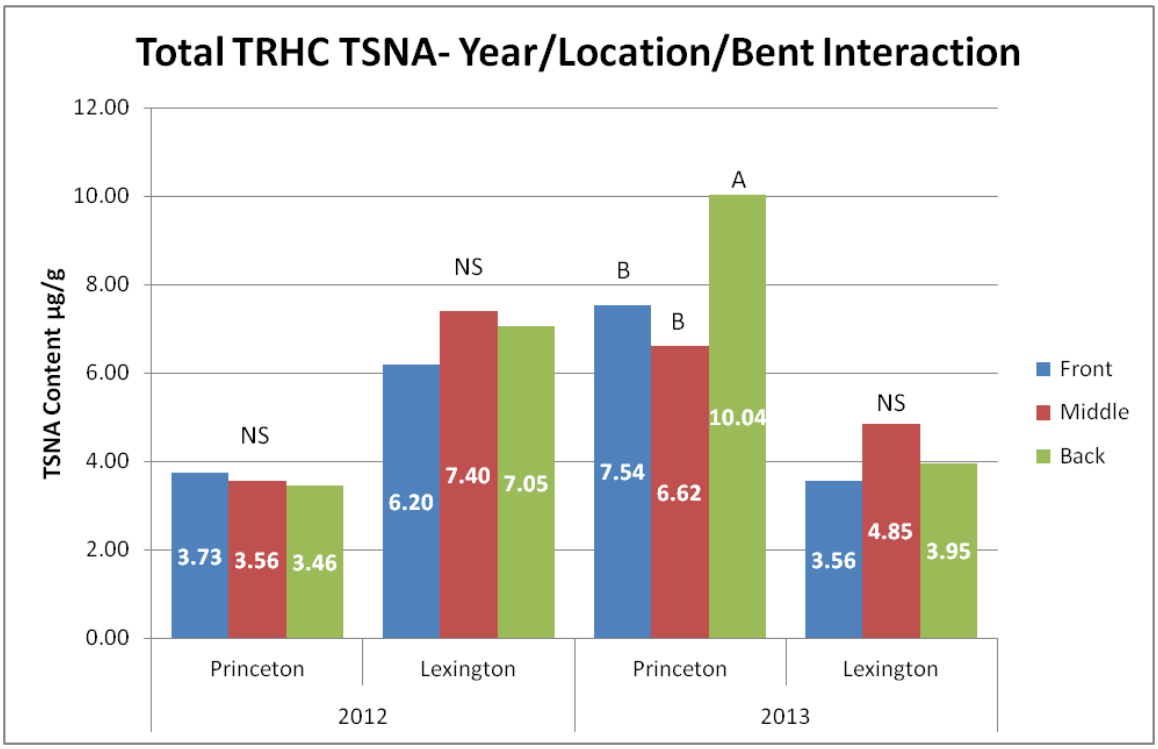
Figure 3.4.4. presents the total TRHC TSNA content year by room interaction when averaged over location. There were no significance differences between rooms in 2012 with average total TRHC TSNA of 5.22 μg g^{-1} across rooms. In 2013, the center room had significantly higher TSNA than the left and right rooms in both barns. The within-barn curing environment does not fully explain the center room having higher TSNA in 2013, although relative humidity was significantly higher in the center and right rooms across both locations in 2013, and numerically highest in the center room. There was no significance of the center room when looking at temperature. Thus, it seems the variability in total TSNA content must be influenced by other factors or environmental data analysis should be improved to better understand and characterize the interaction between temperature and relative humidity on TSNA content.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05, NS= no significant difference.

Figure 3.4.4. Total TRHC TSNA content year by room interaction.

The year by location by bent interaction for total TRHC TSNA is shown in Figure 3.4.5. There were no significant differences for bent within either location in 2012, with the Princeton barn averaging 3.58 µg g^{-1} and the Lexington barn averaging 6.88 µg g^{-1} across bents. In 2013, the Lexington barn had no significant differences between bents but there were significant differences in the Princeton barn, with the back bent having significantly higher TRHC total TSNA than the front or middle bents.



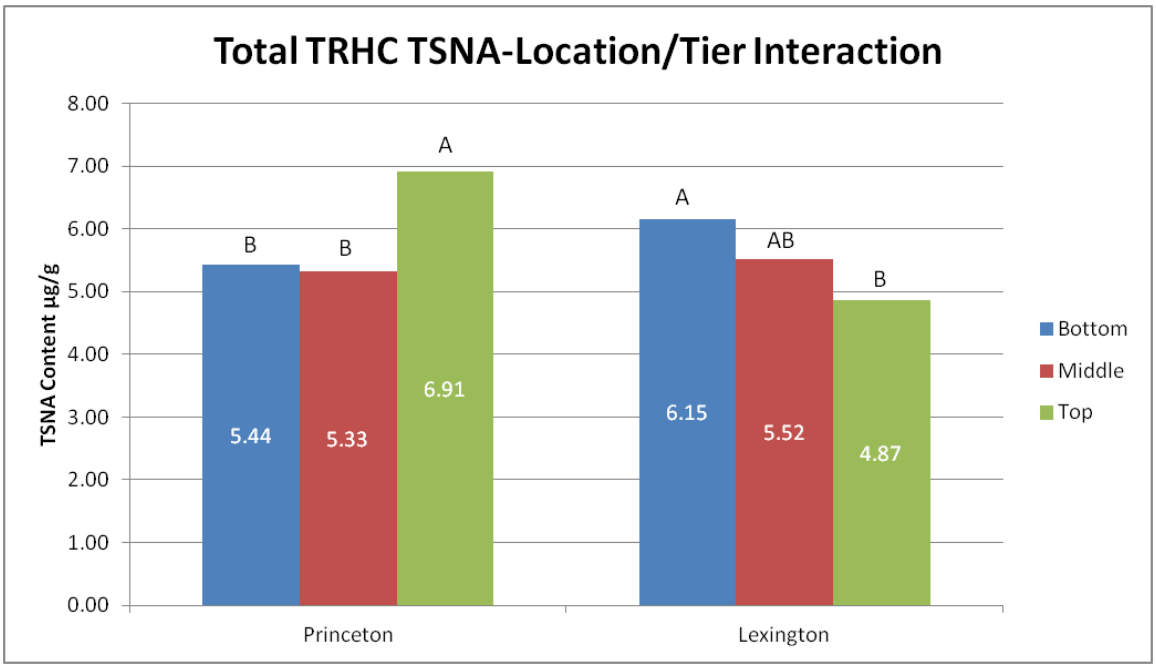
*Means within a year and location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05, NS= no significant difference.

Figure 3.4.5. Total TRHC TSNA content year by location by bent interaction.

Location Effects on TRHC Total TSNA Content

The location by tier interaction averaged over year for TRHC TSNA content is shown in Figure 3.4.6. Effects were different at each location, with higher TSNA content in the top tier at Princeton and higher TSNA content in the bottom tier of the barn at Lexington. Relative humidity also followed this same general pattern for both locations with significantly higher relative humidity in the bottom of the Lexington barn and numerically higher, although not statistically higher, relative humidity in the top of the barn at Princeton.

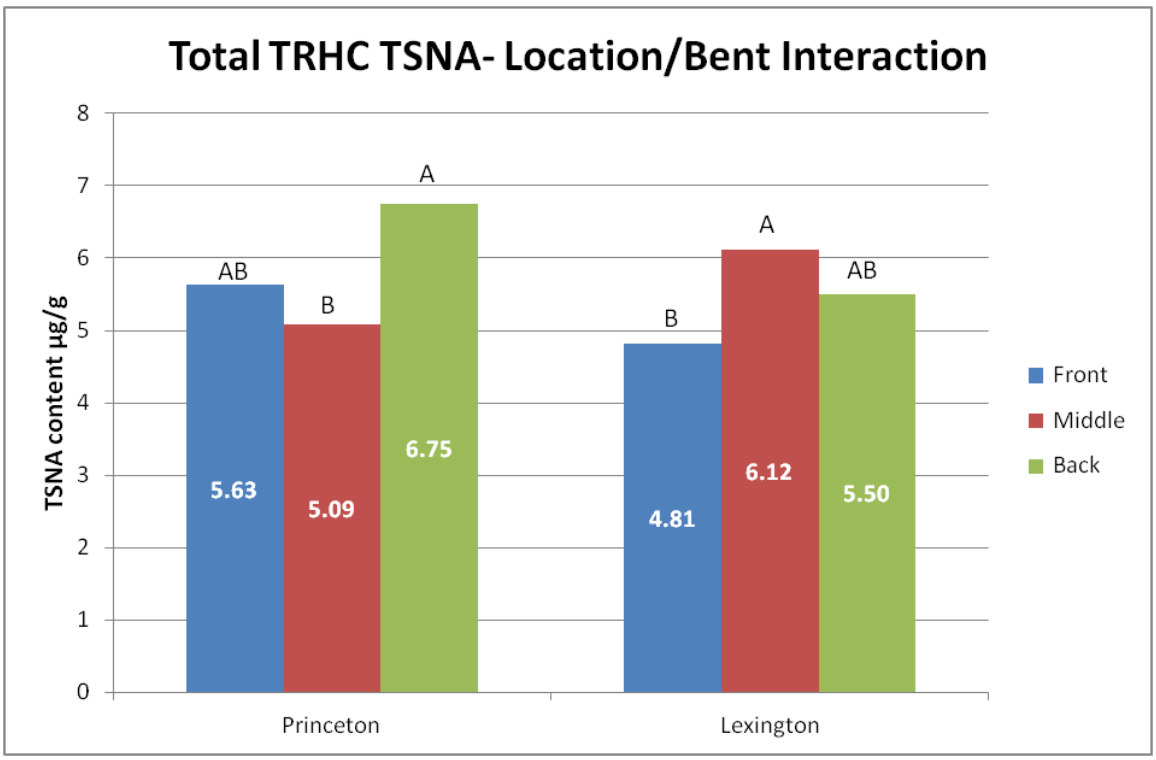
Location effects on TRHC total TSNA.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.4.6. Total TRHC TSNA content location by tier interaction.

The total TRHC TSNA location by bent interaction is shown in Figure 3.4.7. The back bent of the barn at Princeton had significantly higher total TSNA content when compared to the middle bent, but was similar to TSNA in the front bent. Lexington had significantly higher total TSNA content in the middle bent compared to the front, but was similar to the back bent. The curing environment data does not explain this variation well, although relative humidity was numerically highest in the middle bent at Lexington across years and temperature was highest in the back bent at Princeton in 2013.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.4.7. Total TRHC TSNA content location by bent interaction.

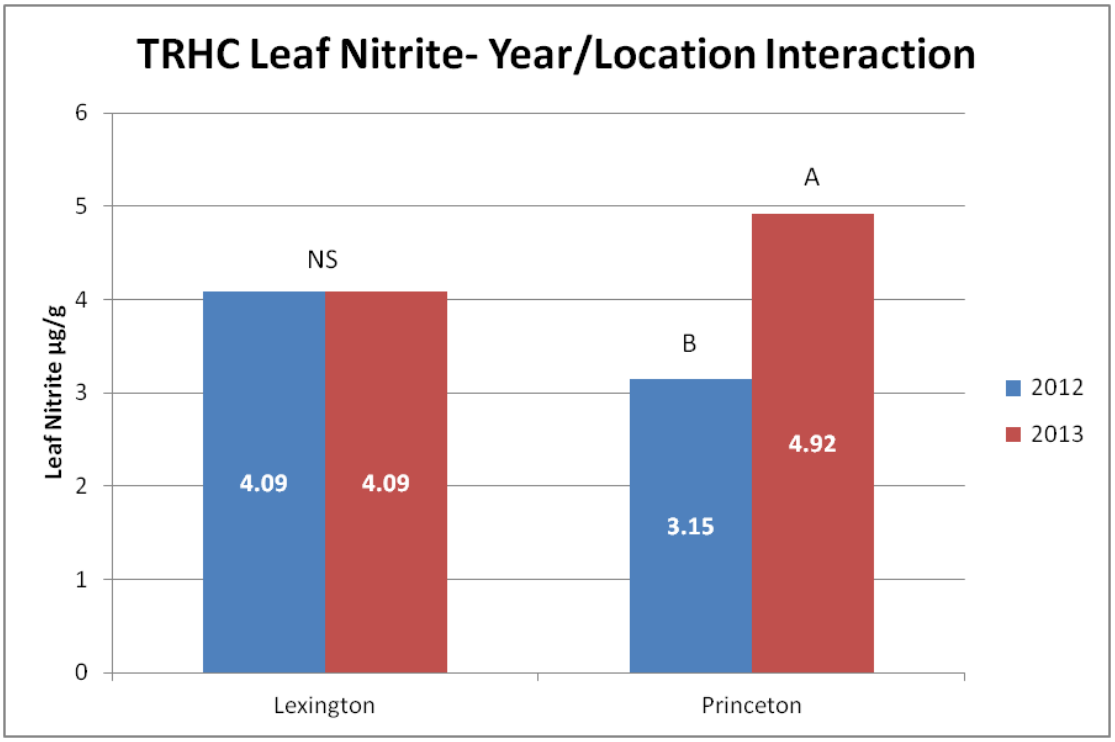
Leaf Nitrite effects on TRHC total TSNA.

TRHC Leaf Nitrite		
Source of Variation	**F Value**	**Pr>F**
Year	5.18	0.0256
Location	0.02	0.8756
Year*Location	5.22	0.0250
Tier	0.95	0.3896
Year*Tier	0.88	0.4178
Location*Tier	1.82	0.1688
Year*Location*Tier	1.54	0.2218
Room	5.15	0.0079
Year*Room	3.30	0.0419
Location*Room	1.42	0.2473
Year*Location*Room	2.65	0.0770
Bent	2.11	0.1277
Year*Bent	1.86	0.1629
Location*Bent	0.21	0.8073

Year*Location*Bent	1.02	0.3645

Table 3.4.2. Analysis of Variance for TRHC Leaf Nitrite.

There was a year by location interaction with TRHC leaf nitrite, as shown in Table 3.4.2 and Figure 3.4.8. In the Princeton barn, 2013 had significantly higher leaf nitrite compared to 2012. This was also observed in total TRHC TSNA, hours above 80% relative humidity, and hours above 24°C temperature.

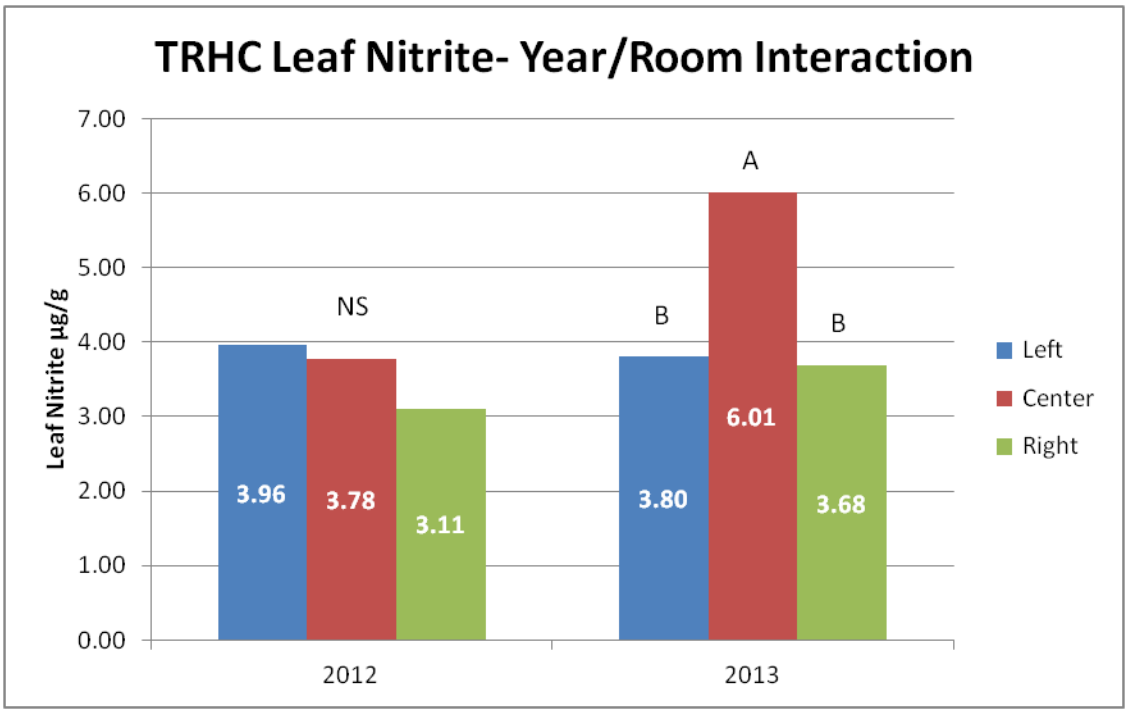


*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05 NS = not significant.

Figure 3.4.8. Year by location interaction total TRHC Leaf Nitrite content.

Within TRHC, there was a leaf nitrite year by room interaction as shown in Figure 3.4.8. There were no significant differences in 2012. In 2013, the center room had significantly higher leaf nitrite when compared to the left and center rooms. These significant differences closely follow the TRHC TSNA year by room interaction. For

both leaf nitrite and TRHC total TSNA, there were no significant differences between rooms in 2012, but the center room was significantly higher than the left and right rooms in 2013. There was no significant year by room interaction for temperature to help explain these data, but relative humidity hours above 80% did follow a similar trend for 2013, with the center room having more hours above 80% than the left room but not different from the right room.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05. NS = not significant.

Figure 3.4.9. TRHC leaf nitrite year by room interaction.

The leaf nitrite simple linear regressions are shown by year and location. The simple linear regression comparing leaf nitrite and TRHC total TSNA at the Princeton location within 2012 is shown in Figure 3.4.9. There was a very positive significant relationship and very good correlation between leaf nitrite and total TSNA in the cured

leaf at the Princeton barn in 2012. This positive relationship has been observed in previous research (Burton et al. 1989b).

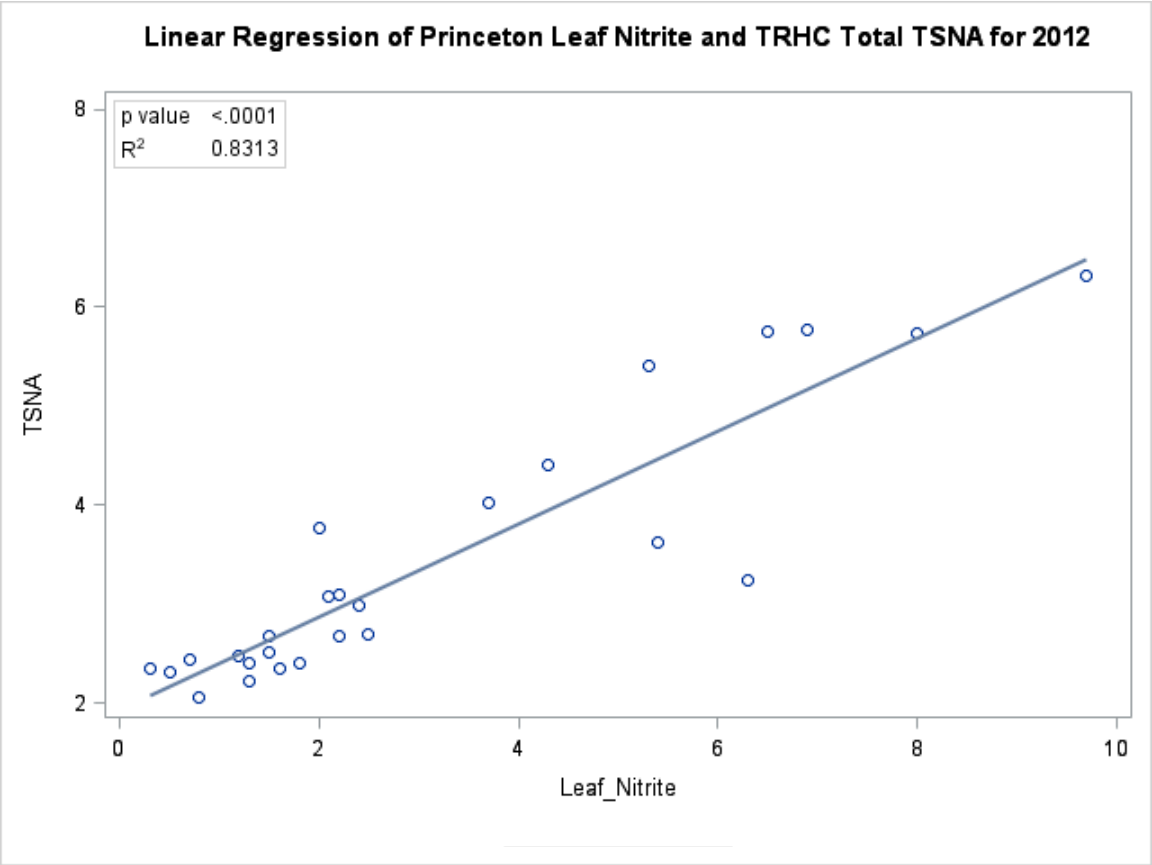


Figure 3.4.10. Simple Linear Regression of Princeton total TRHC TSNA and leaf nitrite in 2012.

There was also a positive relationship between leaf nitrite and TRHC total TSNA observed at the Princeton barn in 2013, as shown in Figure 3.4.10.

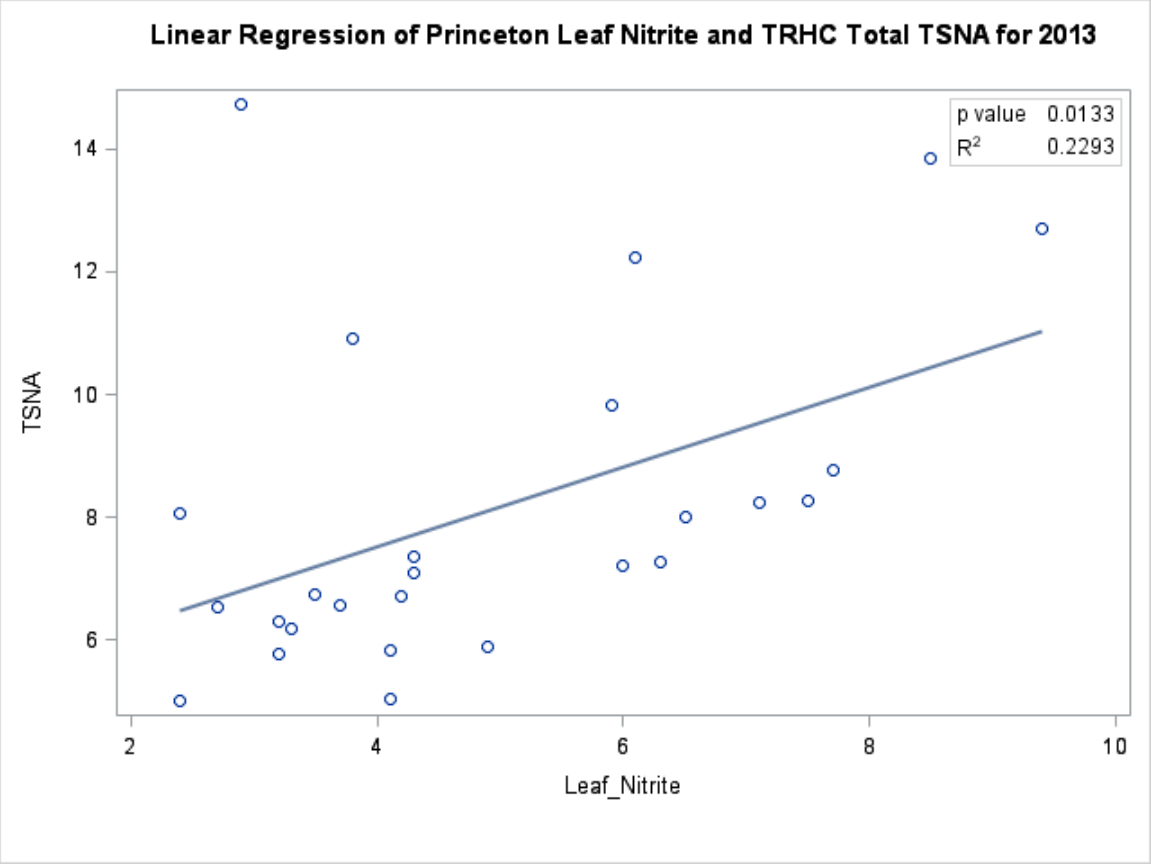


Figure 3.4.11. Simple Linear Regression of Princeton total TRHC TSNA and leaf nitrite in 2013.

There were also significant positive regressions between leaf nitrite and total TRHC TSNA for each year at the Lexington location. The relationship between leaf nitrite and total TSNA for 2012 and 2013 are shown in Figure 3.4.11., and 3.4.12., respectively.

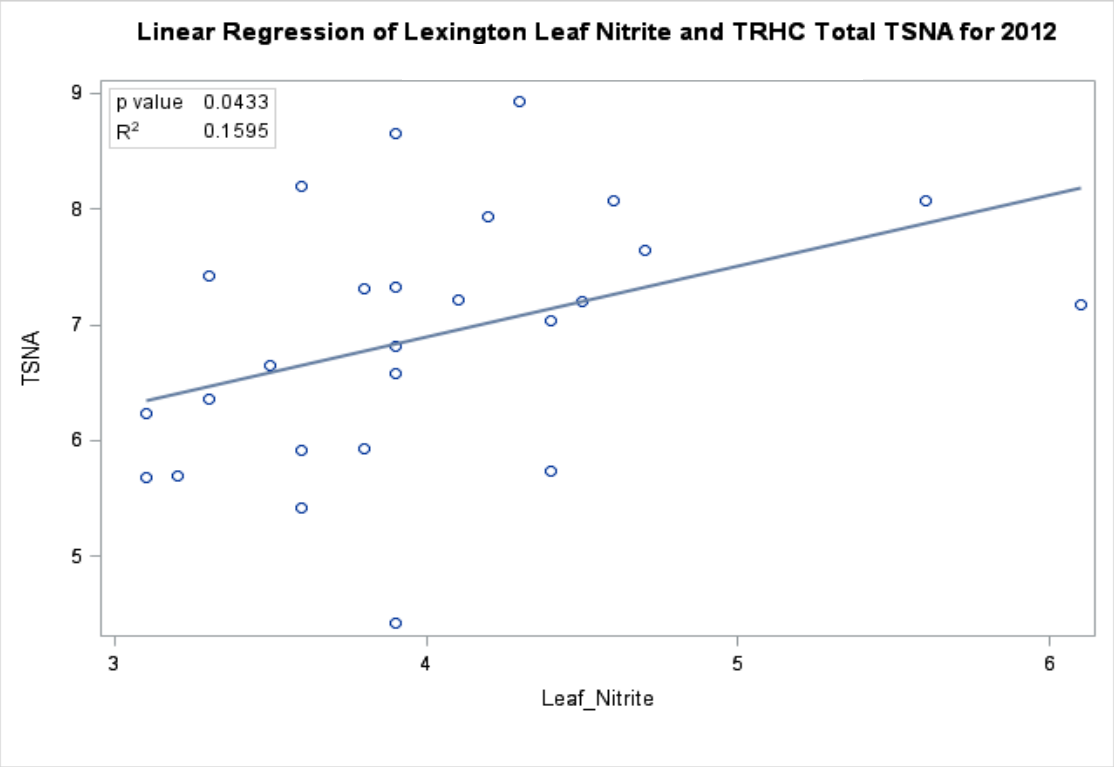


Figure 3.4.12. Simple Linear Regression of Lexington total TRHC TSNA and leaf nitrite in 2012.

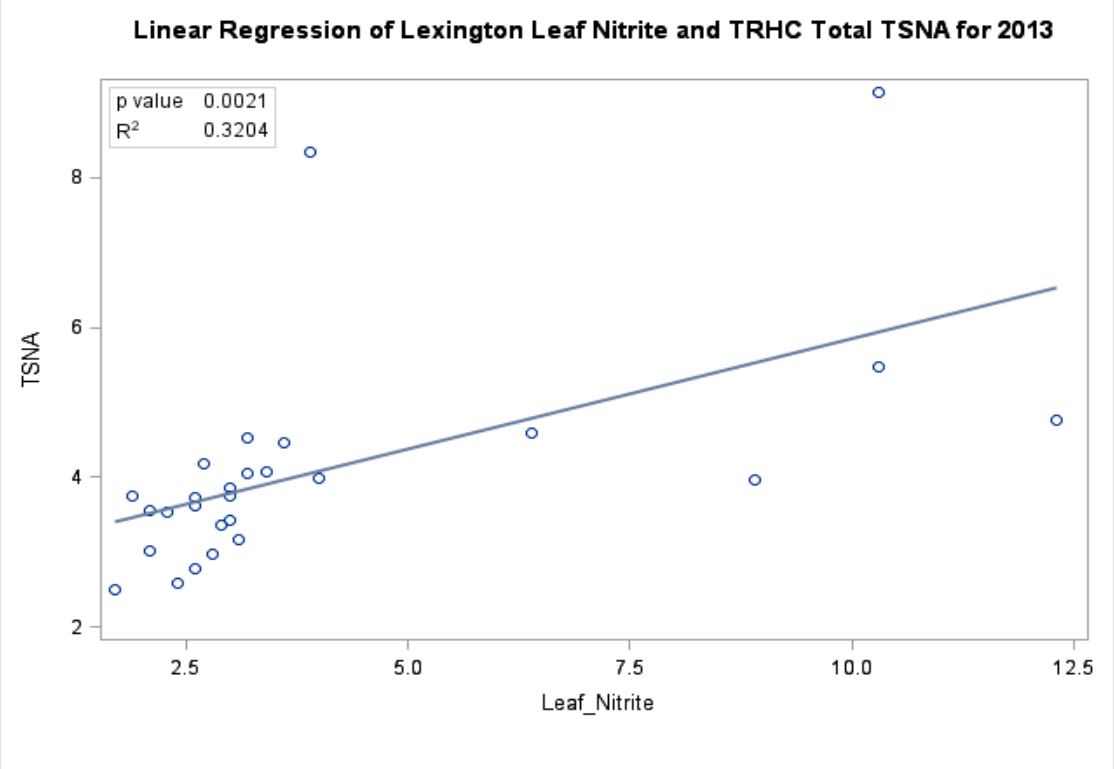


Figure 3.4.13. Simple Linear Regression of Lexington total TRHC TSNA and leaf nitrite in 2013.

TRsc Total TSNA

TRsc Total TSNA		
Source of Variation	**F Value**	**Pr>F**
Year	26.62	<.0001
Location	32.92	<.0001
Year*Location	59.07	<.0001
Tier	2.16	0.1221
Year*Tier	0.98	0.3782
Location*Tier	3.20	0.0460
Year*Location*Tier	0.76	0.4716
Room	5.55	0.0055
Year*Room	3.35	0.0403
Location*Room	5.91	0.0040
Year*Location*Room	0.30	0.7453
Bent	0.76	0.4733
Year*Bent	1.20	0.3077
Location*Bent	2.76	0.0693
Year*Location*Bent	0.93	0.3971

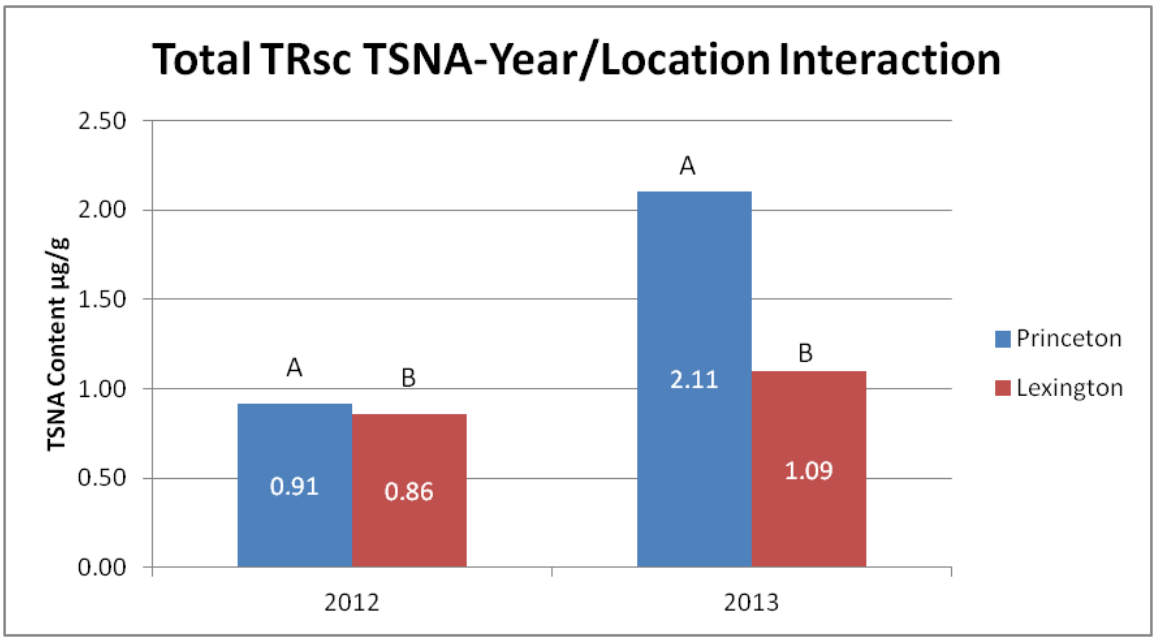
Table 3.4.3. Analysis of Variance for total TSNA for TRsc.

Similar to TRHC total TSNA data, there was an overall year by location interaction for TRsc total TSNA data (Table 3.4.3). Within 3-dimensional barn effects, tier data are shown by location due to a significant location by tier interaction, and room data are presented by year and by location due to significant year by room and location by room interactions. There were no effects of bent in TRsc total TSNA data. Simple linear regression was used to model the relationship of TSNA and hours above 24°C, hours above 80% relative humidity, and leaf nitrite.

Year effects on TRsc total TSNA.

The year by location interaction for TRsc total TSNA is presented in Figure 3.4.13. TSNA content is obviously lower in TRsc compared to TRHC, as expected. Within 2012 and 2013, Princeton had higher total TSNA content when compared to

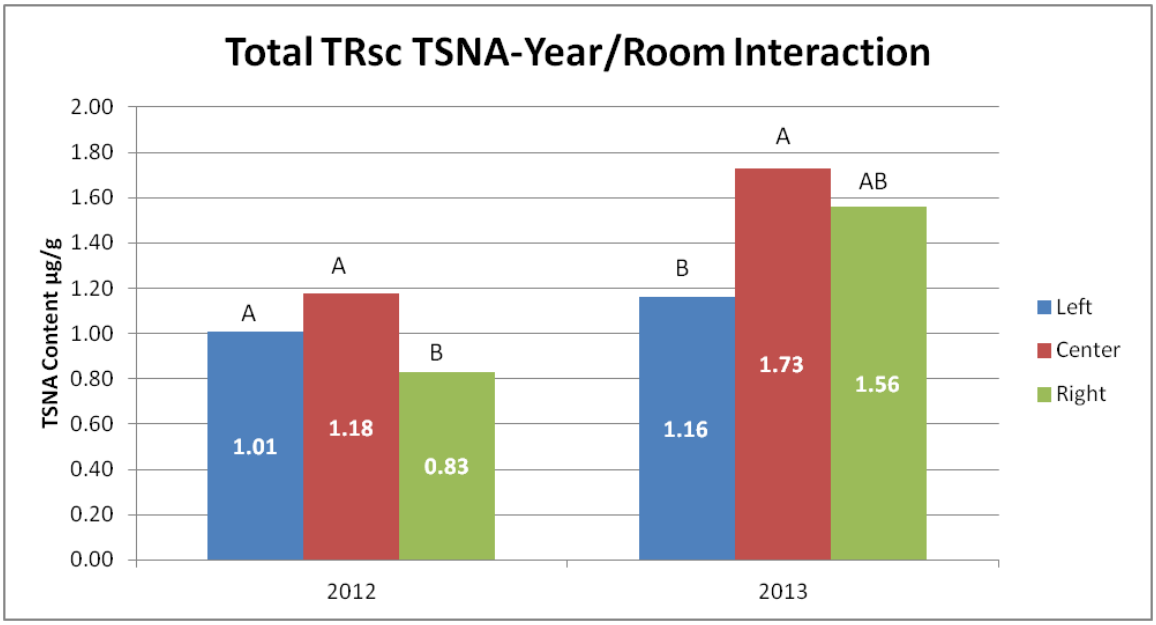
Lexington. Although TRsc total TSNA was different between each location in each year, locations were more similar in 2012 than in 2013.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.4.14. Total TRsc TSNA content year by location interaction.

There was a significant interaction between year and room for total TSNA within the TRsc variety when averaged over location, as shown in Figure 3.4.14. In 2012, the left and center rooms had significantly higher TRsc total TSNA when compared to the right room. In 2013, the center room total TSNA content was significantly higher than the left room, with the right room not significantly different from the left or center rooms. The center room had numerically higher TRsc TSNA each year.



*Means within a year with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 3.4.15. Total TRsc TSNA content year by room interaction.

There was no significant simple linear regression with relative humidity and total TRsc TSNA in 2012. Similar to TRHC total TSNA, there was a significant negative simple linear regression for relative humidity and total TSNA in 2013, as shown in Figure 3.4.15. This negative slope of the regression could be explained by the higher TSNA content observed at Princeton with the lower relative humidity hours above 80%. Lexington had the highest relative humidity and lower total TSNA, suggesting that factors other than relative humidity may be involved.

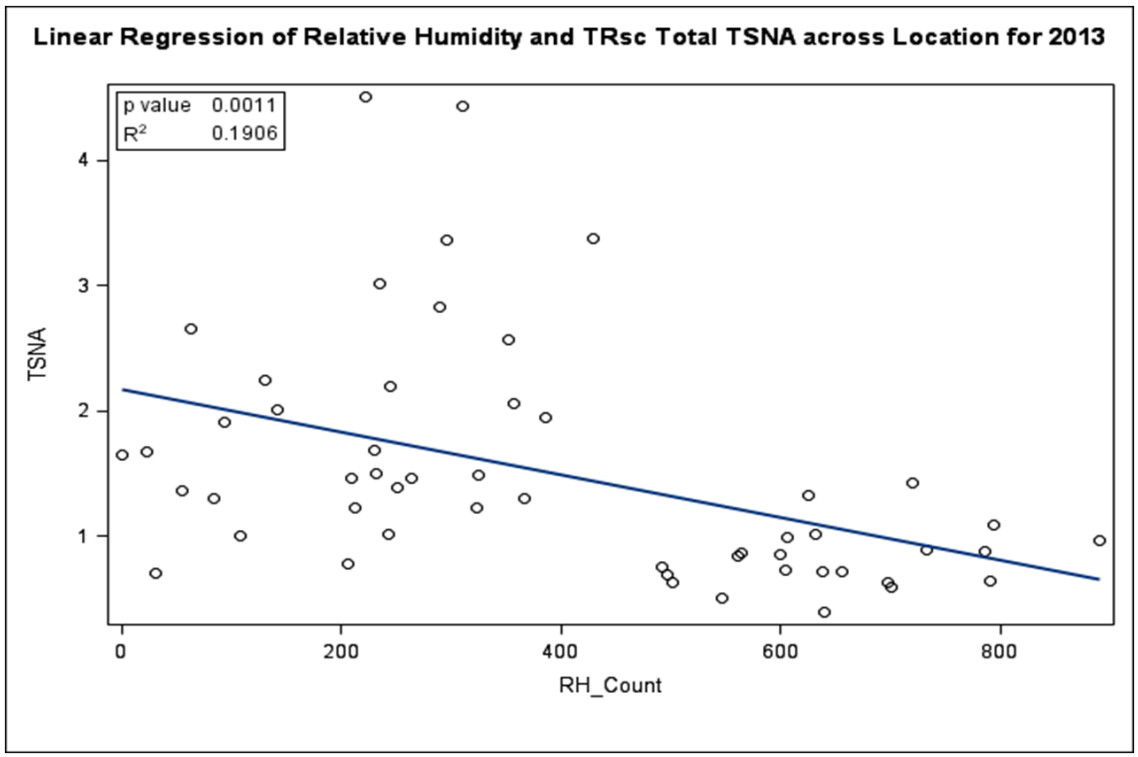
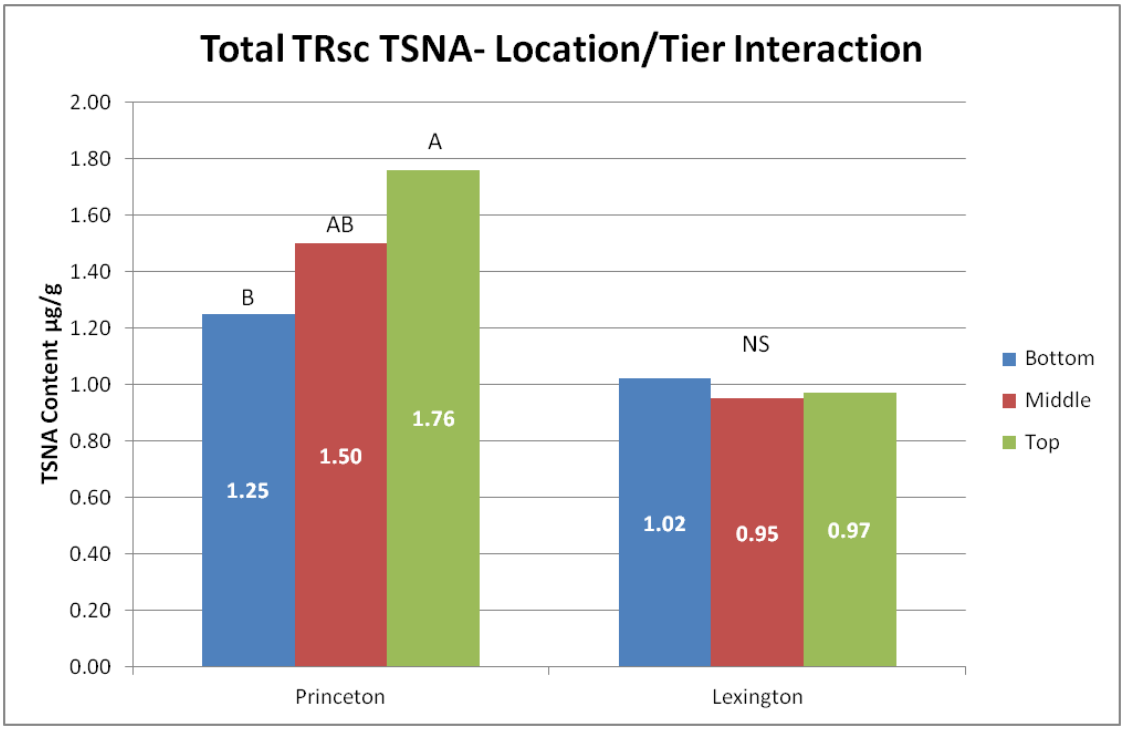


Figure 3.4.16. Simple Linear Regression of total TRsc TSNA and leaf nitrite within 2013.

Location effects on TRsc total TSNA.

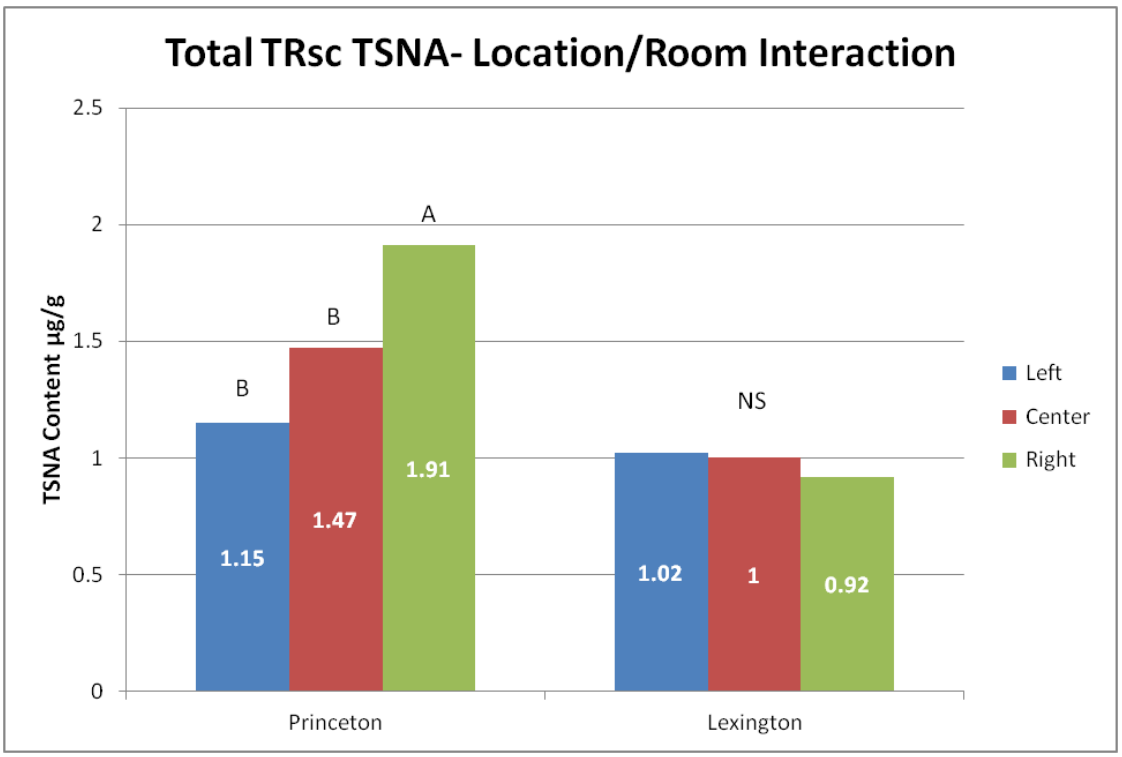
Figure 3.4.16. presents the total TRsc TSNA content location by tier interaction. In Princeton, the top tier was significantly higher than the bottom tier, with the middle not significant from the top or bottom tiers, which was similar to TRHC results at Princeton. There were no significant differences between tiers within the Lexington barn with all tiers accumulating around 1 μg g^{-1} TSNA.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05. NS = not significant

Figure 3.4.17. Total TRsc TSNA content location by tier interaction.

Figure 3.4.17. presents the total TRsc TSNA content location by room interaction. Data for this location by room interaction was very similar to data from the location by tier interaction. In Princeton, the right room was significantly higher than the left and middle rooms. There was no significance of room within the Lexington barn with all rooms accumulating around 1 µg g^{-1} TSNA.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05. NS = not significant

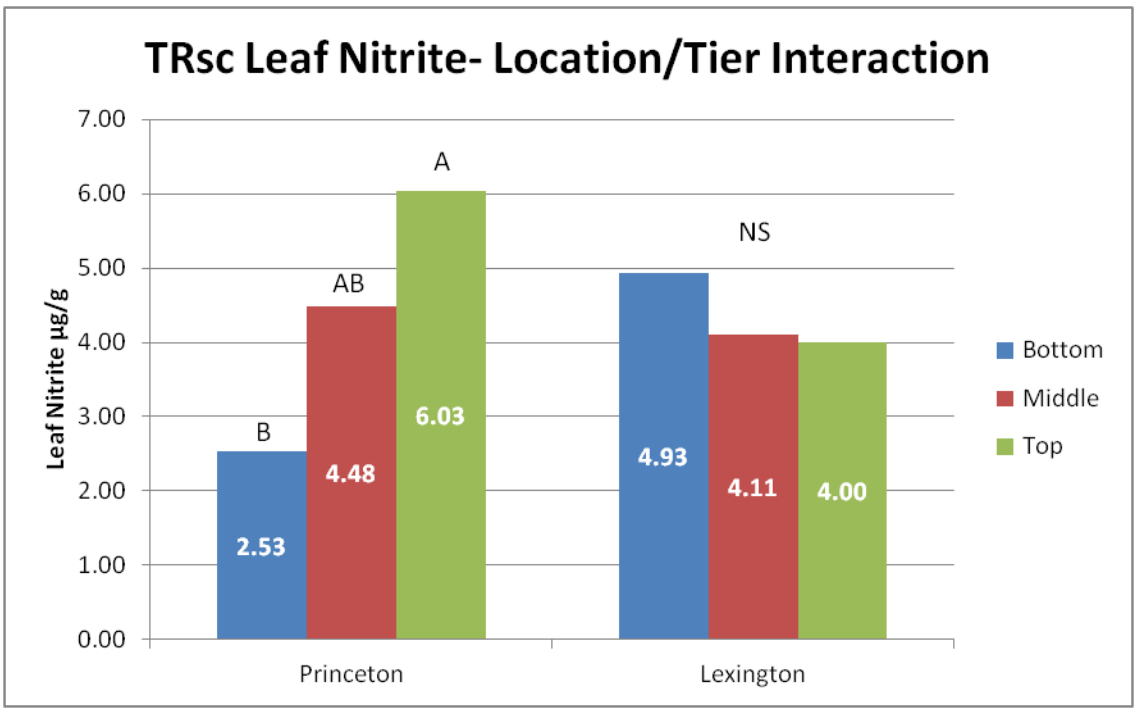
Figure 3.4.18. Total TRsc TSNA content location by room interaction.

3.4.6. Leaf nitrite effects on TRsc total TSNA.

TRsc Leaf Nitrite		
Source of Variation	**F Value**	**Pr>F**
Year	2.30	0.1330
Location	0.00	0.9955
Year*Location	0.00	0.9909
Tier	1.31	0.2757
Year*Tier	0.90	0.4097
Location*Tier	3.99	0.0223
Year*Location*Tier	0.33	0.7228
Room	0.93	0.4007
Year*Room	1.80	0.1725
Location*Room	0.86	0.4288
Year*Location*Room	0.05	0.9537
Bent	0.72	0.4903
Year*Bent	1.77	0.1774
Location*Bent	2.30	0.1070
Year*Location*Bent	1.00	0.3714

Table 3.4.4. Analysis of Variance for TRsc Leaf Nitrite.

There was a significant location by tier interaction for TRsc leaf nitrite, as shown in Table 3.4.4 and Figure 3.4.18. There were no significant differences between tiers within the Lexington location with TRsc leaf nitrite averaging 4.35 µg g^{-1}. In Princeton, the top tier of the barn had significantly higher leaf nitrite than the bottom tier, with the middle tier not different from the top or bottom tiers. This trend was observed in TSNA content for the TRsc variety as well; with the top tier having significantly higher TSNA and leaf nitrite when compared to the bottom tier. This trend was also observed for the temperature above 24°C at the Princeton barn, with the top tier having more hours above 24°C than the bottom tier. Even though there were no significant tier differences in relative humidity between tiers at the Princeton barn, relative humidity was numerically higher in the top tier also.



*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05. NS = not significant

Figure 3.4.19. TRsc leaf nitrite location by tier interaction.

The leaf nitrite linear regressions are shown by year and location. All leaf nitrite and total TSNA regressions for the TRsc variety had a positive relationship, but this relationship was not significant in Lexington for 2012. The simple linear regressions comparing leaf nitrite and TRsc total TSNA at the Princeton location in 2012 is shown in Figure 3.4.19., Princeton in 2013 is shown in Figure 3.4.20, and Lexington in 2013 is shown in Figure 3.4.21.

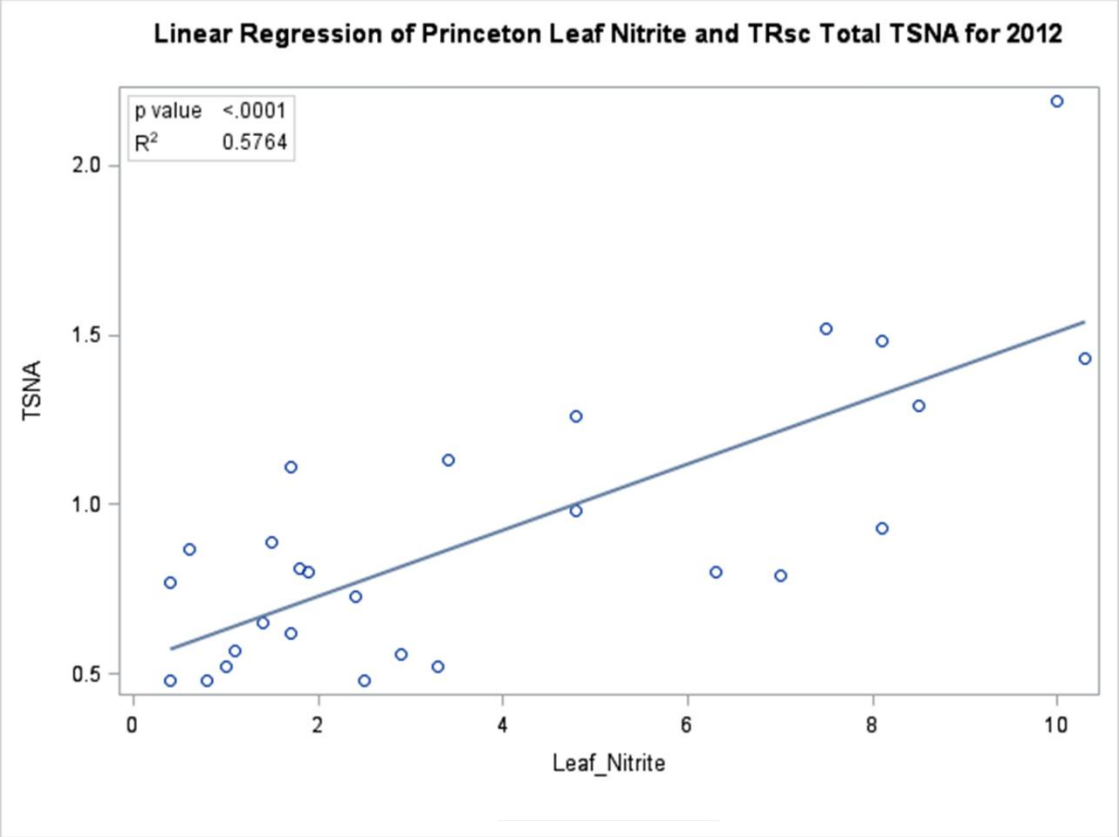


Figure 3.4.20. Simple Linear Regression of Princeton total TRsc TSNA and leaf nitrite in 2012.

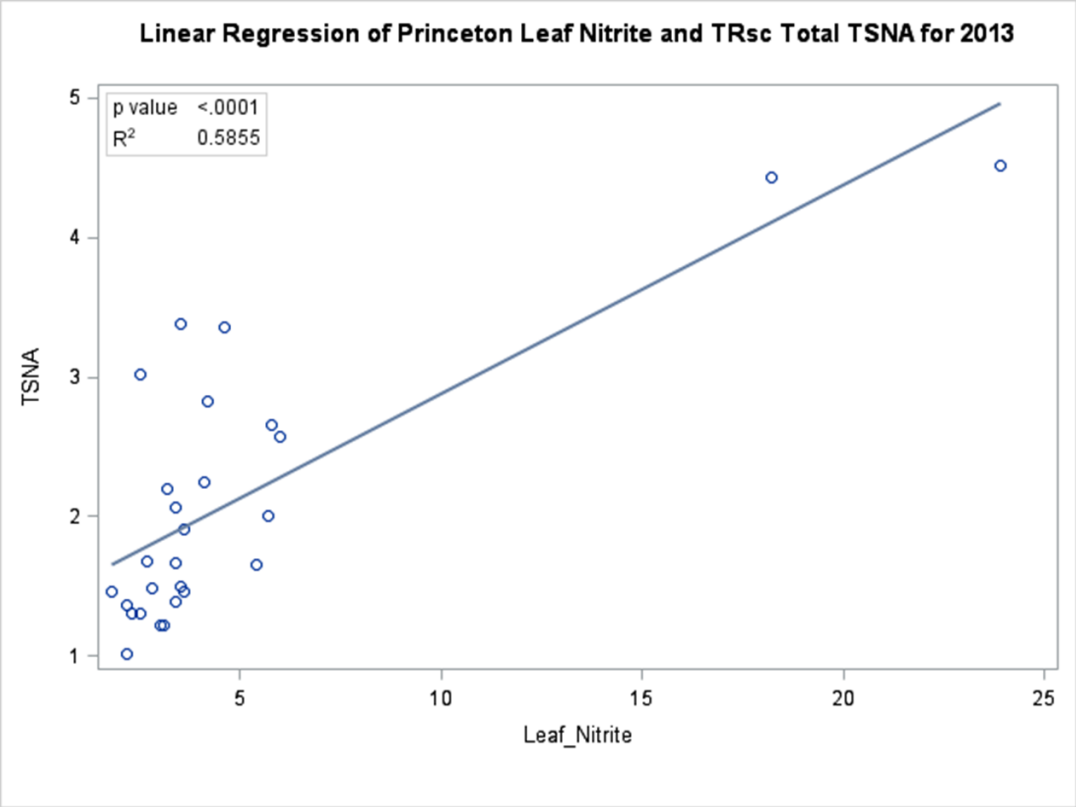


Figure 3.4.21. Simple Linear Regression of Princeton total TRsc TSNA and leaf nitrite in 2013.

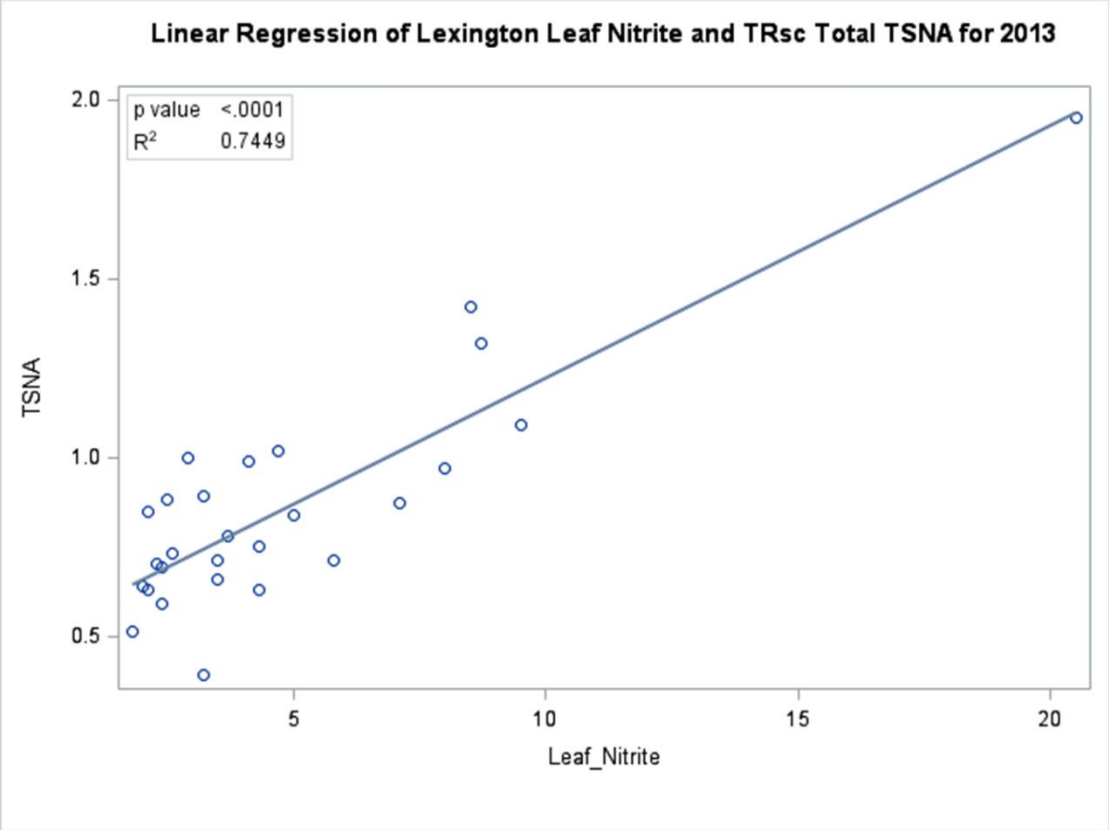


Figure 3.4.22. Simple Linear Regression of Lexington total TRsc TSNA and leaf nitrite in 2013.

3.5 Summary and Conclusions

In summary, several interactions were observed in this study between counts of the number of hours above 24°C temperature, counts of the number of hours above 80% relative humidity, TR Madole high converter (TRHC) and TR Madole screened (TRsc) total TSNA and leaf nitrite (Table 3.1.3).

Temperature above 24°C had significant interactions including: year by location, year by location by tier, year by location by bent, and location by room interactions. The Lexington barn had more hours above 24°C in both years when

compared to the Princeton barn, but this difference was greater in 2012 when compared to 2013 due to difference in housing date. As expected, the top tier of both barns had more hours above 24°C when compared to the middle and bottom tiers.

Relative humidity greater than 80% had significant interactions including: year by location, year by room, year by bent, location by tier, location by room, and location by bent interactions. The Lexington barn had more hours above 80% relative humidity in both years compared to the Princeton barn. The difference was around a 3-fold increase in 2012 and around a 2-fold increase in 2013. The Lexington barn had higher number of hours greater than 80% in the bottom tier compared to the middle and top tiers. There was no significant tier effect within the Princeton barn, but a numerical trend was noticed with increased humidity in the top of the barn.

TRHC total TSNA had significant interactions including: year by location, year by room, year by location by bent, location by tier, and location by bent interactions. The Lexington barn had 2-fold more TRHC total TSNA compared to the Princeton barn in 2012 but the opposite was observed in 2013 with the Princeton barn having 2-fold more TRHC total TSNA than the Lexington barn . The barns had opposite tier effects with increased TRHC total TSNA in the top tier at the Princeton barn and increased TRHC total TSNA in the bottom tier of the Lexington barn.

TRHC cured leaf nitrite had significant interactions including year by location and year by room interactions. The TRHC cured leaf nitrite year by room interaction differences closely followed differences in TRHC total TSNA. For both TRHC total TSNA and cured leaf nitrite , there were no significant differences in 2012, but the center

room was significantly higher than the left and right rooms in 2013. There was no significant year by room interaction but relative humidity hours above 80% did follow a similar trend for 2013. There were several significant simple linear regressions for TRHC total TSNA and leaf nitrite. All significant regressions were positively correlated.

TRsc total TSNA had significant interactions including: year by location, year by room, location by tier, and location by room interactions. For 2012 and 2013, the Princeton barn had higher TRsc total TSNA compared to the Lexington barn. In Princeton, TRsc total TSNA was significantly higher in the top tier than the bottom tier, which was similar to TRHC total TSNA.

TRsc cured leaf nitrite had a significant location by tier interaction. There were no significant differences between tiers within the Lexingtonbarn. In Princeton, the top tier of the barn had significantly higher number of hours above 24°C, cured leaf nitrite, and TRsc total TSNA, and a numerical trend for higher number of hours above 80% relative humidity in the top tier. There were several significant simple linear regressions for TRsc total TSNA and leaf nitrite within years and locations. All significant regressions were positively correlated.

Progress has been made on understanding the formation of TSNA, but it is not completely understood. There are other complex processes that influence accumulation of TSNA. High variability in cured leaf TSNA is still observed. This study had limited significant relationships between temperature and relative humidity effects on TSNA formation which suggests that other factors may be involved. Opposing weather conditions in the two years this experiment was conducted, and differences in harvest and

curing dates between locations in 2012 may have also contributed to opposing results between years and locations. More precise methods of analyzing the within barn environment could help clarify how temperature, relative humidity, and TSNA interact.

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