



TSNA in Air-cured and Fire-cured Tobacco Sub-Group

FINAL CORESTA STUDY GRANT REPORT

**Analysis of Variability in Curing Conditions and TSNA within Barns of
Dark Air-Cured Tobacco**

March 2015

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1. Introduction

Monitoring of curing conditions with environmental data loggers is a common practice in dark tobacco research. Typically only a few (2 to 4) data loggers are placed in each barn at convenient locations in an attempt to measure average conditions. It is well known that TSNA levels can vary tremendously between samples collected within the same barn (Jack et al. 2013). This leads to questions about the spatial variability of curing conditions within air-cured barns and how well the variation in conditions correlates to variations in TSNA accumulation in the cured leaf. This experiment was designed to evaluate within barn variability of curing conditions by placing data loggers at regular intervals throughout two different barns. Cured leaf samples were collected from locations near each data logger and analyzed for TSNA levels to see if they would be correlated with the measured environmental conditions.

2. Material 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 with financial support from a CORESTA study grant. Two dark tobacco lines; TR Madole (TRsc), a line screened for low nicotine to normicotine conversion and TR Madole high converter (TRHC), a line which had been tested and found to have a consistently high conversion of nicotine to normicotine, 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 the bud-early bloom stage to have 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 lines were stalk harvested, allowed adequate field wilting, 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 TRsc area and three from the TRHC area) and analyzed for nitrate. Six green leaf samples (three from TRsc and three from TRHC) were collected and analyzed for nitrite and TSNA content according to the methods used by Morgan et al (2004). Each green leaf sample contained 20 leaves from the 4th leaf position from 20 different plants.

27 HOBO® data loggers (Onset Computer Corporation, Bourne, MA) were placed in each curing barn as the tobacco was housed. Differences in barn dimensions are shown in Figure 1. The Princeton barn was 5 rooms wide and this study occupied the entire barn whereas the Lexington barn was 12 rooms wide and the study occupied only the Northeast corner with

other tobacco hung in the adjacent areas to fill the barn. Each barn was a three-tiered (height) design with five rooms used in the experiment as demonstrated in Figure 2.

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 2. (3 locations x 9 loggers at each location = 27 data loggers). Figure 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. At the end of the curing season cured leaves were sampled from the 4 stalks in the center of the five sticks of each line adjacent to every data logger position. For each stalk the fourth leaf from the top of the plant was sampled. The samples consisting of twenty leaves were freeze dried, and ground and stored in a cold room until they were analyzed.

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 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.

3. Results

Recording curing conditions every hour over the course of a typical cure resulted in over 1500 individual data points for each data logger. For the purposes of this analysis, critical thresholds for temperature and relative humidity were established and used to develop cumulative variables to best represent the entire curing season. Within-barn temperature data were calculated as a cumulative count of the number of hours with the temperature above 24°C at a location over the entire duration of the cure. The temperature 24°C was chosen because previous research has suggested that temperature above 24°C during curing tend to result in higher levels of TSNA in the cured leaves (Wiernik et al, 1995; Burton et al. 1989a.) Within-barn relative humidity data were calculated as a cumulative count of the number of hours above 80% RH over the entire duration of the cure. This 80% threshold RH was chosen because previous research has shown elevated TSNA levels for tobacco cured under conditions of high relative humidity. TSNA data are presented as total TSNA in $\mu\text{g g}^{-1}$, which is the sum of all individual TSNA (NNN, NAT, NAB, NNK). TR-Madole High Converter (TRHC) data were analyzed and are presented separately from TR-Madole screened (TRsc).

There was no significant effect between individual monitoring positions on temperature, relative humidity, TRHC total TSNA, TRsc total TSNA, and leaf nitrite in either barn. The analysis process compared cumulative temperature and RH from each data logger and corresponding TSNA content for each position to all others. This could be visualized as 27 plots per variety within each barn, totalling 54 plots per barn. There was no significant difference between these 27 locations for any line/location combination when comparing each plot to another, due to the overall high variability in TSNA content throughout each barn. This result was not unexpected since the barns at the two locations differ with respect to size, orientation, and construction. This reflects the real world conditions in which many different types of barns and curing structures are often used and each may have its own unique patterns of air movement. It may be inferred from this that the relative curing conditions or accumulation of TSNA cannot be simply predicted based only on the relative position of the curing leaf within the barn.

To look for additional patterns in the data the individual 3-dimensional aspects of the tier (bottom, middle, top), room (left, center, right), and bent (front, middle, back) were investigated. For this analysis tier, room and bent were included as classification variable thus allowing some of the variability to be apportioned to those positional factors. Significant interactions were found for each of the variables tested in this study as shown in Table 1.

3.1 Tier

The impact of tier (height) in the barn was investigated. There was a significant year*location*tier effect for cumulative temperature as shown in Figure 4. Figure 4A shows that in general the number of hours above 24°C temperatures tends to increase with height in the barn for both locations. However, there appear to be differences in the pattern of increase between barns. At the Lexington location the increase was observed with each height increment, but at Princeton the bottom two rails were similar with a significant increase in the top rail only. These results generally follow expectations since heat tends to rise. The cumulative hours of high relative humidity had a significant location*tier interaction as shown in Figure 4B. Since RH is a function of temperature it might be expected that RH would go down as temperature increased. This was generally the case in Lexington but not at Princeton. Total TSNA for the high converter increased with height in Princeton, but decreased with height in Lexington, despite the fact that temperature and relative humidity trends with tier height were similar (Figure 4C). Total TSNA for the screened line also increased with height at Princeton, but was not significantly affected by height in Lexington (Figure 4D).

3.2 Room

There were significant location*room effects for the number of hours above 24°C, number of hours above 80% relative humidity, and total TRsc TSNA, with no significant difference in total TRHC TSNA, as presented by location in Figure 5. Within the Princeton barn, the right room had significantly more hours above 24°C (Figure 5A), more hours above 80% relative humidity (Figure 5B), and higher total TRsc TSNA (Figure 5C) when compared to the left room. At Princeton, both the left and right rooms were along exterior walls as the barn was only 5 rooms wide, the room designated as “right” faced the West. The higher temperature in the right room could be explained by the warming of that side of the barn by the afternoon sun. The TRsc TSNA levels were higher in the right room with the higher temperature and RH variables, but TRHC TSNA levels were not different between rooms. The left room of the Lexington barn had more hours above 24°C and fewer hours above 80% relative humidity. At Lexington, the left room was adjacent to an East facing exterior wall while the right room was near the center of the barn. The tendency toward higher temperatures in the left room may be explained by the warming of the East wall by the morning sun. The right and center rooms of the Lexington barn were more likely influenced by air which may have been cooler and more humid as a result of passing through the large mass of adjacent tobacco. Despite significant difference in curing conditions there were no significant differences in TSNA between rooms at Lexington.

3.3 Bent

There was a significant year*location*bent interaction for hours above 24°C and these data are presented by year in Figure 6A. From Figure 6A, the trend within the Lexington barn was similar for each year with the front bent having significantly higher hours above 24°C and the back room having significantly lower hours above 24°C. There was also a location*bent interaction for relative humidity as shown in Figure 6B. 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 had significantly higher relative humidity than the front bent in

Lexington. The back bent at the Princeton barn was at the east end of the barn and the back bent at Lexington was near the middle of the barn. This could also explain why the middle and back bents were significantly higher in RH 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. There was a location*bent interaction for total TRHC TSNA as shown in Figure 6C. 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 higher in the middle bent at Lexington each year and temperature was the highest in the back bent at Princeton in 2013. There was no significant bent interaction for total TRsc TSNA.

There were many year, location, tier, room, and bent interactions. Therefore, it is probable that all barns will not behave similarly when attempting to characterize barn behavior. Temperature data followed a pattern that was understandable within each year and location. However, relative humidity and TSNA accumulation did not follow similar trends within each barn. These differences in relative humidity within the microenvironments of each barn could be due to differences in barn structure, flooring, directional orientation, dimensions, and/or ventilation structures. These differences in TSNA content could be affected by many other factors in both the field and curing seasons.

4. Discussion

The formation and accumulation of TSNA in curing tobacco is a complex process influenced by many factors. Studies of TSNA formation under controlled environmental conditions have shown that temperature and relative humidity can impact TSNA levels in cured leaves. However, the environmental conditions in air-curing barns are constantly changing and one of the major challenges is trying to adequately characterize the conditions the tobacco is exposed to. Instrumentation used to measure barn environments must be rugged as well as accurate. The HOBO data loggers used in this experiment were found to be reasonably reliable, but variation between individual loggers may be as high as 10% (C. Fisher, personal communication). Placement of loggers is important to get an accurate reflection of the conditions, and loggers in direct contact with leaf material will generally have erroneous readings. For this study small shields were used to keep the loggers from being in direct contact with leaves, however the impact the shields may have had on measurements is unknown. Data loggers placed near the external walls of the barn can experience more fluctuation and influence from ambient conditions outside the barn, particularly if placed near a ventilator.

One thing that is clear from this study is that the effect of within-barn position cannot be easily predicted. For example, it cannot be assumed that the highest TSNA level will always be at the top or bottom or even middle of a barn. Factors such as barn construction, including the location and design of vents, vertical distance between tier rails, and siding materials used will influence the flow of air through the crop. Additional research is needed to determine if there is spatial structure of conditions within curing barns and how to sample the conditions to be able to predict and ultimately manage the barn for optimal curing.

Much of the scientific community share common limitations when executing field research. There are environmental, physical, economical, technical, and logistical factors that influence experimental design and technique. With any TSNA research, inherent variability in TSNA formation, air-curing barns, environmental conditions, and other complex variables are not

easily measured. Correlation between curing environment and TSNA accumulation can be difficult to prove due to these intricate interactions and relationships.

This study was designed to correlate changes in curing conditions within dark air-cured barns to TSNA accumulation in specific areas throughout the barn. The differences between these two barns are tremendous. The distance between these two locations is around 200 miles (320 km). Lexington's elevation above sea level is about 978 ft (298m) when compared to 482 ft (147m) in Princeton. The barns at both locations have different directional orientation, not to mention huge dimensional differences. Therefore, differences were expected between these two locations. Within each barn, the distance between sensors was nearly exact but that total difference equates to around 20 ft (~6m). Increased barn size to allow more distance between sensors and sampling could be a useful technique in attempting to detect variability in curing conditions and TSNA. Placing data loggers on multiple tiers would be the best practice to most accurately characterize environmental conditions within barns.

Progress has been made on understanding the formation of TSNA, but there is still much to learn. There are other complex processes that influence accumulation of TSNA. High variability in cured leaf TSNA is still observed. In this study we found limited significant relationships between temperature and relative humidity on TSNA formation which suggests that other factors may be involved. More precise methods of analyzing the within barn environment could help clarify how temperature, relative humidity, and TSNA interact.

5. References

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Figure 1. Differences in barn dimensions. A) Princeton barn B) Lexington Barn.

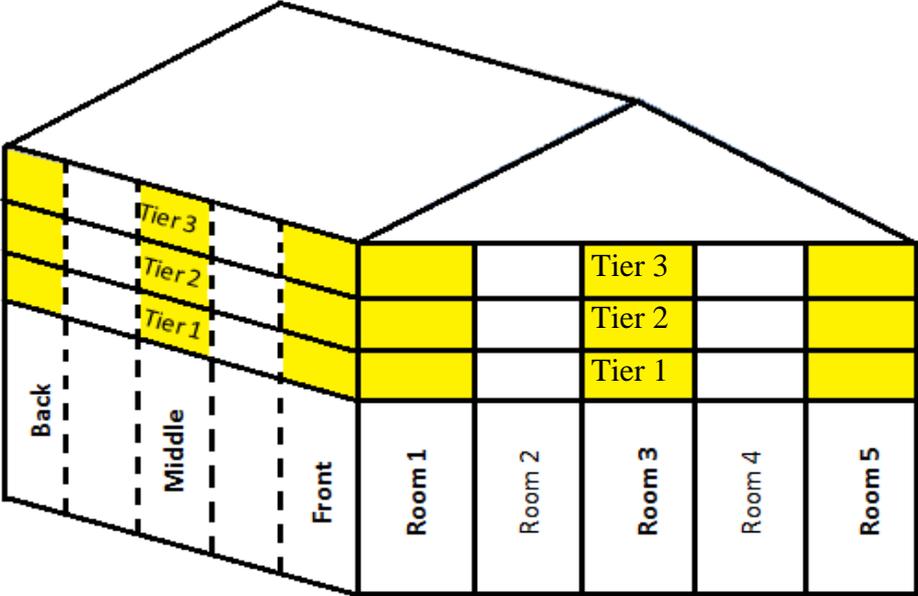


Figure 2. Diagram of long-tier orientation barn demonstrating the 3-Dimensional area that was studied.

Housing and Meter Placement in Barns

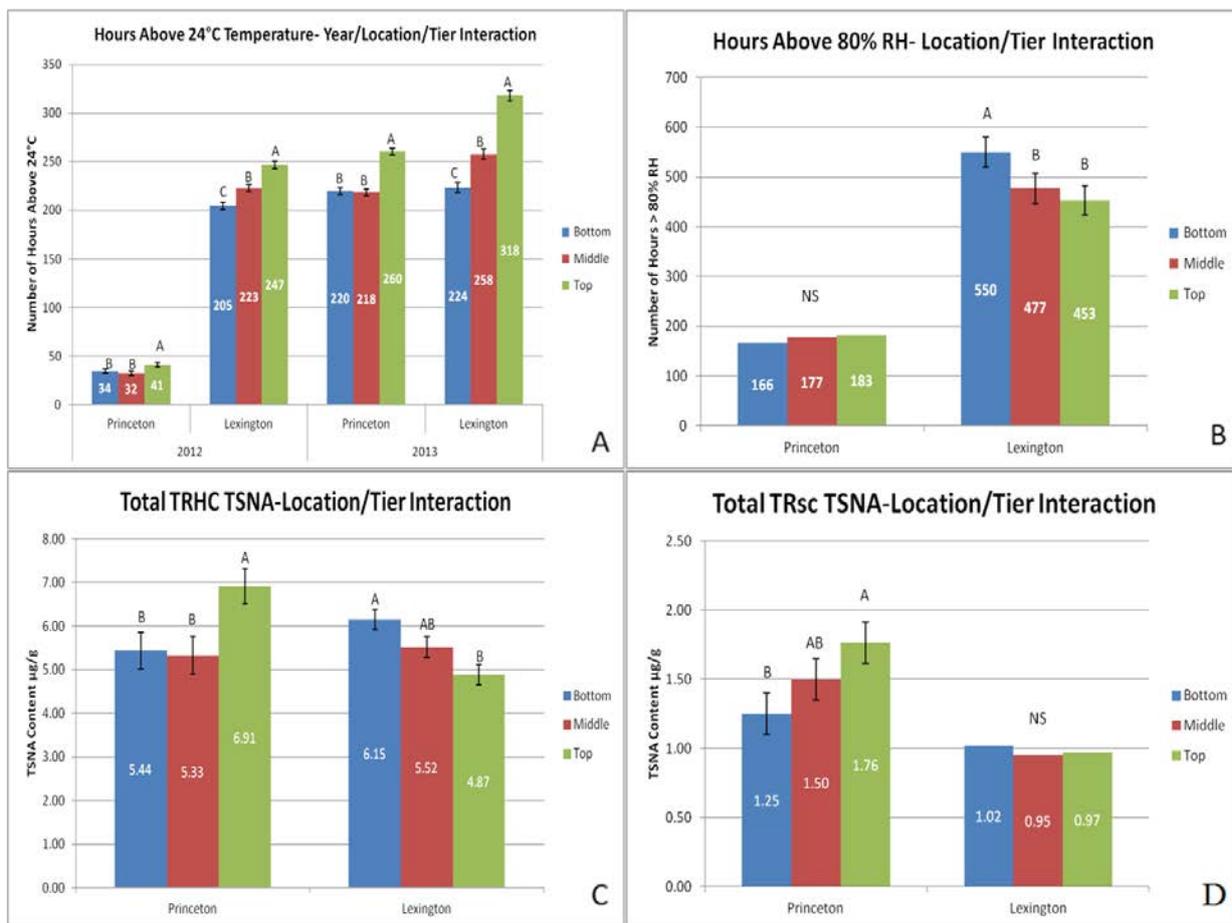
Room 1	Tier3 (top)		5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC
	Tier2 (middle)		5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC
	Tier1 (bottom)		5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC	5 TRsc	5 TRHC	5 TRsc ★ 5 TRHC

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

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

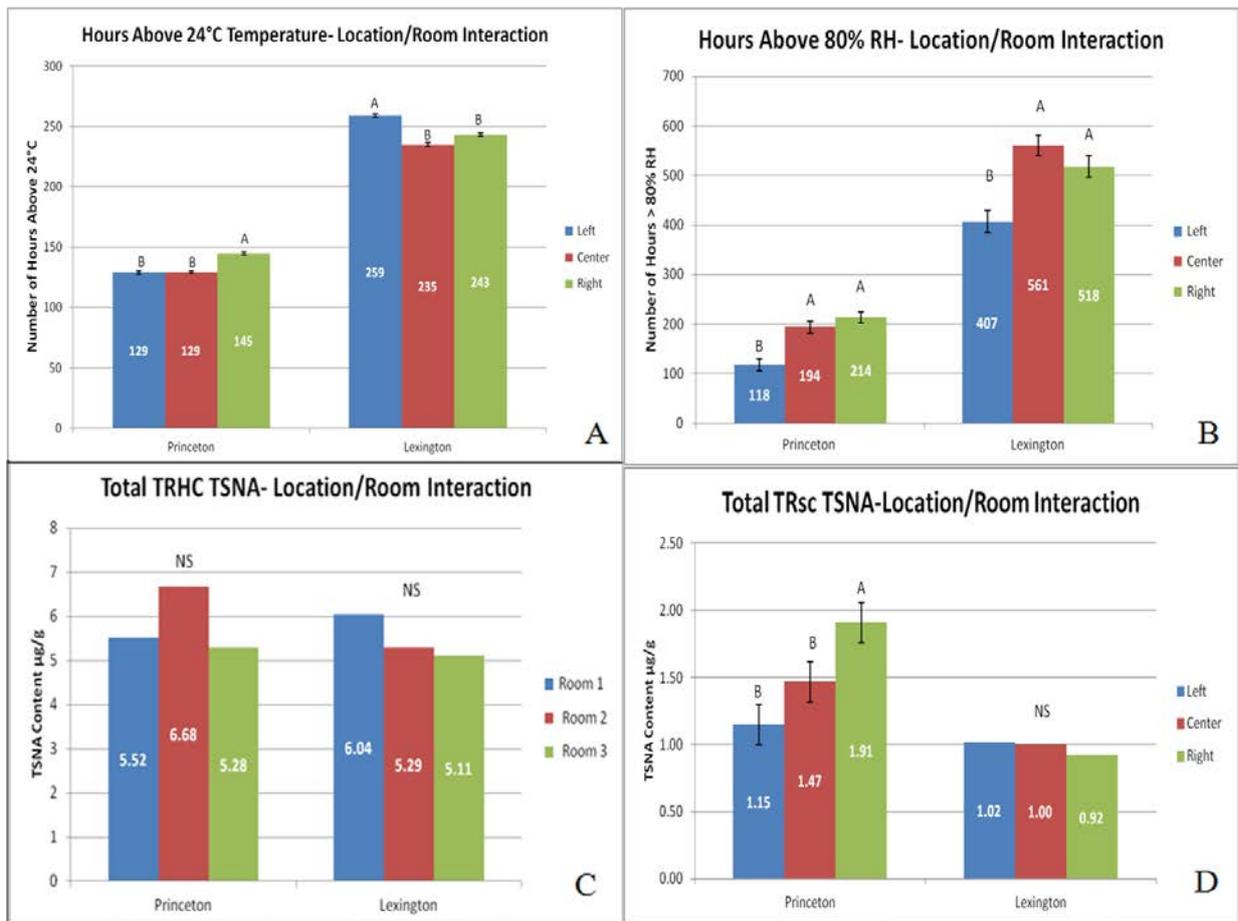
Factor	Temperature	RH	TRHC		TRsc	
			TSNA	Leaf Nitrite	TSNA	Leaf Nitrite
Year	X	X	X	X	X	
Location	X	X			X	
Year*Location	X	X	X	X	X	
Tier	X	X				
Year*Tier	X					
Location*Tier	X	X	X		X	X
Year*Location*Tier	X					
Room	X	X	X	X	X	
Year*Room		X	X	X	X	
Location*Room	X	X			X	
Year*Location*Room				X		
Bent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

Table 1. All significant interactions for within barn curing environment, total TSNA, and leaf nitrite. (X) indicates significance at P<0.05.



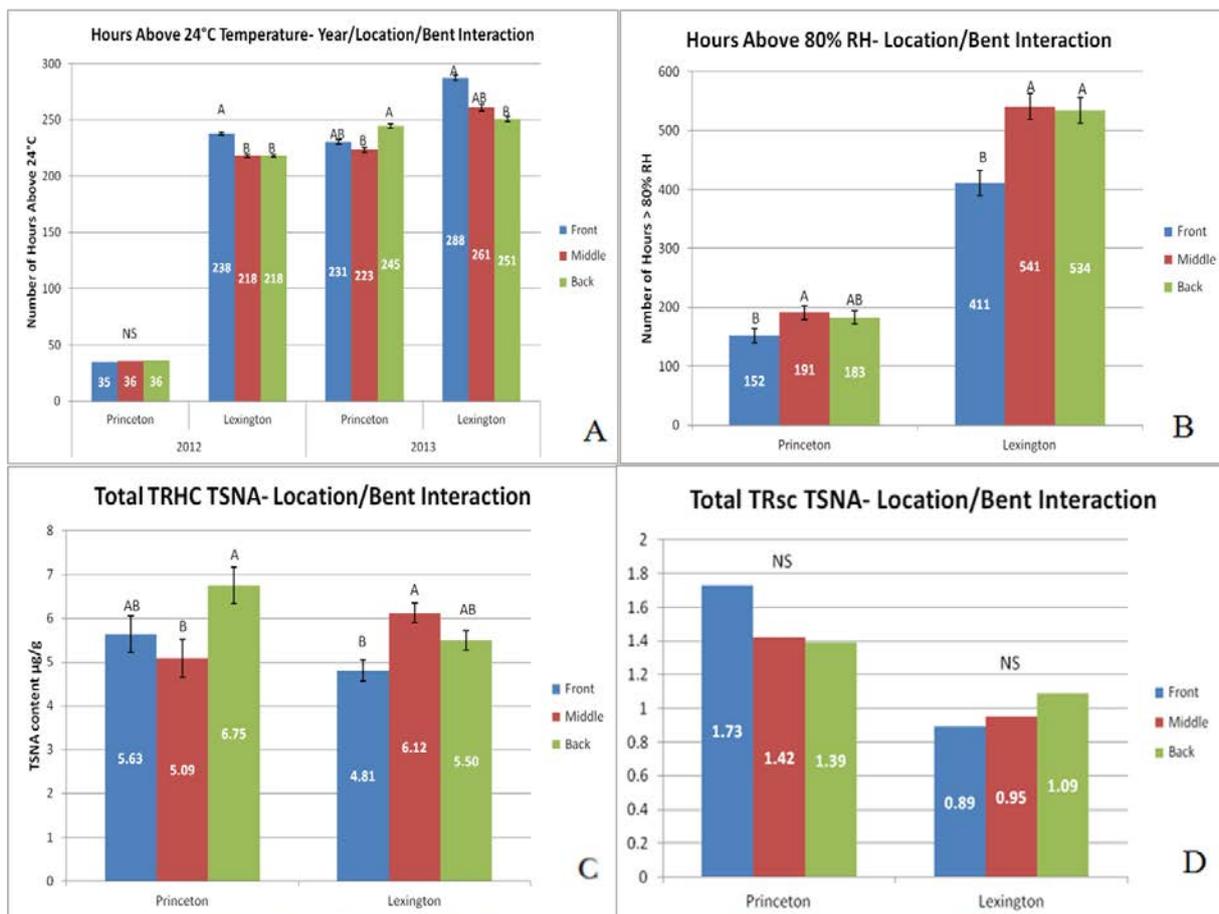
*Means within a location with the same letter are not significantly different according to Fisher's Protected LSD at P = 0.05.

Figure 4 A)Hours above 24°C temperature year by location by tier interaction B) Hours above 80% relative humidity location by tier interaction C) Total TRHC TSNA location by tier interaction D) Total TRsc TSNA location by tier interaction.



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

Figure 5 A) Hours above 24°C temperature location by room interaction B) Hours above 80% relative humidity location by room interaction C) Total TRsc TSNA location by room interaction D) Total TRHC TSNA location by room interaction



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

Figure 6 A) Hours above 24°C temperature location by bent interaction B) Hours above 80% relative humidity location by bent interaction C) Total TRHC TSNA location by bent interaction D) Total TRsc TSNA location by bent interaction