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Hair Cortisol as a Measure of Chronic Stress in Ewes Grazing Either Hardwood Silvopastures or Open Pastures

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Abstract: Hair cortisol is a relatively non-invasive and reliable measure of chronic stress, but it has received limited use, especially in pasture systems. A two-year study was carried out to compare behavioral and physiological (intravaginal temperature, hair, and blood cortisol) responses of ewes (*Ovis aries*) that grazed black walnut (*Juglans nigra*) silvopasture (BSP), honeylocust (*Gleditsia triacanthos*) silvopasture (HSP), or open pastures (OP) treatments. Ewe weights and intravaginal temperatures were recorded once for every 3-week interval. Plasma and hair cortisol concentrations were determined by ELISA. Trail cameras detected animal behavior. Ewe average daily gain was greater in HSP compared with OP ($p = 0.0456$) but did not differ with BSP ($p = 0.4686$) across both years. Ewes on OP had higher ($p < 0.0001$) hair cortisol concentrations than ewes on silvopasture treatments both summers. Ewes on OP had ≥ 0.4 °C higher ($p \leq 0.03$) intravaginal temperatures during portions of the afternoon than ewes managed in silvopasture treatments. Ewes on OP spent 500–700% more ($p < 0.0001$) time standing and 125–150% less ($p < 0.0001$) time lying down compared with ewes on silvopasture treatments. Hair cortisol measures could be an effective and relatively non-invasive technique for determining long-term chronic stress in grazing animals.

Keywords: honeylocust; black walnut; agroforestry; cortisol; heat stress

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1. Introduction

Animal welfare has been a major area of concern for the livestock industry [1]. Heat stress in extensively managed grazing animals can compromise animal welfare and productive performance, causing substantial economic consequences to producers. Silvopasture, which involves the deliberate integration of trees, livestock, and forages into a single management area [2], can be a sustainable means of reducing livestock heat stress in grazing systems. Silvopastures provide shading opportunities that can alter the micro-climatic conditions thus creating a favorable environment for grazing livestock [3–5]. This can help reduce the heat stress level in animals ultimately optimizing their overall health and well-being.

Quantifying physiological benefits for animals in pastoral systems such as silvopasture has been challenging as there are various external forces influencing animals in the system. Although weight gain typically is the default measure of animal productivity, it may not fully assess the effects of acute and chronic exposure to high-temperature environments. Cortisol, a primary stress hormone in ruminants [6], can be an important indicator of stress due to acute and chronic exposure to high temperatures, which can significantly elevate cortisol levels in animals [7]. Blood is a common matrix for assessing cortisol levels in animals, but the sampling procedure requires capturing and restraining animals which itself increases the cortisol level [8,9] potentially confounding the reliability of the assessment. Hair cortisol can be a reliable and relatively less invasive method of accessing long-term chronic stress levels in animals and this measure is not confounded by activities such as handling/restraining [10,11]. However, it has received limited use, especially in grazing systems. Similarly, the rectal temperature has also been commonly used as means of assessing the internal core body temperature of animals. However, there are limitations to its use in extensively managed animals especially for extended periods mainly due to regular fecal matter flow [5]. The use of vaginal temperature sensors could be a reliable and relatively non-invasive technique for assessing the core body temperature of extensively managed animals for an extended period [12].

Heat stress can significantly alter animal behavior and information on animal behavior can help producers understand the responses to heat stress and help them manage them accordingly. Previously, animal behavior and activity were evaluated through direct observation which is time-intensive and can potentially influence the natural behavior and activity pattern of animals [13]. Time-lapse remote cameras can be used to determine the behavior and activity pattern of animals with minimal disturbance [14]. These methods of assessing physiological and behavioral responses of animals in response to heat stress can be advantageous to traditional methods as they are relatively non-invasive to animals and thus may improve the reliability of the assessment. However, only a few studies have used these methods as a means of assessing heat stress in animals in extensive management systems [5,15,16].

Animal welfare is of increasing concern both for producers and consumers, and the adoption of silvopasture systems may help producers address this issue. The integrative, intensive, interactive, and intentional approach of silvopasture also offers a unique opportunity for landowners to manage their land for both short-term (livestock) and long-term (trees/tree products) economic returns along with a suite of different ecosystem services. Regardless of the opportunities and economic and environmental benefits, the adoption of these systems has been slow in the past [17]. Limited knowledge of these systems, site-specific management requirements, and their economic potential have been constraints to the adoption of silvopasture practices [18]. The understanding of the dynamics between shade and animal behavior/productivity is limited. Silvopastures integrating pine trees have been studied to some extent in the southeastern U.S. [3,4,19–23] and more limited work has been conducted with hardwood systems [5,15,16,24], however, none of these studies have measured animal stress response. The objective of this study was to compare the behavioral and physiological responses of ewes grazing open pastures and black walnut- (*Juglans nigra*) and honeylocust- (*Gleditsia triacanthos*) based silvopastures using relatively non-invasive techniques such as hair cortisol. We hypothesized that ewes in silvopastures would have greater average daily gain, lower hair cortisol levels, and cooler intravaginal temperatures when compared with ewes managed in open pastures.

2. Materials and Methods

2.1. Study Site and Its Management

Six-week grazing trials were carried out during the summers of 2020 (late July to early September) and 2021 (early July to mid-August). The studies were carried out at the Whitethorne Agroforestry Demonstration Center at Virginia Tech's Kentland Farm in Blacksburg, VA (37°12′00.6′′ N 80°34′34.8′′ W). Soil series at the site include Berks-Lowell-Rayne complex, Unison and Braddock, and Weaver, with slopes of 25–65%, 15–25%, and 0–5%, respectively. The study site consists of three experimental pasture systems—open pasture (OP), black walnut silvopastures (BSP), and honeylocust silvopastures (HSP). Trees in the silvopastures were established in 1995 by planting into an existing cool-season pasture. Trees were thinned to an approximate 12.3 m $\times$ 12.3 m configuration in 2012. Dominant forage species in both pasture systems include tall fescue (*Schedonorus phoenix*), orchardgrass (*Dactylis glomerata*), Kentucky bluegrass (*Poa pratensis*), and red and white clovers (*Trifolium pratense and T. repens*).

Pastures were mowed to an approximate height of 15 cm with a rotary mower to remove seed heads and were fertilized at a rate of 56-25-50 kg nitrogen-phosphorus-potassium (NPK) per hectare during late spring in 2020 and 56-0-0 kg NPK per hectare during late spring in 2021. For weed management, ProClova (florpyrauxifen + 2,4-D, Corteva Agriscience, Wilmington, DE) was applied at 4.3 L ha^{-1} in 2020 before summer grazing to control stickweed (*Verbesina occidentalis*) and other broadleaf weeds, including creeping thistle (*Cirsium arvense*) and milk thistle (*Silybum marianum*) primarily to BSP systems which had the greater presence of these weeds. Trees in the silvopastures have been trimmed periodically to remove side branches (from ~2.5 to 5 m height) to improve access for farm equipment, allow greater light to the understory, and maintain clear boles. Tree density and tree basal area averaged 91 stems ha^{-1} and 6.1 m^2 ha^{-1} in BSP and 104 stems ha^{-1} and 4.2 m^2 ha^{-1} in HSP.

2.2. Weather Data

Daily mean ambient temperatures (AT), maximum and minimum temperatures, relative humidity (RH), and rainfall data for the research site were downloaded from Virginia Tech WeatherSTEM Data Mining Tool (http://vt-arec.weatherstem.com) for the entire study period for both years (accessed on 21 December 2021). These data were recorded at a weather station located about 500 m from the study site. Average hourly AT and RH for specific dates when intravaginal temperature and behavior data were recorded were downloaded for both years and used to calculate the average hourly Temperature Humidity Index (THI) using the equation developed by Mader et al. [25].

$$THI = [(0.8 \times AT) + (RH/100) \times (AT - 14.4)] + 46.4 \quad (1)$$

2.3. Microclimatic Data

One Spectrum WatchDog 1000 Series Microstation (Spectrum Technologies, Inc., Aurora, IL, USA) was installed in each experimental unit within a replication to collect AT, RH, and photosynthetically active radiation (PAR) data for both summers. These micro stations were preprogrammed to collect measures every 10 min throughout the study period. Data from the loggers were downloaded into a computer using SpecWare 9 Pro software (Spectrum Technologies, Inc., Aurora, IL). To estimate the level of heat stress within the systems, THI was calculated from AT and RH [25].

2.4. Experimental Design, Sheep, and Grazing Management

The experimental systems (OP, BSP, and HSP) were replicated three times and arranged in a randomized complete block design. Thirty-six (36) open Katahdin ewes from Virginia Tech's Southwest Virginia Agricultural Research and Extension Center, Glade Spring, VA were used for the study each year. In 2020, ewes used for the study were 3 to 6 years old with a mean initial body weight of 53 kg $\pm$ 0.9 kg. In 2021, ewes used for the study were 4 to 7 years old with a mean initial body weight of 52 kg $\pm$ 1.1 kg. These animals are part of the University's breeding flock and are raised on pasture and supplemented with grain only during the late gestation/lactation (March to early May). Prior to the study, ewes were vaccinated for *Clostridium perfringens* type C and D and *C. tetani* and were dewormed with Cydectin for each year. Ewes were checked for anemia levels by using a FAMACHA protocol [26] prior to the study and at 3-week intervals thereafter. Ewes were stratified by body weight, age, and coat color and four ewes then were randomly assigned to one of the nine experimental units (EU). Each 0.27-ha EU was subdivided into four permanent subpaddocks. Each subpaddock was further subdivided using electrified net fencing to create four approximately equal sections within each subpaddock and ewes were rotational stocked when the forage residual height reached about 7 cm.

2.5. Forage Analysis

Pre-and post-grazing forage biomass within each EU was estimated with a double-sample technique using a rising plate meter (Jenquip, Fielding, NZ) and quadrats [27,28]. Two forage grab samples were also collected randomly by walking across each experimental treatment at the beginning of each season and dried in an oven at 60 °C for 72 h. Samples were ground in a Wiley mill (Thomas Wiley, Philadelphia, PA) to pass through a 2 mm screen and then ground to pass through a 1 mm screen in a cyclone mill (Udy Mill, (UDY Corporation, Fort Collins, CO, USA). Ground samples were scanned in NIRS DS2500F using ISIScan Nova v. 8.0.6.2 (Foss North America, Eden Prairie, MN) to estimate crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) content. Equations for the forage nutritive analysis were standardized and checked for accuracy using the grass hay equation developed by the NIRS Forage and Feed Consortium [29].

2.6. Animal Weight Gain

Animal body weight (BW) of all ewes was measured on two consecutive days at the beginning (days −1 and 0) and end (days 41 and 42) of the study and averaged to determine ewes' beginning and ending BW. The average daily gain (ADG) of ewes was determined by dividing the total BW gain by the number of days animals were stocked on the treatment pastures.

2.7. Intra-Vaginal Temperatures

Ewe intra-vaginal temperatures were measured with a Star Oddi Data Storage Tag (DST) micro-T temperature logger (Star Oddi, Iceland; sensitivity- 0.2 °C). These loggers were attached to a blank controlled internal drug release (CIDR) device (Eazi-Breed, Zoetis, Parsipanny, NJ, USA) by removing about a 2 cm segment from the middle of the CIDRs, replacing it with the logger, and wrapping the assembled pieces with vinyl electrical tape. CIDRs with loggers were inserted into the vagina of two ewes within each EU for two consecutive days at mid- (days 19 and 20) and end (days 40 and 41) of the study using a CIDR sheep applicator for both years. The loggers were set to collect temperature data at every 10 min interval. Data collected were downloaded through a communication box and Mercury software (Star Oddi, Iceland) and was exported to Microsoft Excel for further analysis.

2.8. Hair and Plasma Collection, Cortisol Extraction, and Analysis

On day 0 of the experiment, hair samples were collected from an approximate 15 cm × 15 cm site in the loin region of all ewes by clipping close to the skin with an electric clipper (900cl Cordless Clipper with Eagle 30 Small Clipper Blade Set, Premier 1 Supplies, Washington, IA, USA). This sample served as a baseline measure of hair cortisol. The same site was trimmed again on days 21 and 42. Hair samples were wrapped in aluminum foil and stored at room temperature until analysis. Cortisol in hair samples was extracted using methanol [11]. Briefly, 250 mg of hair was washed 3 times with a 5-mL aliquot of 100% isopropanol and was left to dry at room temperature for 72 h. Dried hair samples were ground with a mini bead beater (BioSpec Products, Inc., Bartlesville, OK, USA) for 5 min at 30 Hz. Cortisol was extracted from ground hair samples by adding 1 mL of 100% methanol in a microcentrifuge tube with 50 mg of ground hair and rotating in an orbital shaker for ~24 h. Samples were centrifuged and 600 µL of liquid supernatant was aliquoted to a new tube. Methanol was dried off and samples were reconstituted with ELISA buffer. Cortisol concentration was quantified with a commercial salivary cortisol ELISA (Enzyme-Linked Immunosorbent Assay) kit (Cayman Chemical, MI, USA) according to the manufacturer's instructions. Along with the hair samples, blood samples were also collected from all ewes by jugular venipuncture on days 0, 21, and 42. Samples were kept in a cooler with ice until plasma was separated from blood samples by centrifugation at 3400× *g* at room temperature for 15 min. The plasma samples collected were stored at −70 °C until further analysis. Cortisol was extracted by adding 5× volume of ethyl ether into the plasma sample, drying the ethyl ether overnight, and reconstituting with ELISA buffer [30]. The plasma cortisol level was determined using the same commercial cortisol ELISA test kit (Cayman Chemical, MI, USA).

2.9. Animal Behavior Data

Time-lapse imagery was collected simultaneously with measures of intravaginal temperature (two consecutive days every 3 weeks) using Moultrie D-500 trail cameras (EBSCO Industries, Inc., Birmingham, AL). One camera was set up in each EU in such a way that it could capture images of the entire section of the sub paddock where ewes were stocked. Two (2) ewes in each EU with temperature loggers were sprayed with orange or blue color fluorescent paint (one color per ewe) for easy visual identification. Images were captured from morning (0700 or 0800) to evening (2100 h) at one-minute intervals. Animal behaviors (grazing, lying, standing up, drinking water, and eating salt) were recorded manually for each captured image, and the total time spent in each activity by each ewe was summed.

2.10. Statistical Analysis

A regression equation of plate meter reading against forage mass was calculated with PROC REG in SAS Studio, v. 3.5 (SAS Inst., Cary, NC, USA) and the best fit was determined with a quadratic equation. A mixed-effect analysis of variance test was carried out to determine the difference in ADG, forage nutritive value, pre-and post-grazing forage biomass, cortisol measures, intravaginal temperature, and animal behavior using PROC MIXED in SAS Studio, v. 3.5. The study was conducted as a randomized complete block design with three replications; year was included as a random effect. Repeated measures analysis by period was used with a standard variance-covariance structure for the analysis of hair cortisol and intravaginal temperatures data. A compound symmetry variance-covariance structure was used for the analysis of plasma cortisol and ewes' behavior data. Repeated measures analysis by sampling data was used with a compound symmetry variance-covariance structure for the analysis of pre-and post-grazing forage biomass and herbage disappearance data. Variance-covariance structure for the analysis was selected based on the lowest AIC value. Microclimatic data for both summers were analyzed using a one-way analysis of variance test using PROC GLM in SAS Studio, v.3.5. LS- means and Tukey's adjusted differences were calculated. Differences were considered significant when $p < 0.05$ and was reported as trends when $0.05 < p < 0.10$.

3. Results

3.1. Weather Data

The mean daily AT throughout the study period in 2020 and 2021 was 22.6 °C and 25.2 °C, respectively. Mean minimum and maximum AT were 18.3 °C and 29.0 °C in 2020 and 17.4 °C and 29.7 °C, respectively, in 2021 (Table 1). In 2020 and 2021, the study site received 7.7 and 1.8 cm of total precipitation throughout the respective study periods. The temperature-humidity index at the study site was above 72 during the late morning and afternoon hours (1000 to 1900 h) during the intravaginal temperature and behavior data collection dates for both years (Figure 1).

Table 1. Mean daily temperature (°C), minimum temperature (°C), maximum temperatures (°C), and total precipitation (cm) throughout the study period during the summers of 2020 and 2021 at Whitethorne Agroforestry Demonstration Center, Blacksburg, VA, USA.

Year	Temperature °C			Precipitation (cm)
	Max [1]	Min	Mean	
2020	29.0	18.3	22.6	7.7
2021	29.7	17.4	25.2	1.8

[1] Max—Maximum; Min—Minimum.

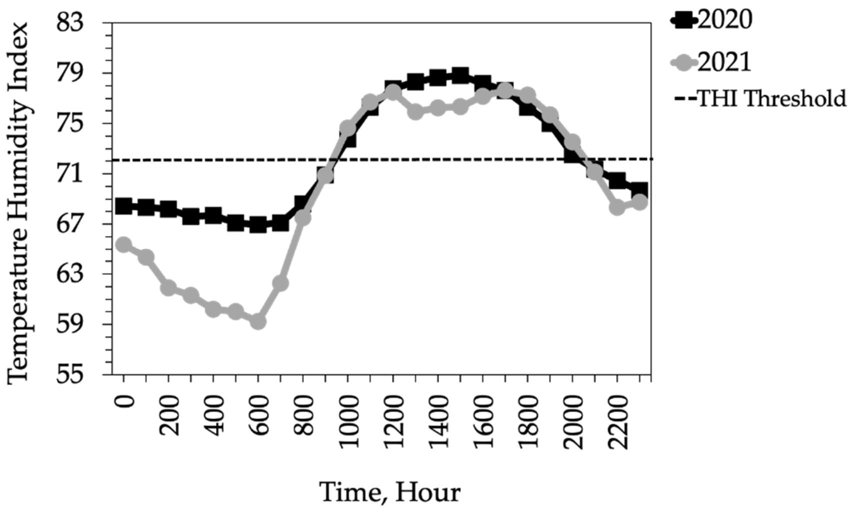

Figure 1. Average temperature-humidity index (THI) by hour during the intravaginal temperature and ewes' behavior data collection dates for the summers of 2020 and 2021.

3.2. Microclimatic Conditions

In 2020, average daily AT in OP tended ($p = 0.0599$) to be greater than in BSP whereas it was similar compared with HSP ($p = 0.8768$; Table 2). In 2021, the average daily AT did not differ among treatments ($p \geq 0.4943$). In 2020, RH did not differ among treatments, whereas in 2021, RH was higher in OP than in silvopasture treatments ($p \leq 0.0375$). The PAR was higher in OP compared with silvopasture treatments for both years ($p < 0.0001$).

Table 2. Microclimatic conditions in an open pasture (OP), black walnut silvopasture (BSP), and honeylocust silvopasture (HSP) treatments throughout the study period during the summers of 2020 and 2021 at the Whitethorne Agroforestry Demonstration Center, Blacksburg, VA, USA.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
			Temperature, °C				
2020	21.8	22.6	22.9	0.34	0.2159	0.0599	0.8081
2021	22.7	23.0	23.2	0.27	0.7945	0.4943	0.8768
			Relative Humidity, %				
2020	84.4	86.5	86.1	1.26	0.4758	0.6128	0.9733
2021	77.1	76.0	80.7	1.03	0.7461	0.0375	0.0047
			PAR [2], $\mu Molm^{-2} s^{-1}$				
2020	121	265	364	10.6	<0.0001	<0.0001	<0.0001
2021	188	346	517	11.8	<0.0001	<0.0001	<0.0001
			THI [3]				
2020	69.6	70.9	71.3	0.54	0.1971	0.0687	0.8653
2021	71.0	71.3	72.0	0.44	0.8784	0.2351	0.4853

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture. [2] PAR—Photosynthetically active radiation; [3] THI—Temperature humidity index.

3.3. Forage Measures

3.3.1. Forage Biomass

The year x treatment interaction was significant for pre-grazing forage biomass and herbage disappearance ($p < 0.0001$) and non-significant for post-grazing forage biomass ($p = 0.1634$). Pre-grazing forage biomass in OP was greater ($p < 0.0001$) compared with BSP for both years whereas it was greater ($p < 0.0001$) in OP compared with HSP in 2020, but not in 2021 ($p = 0.4373$; Table 3). Post-grazing forage biomass was greater ($p < 0.0001$) in OP compared with silvopasture treatments across both years and it was greater ($p = 0.0031$) in HSP compared with BSP treatment across both years. In 2020, forage disappearance did not differ ($p \geq 0.3867$) among treatments whereas in 2021, HSP had greater ($p < 0.0001$) herbage disappearance followed by BSP and OP treatments.

Table 3. Estimated pre- and post-grazing forage biomass and herbage disappearance (difference) in an open pasture (OP), black walnut silvopasture (BSP), and honeylocust silvopasture (HSP) treatments during the summers of 2020 and 2021 at the Whitethorne Agroforestry Demonstration Center, Blacksburg, VA, USA.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
			Pre-grazing [2], kg ha^{-1}				
2020	3090	3380	3970	61.3	0.0017	<0.0001	<0.0001
2021	3400	4020	4090	69.9	<0.0001	<0.0001	0.4373
			Post-grazing, kg ha^{-1}				
2020	2130	2430	3180	80.2	0.0074	<0.0001	<0.0001
2021	2400	2730	3310	66.6	0.0056	<0.0001	<0.0001
Average	2270	2570	3240	53.9	0.0031	<0.0001	<0.0001
			Difference [2], kg ha^{-1}				
2020	920	910	810	86.0	0.9718	0.3867	0.4060
2021	990	1290	770	43.4	<0.0001	0.0013	<0.0001

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture; [2] Significant year x treatment interaction (*p*-value: Pre-grazing < 0.0001; Difference = 0.0022).

3.3.2. Forage Nutritive Value and Digestibility

There was no year x treatment interaction for forage CP ($p = 0.5887$), ADF ($p = 0.7700$), and NDF ($p = 0.2217$) data. Forage CP concentration did not differ ($p \geq 0.7346$) between treatments across both years (Table 4). Forage from BSP had greater ($p = 0.0342$) ADF and tended ($p = 0.0979$) to have greater NDF than OP across both years.

Table 4. Forage nutritive value in an open pasture (OP), black walnut silvopasture (BSP), and honeylocust silvopasture (HSP) treatments during the summers of 2020 and 2021 at the Whitethorne Agroforestry Demonstration Center, Blacksburg, VA, USA.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
			CP [2], %				
2020	18.4	18.6	19.0	0.85	0.8956	0.6451	0.7388
2021	20.8	21.3	20.8	0.85	0.6835	0.9744	0.7067
Average	19.6	19.9	19.9	0.67	0.7346	0.7600	0.9730
			ADF [2], %				
2020	31.9	31.6	30.5	0.49	0.6675	0.0924	0.1725
2021	28.6	27.7	26.5	0.96	0.5337	0.1683	0.3997
Average	30.3	29.7	28.5	0.45	0.3835	0.0342	0.1238
			NDF [3], %				
2020	60.2	59.6	58.8	0.69	0.5515	0.2096	0.4682
2021	56.4	54.0	52.5	1.63	0.3503	0.1444	0.5306
Average	58.3	56.8	55.6	0.95	0.3123	0.0979	0.4251

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture. [2] CP—Crude protein; ADF—Acid detergent fiber; [3] NDF—Neutral detergent fiber.

3.4. Animal Gain

There was no year x treatment interaction for ADG ($p = 0.1781$). The overall ADG of ewes in OP, BSP, and HSP was 51.3, 73.6, and 85.9 g d^{-1}, respectively. Greater ADG was measured in HSP ($p = 0.0457$) compared with OP across both years (Table 5). The ADG of ewes in BSP did not differ from that of ewes on HSP ($p = 0.4686$) and OP ($p = 0.1902$) across both years.

Table 5. Average daily gain (g day^{-1}) of Katahdin ewes that grazed either open pasture (OP), black walnut silvopasture (BSP), or honeylocust silvopasture (HSP) treatments during the summers of 2020 and 2021.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
2020	12.6	30.0	19.9	21.50	0.4360	0.7434	0.6495
2021	134.6	141.9	82.8	16.73	0.7641	0.0361	0.0203
Average	73.6	85.9	51.3	11.87	0.4686	0.1902	0.0457

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture.

3.5. Intravaginal Temperature

There was no year x treatment interaction ($p \geq 0.1089$) for intravaginal temperature data. At 1000 h, the intravaginal temperature of ewes on OP tended to be higher ($p = 0.0730$) compared with ewes on silvopasture treatments (Figure 2). Ewes on OP had higher ($p \leq 0.0343$) intravaginal temperatures than ewes on both silvopasture treatments between 1100 h–1700 h and ewes on HSP between 1800 h–2000 h. The intravaginal temperature of ewes on OP tended to be higher than ewes on HSP at 2100 h ($p = 0.0980$) and ewes on BSP at 1800 h ($p = 0.0804$).

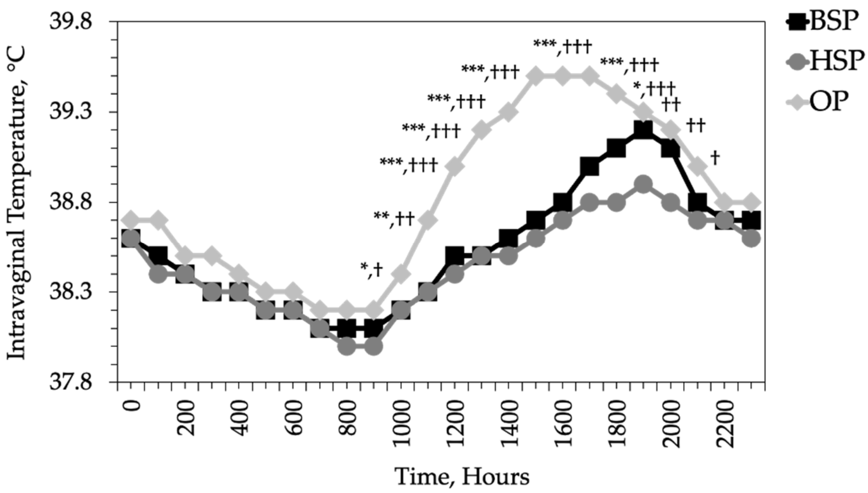

Figure 2. Mean vaginal temperatures (SE = 0.1) of Katahdin ewes by hour that grazed either open pasture (OP), black walnut silvopasture (BSP), or honeylocust silvopasture (HSP) treatments. * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$—Indicates a trend or significant difference between BSP and OP † $p < 0.1$, †† $p < 0.05$, ††† $p < 0.01$—Indicates a trend or significant difference between HSP and OP.

3.6. Plasma and Hair Cortisol Concentration

There was no year x treatment interaction for plasma cortisol data ($p = 0.3613$) whereas there was a significant year x treatment interaction for hair cortisol data ($p = 0.0356$). Plasma cortisol level was greater ($p = 0.0400$) in ewes on OP compared with BSP whereas there was no difference in plasma cortisol level in ewes on HSP and BSP ($p = 0.6954$). There was a trend ($p = 0.0983$) toward greater plasma cortisol level of ewes on OP compared with HSP treatment across both years (Table 6). Ewes on OP had a greater hair cortisol level compared with ewes on silvopasture treatments both in 2020 and 2021 ($p < 0.0001$). In 2021, ewes on BSP had lower ($p = 0.0236$) hair cortisol levels compared with ewes on HSP.

3.7. Animal Behavior

There was a trend toward significant year x treatment interaction for time ewes spent grazing ($p = 0.0611$) and a significant year x treatment interaction for time ewes spent eating salt ($p < 0.0001$). There was no year x treatment interaction ($p \geq 0.2747$) for standing, lying, and drinking water behavior categories. In 2020, ewes on BSP spent about 20% more time grazing than ewes on OP (p = 0.0054) and HSP (p = 0.0106) whereas in 2021 ewes on BSP spent about 36% more time (p = 0.0014) grazing than ewes on HSP and ewes on OP spent about 20% more time (p = 0.0192) grazing compared with ewes on HSP (Table 7). Ewes on OP spent 400% more time standing than ewes on BSP ($p < 0.0001$) and 750% more time standing than ewes on HSP ($p < 0.0001$). Ewes on OP spent 20% less time lying down compared with ewes on BSP ($p < 0.0001$) and 33% less time lying down compared with ewes on HSP ($p < 0.0001$). Ewes on silvopasture treatments showed some trend toward 70% less time ($p \leq 0.0599$) drinking water compared with ewes on OP.

Table 6. Plasma cortisol level (ng ml^{-1}) and hair cortisol level (pg mg^{-1}) of Katahdin ewes that grazed either open pasture (OP), black walnut silvopasture (BSP), or honeylocust silvopasture (HSP) treatments during the summers of 2020 and 2021.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
			Plasma cortisol, ng ml^{-1}				
2020	20.2	19.3	21.1	1.54	0.6534	0.6833	0.3925
2021	11.2	13.3	17.9	2.34	0.5338	0.0489	0.1883
Average	15.6	16.4	19.6	1.40	0.6954	0.0400	0.0983
			Hair cortisol [2], pg mg^{-1}				
2020	21.5	22.0	38.9	2.06	0.8642	<0.0001	<0.0001
2021	24.6	33.0	51.1	2.95	0.0236	<0.0001	<0.0001

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture. [2] Significant year x treatment interaction (p = 0.0356).

Table 7. Percent of time (0700–2100 h) spent by ewes within different behavior categories in either open pasture (OP), black walnut silvopasture (BSP), or honeylocust silvopasture (HSP) treatments during the summers of 2020 and 2021.

Year	Treatments [1]				Tukey's Adjusted *p*-Value		
	BSP	HSP	OP	SE	BSP vs. HSP	BSP vs. OP	HSP vs. OP
			Grazing [2], %				
2020	40.5	34.8	33.7	1.46	0.0106	0.0054	0.6264
2021	34.1	25.1	31.4	1.80	0.0014	0.2950	0.0192
			Standing, %				
2020	5.7	3.3	22.9	1.22	0.1872	<0.0001	<0.0001
2021	4.9	2.8	22.9	1.31	0.2630	<0.0001	<0.0001
Average	5.3	3.1	22.5	1.36	0.2434	<0.0001	<0.0001
			Lying, %				
2020	53.5	61.7	43.2	1.77	0.0032	0.0008	<0.0001
2021	60.9	72.1	45.4	1.76	0.0001	<0.0001	<0.0001
Average	57.3	66.9	45.1	1.80	0.0004	<0.0001	<0.0001
			Drinking Water, %				
2020	0.07	0.05	0.09	0.027	0.5302	0.6214	0.2864
2021	0.00	0.01	0.14	0.034	0.8306	0.0095	0.0158
Average	0.03	0.03	0.14	0.039	0.9506	0.0599	0.0542
			Eating Salt [3], %				
2020	0.24	0.17	0.10	0.061	0.3995	0.1251	0.4350
2021	0.03	0.09	0.11	0.042	0.3380	0.1888	0.7232

[1] Treatments: BSP—Black walnut silvopasture; HSP—Honeylocust silvopasture; OP—Open pasture. [2] A trend towards significant year x treatment interaction (p = 0.0611). [3] Significant year x treatment interaction ($p < 0.0001$).

4. Discussion

4.1. Microclimatic Condition

Microclimatic measures (ambient temperature, relative humidity, soil moisture, and PAR) indicate environmental conditions in OP were more stressful than in silvopasture treatments. Ambient temperature and PAR tend to be lower under tree canopies compared with open systems [31], and past studies have reported cooler microclimatic conditions in silvopasture versus OP [3,4,32,33]. The BSP and HSP silvopasture systems used in this study provided shade and lowered the PAR and ambient temperature within the system compared with OPs with no canopy cover. Differences in tree species also were observed, as PAR was greater in HSP compared with BSP. This is likely due to the differences in tree and leaf size and structure, and differences in canopy architecture between honeylocust and black walnut trees. Although we did not make direct measures of tree, leaf, and canopy size in BSP and HSP treatments, past measures and visual observation indicated black walnut trees generally were larger, with larger canopies, larger leaves and leaflet and denser canopies. These differences translated to lower light levels within the BSP system.

4.2. Forage Measures

4.2.1. Forage Biomass

The impact of trees on forage productivity in silvopasture is dependent on various factors such as forage and tree species utilized, age of the system, and tree arrangement [34]. Pre-grazing forage biomass was 20% less in BSP compared with OP. This corresponded with reduced levels of PAR in that system but may also reflect changes in forage species that have been observed under BSP at this site [5,15,16,35]. Changes in forage biomass in response to increasing shade levels are not linear [36] but yield generally declines substantially when PAR is reduced to less than 50% of full sun. Similar yield reductions have been observed in pine-walnut silvopastures in Missouri when compared with OP systems [23]. In contrast, the smaller honeylocust trees, with thinner canopy cover, allowed more PAR to penetrate to the forage understory; thus, forage production in HSP was similar to that in OP. There has been no significant reduction in forage productivity of cool-season grasses with up to 50% shade in some studies [37,38]. These results underscore the importance of light and tree canopy management for maintaining forage productivity under silvopasture systems.

Greater forage disappearance in silvopasture treatments compared with OP might be attributed to greater intake by ewes in silvopasture treatments. However, actual forage intake by ewes was not measured in this study, and changes in the rate of disappearance might also be due to the underestimation of residual forage mass in silvopasture treatments. Greater intake by lambs in BSP compared with OP (as estimated from herbage disappearance) has been observed at this research site [39]. Intake estimates of animals in silvopasture systems have received very limited study and require more investigation to better understand the efficiency of these systems.

4.2.2. Forage Nutritive Value and Digestibility

The trees within silvopasture systems can significantly impact understory forage nutritive value through botanical, morphological, and physiological adaptations [37,40]. Past studies on both cool-season [41] and warm-season [42] forages have reported improvement to forage nutritive value and digestibility underneath trees or shade as compared with open systems, although equal or lower digestibility of forages underneath the shade has also been reported [43,44]. Forage CP content did not differ among treatments whereas forage ADF and NDF content were greater in BSP compared with OP across both years in the present study. A study by Fannon et al. [35] also reported no difference in forage CP and greater forage ADF, NDF, and lignin content in BSP compared with OP during summer at this site. Higher fiber concentration in BSP likely reflects the differences in vegetation composition between treatments as the competition of warm-season grass (nimblewill' *Muhlenbergia scherberi*) was greater in BSP compared with OP [35].

4.2.3. Animal Gain

Animal productivity in grazing systems is directly influenced by a number of variables. Ambient temperature, relative humidity, and solar radiation are the key climatic variables that affect animal production [45] and these climatic conditions can severely impact the overall health, metabolism, and physiology of animals [46,47]. The impacts of climatic variables may be lessened or eliminated by integrating trees within the system to promote animals' comfort and help improve animal productivity [48,49]. In some cases, the reduction in stress associated with grazing under trees could compensate for the reduction in available forage. The average daily gain of ewes in OP was lower compared with ewes in HSP but did not differ with BSP treatment. This is likely due to greater intake by ewes in HSP as the herbage disappearance in HSP was higher than OP. Additionally, a cooler ambient condition in HSP due to tree shade may have reduced heat stress in ewes thus improving their overall productivity. The animal gain was low during the first summer, but ADG improved significantly in the second year. Forage productivity in silvopasture treatments during the second year was about 10% more along with greater CP and lower fiber content of forages compared with the first year which may have created a better opportunity for ewes gain in the second year compared with the first year. The ewes used for the study were completely new to the environment and may have been stressed due to herd separation which may have significantly impacted their behavior and diet selection during the first year of the study. The literature suggests that sheep when transferred to a new environment significantly modify their behavior and diet selection [50,51] thus influencing their overall performance. However, the same ewes were used during the second year of the study, and they were more familiar with the environment, which may have also helped improve their overall performance.

4.2.4. Intravaginal Temperatures

The intravaginal temperature loggers used to measure ewe core body temperatures in the present study can collect data for an extended period with little disturbance to the animals [12]. Intravaginal temperatures are well correlated with rectal temperatures and other measures of core body temperatures [12]. Ewes on silvopasture treatments were cooler than ewes on OP, especially during periods of greater ambient temperature in the OP systems. A study by Pent et al. [5] also reported a 0.3–0.5 °C reduction in the intravaginal temperature of lambs in BSP compared with lambs in OP during the afternoon hours (1200–1900 h). This difference in core body temperature of ewes between treatments especially during the afternoon hours indicates a stronger response to the ambient condition by ewes in OP than for those in silvopasture treatments. These findings support the work of others [5,52] who found greater core body temperatures in animals without shade during the afternoon hours, corresponding to the hottest part of the day. Shade within the silvopasture systems helped lower the core body temperature of ewes thus reducing their heat load and overall stress compared with ewes on OP.

4.2.5. Plasma and Hair Cortisol Concentration

Measures of plasma cortisol did not differ between ewes that grazed silvopasture or OP treatments, and the data were highly variable. The sampling procedure for this assay required that animals be moved from treatment pastures to a central pen and subsequently handled and restrained to draw blood. Such procedures can cause acute stress, thus this procedure itself likely increased the blood cortisol levels in our animals [52] and would potentially confound the reliability of the assessment. This may have resulted in high variability of data in an acute measure and thus a nonsignificant difference in plasma cortisol levels among treatments in the current study.

Hair cortisol levels were lower in ewes on silvopasture treatments compared with ewes on OP. Blood cortisol diffuses into the hair and accumulates over time as animals are exposed to stressors such as elevated temperature. Hair cortisol thus reflects long-term chronic stress levels in animals over weeks to months [53] and is not confounded by activities such as animal handling and restraint [10,11], and various studies have reported a positive correlation between cortisol level in hair and stress [54,55]. Thus, hair cortisol could provide a more reliable means of assessing long-term chronic heat stress of animals in extensive grazing systems. This approach also is relatively less invasive, collecting hair samples is simple, and the samples can be stored at room temperature for long periods [53].

Lower hair cortisol in ewes on silvopasture treatments likely reflects milder microclimatic conditions in silvopasture treatments compared with OP and the lower body temperatures maintained through the hottest part of the day. Shade minimizes heat stress in ewes [56] and, in past work, sheep on this site were seen to actively move to access shade during the day [15].

4.2.6. Animal Behavior

Ewes on BSP spent greater time grazing compared with ewes of HSP and OP treatments Likely due to more limited forage availability. Animals spend less time grazing when there is plenty of forage available and is of good quality whereas animals spend more time grazing when the quantity or quality of forages is limited [57]. Ewes in silvopastures experienced less heat stress and spent more time lying down than ewes in OP. In contrast, ewes on OP were more stressed and thus spent more time standing up. Time spent lying down is considered a traditional metric of animal comfort [58,59] whereas standing up or loafing is considered a behavioral response to heat stress [60,61]. Standing improves airflow and increases the effectiveness of convection heat loss from the body in animals under heat stress, thus ewes on OP may have spent more time standing as a strategy to reduce head load [62]. More time spent standing could also be an adaptive mechanism to limit heat gain from the ground surface. Higher solar radiation and ambient temperature may have increased the surface temperature in OP compared with silvopasture treatments, limiting ewes' ability to lie down and lose heat through conduction. However, in silvopasture treatments, cooler ambient conditions due to shade may also have reduced surface soil temperatures, thus encouraging ewes to lie down and lose heat through conduction to the soil. Ewes on OP also spent more time drinking water compared with ewes on silvopasture treatments. This might also be a response of ewes to a stressful environment, as drinking water can help reduce heat load in animals [63]. The differences observed in ewes' behavior among treatments likely reflect both direct effects of altered microclimatic conditions (and greater heat stress in OP systems) as well as indirect effects in terms of changes in forage composition and productivity (which affected grazing behavior, particularly in BSP).

5. Conclusions

Animal welfare is a major concern for the livestock industry. Heat stress in grazing systems can compromise animal welfare and productive performance, causing economic losses to livestock producers. Silvopastures provide shading opportunities that can help reduce heat stress and improve the health and well-being of grazing animals. Black walnut and honeylocust trees in these silvopasture systems altered micro-climatic conditions and created a more favorable environment for sheep. Ewes grazing HSP had greater gains compared with those in OP. Ewes in silvopasture systems had cooler intravaginal temperatures and lower hair cortisol level concentrations, indicating lower stress levels. Because blood cortisol gets elevated quickly in response to acute stressors such as handling and restraint, hair cortisol provided a more reliable and relatively non-invasive method of assessing animal stress in these extensive grazing systems. This approach may be valuable where measures of chronic (vs. acute) stress are appropriate for the given research. Although both tree species used in the study moderated ambient conditions and helped reduce stress levels in ewes, tree species had variable effects on animal physiology and overall productivity of the system, which should be a key consideration for producers adopting silvopasture systems.

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