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Antimicrobial Resistance in Bacteria Isolated from U.S. Goat, Sheep, and Lamb Cecal Content Samples: A FSIS NARMS Study

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Summary

The demand for goat, sheep, and lamb meat is increasing in the U.S. and globally. Unfortunately, in these animals, the prevalence of bacterial antimicrobial resistance (AMR) at slaughter is poorly understood. AMR is a global public health threat that may result in treatment failure and deaths in humans and animals. To address knowledge gaps, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) conducted this first nationwide cecal (intestinal) sampling study under the National Antimicrobial Resistance Monitoring System (NARMS). From February 2020 to September 2022, FSIS collected a total of 1,025 cecal samples from goat, sheep and lamb from 449 FSIS-regulated slaughter establishments. The recovery of *Salmonella* of public health importance was low. Analysis showed that 91% of *Salmonella*, 23% of *Campylobacter*, 61% of *Enterococcus*, and 48% of generic *E. coli* found were not resistant (were pan-susceptible) to the antimicrobials tested. Resistance to 1-2 antimicrobial classes was highest in *Campylobacter* (74%), followed by *Enterococcus* (52%), generic *E. coli* (30%), and *Salmonella* (8%). Resistance to quinolones (ciprofloxacin and nalidixic acid) and/or tetracycline was exhibited in *Campylobacter*. Resistance to tetracycline was highest among *Salmonella*, generic *E. coli*, and *Enterococcus*. Multi-drug resistance (resistant to three or more classes of antimicrobial drugs) was highest in generic *E. coli* (9%), followed by *Campylobacter* (3%), *Salmonella* (≤ 1%), and *Enterococcus* (≤ 1%). A host-adapted *Salmonella* IIIb 61:k:1,5,(7) (enterica subspecies *diarizonae*) that can cause serious illnesses in sheep and lamb, was recovered in disproportion-

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ally higher numbers from cecal samples of sheep (63%) and lamb (52%) compared to goats (2%). More than 40% of cecal *Campylobacter* isolates from goat and sheep were resistant to quinolones (ciprofloxacin and nalidixic acid). This study provides a representative national snapshot of AMR occurrence in pathogens (*Salmonella*, *Campylobacter*) and indicator bacteria (generic *E. coli*, *Enterococcus*) from goat, sheep, and lamb collected from cecal content at the time of slaughter.

Key Words: Antimicrobial Resistance; National Antimicrobial Resistance Monitoring System; *Salmonella* IIIb 61: K: 1,5, (7); *Campylobacter*; generic *E. coli*; *Enterococcus*.

Introduction

Food-producing animals are a valuable source of macronutrients, including protein, micronutrients, and a variety of edible and inedible byproducts. In the U.S. alone, animal-derived foods currently provide energy (24% of total), protein (48%), essential fatty acids (23-100%), and essential amino acids (34-67%) in people's diet (White and Hall, 2017). Based on data from 2022, compared to the per capita U.S. consumption of major meat sources such as pork (56 lbs.), poultry (113 lbs.) or beef (59 lbs.), the per capita consumption of goat, sheep, and lamb meat is significantly lower at 0.25 lbs. for goat meat and 1.3 lbs. for lamb and mutton (Statista — beef, pork, poultry, lamb, and mutton). It is noteworthy that the popularity and demand of sheep and lamb is growing among U.S. ethnic populations in urban areas (Harvest Returns, 2023). The demand for and consumption of goat, sheep, and lamb meat are also increasing globally. By 2030, sheep meat as a source of dietary protein is expected to grow by 15.7% (OECD-FAO 2021). According to Mazinani, global sheep production is nearing 9 million tons, and ranks fourth after pork, poultry, and beef (Mazinani, 2020).

While food-producing animals are important sources of nutrients they can also be reservoirs for zoonotic pathogens. According to the Centers for Disease Control and Prevention (CDC), estimates are that animals spread more than 6 out of every 10 known human infectious diseases and 3 out of every 4 new or emerging infectious diseases in people come from animals (CDC, About Zoonotic Diseases). Some of these pathogens can cause foodborne infections and may be resistant to antimicrobials (*i.e.*, exhibit antimicrobial resistance, or AMR). Infections with AMR pathogens in humans are difficult to treat and can result in unexpected treatment failures and even death (CDC, 2019).

To protect the health of people and animals, zoonotic foodborne pathogens and AMR need to be managed effectively with the goal of reducing AMR to meet national and international AMR reduction targets (WHO, 2021). In food-producing animals, this requires a One Health type approach that encompasses 'farm to fork' components of farming, processing, distribution, and consumption to prevent, detect, and control hazards from pathogens of animal origin (Abebe et al., 2020) (WOAH, n.d.). This requires robust, well-designed, multifaceted national surveillance systems for detecting pathogens and AMR. Countries with well-designed national level AMR surveillance systems include the U.S., the European Union (European Commission, 2023), Canada (CARSS, 2023), Australia (AUS, 2019) and New Zealand (New Zealand Ministry of Health, 2017). While some of these surveillance systems use a unified farm to fork approach (such as the Canadian Integrated Program for Antimicrobial Resistance)(CIPARS), other countries (such as the U.S.) use separate surveillance systems designed to capture pathogen and AMR trends at different points from farm to fork.

In the U.S., the U.S. Department of Agriculture (USDA) monitors AMR and animal pathogens with the Animal and Plant Health Inspection Service (APHIS) responsible for studies of on-farm pathogens, and AMR through the National Animal Health Monitoring System (NAHMS) (APHIS, 2022, 2024). The Food Safety and Inspection Service (FSIS) studies pathogens and AMR from cecal and food samples collected from poultry, swine, and cattle at slaughter and processing (FSIS, 1996). FSIS analyzes pathogens and AMR from cecal and food samples in collaboration with the National Antimicrobial Resistance Monitoring System (FSIS NARMS, n.d.). Within the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration NARMS (FDA NARMS, n.d.) program studies pathogens and AMR in retail samples of meat products from poultry, swine, and cattle, the final stage in "farm to fork." While these AMR studies are in different populations and at different stages of livestock and poultry production, together they provide a national snapshot of AMR in food-producing animals and animal-derived foods in the U.S. Studies at the regional or local level help to assess and address changes in pathogens and AMR (EFSA et al., 2021; Herawati et al., 2023).

In the U.S., national level surveillance for zoonotic pathogens and AMR has historically focused on the major meat producing species of poultry, swine, and cattle. APHIS periodically studies zoonotic pathogens and AMR in fecal samples of goat, sheep, and lamb through NAHMS focusing on farm level production. Studies of the minor meat producing species of goat, sheep, and lamb at slaughter are limited, leaving a data gap in this area. Recognizing this data gap, in 2017, FDA's Science Board recommended that the microbial hazards of concern in these food-producing animals and their potential risk to human health and food safety be studied further (FDA, 2017). In February 2020, in collaboration with FDA, FSIS initiated the NARMS expansion surveillance projects. These included a study of AMR in *Salmonella*, *Campylobacter*, generic *Escherichia coli* (*E. coli*), and *Enterococcus* spp. isolated from cecal samples collected from goat, sheep, and lamb. This was the first nationwide AMR study in these minor species at slaughter.

Materials and Methods

Sampling Design

The FSIS Annual Sampling Plan (FSIS, 2024a) outlines the Agency's overall strategy for directing sampling resources in a given fiscal year. It identifies changes planned for various sampling programs and aligns goals and measures with sampling activities and results. The FSIS NARMS part of the sampling program is based on classes of animals slaughtered and annual slaughter volumes. For cecal sampling, FSIS

NARMS uses a statistical design based upon establishment slaughter volume and predicted positive rates to reach a target number of bacterial isolates. In this study, sampling task frequencies were assigned based upon 12 months of slaughter volume data for each class and included up to four samples per month for the establishments with the top 25% (≥ 75%) of slaughter volume, up to two per month for the next 25% (≥ 50% and < 75%), and up to one sample per month for the remaining 50% of eligible establishments (< 50%) (FSIS, 2024b). For this cross-sectional study, cecal samples were collected from goat, sheep, and lamb at FSIS-regulated establishments throughout the U.S. that slaughter at least 10 animals/year/slaughter class. Sampling occurred from February 2020 to September 2022. Due to COVID-19 pandemic disruptions to staffing availability, cecal samples were not collected in April, May, and part of June 2020. This study provided 1,025 goat, sheep, and lamb cecal samples collected from 449 FSIS-regulated establishments.

Bacterial Isolation and Confirmation

Samples were collected from the cecum (pl. ceca), a small blind pouch located at the intersection of the small and large intestine and sent to the FSIS Eastern Laboratory for microbiological analysis (FSIS, 2022b). The number of samples screened for each organism varied due to differences in expected recovery rates. Recovery and isolation of pathogens from cecal samples are described in the FSIS Microbiology Laboratory Guidebook Chapter 31 (FSIS, 2024c.) with a summary of methods used provided here. Cecal contents were enriched in Buffered Peptone Water (BPW) and incubated overnight. For *Salmonella*, enriched cecal samples were screened through a BAX® system real-time PCR Assay Kits (Dupont Nutrition and Health) and presumptive positives were carried forward to selective enrichment and plating media. For *Campylobacter*, enriched BPW was inoculated into double-strength Bolton enrichment broth, incubated, streaked to a Modified Charcoal-Cefoperazone-Deoxycholate Agar plate, and screened for typical colonies. For generic *E. coli*, an aliquot from BPW was streaked on Eosin Methylene Blue Agar media and screened for typical colonies. For *Enterococcus*, an aliquot of the enriched BPW was transferred into Enterococcusel™ broth, incubated, streaked to Enterococcosel™ agar, and screened for typical colonies. For each of the four enteric bacteria, a single presumptive positive isolate was streaked to Trypticase Soy Agar with 5% Sheep Blood plates and confirmed by Bruker® MALDI Biotyper. Bacterial isolates were further characterized for AMR.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed using the Clinical and Laboratory Standard Institute methods (CLSI, 2018 and 2020). Susceptibility testing was performed through broth microdilution (Sensititre System™, Thermo Fisher Scientific) using antibiotic panels CMV5AGNF for *Salmonella* and generic *E. coli*, CMVCAMPY for *Campylobacter* and CMV4AGP4 for *Enterococcus* that includes antimicrobial drugs selected based upon their importance in human and veterinary medicine. The interpretation of minimal inhibitory concentrations (MICs) was based upon the Clinical and Laboratory Standards Institute (CLSI) M100 (CLSI, 2020) clinical breakpoints. For ciprofloxacin, isolates with decreased susceptibility (MIC ≥ 0.12 µg/mL) were also included in total resistance calculations. For those without CLSI breakpoints, NARMS provisional cutoffs were used: streptomycin (generic *E. coli* and *Salmonella*, MIC ≥ 32 µg/mL), azithromycin (MIC ≥ 32 µg/mL), and tigecycline (MIC > 0.25 µg/mL). For *Campylobacter*, epidemiological cutoff values (ECOFFs) were based upon EUCAST recommendations (EUCAST, n.d.). The interpretive criteria used for susceptibility testing are in Appendix A, Tables A1-A3; susceptibility definitions are in Appendix B.

Statistical Analyses

Basic descriptive analyses, including contingency tables, simple proportions, pie charts and bar graphs, were used to portray the distribution of antimicrobial susceptibility detected for the four targeted bacteria (*Salmonella*, *Campylobacter*, generic *E. coli*, and *Enterococcus*) and their antimicrobial susceptibility patterns.

Results

Sample distribution based upon volume of slaughter facility

The distribution of samples collected by establishment slaughter volume is shown in Table 1. The distribution of collected samples based on the establishment's slaughter volume was 90% for the top 25%, 7% for the next 25%, and 3% for the bottom 50%. A total of 1,025 cecal samples were collected: 349 goat, 319 sheep, and 357 lamb samples.

Recovery of Bacteria

Cecal samples were screened for the microbes listed in Table 2 for goat, sheep and lamb. *Salmonella* was recovered at 12% (n=43) in goat, 34% (n=107) in sheep, and 21% (n=75) in lamb. A greater number of cecal samples were positive for *Campylobacter* than *Salmonella* with 26% (n=46) goat, 36% (n=58) sheep, and 38% (n=70) lamb samples being *Campylobacter* positive. Levels of generic *E. coli* and *Enterococcus* were high (≥ 78%).

Table 1. Distribution of number of samples and percent based on establishment slaughter volume, 2020-2022.

Slaughter volume	Number of establishments sampled[1]	Number of samples	Percentage of total
Top 25%	104	926	90%
Next 25%	56	73	7%
Bottom 50%	25	26	3%
Total	185	1,025	100%

[1] There are 27 establishments that are counted more than once because they were categorized differently based on commodity and year.

Table 2. Number of positive isolates per number of samples screened for each organism and slaughter class, 2020-2022.

Organism	Goat			Sheep			Lamb		
	No. of samples screened[1]	No. of positives	% positive	No. of samples screened[1]	No. of positives	% positive	No. of samples screened[1]	No. of positives	% positive
Salmonella	349	43	12%	319	107	34%	357	75	21%
Campylobacter	175	46	26%	159	58	36%	186	70	38%
Generic *E. coli*	103	84	82%	87	72	83%	105	90	86%
Enterococcus	98	79	81%	84	69	82%	100	78	78%

[1] Not all samples collected were screened for all organisms; hence, the number of samples screened vary. For generic *E. coli* and *Enterococcus*, lower number of samples were screened due to their high rate of recovery (percent positive) while recovery of *Salmonella* and *Campylobacter* was relatively lower.

Distribution of *Salmonella* Serotypes

The distribution and diversity of *Salmonella* serotypes by slaughter class is shown in Table 3 and Figure 1. Nine predominate serotypes (each comprising ≥2% and ≥3% of the total serotypes isolated in sheep and lamb, respectively) were recovered from sheep and lamb. *Salmonella* enterica subsp. diarizonae serotype IIIb 61:k:1,5,(7) (herein referred to as *Salmonella* serotype IIIb 61:k:1,5,(7)) was the most frequent serotype isolated (63% of *Salmonella* isolates, n=67) in sheep and (52% of *Salmonella* isolates, n=39) in lamb. Other serotypes were observed at lower levels: for sheep, Muenster (6%, n=6) and Typhimurium (4%, n=4), for lamb, Typhimurium (7%, n=5) and I 4,[5],12:i:- (5%, n=4). In total, the top three serotypes accounted for over half of the total number of *Salmonella* isolates, 73% in sheep and 64% in lamb.

Twelve predominant *Salmonella* serotypes (each comprising ≥5% of the total serotypes isolated) were recovered in goat cecal samples with the top three serotypes recovered being: Muenster (16%, n=7), Montevideo (9%, n=4), and Anatum (7%, n=3) that accounting for 32% of the total number of *Salmonella* isolates. The diversity and the distribution of *Salmonella* serotypes are shown in Figure 1.

Distribution of *Campylobacter* Species

The distribution of *Campylobacter* species by slaughter class is shown in Table 4. *C. coli* was the predominant species accounting for 65% (n=30) of *Campylobacter* isolates in goat, 69% (n=40) in sheep, and 53% (n=37) in lamb. *C. jejuni* was present in goat (35%, n=16) and sheep (31%, n=18) with a higher proportion of lamb samples 47% (n=33) tested having *C. jejuni* (Table 4).

Distribution of *Enterococcus* species

The distribution of *Enterococcus* species by slaughter class is shown in Table 5. The most frequent species observed among all slaughter classes was *Enterococcus* hirae with similar percentages: 47% (n=37) in goat, 42% (n=29) in sheep, and 49% (n=38) in lamb. *Enterococcus faecalis* ranked second in goat

Table 3. *Salmonella* serotype distribution for goat, sheep, and lamb, 2020-2022.

Goat (N=349)			Sheep (N=319)			Lamb (N=357)		
Serotype	**n**	**%**	**Serotype**	**n**	**%**	**Serotype**	**n**	**%**
Muenster	7	16%	IIIb 61:k:1,5,(7)	67	63%	IIIb 61:k:1,5,(7)	39	52%
Montevideo	4	9%	Muenster	6	6%	Typhimurium	5	7%
Anatum	3	7%	Typhimurium	4	4%	I 4,[5],12:i:-	4	5%
Infantis	3	7%	I 4,[5],12:i:-	4	4%	Anatum	2	3%
Typhimurium	2	5%	Anatum	3	3%	Altona	2	3%
Altona	2	5%	Montevideo	2	2%	Reading	2	3%
Bredeney	2	5%	Altona	2	2%	Muenchen	2	3%
Agona	2	5%	Muenchen	2	2%	Derby	2	3%
Panama	2	5%	Newport	2	2%	Chester	2	3%
Kiambu	2	5%	-	-	-	-	-	-
Kentucky	2	5%	-	-	-	-	-	-
Adelaide	2	5%	-	-	-	-	-	-
Others	10	23%	Others	15	14%	Others	15	20%
Total	43	100%	Total	107	100%	Total	75	100%

N = total number of samples screened, n= number of isolates, Others = include serotypes with a single occurrence

Figure 1. *Salmonella* serotype diversity for goat, sheep, and lamb, 2020-2022.

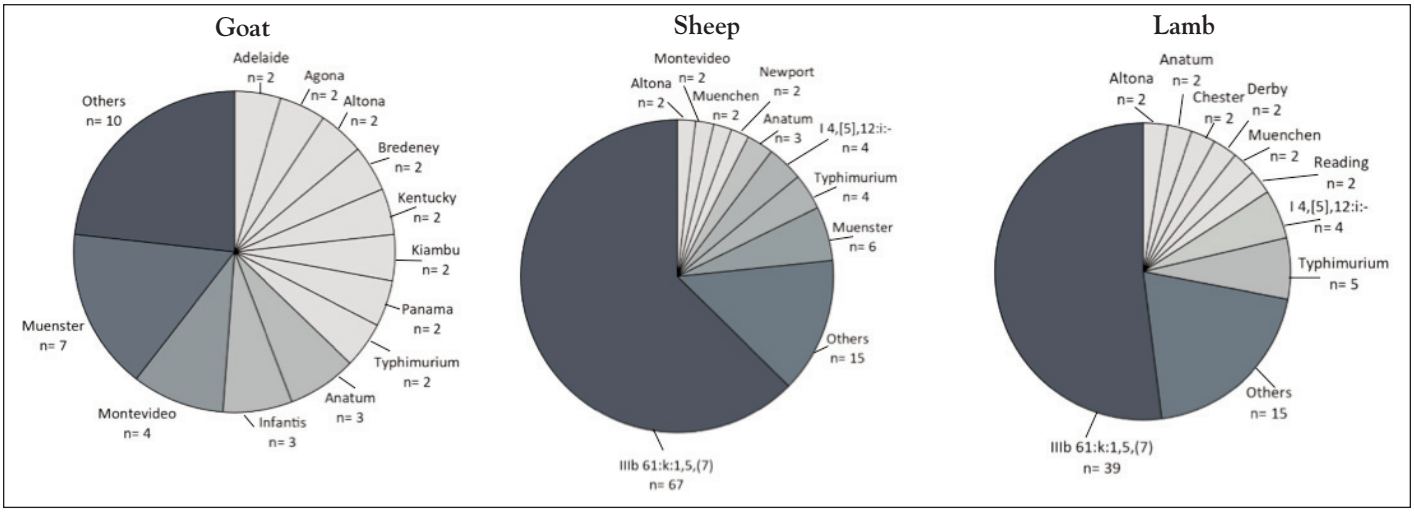

Table 4. *Campylobacter* species distribution for goat, sheep, and lamb, 2020-2022.

	Goat (N=175)		Sheep (N=159)		Lamb (N=186)	
***Campylobacter* Species**	**n**	**%**	**n**	**%**	**n**	**%**
coli	30	65%	40	69%	37	53%
jejuni	16	35%	18	31%	33	47%
Total	46	100%	58	100%	70	100%

N = total number of samples screened, n= number of isolates

(19%, n=15) and lamb (15%, n=12) and third in sheep (19%, n=13). *Enterococcus gallinarum* ranked second in sheep (23%, n=16) and third in goat (16%, n=13) and lamb (12%, n=9).

AMR in Microbes Recovered from Cecal Samples

The distribution of bacterial isolates and antimicrobial resistance for goat, sheep, and lamb are shown in Figure 2 and Table 6. Most *Salmonella* isolates from the three slaughter classes combined (91%, n=204) were pan-susceptible, with 8% (n=18) resistant to 1-2 classes of antimicrobials and 1% (n=3) showing multi-drug resistance (MDR). A similar trend was observed for pan-susceptible *Salmonella* isolates in individual slaughter classes: 88% (n=38) in goat, 93% (n=99) in sheep, and 89% (n=67) in lamb (Table 6). One MDR *Salmonella* isolate was found in sheep, two in lamb, and none in goat (Table 6).

In contrast to *Salmonella*, most (74%, n=128) *Campylobacter* isolates from goat, sheep, and lamb tested were resistant to 1-2 classes of antimicrobials while 23% (n=40) were pan-susceptible, and only 3% (n=6) were MDR (Figure 2). Resistance to 1-2 classes of antimicrobials among the individual slaughter classes was similar: 76% (n=35) in goat, 72% (n=42) in sheep, and 73% (n=51) in lamb (Table 6).

A majority (61%, n=150) of generic *E. coli* isolates were pan-susceptible for goat, sheep, and lamb combined, followed by 30% (n=73) of the isolates being resistant to 1-2 classes and 9% (n=23) being MDR (Figure 2). When generic *E. coli* was examined individually in goat, sheep, and lamb, pan-susceptibility was 62% (n=52) in goat, 63%

Table 5. *Enterococcus* species distribution for goat, sheep, and lamb, 2020-2022.

Goat (N=98)			Sheep (N=84)			Lamb (N=100)		
Species	**n**	**%**	**Species**	**n**	**%**	**Species**	**n**	**%**
faecalis	15	19%	gallinarum	16	23%	faecalis	12	15%
gallinarum	13	16%	faecalis	13	19%	gallinarum	9	12%
durans	7	9%	faecium	5	7%	faecium	7	9%
casseliflavus	3	4%	casseliflavus	3	4%	casseliflavus	6	8%
faecium	3	4%	mundtii	2	3%	durans	3	4%
mundtii	1	1%	durans	1	1%	mundtii	3	4%
Total	79	100%	Total	69	100%	Total	78	100%

N = total number of samples screened, n = number of isolates

Figure 2. Distribution of aggregated bacterial AMR categories for goat, sheep, and lamb combined, 2020-2022.

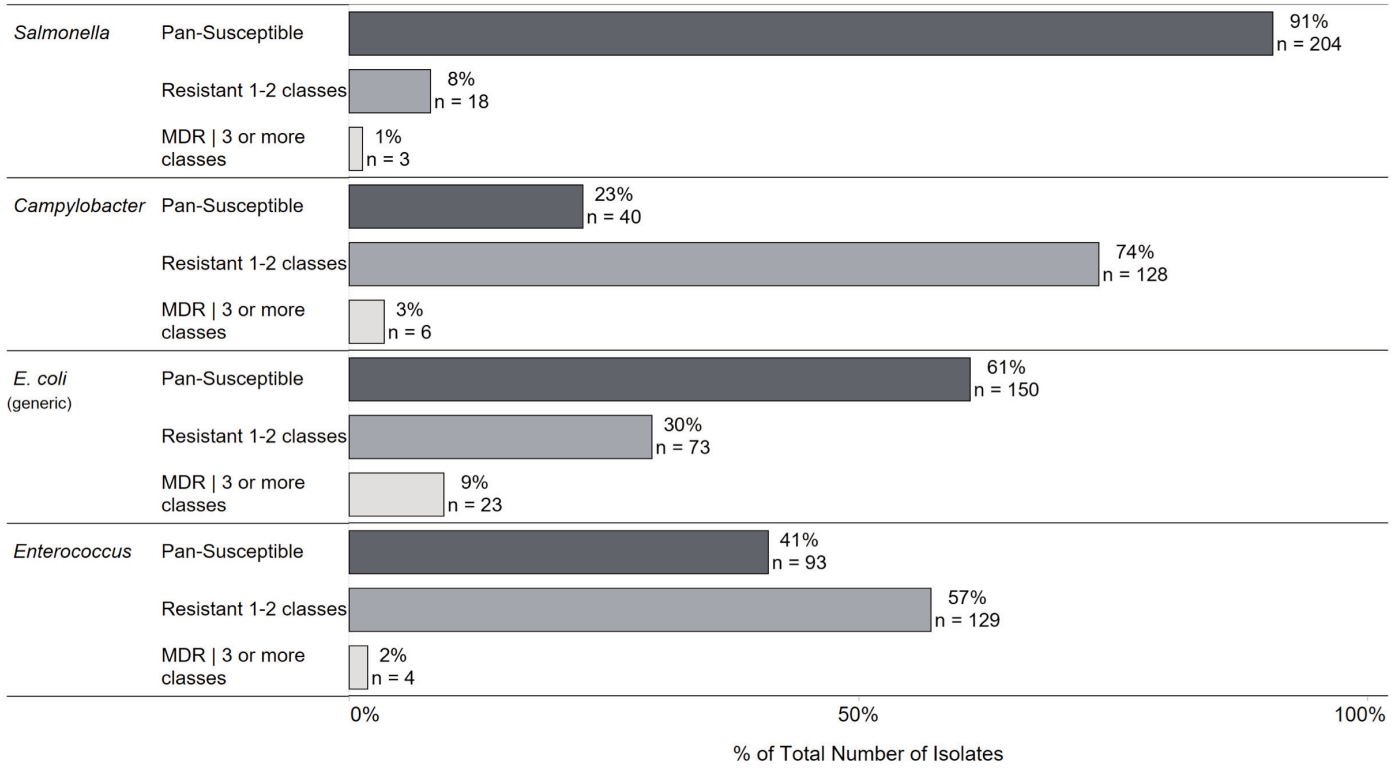

Table 6. Distribution of bacterial isolates by slaughter class and AMR category, 2020-2022.

	Goat (n=43)		Sheep (n=107)		Lamb (n=75)	
***Salmonella* (N=225)**	**n**	**%**	**n**	**%**	**n**	**%**
Pan-Susceptible	38	88%	99	93%	67	89%
Resistant 1-2 classes	5	12%	7	6%	6	8%
MDR (3 or more classes)	0	0%	1	1%	2	3%
	Goat (n=46)		**Sheep (n=58)**		**Lamb (n=70)**	
***Campylobacter* (N=174)**	**n**	**%**	**n**	**%**	**n**	**%**
Pan-Susceptible	8	17%	13	22%	19	27%
Resistant 1-2 classes	35	76%	42	72%	51	73%
MDR (3 or more classes)	3	7%	3	5%	0	0%
	Goat (n=84)		**Sheep (n=72)**		**Lamb (n=90)**	
Generic *E. coli* (N=246)	**n**	**%**	**n**	**%**	**n**	**%**
Pan-Susceptible	52	62%	45	63%	53	59%
Resistant 1-2 classes	22	26%	22	31%	29	32%
MDR (3 or more classes)	10	12%	5	7%	8	9%
	Goat (n=79)		**Sheep (n=69)**		**Lamb (n=78)**	
***Enterococcus* (N=226)**	**n**	**%**	**n**	**%**	**n**	**%**
Pan-Susceptible	36	46%	29	42%	28	36%
Resistant 1-2 classes	40	51%	39	57%	50	64%
MDR (3 or more classes)	3	4%	1	1%	0	0%

N= total number of isolates for slaughter classes combined; n = number of isolates in each slaughter class

(n=45) in sheep, and 59% (n=53) in lamb. For generic *E. coli*, resistance to 1-2 antimicrobial drug classes was 26% (n=22) in goat, 31% (n=22) in sheep, and 32% (n=29) in lamb (Table 6). In goat, sheep, and lamb, MDR in generic *E. coli* was 12% (n=10), 7% (n=5) and 9% (n=8), respectively (Table 6).

Overall, 41% (n=93) of the *Enterococcus* isolates were pan-susceptible, 57% (n=129) were resistant to 1-2 classes, and 2% (n=4) of the *Enterococcus* isolates were MDR (Figure 2). The distribution of pan-susceptible *Enterococcus* isolates in goat, sheep, and lamb was 46% (n=36), 42% (n=29), and 36% (n=28), respectively. *Enterococcus* isolates resistant to 1-2 antibiotics were highest in lamb (64%, n=50), followed by sheep (57%, n=39) and goat (51%, n=40) (Table 6). Goat samples contained three *Enterococcus* isolates (4%) with MDR, while sheep had one MDR isolate (1%), and lamb had 0 MDR isolates (Table 6).

Antimicrobial susceptibility for bacterial isolates is shown in Table 7. Tetracycline resistance was found in 12% (n=5) of goat, 8% (n=8) of sheep, and 11% (n=8) of lamb *Salmonella* isolates.

Table 7. The number of bacterial isolates from goat, sheep, and lamb, and antimicrobial susceptibility testing results, 2020-2022.

Antimicrobial Class	Antimicrobial	*Salmonella*			*Campylobacter*			Generic *E. coli*			*Enterococcus*		
		Goat n=43	Sheep n=107	Lamb n=75	Goat n=46	Sheep n=58	Lamb n=70	Goat n=84	Sheep n=72	Lamb n=90	Goat n=79	Sheep n=69	Lamb n=78
Aminoglycosides	Gentamicin (C)	-	-	-	0	1	0	0	1	1	0	0	0
	Streptomycin (C)	-	-	-	-	-	-	-	-	-	2	1	0
β-Lactam/ β-Lactamase Inhibitor Combinations	Amoxicillin/ Clavulanic Acid (C)	0	0	0	-	-	-	0	0	0	-	-	-
Carbapenems	Meropenem (C)	0	0	0	2	1	0	0	0	0	-	-	-
Cephems	Cefoxitin (H)	0	0	0	-	-	-	0	0	0	-	-	-
	Ceftriaxone (C)	0	0	0	-	-	-	1	0	1	-	-	-
Folate Pathway Inhibitors	Sulfisoxazole (I)	3	3	3	-	-	-	19	7	14	-	-	-
	Trimethoprim/ Sulfamethoxazole (C)	0	1	0	-	-	-	4	1	0	-	-	-
Glycopeptides	Vancomycin (C)	-	-	-	-	-	-	-	-	-	0	0	0
Glycylcycline	Tigecycline (C)	-	-	-	-	-	-	-	-	-	0	0	0
Lincosamides	Clindamycin (H)	-	-	-	2	2	4	-	-	-	-	-	-
	Lincomycin (NC)	-	-	-				-	-	-	0	0	0
Lipopeptides	Daptomycin (C)	-	-	-				-	-	-	2	0	1
Macrolides	Azithromycin (C)	0	1	0	0	2	1	1	0	0	-	-	-
	Erythromycin (C)	-	-	-	0	1	0	-	-	-	4	0	1
Nitrofurans	Nitrofurantoin (H)	-	-	-	-	-	-	-	-	-	0	2	2
Orthosomycin	Avilamycin (NC)	-	-	-	-	-	-	-	-	-	0	0	0
Oxazolidinones	Linezolid (C)	-	-	-	-	-	-	-	-	-	0	0	0
Penicillins	Ampicillin (H)	0	1	2	-	-	-	7	3	6	0	0	0
Phenicols	Chloramphenicol (H)	0	1	0	-	-	-	9	4	8	1	0	0
	Florfenicol (H)	-	-	-	0	0	0	-	-	-	-	-	-
Polymyxin	Colistin (C)	0	0	0	-	-	-	0	0	0	-	-	-
Quinolones	Ciprofloxacin (C)	0	1	1	21	27	17	3	1	1	1	0	01
	Nalidixic Acid (C)	0	0	1	21	26	17	1	1	0	-	-	-
Streptogramins	Quinupristin/ Dalfoprisitn (H)	-	-	-	-	-	-	-	-	-	25	24	30
Tetracyclines	Tetracycline (H)	5	8	8	33	38	47	32	27	36	30	24	31

Note: Blank fields (no values) represent/denote antibiotics not tested for specific bacteria. FDA classifies antimicrobials into critically important (C), highly important (H), important (I) and not classified (NC) based on their human medical importance. See FDA's Guidance For Industry #152 for additional information. For ciprofloxacin resistance, isolates with decreased susceptibility (MIC > 0.12 μg/mL) were also included in total resistance calculations.

Tetracycline resistance was found in 72% (n=33) of goat, 66% (n=38) of sheep, and 67% (n=47) of lamb *Campylobacter* isolates; 38% (n=32) of goat, 38% (n=27) of sheep, and 40% (n=36) of lamb *E. coli* isolates; and 38% (n=30) of goat, 35% (n=24) of sheep, and 40% (n=31) of lamb *Enterococcus* isolates.

Campylobacter had the highest percent of isolates resistant to the quinolones with 46% (n=21) of goat, 47% (n=27) of sheep, and 24% (n=17) of lamb isolates resistant to ciprofloxacin and 46% (n=21) of goat, 45% (n=26) of sheep, and 24% (n=17) of lamb isolates resistant to nalidixic acid.

For generic *E. coli*, sulfisoxazole resistance was 23% (n=19) of goat, 10% (n=7) of sheep, and 16% (n=14) of lamb isolates. *Enterococcus* resistance to quinupristin/dalfopristin was observed at 32% (n=25) of goat, 35% (n=24) of sheep , and 38% (n=30) of lamb isolates.

Discussion

In the U.S., goat, sheep, and lamb are considered to be minor species of food-producing animals (New Animal Drugs for Minor Use and Minor Species, 2022). Globally, these animals are important sources of meat as well as milk and fiber. Minor species meat consumption varies greatly and is influenced by cultural, dietary, economic, social, and geographic factors (Mazinani, 2020). Given the importance of minor species to U.S. agriculture, APHIS periodically conducts voluntary on-farm national studies under the NAHMS program. These studies gather health and management related information including antimicrobial use (AMU). A proportion of studies include AMR testing from fecal samples collected from animals on the operations participating in the study (APHIS, 2023, 2024; Dargatz et al., 2015; Gensler et al., 2024). In addition, several regional or convenience AMU/AMR studies have been conducted with goat, sheep, and lamb (Atlaw et al., 2022; CDFA, 2019; Cheney et al., 2015; Roug et al., 2013; Xia et al., 2019). While the APHIS on-farm studies provide a national snapshot of AMR and the convenience studies do the same at a state or regional level, a representative national snapshot of AMR in goat, sheep, and lamb at slaughter or in retail meats in the U.S. was lacking. This FSIS NARMS study is the first of its kind to provide national AMR information from FSIS-regulated slaughter establishments for goat, sheep, and lamb. This paper examined cecal samples from federally regulated goat, sheep, and lamb slaughter establishments for *Salmonella*, *Campylobacter*, generic *E. coli*, and *Enterococcus* and associated AMR.

The FSIS cecal sampling program, administered under FSIS NARMS, provides a nationally representative means to monitor trends in AMR in pathogens (*Salmonella*, *Campylobacter*) and indicator organisms (generic *E. coli* and *Enterococcus* spp.). FSIS NARMS routinely includes major meat-producing animals (poultry, swine, and cattle). This study expanded the ability of FSIS to monitor minor meat-producing animals such as goat, sheep, and lamb for trends in AMR or pathogens at the point of slaughter.

Both NAHMS and NARMS studies gather AMR information from sheep and goats; however, each has its own method of collecting data, selecting animals, and testing samples that fits their respective purpose. NAHMS conducts nationally representative and voluntary on-farm studies examining animal health and management practices. Operations that complete two questionnaires and meet the size requirements are eligible to participate in the animal testing phase, during which up to 25 sheep or goats meeting specific age and breeding class requirements are sampled. The Goat 2019 Study was conducted in 24 states that represented 76% of U.S. goat operations with >5 adult goats and 80% of the adult goats in the U.S. (APHIS, 2019). The 2011 Sheep Study was conducted in 22 states that represented 86% of the U.S. ewe inventory and 70% of U.S. farms with ewes (APHIS, 2013). Only operations with 20 or more ewes on January 1, 2010 and that completed two questionnaires were eligible to participate in biologic collection. FSIS NARMS studies collect cecal samples at slaughter from establishments that slaughter at least 10 animals per slaughter class of goat, sheep, or lamb per year nationwide. The NAHMS and FSIS NARMS studies are representative and provide a means to monitor AMR trends in goat, sheep, and lamb on-farm and at slaughter.

CDC estimates that *Salmonella* causes about 1.35 million infections, 26,000 hospitalizations, and 420 deaths in the U.S. every year, with food identified as the source of most of these illnesses (CDC *Salmonella*). Although the number of reported *Salmonella* outbreaks and illnesses related to consumption of goat, mutton, and lamb meat is very low, additional work is needed to assess public health risks (CDC NORS). The FSIS NARMS expansion study found that *Salmonella* serotype IIIb 61:k:1,5,(7) accounted for over half of the isolates in sheep (63%) and lamb (52%). A 2011 NAHMS on farm sheep study collected 1,133 composite fecal samples (up to six animals per sample, five samples per farm), of which 370 (32.7%) were positive for *Salmonella*. The *Salmonella* serotype IIIb 61:-:1,5, [7] accounted for 94.6% of the isolates (APHIS, 2013). This serotype is thought to be host adapted to sheep. In other sheep studies, *Salmonella* serotype IIIb 61:k:1,5,(7) was found to cause chronic proliferative rhinitis (Lacasta et al., 2012; Meehan, 1992), orchitis and epididymitis (Ferreras et al., 2007), and stillbirths in sheep (Davies et al., 2001). *Salmonella* serotype IIIb 61:k:1,5,(7) has been found in higher numbers in sheep than goats (Alvseike and Skjerve, 2002; Bonke et al., 2012) and has differences in regional and seasonal prevalence (Davies et al., 2001). Although *Salmonella* IIIb 61:k:1,5,(7) infections in humans are not common, reports show that this serotype is capable of causing humans illness. *Salmonella* IIIb 61:k:1,5,(7) infections have been reported in individuals who traveled internationally (Hall, 1992), immunocompromised individuals (Hoag and Sessler, 2005), and individuals who have handled reptiles (CDC, 2003; Parihar, 2020). In 2009, there were 86 laboratory-confirmed *Salmonella* IIIb 61:k:1,5,(7) isolates from human sources reported to the CDC compared with over 30,000 subspecies enterica isolates (CDC, 2011).

Salmonella recovered from goats in the FSIS NARMS expansion study were more diverse than in lamb and sheep, with Muenster (16%), Montevideo (9%), Anatum (7%), and Infantis (7%) making up the top four serotypes. Similarly, the NAHMS goat 2019 study of 4,918 fecal samples from 332 farm operations in the U.S. showed a low prevalence of *Salmonella* (0.7%) and a broad range of serotypes in goat species. The

top five *Salmonella* serotypes in the NAHMS study included Bareilly, Uganda, Newport, Poona, and Manhattan (Hempstead, et. al., 2022). These findings differ from those of the FSIS NARMS expansion study. These differences may be due to multiple factors, including (but not limited to) sample selection, sample sources (fecal vs. cecal samples), and laboratory methodology.

The CDC estimates that 1.5 million people in the U.S. become ill from *Campylobacter* infection every year. (CDC, 2024). *Campylobacter jejuni* is one of the most common bacterial causes of human foodborne illness (UW Madison, 2015) and, according to the CDC, *C. jejuni* causes 90% of human cases of *Campylobacter* (CDC, 2024). Less common species, such as *C. coli*, *C. upsaliensis*, *C. fetus*, and *C. lari* also infect people. In this FSIS NARMS study, *C. coli* was found in the majority of goat, sheep, and lamb samples (>50% of the *Campylobacter* organisms isolated). In the 2011 APHIS NAHMS on-farm study, among the *Campylobacter* species isolated from sheep and lamb fecal samples, the predominant species was *C. jejuni* (APHIS, 2014). In one study of retail meat, *C. coli* was found to be the most prevalent *Campylobacter* isolated from goat meat (Rahimi et al., 2010), while other studies found *C. jejuni* to be more prevalent in sheep and goat meat (Gensler et al., 2024; Lazou, 2014; Mpalang et al., 2014; Scates et al., 2003). The differences in *Campylobacter* species (*C. coli*, *C. jejuni*) recovered in different studies are influenced by multiple factors as seen in *Salmonella*.

Generic *E. coli* and *Enterococcus* species are normal bacteria in the gastrointestinal tract. The NARMS program has historically used these bacteria as indicators to monitor for emerging trends in antimicrobial resistance in enteric bacteria. These bacteria have been found to play a role in the horizontal transfer and spread of antibiotic-resistant genes (ARG) and mobile elements, in both internal (intestinal) and shared external environments. The internal and external environments provide an opportunity for horizontal gene transfer where genetic determinants of AMR may be exchanged between commensals and opportunistic pathogens (Lerner et al., 2017). A cause-and-effect relationship between antimicrobial usage and AMR should not be automatically assumed since the transfer and spread of AMR may be mediated through mobile genetic elements; these may spread among microbial populations through triggers not directly related to antimicrobial use.

The inclusion of *Enterococcus* and generic *E. coli* testing in this study provides insight into the presence of AMR in goat, sheep, and lamb. These bacteria were isolated from >80% of cecal samples in all three classes of animals compared to 12% positive for *Salmonella* and 38% positive for *Campylobacter* and may provide insight into the presence of AMR.

Antimicrobial Resistance

Despite some gains in combating AMR, the CDC 2019 Antibiotic Resistance Threats Report (CDC, 2019) shows additional actions are needed to protect against AMR. There are over 2.8 million antibiotic-resistant human infections and 35,000 deaths attributed to antibiotic resistance each year. AMR is recognized as an increasing global public health threat. In this study, we found that there was a high proportion of pan-susceptibility among *Salmonella* (88% - 93%) and generic *E. coli* (60% - 63%) isolates from all three minor species. This proportion was lower among *Campylobacter* isolates (8% - 27%) and varied between goat (8%), sheep (22%), and lamb (27%), with a greater proportion of isolates (72%-76%) showing resistance to 1-2 antimicrobial drug classes. The proportion of AMR in *Enterococcus* isolates was somewhat evenly distributed across all three species between pan-susceptible (44%-51%) and resistant to 1-2 antimicrobial drug classes (48%-56%). The highest proportion of MDR was observed in *Campylobacter* (7%) and generic *E. coli* (8%) in goat cecal samples.

Resistance to tetracycline was the most common finding among the three minor species and among the four bacteria. Tetracycline resistance was higher in *Campylobacter*, generic *E. coli*, and *Enterococcus* isolates and lower in *Salmonella* isolates. Chopra and Roberts reported that increasing resistance to tetracycline was seen in a number of pathogenic, opportunistic, and commensal bacteria (Chopra and Roberts, 2001). The authors opined that this was mostly mediated by the genetic acquisition of tet genes and that this phenomenon followed the introduction of tetracyclines in the mid-20th century for clinical, veterinary, and agricultural use.

Resistance to the critically important quinolone antibiotics, ciprofloxacin and nalidixic acid, was observed in approximately half of the *Campylobacter* isolates from goat and sheep. In addition to tetracycline, resistance to other important antimicrobial drugs were seen, including chloramphenicol in *Salmonella* (sheep), generic *E. coli* (goat, sheep, and lamb), and *Enterococcus* (goat) and quinupristin/dalfopristin in *Enterococcus* (goat, sheep, and lamb). Whereas *Salmonella* showed resistance in 8% and MDR in 1% of isolates, generic *E. coli* showed resistance in 30% and MDR in 9% of isolates. *Campylobacter* and *Enterococcus* were similar to each other with 74% and 52% resistant and 3% and 0% MDR, respectively. Tetracycline resistance was high in all slaughter classes and *Campylobacter*, *Enterococcus* and generic *E. coli* showed higher levels of resistance to sulfisoxisole, penicillins and phenicols.

In the NAHMS goat 2019 study conducted by APHIS, 4,917 fecal samples were collected from 332 operations tested for *Salmonella* and AMR (Hempstead, et al., 2022). In this on farm study, fecal *Salmonella* prevalence was low (0.7%), and all the *Salmonella* tested were pan-susceptible. While *Campylobacter* and generic *E. coli* isolates showed varied degrees of pan-susceptibility (42.3% and 84.7%, respectively), the most frequent resistance seen in these organisms was to tetracycline (APHIS, 2023; Gensler et al., 2024).

In collaboration with the California Department of Agriculture goat operations in California were oversampled and thus represent a state-level subset of the NAHMS goat 2019 study operations (CDFA, 2019). Nearly 50 goat operations in California voluntarily submitted fecal samples for AST using a panel of drugs important to human health. In the study, fecal recoveries of *Salmonella* (2%) and *Campylobacter* (10%) were relatively low. Almost all the *Salmonella* isolates in these studies were pan-susceptible and only a few *Campylobacter* isolates exhibited resistance to ciprofloxacin and nalidixic acid, although these drugs are not used in goats. Compared to this study, we found 88% of *Salmonella* iso-

lates to be pan-susceptible and 17% of *Campylobacter* to be pan-susceptible. The difference in levels of pan-susceptible *Campylobacter* reflected an increase of isolates resistant to one or more classes of antibiotics (78%) and MDR (8%). Compared to the above study, 46% of *Campylobacter* showed resistance to the WHO highest priority critically important antimicrobials ciprofloxacin and nalidixic acid.

The differences in recovery of *Salmonella*, *Campylobacter*, *E. coli*, or *Enterococcus* among the different studies of minor species could be due to multiple factors. These include, but are not limited to, the points of sampling along the farm to fork continuum, type of sample (fecal vs. cecal samples), and the differences in testing methodologies.

Conclusion

The NARMS minor species cecal AMR study is the first study of its kind to address the AMR data gap at slaughter for goat, sheep, and lamb. Unlike the regional or convenience studies, this study provides a representative national snapshot of cecal AMR and enables comparisons between goat, sheep, and lamb at a national level. Although *Salmonella* and *Campylobacter* and their AMR can be major concerns in foods and food-producing animals, findings from this study indicate that in minor species, a vast majority of *Salmonella* and roughly half of the *Campylobacter* isolates were pan-susceptible. In addition, MDR in both *Salmonella* and *Campylobacter* was minimal to low. A notable resistance to quinolones (ciprofloxacin/nalidixic acid) in *Campylobacter* will need further study. Periodic monitoring of *Salmonella* serotypes in minor species is important to maintain awareness of AMR trends. From the AMR surveillance perspective, inclusion of generic *E. coli* and *Campylobacter* provide a good assessment of the potential for MDR and quinolone resistance. While this national cecal sample study of minor species helps to address the AMR data gap at the initial point of slaughter, a similar nationally representative study with minor species derived food products (meat) a similar nationally representative study with minor species derived food products (meat) would shed light on AMR in finished products.

Limitations of this cross-sectional study include the relatively small numbers of bacterial isolates recovered and AMR findings. Also, bacterial isolates and AMR from the final products or retail meats were not evaluated. A follow-up study of cecal and retail meats conducted in conjunction with an on-farm study may provide the opportunity to determine if findings from this study persist over time and if there is any association with the on farm and at retail findings. However, we acknowledge that differences between on farm, at slaughter, and at retail samples; the types and ages/stages of animals sampled; and the interim influences between animals on farm and animals at the end of the slaughter process may make these correlations challenging.

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Appendix A

Table A1. Interpretive Criteria Used for Susceptibility Testing of *Salmonella* and Generic *E. coli*.

			Breakpoints (µg/ml)		
Antimicrobial Class	**Antimicrobial Agent**	**Ranking[1]**	**Susceptible**	**Intermediate**	**Resistant**
Aminoglycosides	Gentamicin	C	≤ 4	8	≥ 16
Aminoglycosides	Streptomycin	C	≤ 16	8	≥ 16
β-Lactam/ β-Lactamase Inhibitor Combinations	Amoxicillin-Clavulanic Acid	C	≤ 8 / 4	N/A	≥ 32
Carbapenems	Meropenem	C	≤ 1	16 / 8	≥ 32 / 16
Cephems	Cefoxitin	H	≤ 8	2	≥ 4
Cephems	Ceftriaxone	C	≤ 1	16	≥ 32
Folate Pathway Inhibitors	Sulfisoxazole	I	≤ 256	2	≥ 4
Folate Pathway Inhibitors	Trimethoprim-Sulfamethoxazole	C	≤ 2 / 38	N/A	≥ 512
Macrolides	Azithromycin	C	≤ 16	N/A	≥ 4 / 76
Penicillins	Ampicillin	H	≤ 8	N/A	≥ 32
Phenicols	Chloramphenicol	H	≤ 8	16	≥ 32
Polymyxin	Colistin	C	N/A	16	≥ 32
Quinolones	Ciprofloxacin	C	≤ 0.06	≤ 2	≥ 4
Quinolones	Nalidixic acid	C	≤ 16	0.12-0.5	≥1
Tetracyclines	Tetracycline	H	≤ 4	N/A	≥ 32

[1] Ranking according to FDA's Guidance for Industry #152 (FDA, 2023): C - Critically important, H - Highly important, I - Important, NC - Not classified

Table A2. Interpretive Criteria Used for Susceptibility Testing of *Enterococcus*.

			Breakpoints (µg/ml)		
Antimicrobial Class	**Antimicrobial Agent**	**Ranking[1]**	**Susceptible**	**Intermediate**	**Resistant**
Aminoglycosides	Gentamicin	C	≤ 500	N/A	>500
	Streptomycin	C	≤ 512	N/A	≥ 1000
Glycopeptides	Vancomycin	C	≤ 4	8 -16	≥ 32
Glycylcycline	Tigecycline	C	≤ 0.25	N/A	≥ 0.5
Lipopeptides	Daptomycin (*E. faecium* only)	C	≤ 4	N/A	≥ 8
	Daptomycin (*Enterococcus* species other than *E. faecium*)	C	≤ 2	4	≥ 8
Macrolides	Erythromycin	C	≤ 0.5	1 - 4	≥ 8
Nitrofurans	Nitrofurantoin	H	≤ 32	64	≥ 128
Oxazolidinones	Linezolid	C	≤ 2	4	≥ 8
Orthosomycin	Avilamycin	NC	≤ 2	4	16
Penicillins	Ampicillin	H	≤ 8	N/A	≥ 16
Phenicols	Chloramphenicol	H	≤ 8	16	≥ 32
Quinolone	Ciprofloxacin	C	≤ 1	2	≥ 4
Streptogramins	Quinupristin/Dalfopristin	H	≤ 1	2	≥ 4
Tetracyclines	Tetracycline	H	≤ 4	8	≥ 16

[1] Ranking according to FDA's Guidance for Industry #152 (FDA, 2023). C - Critically important, H - Highly important, I - Important, NC - Not classified

Appendix B

The following categories were used to describe the susceptibility or resistance of enteric bacterial isolates to antimicrobial drug classes tested.

- *Pan-susceptible:* bacterial isolates that are susceptible to all antimicrobial drugs included in the NARMS testing panels.
- *Resistant 1-2 classes:* bacterial isolates resistant to antimicrobials in one or two drug classes.
- *Multi-Drug Resistant (MDR):* bacterial isolates resistant to antimicrobials in three or more drug classes.

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From Wild to Watchful: Integrating BLM Donkeys (Burros) for Sheep Ranch Protection

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Summary

The historical challenge of protecting sheep from predation has often been addressed through non-lethal measures, notably the employment of Livestock Guardian Animals (LGAs). Among LGAs, donkeys have been underutilized and understudied compared to other protection animals such as dogs. This study evaluated the effectiveness of using feral Bureau of Land Management (BLM) burros (henceforth referred to as donkeys) as LGAs focusing on their acclimation and integration into sheep flocks. Four donkeys were adopted in October 2023 and observed for integration success in spatially separate pastures and their corresponding cohort of ewes (without lambs). The integration timeline varied, with a notable polynomial quadratic relationship between time and distance to the nearest sheep ($P < 0.001$; $R^2 = 0.45$), indicating approximately 5 weeks for full integration across subjects. Individual differences were pronounced; one donkey integrated without intervention, while another required relocation to a simpler environment for successful integration. Sheep did not display high or different levels of vigilance ($\bar{x}$ = 2.2% ± 1.4 of observations; $P = 0.192$) but donkeys did display high levels of vigilance (ranged from 9.1% to 47.2% [$\bar{x}$ = 25.7% ± 9.3)] with significant inter-individual variation between donkeys ($P = 0.019$). Challenges in the acclimation and integration of donkeys as LGAs often arose from overly large and complex pasture environments, as well as the presence of distracting equine neighbors. Nevertheless, with meticulous management of pasture size and complexity, we successfully integrated naive BLM donkeys with sheep flocks in a timeframe of less than six weeks. This process underscores the importance of environmental considerations in the effective utilization of donkeys as non-lethal deterrents against predation.

Key Words: *Equus asinus*, Livestock Guardians, Loss, Mortality, Predators, Vigilance

Abbreviations: BLM, Bureau of Land Management; LGA, Livestock Guardian Animal

Introduction

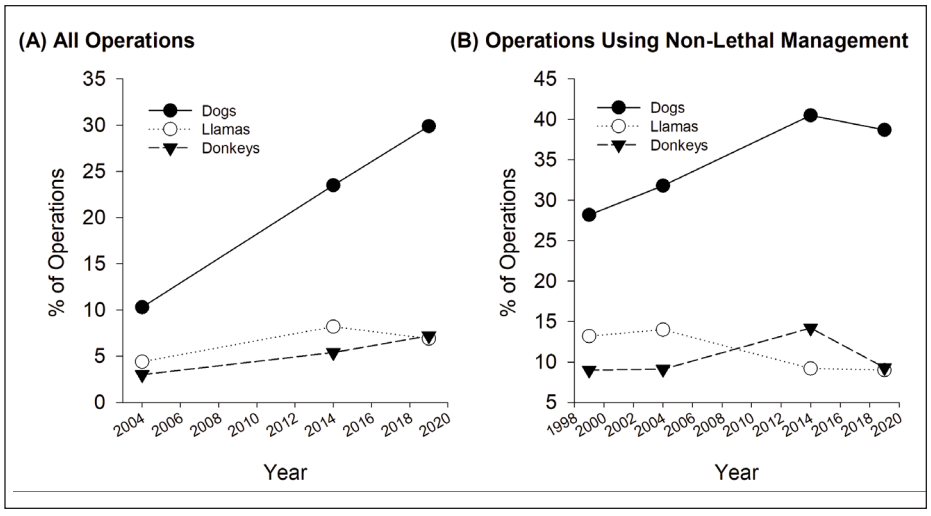
Sheep predation has been a persistent global issue since antiquity, with documented efforts to protect these animals dating back to around 1000 BC, as illustrated by biblical accounts. This problem continues to affect modern sheep production, both intensive and extensive, leading to significant economic impacts (Muhly and Musiani 2009; Mattiello et al. 2012; Scasta et al. 2018). For instance, in 2019, the western United States (AZ, CA, CO, ID, MT, NM, NV, OR, UT, WA, WY), experienced sheep losses valued at approximately $121.6 million with predation accounting for a considerable portion of both adult sheep (32.6%) and lamb mortality (40.1%), predominantly by canids such as coyotes and dogs (APHIS 2020; Western Livestock Journal 2022).

Given the ongoing challenge of predation on sheep and goats, it is crucial to explore various mitigation strategies. These strategies are broadly separated into lethal and non-lethal options. Lethal options include trapping, snaring, and shooting. Non-lethal options include fencing, herding, night-penning, lambing in a shed, repellents and fright tactics, removing carrion, culling older sheep, changing bedding grounds, frequent checks, changing breeding and lambing timing, and Livestock Guardian Animals or LGAs (Shivik 2004; APHIS 2020). The shift towards non-lethal methods has been notable, with their usage increasing significantly among sheep operations from 58% in 2014, to 77.1% in 2019 (APHIS 2020). Among these, LGAs have emerged as a promising solution, with a historical precedent and archaeological evidence supporting their effectiveness, particularly dogs (Smith et al. 2000; Urbigkit and Urbigkit 2010; Scasta et al. 2017). The use of LGAs may also be of increasing interest due to various prohibitions on lethal control including regulatory, legislative, and social including such provisions as the Endangered Species Act.

In the context of non-lethal methods for protecting sheep and goats from predators, LGAs such as dogs, llamas, and donkeys have been employed with varying degrees of adoption (Andelt 2004). Dogs have historically been the most commonly used LGA, with their usage in the United States increasing from 28.2% in 1999 to 38.7% in 2019 (Figure 1). Llamas and donkeys, while less common, have also played significant roles in predator deterrence. The use of llamas fluctuated slightly, peaking at 14.0% in 2004 before dropping to 9.0% in 2019, whereas donkeys saw an increase from 9.0% in 1999 to 14.2% in 2014, stabilizing at 9.3% in 2019, see Figure 1 (Walton and Field 1989; APHIS 2020). Internationally, donkeys have been utilized as LGAs in diverse regions including Australia, Brazil, Canada, Mexico, Namibia (cattle specifically; see Marker et al. 2005), Switzerland, the United States, and Uruguay, demonstrating their global relevance. (Landry et al. 1999; Jenkins and Noad 2003; Bough 2016; Rodrigues et al. 2021). Notably, their successful adoption by Australian ranchers to combat wild dog predation suggests potential lessons for similar challenges in the western US (Bough 2016). While some countries like Germany and Norway have recommended, rather than reported, the explicit use of donkeys as LGAs (Linnell et al. 1996; Reinhardt et al. 2012), historical evidence by Pitt (1988) stated "numerous engravings and pastoral stories, the donkey is found in the middle of the sheep" and contemporary evidence underscores their effectiveness, particularly their innate aversion to canids (Walton and Field 1989; Landry 1999; Smith et al. 2000).

However, there is generally scant information about donkeys as LGAs (Walton and Field 1989; Smith et al. 2000) and according to Bough (2016) "There has been no systematic research into guardian donkeys and how they operate". Very specifically we note there is limited to no empirical information about the acclimation of feral BLM donkeys to sheep as potential LGAs, including the potential factors that could hinder bonding and integration. Despite being rated less effective than dogs and llamas (Andelt 2004), donkeys present unique advantages as LGAs. These advantages include a lower initial purchase price, lesser upkeep compared to dogs, suitability to existing fencing and handling facilities, and similar forage-based dietary composition to the livestock they protect (Walton and Field 1989). Disadvantages include anecdotal reports or difficulty managing obesity and trimming feet. Their ability to coexist with standard farm practices, coupled with a long working life and minimal supervision requirements, positions them as a viable option for predator control (Wilbanks 1995; Smith et al. 2000; Jenkins and Noad 2003). However, the literature reveals a notable gap in systematic research on donkeys' effectiveness and operational dynamics as LGAs, particularly regarding their acclimation and integration with sheep flocks (Walton and Field 1989; Smith et al 2000; Jenkins

Figure 1. Sheep operation use of dogs, llamas, and donkeys as Livestock Guardian Animals (LGAs) for (A) all operations and (B) operations using non-lethal management. Data from the United State Department of Agriculture – Animal and Plant Health Inspection Service (USDA – APHIS) – Sheep Death Loss in the United States 2020.



and Noad 2003; Bough 2016). Wilbanks (1995) stated "Because individual differences in guarding abilities exist among donkeys, management practices may need to be tailored to capitalize on the particular qualities of a donkey".

This study aimed to fill this void by examining the adaptation process of feral Bureau of Land Management (BLM) donkeys to sheep flocks in Wyoming, USA, considering their individual variability and potential in predator deterrence. The study objectives were to explore acclimation and integration processes to better understand how these donkeys can be effectively integrated into livestock protection strategies, acknowledging the nuanced and variable nature of their guardian abilities.

Given the potential advantages in some situations of donkeys over dogs, and the reported adoption of BLM donkeys for use as LGAs, and limited systematic research on the topic, we sought to develop quantitative information about how BLM donkeys acclimate and integrate to sheep flocks in Wyoming, USA. Regarding the concept of acclimation and integration and in the context of this manuscript, we refer to the definitions by the Cambridge dictionary whereas ***acclimation*** is defined as *"the process of changing to suit different conditions of life, weather, etc., or the act of making someone or something do this"* and whereas ***integration*** is defined as *"the action of process of successfully joining or mixing with a different group…"*.

Methods

All animals and property where this project was conducted are owned and operated by the University of Wyoming–Agricultural Experiment Station (AES) at 2,195 m elevation near Laramie, Wyoming at the Laramie Research and Extension Center (LREC). The pastures used in this study included irrigated hay meadows dominated by exotic grasses and native rangeland dominated by native grasses and shrubs. The commercial western white-face sheep used in the demonstration research were mixed age ewes 1 to 6 yrs of age managed in four separate grazing management cohorts. In early October 2023, LREC acquired four female BLM donkeys (originating from California and straight off the range other than general sorting, processing, and transport according to our knowledge of their history), including three yearlings and one aged four years, each assigned a distinct numerical identifier (Refer to Figure 2 for Donkey Identifications: 7100, 7107, 6891, and 7092). These donkeys were individually introduced to separate flocks and pastures, and their interactions were monitored over a 43-day period from October 2, 2023, to November 13, 2023. Observations of ~5 minutes were made daily, either in the morning or mid-afternoon during active grazing periods, to visually estimate the proximity of each donkey to the sheep using a combination of a Bushnell Prime 1300 laser range finder, Vortex 15 × 56 mm Diamondback® HD binoculars, and the Google Earth measure tool. During these observations, the activities of the sheep and donkeys were qualitatively assessed, along with the donkey's relative position to the flock, categorized as 'in', 'near', or 'away' (as described in Figure 3). Subsequent spot checks continued daily until the end of the 2023 calendar year to monitor if the donkeys remained with the flocks or if any issues arose. All animal care and use complied with the guidelines outlined in the "Guide for the Care and Use of Agricultural Animals in Research and Teaching" (McGlone 2010).

To analyze the operational acclimation and integration of the four donkeys to unique flocks and pastures, polynomial regression analysis was employed using a quadratic trendline to model the number of days since donkeys introduction (x) to predict mean distance of all four donkeys to the nearest sheep (y). This analysis was performed with all donkey-flock pairs in a single model using SigmaPlot version 12.3. The variability among these pairings was further explored by detailing pasture and flock sizes, pasture complexity, and any necessary adjustments. Additionally, a daily binary indicator (0 or 1) reflecting each donkey's association with its flock (cate-

Figure 2. Individual donkeys and identification numbers acquired by the University of Wyoming from the Bureau of Land Management in 2023.

Figure 3. Qualitative assessment of burro proximity to the flock relative to general distance, awareness, and behavior used in assessing burro acclimation and fidelity as (A) 'in', (B) 'near', or (C) 'away' from sheep in Laramie, WY, USA.

(A) 'IN': Burro is 'in' the flock actively grazing and behaving similar to the sheep. In this example, the burro is grazing within the flock and often within <10 meters of individual sheep and moves within the flock.

(B) 'NEAR': Burro is 'near' the flock where it is aware of the presence of sheep and generally is moving through the landscape with the flock. In this example, the burro is often within 10 to 30 meters of the sheep and remains within that distance but on the perimeter.

(C) 'AWAY': Burro is 'away' from the flock where it is oblivious to the sheep and is moving through the landscape independent of the flock. In this example, the burro is often >100 meters from the flock and may be paying particular attention to other influences like other equids nearby.

gorized as 'in', 'near', or 'away') was established, and a Wilcoxon signed-rank test was applied for pairwise comparisons among the donkeys based on their proximity to the flock using JASP version 13.1 (Love et al. 2019). The results were then represented as stacked bar charts for each donkey. Finally, behavioral observations of both sheep and donkeys were classified as either 'non-vigilant' (including activities such as grazing, resting, and drinking) or 'vigilant' (including standing, walking, and vocal socialization, such as braying at donkeys in other pastures). The frequency of these behaviors was calculated and subjected to an arcsine transformation to satisfy normality assumptions. One-sample and paired-sample t-tests were then applied to examine the behavioral variations within and between donkeys and flocks, respectively, regarding vigilant and non-vigilant behaviors, using JASP version 13.1 for statistical analysis (Love et al. 2019).

Results and Discussion

Generalizable Integration Dynamics for all Four Donkeys

There was considerable variation among the donkeys regarding their proximity to the nearest sheep (Figure 4A). When assessing the operation-level acclimation and integration based on distance to the nearest sheep, it took approximately five weeks for all four donkeys to fully integrate with the sheep (Figure 4B). The data displayed a significant and correlated ($P < 0.001$; R2 = 0.45; Figure 4B) polynomial quadratic response (y = -0.3523x2 + 9.3x + 135.5), showing an initial increase in distance within the first two weeks followed by a rapid decrease between weeks 2 and 5 (Figure 4B). By the end of week 4, the mean distance to the nearest sheep for the operation was consistently less than 50 meters, with the quadratic trendline crossing zero around day 38 (Figure 4B). This aligns with the timeframe reported by Green (1989) of 4-6 weeks for a naive donkey to bond with sheep. Subsequent spot checks until the end of December 2023 confirmed the donkeys' continued affinity to be in or near the sheep, even amidst pasture changes and flock mixings.

Individual Variability and Responses

Immediate Integration: Donkey 7092

This yearling jenny bonded immediately upon introduction to the flock with no intervention, displaying consistent proximity to the sheep throughout the study period (Figure 4A). This donkey exhibited high fidelity to the flock, remaining in or near it during over 90% of observations (Figure 5). Noteworthy to Donkey 7092 was the 17 acre pasture and the flock of 102 mature ewes with a sheep density of 6.0 sheep per acre, which was the highest density of all flock-pasture combinations. There was no other equine sharing the fence line in this pasture but there was one donkey across the road to the north. On average this donkey was 7 m from the nearest sheep and never measured more than 30 m from the nearest sheep (Figure 4A).

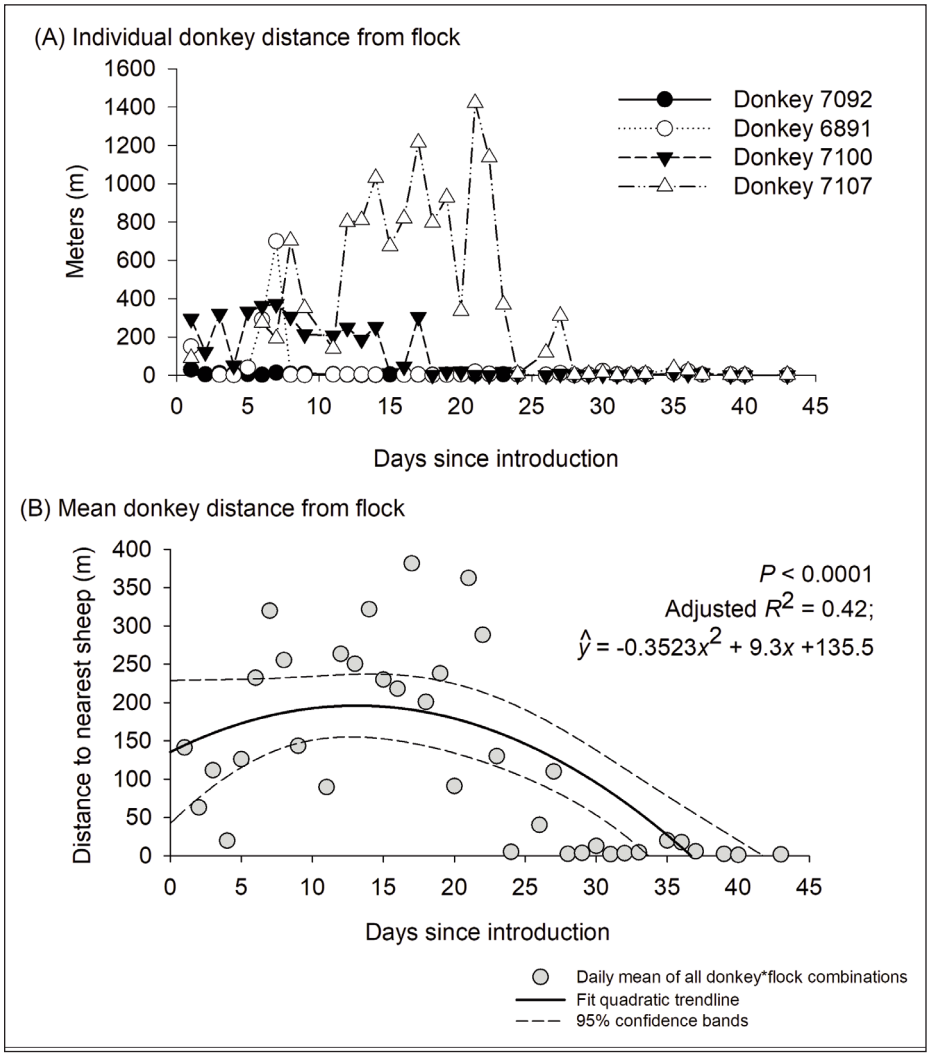
Took Time (Concern with Donkey Across the Road): Donkey 7100

This yearling jenny initially struggled to bond with the flock due to fixation on another donkey (7092 described above) across the road (an issue reported by Green 1989) (Figure 4A). She was placed in a 55 acre pasture with 105 yearling ewes yielding a sheep density of 1.9 sheep per acre which was the second highest density of all flock-pasture combinations. It took approximately two weeks (16 days) for this donkey to integrate fully, with subsequent high fidelity to the flock, albeit with occasional periods away during the initial period of introduction but never more than 20 m away (Figure 4A). Still, on average this donkey was 101 m from the nearest sheep; yet during the early period, one observation was found 372 m 'away' (Figure 4A). During the study period, this donkey was 'away' from the flock 38% of observations and was found 'in' or 'near' the flock 51% and 11% of observations respectively (Figure 5).

Too Many Equine Neighbors (Intervention Needed): Donkey 6891

This yearling jenny was placed in a 102 acre pasture with 114 ewes yielding a sheep density of 1.1 sheep per acre which was the third highest density of all flock-pasture combinations. This pasture created a difficult scenario for this donkey due to the presence of 16 horses in the pasture to the northeast and 15 horses in the pasture to the south. The equine manager recognized the social challenges for this donkey and used a hobbling treatment overnight (1 night) in the pen with sheep, side hobbling, and penning again with the flock prior to turn out and herding together. Within 1 week, the donkey integrated with the sheep and was no longer distracted by

Figure 4. (A) Individual donkey distance from flock and (B) mean of all donkeys (n = 4) distance to flock as an indication of operational mean time to integration across all four donkeys.



the equine neighbors across the fence. On average, this donkey was 39 m from the nearest sheep, but early observations found the donkey 700 m away before interventions (Figure 4A). However, once the donkey bonded after interventions it was never found more than 25 m away from the nearest sheep (Figure 4A). This donkey was recorded 'in' or 'near' the flock for more than 90% of observations and only 9% of observations during the study period found the donkey 'away' from the flock (Figure 5).

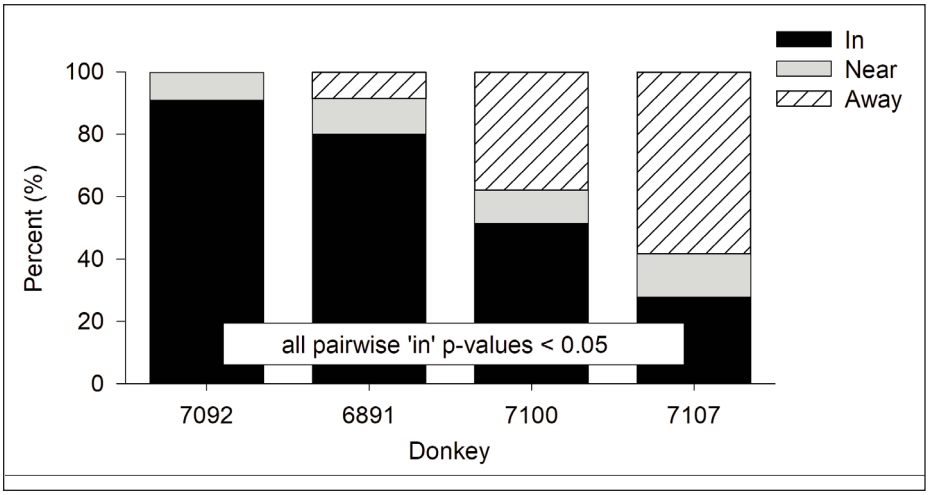
Pasture Too Big and Complex: Donkey 7107

This 4 year old jenny was placed in a 779 acre pasture with 50 mature ewes yielding the lowest sheep density of all flock-pasture combinations at 0.1 sheep per acre. In addition to the vast size of the pasture and the lower density of sheep, complexities within this pasture included: two cross fences (one with an open gate and one with an incomplete section in a marshy area), multiple water points (troughs for livestock water but also access to the Laramie River whereas the other three pastures only had a single water point), presence of 40 cows, 15 horses situated to the east across the road, and occasional visits by an older Great Pyrenees dog. Furthermore, this pasture featured slight undulations and shrubs, which present a more heterogeneously complex environment relative to the topographical cover utilized by predators (Jenkins and Noad 2003). The donkey alternated between spending time near the road, observing the neighboring horses, and mingling with the cattle. As the sheep primarily occupied the distant sections of the pasture, the donkey failed to integrate with them. After 25 days, the managers relocated the donkey to an 18 acre meadow containing 30 ewes and 1 ram, and within 5 days, the donkey formed a bond with the sheep. This smaller pasture had a sheep density of 1.7 sheep per acre, potentially expediting the acclimation and integration process. Despite the presence of the same equine neighbors adjacent to the fence in the new pasture, the donkey appeared to have formed a strong bond with the sheep. On average, this donkey was 383 m from the nearest sheep and during one observation was found >1,422 m 'away' (Figure 4A). During a few attempts to quantify distance from nearest sheep, the donkey was likely even further away because we could not visually locate the sheep due to the pasture size. The observations away from the flock were in the initial extensive and complex pasture as described above and once the donkey bonded with the sheep in the new smaller and simpler pasture, it was never found more than 40 m away and it remained here for the duration of the study (Figure 4A). During the study period, this donkey was 'away' from the flock 58% of observations and was found 'in' or 'near' the flock 28% and 14% of observations respectively (Figure 5).

Sheep and Donkey Activity

Sheep flocks were observed engaging in non-vigilant activities, such as grazing and resting, for the majority of observations (mean = 97.8% ± 1.4), with only a small percentage of observations showing vigilant activities (mean = 2.2% ± 1.4) (Table 1). There were no statistical differences between flocks in their expression of vigilant behaviors ($P = 0.192$) but there were for non-vigilant behaviors ($P < 0.001$) (Table 1). Donkeys exhibited variations in both non-vigilant and vigilant activities, with statistical differences observed between donkeys for both types ($P = 0.002$ and $P = 0.019$, respectively; see Table 1). Non-vigilant activities ranged from 52.8% to 90.9% (mean = 74.3% ± 9.3), while vigilant activities ranged from 9.1% to 47.2% (mean = 25.7% ± 9.3) (Table 1). Comparatively, sheep showed statistically lower vigilance than non-vigilant activity ($P = 0.002$), whereas

Figure 5. Individual donkey proportional qualitative association with flock location ('in', 'near', 'away'; Figure 3).



donkeys did not display significant differences ($P = 0.088$). Sheep did have statistically lower vigilance and higher non-vigilance than donkeys (both *P-values* = *0.029*; Table 1). Donkeys showed higher levels of vigilance, particularly in standing vigilance compared to grazing, but anecdotally time spent grazing or drinking increased (or vigilance decreased) after integration was achieved. Additionally, donkeys that took longer to acclimate and integrate exhibited heightened vigilance.

Additional Observations

Our utilization of donkeys as guard animals for sheep aligns with the guidelines provided by Bough (2016), which suggest maintaining a donkey-to-sheep ratio not exceeding 1:200. It is worth noting that while Green (1989) proposes a maximum ratio of 1:200-300, Walton and Field (1989) advocate for a maximum ratio of 1:400, with 1:200 being considered ideal. Furthermore, our findings support the recommendation against using pastures larger than 600 acres, as observed difficulties with donkey 7107 align with this advice (Green 1989; Walton and Field 1989). Throughout the study period, we encountered various observations worth noting. Firstly, a visiting rancher reported witnessing donkeys chasing ravens, an important deterrence given the concern ranchers have expressed about protected predatory birds as discussed by Windh et al. (2019). Additionally, a neighbor reported the presence of a coyote in a pasture containing a donkey, although no depredation incidents occurred. Moreover, there were two instances of sheep depredation observed in flocks lacking integrated donkeys. Anecdotally, during this time period in 2022 approximately 15 depredation incidents were recorded, contrasting with only two incidents in 2023, as previously mentioned (and sheep were similarly managed and distributed across the landscape). Lastly, it is important to consider that while herding dogs were routinely used to gather sheep, some instances were noted where donkeys exhibited defensive behaviors against these herding dogs.

Conclusion

The integration of donkeys can vary depending on the individual donkey, but it is significantly influenced by factors such as pasture size and conditions. A realistic timeline for integration typically falls within the range of 4-6 weeks, as suggested by Green (1989) and Jenkins and Noad (2003). Additionally, the presence of other equids and cattle nearby may initially hinder the acclimation and integration process with sheep, as indicated by Wilbanks (1995). Donkeys' aversion to canids makes them particularly suitable for guarding sheep, especially in environments where the primary predators are smaller canids, such as those found at the LREC farm, as noted by Green (1989) and Bough (2016). In our study, success was more quickly realized as pasture size went down but more importantly as sheep density relative to land area went up. It is crucial for producers to consider the size and complexity of the initial pasture for each donkey's integration. If successful integration is not achieved early on, adjustments should be made by moving the donkey to a smaller, less complex pasture. Additionally, there is a need to further measure the daily activity budgets of acclimated donkeys and utilize

Table 1. Pooled Sheep and Donkey Activity Categories: Non-vigilant and vigilant behaviors analyzed using one-sample t-tests for intra-flock and intra-donkey variation, and paired sample t-tests for sheep vs. donkey comparisons.

Animal Activity Estimate	6891	7092	7100	7107	Mean	One Sample t-test
Sheep - Non-Vigilance (%)	97.1	100.0	100.0	94.1	97.8 ± 1.4	p < 0.001, t = 23.740, df = 3
Sheep - Vigilance (%)	2.9	0.0	0.0	5.9	2.2 ± 1.4	p = 0.192, t = 1.678, df = 3
Donkey - Non-Vigilance (%)	88.6	90.9	64.9	52.8	74.3 ± 9.3	p = 0.002, t = 9.624, df = 3
Donkey - Vigilance (%)	11.4	9.1	35.1	47.2	25.7 ± 9.3	p = 0.019, t = 4.638, df = 3
Paired Samples t-tests						
Test: Sheep Vigilance vs. Non-Vigilance	p = 0.002, t = -11.03, df = 3					
Test: Donkey Vigilance vs. Non-Vigilance	p = 0.088, t = -2.493, df = 3					
Test: Sheep vs. Donkey Vigilance	p = 0.029, t = -3.959, df = 3					
Test: Sheep vs. Donkey Non-Vigilance	p = 0.029, t = 3.959, df = 3					

technology to better quantify their spatial relationships with flocks such as GPS collars on sheep and donkeys. This could potentially involve documenting nighttime protection activity. The current study simulated pastoral conditions at the semi-extensive LREC sheep production site, utilizing dormant hay meadows during a period of the year when predation risks are higher which is similar to many sheep operations in the region. Further investigations in working production systems need to quantify whether donkeys effectively reduce and mitigate predation, especially in relation to different flock sizes, and if once donkeys are successfully acclimated in small pastures does the bond hold in larger pastures. Jenkins and Noad (2003) suggest that donkeys are most effective in flocks with fewer than 50 head yet this statement should perhaps be quantified by the density of the grazing cohort relative to pasture size. However, Bergman et al. (1998) reported that producers in North Dakota used donkeys in flocks with an average of 405 head, while those in Texas had an average of 213 head. Additionally, it is important to assess whether routine production activities such as shearing and resorting of sheep management groups (e.g., breeding groups) impact the acclimatization and integration process of donkeys and should be further evaluated. The use of BLM donkeys as LGAs, which has been reported to range from 62-79% in the US, may provide additional value and utility of these animals in other countries (Bough 2016; Smith et al. 2000). However, some producers may be skeptical about adopting a feral animal that is unproven as opposed to a donkey that has some experience with sheep (Bergman et al. 1998). Finally, future work also needs to address the efficacy against specific predator species including larger carnivores (such as mountain lions, wolves, and bears) which have been noted to prey on donkeys (Wilbanks 1995; Reinhardt et al. 2012).

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Variation in Wool Cortisol, Progesterone, and Testosterone in Targhee Ewes Across Physiological States and Varying Production Levels[1,2]

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Summary

Determining the pregnancy status of ewes prior to lambing is important for sheep producers to properly manage limited resources and improve profitability. We tested the hypothesis that steroid hormone (progesterone, cortisol, and testosterone) concentrations change through a production cycle and may be used as a pre-lambing pregnancy test. Twenty multiparous (4.5 ± 1.5 y) purebred Targhee ewes were enrolled in this study before the breeding season. Wool samples were collected at four time points beginning with a sample prior to breeding, at 30-d gestation; 110-d gestation; and approximately 40-d postpartum. Wool production data, including fiber diameter and staple length, and lamb birth data, were collected to test linear regression associations between hormone concentration and production. Using a repeated measures ANOVA, we found differences ($P < 0.05$) between time points and a post-hoc analysis showed that the 40 days post-lambing was statistically different (Bonferroni adjusted $P < 0.05$) from the previous three samples points. There were no associations between wool hormone concentrations prior to breeding, at 30-d gestation, or 40-d postpartum with production metrics ($P > 0.05$). An association was observed between progesterone levels at 110 days of gestation and litter size. These findings suggest that wool hormones could serve as valuable tools for researchers assessing animals post-lambing, although their utility as a diagnostic tool for producers may be limited. Nonetheless, further research is necessary to ascertain the potential of wool hormone monitoring in predicting other economically relevant flock performance metrics.

Key Words: Hormones, Pregnancy, Non-invasive Sampling, Ovis Aries

Introduction

Accurate and reliable health and production monitoring is an integral component to a well-managed and sound flock. Most sheep producers currently rely on production measures, veterinary diagnostics, and their own observations to determine the current status and outlook of their flock. Unfortunately, a number of these diagnostics may be expensive, time-consuming, and labor-intensive reducing their usefulness to individual producers. This challenge reflects a need for non-invasive and cost-effective tools to improve monitoring of sheep throughout a production cycle.

Unique to fiber-producing animals, wool is a complex, naturally renewable fiber that continuously grows through a sheep's life and may serve as an important resource for monitoring longitudinal effects on health and production levels. Given that wool grows year-round, wool represents an optimal sample for precise retrospective monitoring without sampling biases that exist with other biological matrices (Palme, 2012; Fürtbauer et al., 2019). Hair and wool are commonly used as tissue specimens for evaluation of chronic stress via cortisol measurements in many livestock species (Stubsjøen et al., 2015; Duran et al., 2017; Heimbürge et al., 2019; Sawyer et al., 2019). This represents a non-invasive sampling technique that producers routinely use for wool trait characterization (Scobie et al., 2015). While measuring wool cortisol is typically restricted to research uses, the potential exists for its application in the commercial industry.

Wool has also been found to incorporate the steroid hormones such as cortisol and progesterone, which are important biomarkers for stress and pregnancy status, respectively (Sawyer et al., 2019). It takes approximately 14 days for glucocorticoids in the circulation to be observed in the wool (Weaver et al., 2021). Elevated testosterone and cortisol levels in wool post-lambing were previously associated with litter size (Alon et al., 2021). Together these hormones could be measured throughout a production cycle to evaluate pregnancy status and chronic stress; however, their association to other aspects of lamb and wool production currently remains unknown.

The objectives of this study were to characterize the wool progesterone, cortisol, and testosterone profiles throughout a production cycle and determine if these measurements could be used as a producer diagnostic tool.

Materials and Methods

All animal procedures were approved by the Montana State University Agricultural Animal Care and Use Committee (Protocol # 2021-AA14).

Animals and Wool Sample Collection

Twenty multiparous purebred Targhee ewes (4.5 ± 1.5 y) from the Montana Agricultural Experiment Station flock were enrolled in the study prior to breeding season. Ewes were estrus synchronized using CIDR devices inserted and kept in place for 10 days prior to exposure to rams. Ewes were naturally exposed to rams for 30 days before rams were removed but conceived on the first cycle due to synchronization. Ewes were confirmed pregnant via trans-abdominal ultrasound on January 31st, 2022. Wool samples used for hormone extraction were collected from the rump using an electric shearing machine (Heiniger, Switzerland) with a 13-tooth comb as close to the skin as possible and were an approximately 5 x 5 cm square. Samples were placed into a Ziplock or brown paper bag and stored in a climate-controlled area at standard room temperature and humidity and out of sunlight until steroid extraction.

Wool samples were collected from each animal at four time points: prior to breeding (taken prior to CIDR insertion), at 30-d gestation, 110-d gestation, and approximately 40 days postpartum, as shown in Table 1. These time points were selected because they coincide with typical management practices occurring on western U.S. range sheep operations when producers would be able to easily collect a wool sample. A sampling schematic and corresponding management events can be found in Figure 1.

Figure 1. Sampling timeline. The dates, physiological stage, and sample collected are represented above the bar. Pregnancy is represented by the blue box. Western U.S. sheep industry management events are noted below the bar and correspond to sampling time points.

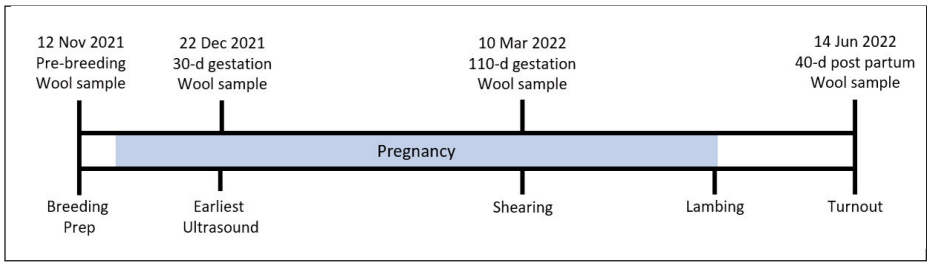

Table 1. Wool sampling dates, characteristics, and mean values ± S.E. for wool hormones

	Sampling date and status at time of sampling			
	12 Nov 2021	22 Dec 2021	10 Mar 2022	14 June 2022
Hormone pg/mg[1]	Pre-breeding	30-d gestation	110-d gestation	40-d post-partum
Cortisol (*n*=14)	1.94 ± 0.27^{ab}	2.78 ± 0.44^{a}	1.22 ± 0.24^{b}	7.97 ± 0.73^{c}
Progesterone (*n*=20)	7.17 ± 1.39^{ab}	12.4 ± 1.88^{a}	5.52 ± 0.67^{b}	33.8 ± 2.62^{c}
Testosterone (*n*=16)	1.36 ± 0.22^{a}	2.55 ± 0.45^{a}	1.44 ± 0.26^{a}	6.40 ± 0.56^{b}

[1] Different superscripts in a row indicate significant differences between sampling times at the Bonferroni adjusted *P*-value of 0.05.

Hormone Extraction and Detection

Cortisol, progesterone, and testosterone were extracted from wool samples as previously described with the following modifications (Sawyer et al., 2019; Alon et al., 2021). 250 mg samples were washed in 50mL conical tubes with 5 mL of 100% 2-propanol overnight and subsequently dried for a minimum of 24 hours. Following drying, a 50 mg sample of the most proximal portion, the approximately 1 cm closest to the skin, of the wool staple was isolated, placed into 1 mL of 100% methanol, and left to soak for 48 hours. The methanol layer was aliquoted and placed in a 0.5 mL microcentrifuge tube and allowed to evaporate in a fume hood at room temperature for a minimum of 24 hours. Once all the methanol was evaporated, the remaining residue was reconstituted in 400 µL of ELISA assay diluent. Hormone concentrations were quantified in duplicate using commercially available ELISA kits following manufacturer's protocols (Salimetrics; Ann Arbor, MI, USA) as previously reported (Fürtbauer et al., 2019; Alon et al., 2021). Extracted wool samples were diluted 1:10 and 1:15 for progesterone and testosterone, respectively. The extractions for evaluating cortisol were not diluted. Plates were read on a BioTek Epoch 2 plate reader at 450 nm and absorbance data captured using the BioTek Gen5 Data Analysis software (Agilent Technologies, Inc; Santa Clara, CA, USA). Intra-assay CV was 3.1%, 3.5%, and 3.5% for cortisol, progesterone, and testosterone respectively.

Production Data

Wool samples from the mid-side of the sheep at 110-d gestation, corresponding with a full-length staple and industry standards in a commercial setting, were used to evaluate fiber characteristics including fiber diameter, fiber diameter coefficient of variation, staple length, and curvature using the Optical Fiber Diameter Analyzer 2000 at the Montana Wool Lab. Wool samples were analyzed along the length of the wool staple. Gross fleece weights were also recorded at 110-d gestation. Lamb birth data collected included litter size and birth weights.

Statistical Analysis

All statistical analyses were performed using R version 4.0.4 (R Core Team, 2021). Data were analyzed for possible outliers defined as values more than three times above or below the interquartile range and assessed for normality by a Shapiro-Wilk test. For each hormone, a repeated measures ANOVA was conducted with sampling date as the independent variable and the hormone measures as the dependent variables. For the repeated measures ANOVA, only complete cases, those with all four time points measured for each hormone were used in the analysis. Post-hoc pairwise paired t-tests were conducted within each hormone to determine which time points were statistically different from one another. A Bonferroni adjusted p-value < 0.05 was considered statistically significant.

For associations with the production traits, a univariate linear regression was performed for each trait and each individual time point hormone value to determine if the level of steroid hormone in wool influences production traits.

Results and Discussion

Cortisol, Progesterone, and Testosterone measures

For each hormone repeated measures ANOVA, a difference was found ($P < 0.05$). Following post-hoc analysis there were no differences detected between the first two time points (Bonferroni adjusted $P > 0.05$) across all three hormones. Cortisol and progesterone concentrations decreased at the 110-d of gestation time point (Bonferroni adjusted $P < 0.05$). All hormone measurements increased at the 40-d postpartum sampling date (Bonferroni adjusted $P < 0.05$). Cortisol, progesterone, and testosterone, had approximately 4.03, 4.05, and 3.60 times the hormone level at 40-d postpartum compared to the average of the first three sampling time points, respectively. These elevated hormone concentrations in the wool at this time point reflect the last approximately 40 days of gestation and first 25 days of lactation. As previously shown in ruminants, concentrations of cortisol, progesterone, and testosterone rapidly increase in matrixes such as serum and milk towards the end of gestation [11-13]. Hormone concentrations for each sampling point can be found in Table 1. It is important to note the lag time between wool and blood hormone measurements and understand that the two measurements will not be identical when comparing to previous literature. As previously reported it takes approximately 14 days for glucocorticoids to incorporate into the wool if the hormones are elevated for sustained periods of time (Weaver et al., 2021).

It was unexpected that the concentrations of cortisol, testosterone, and progesterone were not increased at approximately 110-d in gestation or about 45 days before lambing compared to the prior two time points. This contrasts with results presented from maiden Australian merino ewes which showed progesterone and cortisol concentrations to be higher about two weeks prior to lambing (Sawyer *et al.*, 2019). This difference may be due to sampling time differences. In the present study, samples were collected approximately four weeks earlier than in Sawyer et al. 2019, using wool representing lower circulating concentrations of progesterone, cortisol, and testosterone during mid-gestation as opposed to late gestation (Fylling, 1970; Gaiani et al., 1984; Fowden et al., 1998). All three hormones showed a numerical decrease in concentration for the wool sample collected at 110-d of gestation compared to the 30-d gestation sample indicating there may be a difference from this sample than others. The 110-d samples were collected during the regular shearing process so could possibly have contributed to this unexpected result.

This result likely reduces the utility of wool hormone testing as a possible producer-oriented pregnancy diagnostic tool, given elevated hormone concentrations in the wool are not appearing early enough in gestation to be more valuable to the producer compared to other available tools. Given there are other pregnancy diagnostic tools available, such as blood testing for pregnancy associated glycoproteins and transabdominal ultrasound, which diagnose pregnancy much earlier in gestation, evaluating hormone status using a non-invasive wool sample may not be practical for the U.S. sheep industry. However, this type of testing could be used to determine if a ewe was

pregnant when ewes are lambing without supervision in a range or other extensive setting or ewes are not evaluated by producers shortly after lambing opening doors for monitoring in more extensive settings.

Associations with Production Metrics

The OFDA2000 results showed the ewes had a mean gross fleece weight of 3.22 ± 0.15 kg, mean fiber diameter of 19.9 ± 0.18 microns, mean coefficient of variation of 18.35 ± 0.43 percent, mean staple length of 78.75 ± 1.80 mm, and a mean curvature of 101.1 ± 3.09 degrees/mm.

Ewes lambed began lambing on April 22, 2022 and had a standard error of 1.10 days. Twenty ewes delivered 31 lambs with eleven sets of twins and the remaining lambs being born as singles. Sixteen lambs were male and fifteen were female. Lambs weighed 4.5 ± 0.14 kg at birth.

We did not observe an association between concentrations of post-partum wool testosterone or cortisol and litter size as previously reported (Alon et al., 2021; Zeinstra et al., 2023). This may be due to the fact that the largest litter size in our study population was only two lambs, whereas Alon et al. reported litter sizes of three and four lambs, though Alon et al. reported no statistical difference between those carrying singles and those carrying twins but found a statistical difference between singles and multiples when triplets and quadruplets were included (Alon et al., 2021). However other reports only found this association between large (3-4 lambs per litter) and small (1-2 lambs per litter) comparisons (Zeinstra et al., 2023). It is possible that these differences exist in triplet and quadruplet bearing rangeland type ewes, such as the Targhee breed studied here, but larger litter sizes are less common in a rangeland setting and more data is needed to validate that association in this setting. An association ($P < 0.05$) between wool progesterone level at 110-d gestation and litter size was observed, with ewes carrying singles (6.82 ± 0.90 pg/mg) having a higher level of progesterone than ewes carrying twins (3.96 ± 0.80). This is unexpected given reports in the literature typically show increasing blood progesterone concentrations with increasing litter size in sheep, though not consistent enough to use as a diagnostic for litter size determination (Stabenfeldt et al., 1972; Butler et al., 1981; Karen et al., 2006; Roberts et al., 2017). However, given the wide individual variation reported in progesterone concentrations among pregnant ewes, the relatively small sample size in the present study, and different biological material being tested, additional research is needed to further prove or dispute the present results.

There was no relationship identified between cortisol concentrations and fiber diameter as reported in Australian Merinos (Sawyer et al., 2021). This may be explained by the previous reported study using the topknot of wool for characterizing wool characteristics instead of the mid-side sample in the present study. While the topknot may be a convenient sample, there is variability in fiber diameter and characteristics across the sheep's body making a mid-side sample most appropriate for representing the entire fleece (Scobie et al., 2015). We did not observe any other relationships between wool traits and testosterone, progesterone, or cortisol measured at any other sample point.

This preliminary work shows that wool hormone concentrations do differ at different times over a production cycle. Monitoring wool hormones may be a useful tool for researchers and evaluating animals post-lambing, but may not be beneficial as a producer diagnostic tool prior to lambing given the lack of associations seen in the present study. However, given the small samples size and lack of open ewes in the present study, further work is warranted to determine the utility of wool hormone monitoring for possible predictive ability of long-term performance, longevity, or other economically relevant metrics not measured in this project.

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Melatonin-Implanted Pregnant Ewes Produce Lambs that have Higher Average Daily Growth Rates and Live Weights at Weaning

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Summary

The aim of this study was to quantify differences between lambs from birth to weaning born from ewes that received a melatonin implant before lambing and those that were not implanted. Forty d before lambing, 457 pregnant ewes either did or did not receive a melatonin implant. Subsequently, lambs were divided into two groups: lambs whose mothers received melatonin (MEL, n=248, 166 males, 161 females), and lambs whose mothers were non-treated (CTR, n=327, 128 males, 120 females). Lambs were weighed (kg) at birth (LW0) and at weaning (LWW) and age at weaning (AW, d) was recorded. Average daily growth rate (g/d) (AGR) was calculated as [(LWW-LW0)/AW]. MEL lambs had a mean LWW significantly ($P<0.05$) higher than CTR lambs. In particular, male MEL lambs had a significantly ($P<0.05$) higher LWW and AGR than male CTR lambs. Singleton male MEL lambs had higher LW0, LWW, and AGR, and lower AW at the day of weaning than the other lambs, and differences with singleton male CTR lambs were significant ($P<0.05$). LW0, LWW, and AGR were negatively correlated ($P<0.05$) with the implanting-weaning interval (IWI). Lambs with the shortest IWI had the highest LWW ($P<0.05$) and AGR ($P<0.01$), and the lowest AW ($P<0.01$). Treatment of pregnant ewes with melatonin before lambing increased lamb performance until weaning, and the effect was most pronounced in singleton male lambs, which had the highest growth rate. It remains to be elucidated what is the minimal interval between implantation in the pregnant ewes and parturition that maximizes the growth of lambs during lactation.

Key Words: Lambs, Melatonin, Growth, Live Weight

Introduction

Sheep productivity is constrained by sexual seasonality, which is governed by photoperiod (Yeates, 1949); i.e., the endocrine system receives photoperiodic information from the hormone melatonin, which dictates the timing of reproduction. Melatonin is secreted at night, and subcutaneous melatonin implants can be used to artificially manage estrus in sheep, which causes a brief daytime-like response without inhibiting endogenous production (O'Callaghan et al. 1991; Malpaux et al. 1997). In Spain, the anestrous period covers the late winter/early spring (Feb-Mar) to early- or mid-summer period (June-July); this seasonal breeding pattern results in a clear period of lambing, which in turn causes a seasonal pattern of product prices, with prices being lowest when the supply of meat is the highest (late spring to early fall) and vice versa. If farmers were able to produce products "out of-season", they could take advantage of higher prices for these during the winter by inducing estrous cycles during the seasonal anestrus. In this context, melatonin implants play an important role to obtain out-of-season lambs at high prices. Spain has reached the highest market share of the melatonin implants for sheep in the world, with more than 500,000 treatments per year applied in the ovine population, which is about 14 million heads; it means that around 1 out of 30 ewes in Spain have been treated with melatonin.

Recently, various uses of melatonin have been used in small ruminants, apart from the traditional reproductive control, with most of the focus on the survival and growth of offspring, and the improvement of colostrum and milk quality. Melatonin implants given between 70 and 120 d of pregnancy reduced neonatal mortality and increased survival rates at weaning, which were associated with increases in survival of twins and tolerance for prolonged parturition in sheep flocks that had been intensively managed (Flinn et al. 2020a, 2020b). Melatonin rapidly crosses the blood-brain barrier and the ovine placenta (Yellon and Longo 1987; Aly et al. 2015), which allows for maternal supplementation as a means for providing melatonin to the fetus before birth. Elsewhere (Abecia et al., 2020), we demonstrated that, in the fourth month of pregnancy, melatonin implants in ewes improved the quality of the colostrum produced, and lambs born of ewes that received exogenous melatonin had more IgG than did lambs from ewes that did not receive an implant. Furthermore, treatment with melatonin in ewes at lambing increased the growth rates in their lambs and the fat content of the milk (Abecia et al., 2021), and newborn lambs from ewes that had received a subcutaneous melatonin implant at day 120 of pregnancy had higher rectal temperatures and higher average and minimum body surface temperatures of the shoulder, mid loin, and hips than did control lambs (Canto et al., 2023). Recently, we showed that ewes that received a melatonin implant 40 d before lambing produced colostrum that had higher IgG concentrations, produced more milk, which had a lower somatic cell count (SCC), than did non-implanted ewes. A second melatonin implant prolonged the effect on SCC (Canto et al., 2022). In Lacaune dairy sheep, although exogenous melatonin treatment in late pregnancy did not have an effect on milk yield, it did affect milk composition; specifically, increasing milk fat concentrations and decreasing milk protein and lactose (Canto and Abecia, 2022). Collectively, the evidence indicates that administering melatonin implants to pregnant sheep might increase their economic return by improving the performance of their lambs through an increase in milk quantity and quality, and or an increase in lamb survival.

The Spanish sheep and goat sector accounts for approximately 10% of Spain's final livestock production, when considering the entire meat and dairy subsector. With a sheep population of around 14 million in the last five years, our country holds the top position in importance within the European Union (MAPA, 2023).

The objective of this study was to quantify the differences from birth to weaning between Rasa Aragonesa lambs born from ewes that had received an exogenous melatonin implant before lambing and those that were not.

Material and Methods

The experiment followed a protocol (PI29/21) approved by the Ethics Committee of the University of Zaragoza, which met the requirements of the European Union for Scientific Procedure Establishments.

The study involved 575 Rasa Aragonesa lambs (294 males, 281 females; 199 singles, 307 twins, 69 triplets) born on two commercial sheep farms (Farm1, n=354; Farm2, n=221) in Zaragoza, Spain. The farms were members of the Cooperative "Oviaragón", which produce the local lamb "Ternasco de Aragón" under the European Protected Geographical Indication (PGI). The Rasa Aragonesa sheep breed is a native breed of Spain that has been traditionally raised in Northeastern Spain; this breed has been recognized for its resistance, adaptability to various environments and its role in meat and wool production. After weaning in the farms, at an age of 45 d, lambs are housed in feed lots to achieve the slaughter weight (18-24 kg; 70-90 d of age). The farms applied the same management of the animals for the breed (Rasa Aragonesa), because farms that are involved in the production of that PGI lamb must meet several quality standards.

Approximately 40 d (mean ± S.D.= 39 ± 7 d; range 18-60 d) before the expected time of lambing (lambing season: 2 May-9 Jun), 457 pregnant ewes were either treated or not with a single melatonin implant (18 mg melatonin; Melovine, CEVA Salud Animal, Barcelona, Spain), which produced lambs that were assigned to one of two groups for analytical purposes: lambs whose mother had received a melatonin implant (group MEL, n=248) and lambs whose mothers were non-treated (group CTR, n=327). Lambs were weighed (kg) at birth (LW0) and at weaning (47 ± 8 d of age) (LWW). Age at weaning (AW, d) was recorded, and the average daily growth rate (g/d) (AGR) was calculated as [(LWW-LW0)/AW]. The weaning date is decided by the farmer when the lambs begin to reach a LW of about 12 kg, and the whole group of lambs is weaned. The effects of farm, sex of the lamb, type of parturition (single or multiple), and treatment with melatonin (MEL or CTR), were evaluated statistically based on a multifactorial model. It included farm, sex of the lamb, type of parturition, and treatment as fixed effects, and the Least Squares Method of the GLM procedure in SPSS v.26 (IBM

Corp. Released, 2019) was used. Sire effects were not considered since no information about mating is available in our farms when natural mating is used. Within fixed effects, significant differences were identified by an ANOVA. Pearson correlation coefficients among the implantation-weaning interval (IWI) and the lamb performance (LW0, LWW, AGR and AW) were calculated. A regression analysis was conducted between IWI and AGR.

To identify the optimal time before parturition to insert a melatonin implant in the ewes, the interval between melatonin implantation and lambing (IIL) was divided into four quartiles based on 'visual binning' (SPSS), which provides an interactive means of choosing how to transform a quantitative variable into a categorical variable. Differences in LW0, LWW, AGR, and AW among quartiles and the control group were assessed statistically by an ANOVA and the Least Squares Method.

Results and Discussion

Farm, sex of the lamb, type of parturition, and treatment with melatonin of the mothers had a significant (at least $P<0.05$) effect on LW0 and LWW (Table 1), and the interaction between type of parturition and treatment with melatonin of the mothers had a significant ($P=0.01$) effect on LW0. Farm, type of parturition, and their interaction had a significant ($P<0.001$) effect on AGR, and the interaction between sex and treatment was significant ($P=0.05$). Farm and type of parturition, but not melatonin treatment of the mothers, had a significant effect on AW.

MEL lambs had a higher mean (± S.E.) LWW (12.26 ± 0.10 g/d) than did CTR lambs (12.00 ± 0.08 g/d) ($P<0.05$). In particular, male MEL lambs had a higher LWW and AGR than did male CTR lambs ($P<0.05$), but there were no significant differences between female MEL and CTR lambs (Table 2). Singleton male MEL lambs had the highest LW0, LWW, and AGR, and the lowest AW, and the differences with singleton male CTR lambs were significant ($P<0.05$) (Table 3). Male MEL and CTR lambs with littermates, and female MEL and CTR lambs with or without littermates did not differ significantly (Table 3).

LW0, LWW, and AGR were significantly negatively correlated with IIW ($P<0.01$ for LW0, AGR, and AW, and $P<0.05$ for LWW) (Table 4). The linear regression analysis between IIW and AGR had a high coefficient of determination with AGR (0.3512), and a negative slope (-2.3459) (Figure 1), which reflected the negative relationship between the IIW and the AGR of the lambs. The lambs that had the shortest IIW had the highest LWW ($P<0.05$) and AGR ($P<0.01$), and the lowest AW ($P<0.01$) (Table 5).

The experiment in this study demonstrated that lambs born from ewes that had received a melatonin implant in the last third of pregnancy had the highest LW during lactation and grew faster than did lambs born of non-implanted ewes. Their mother's milk was the lamb's only source of nourishment; therefore, the melatonin implants increased the quantity and or quality of the milk. Previously, we (Canto et al., 2022) demonstrated that melatonin implants in pregnancy had a significant effect on milk quality; specifically, ewes that had received a melatonin implant 40 d before lambing produced the most milk, which had the highest fat content. In another study (Abecia et al., 2021), ewes that had received a melatonin implant at lambing produced milk that had the fattest content, and their offspring had the highest growth rate In goats, melatonin implants inserted seven weeks before kidding had a significant effect on milk production in the subsequent lactation and improved the daily weight gain of their suckling kids (Avilés et al., 2019). Melatonin membrane receptors MT1 and MT2 are expressed in the mammary glands of goats throughout lactation (Zhang et al., 2019), which suggests that melatonin has a direct role in the regulation of mammary physiology.

In our study, the effects of treating with melatonin ewes in pregnancy were

Table 1. *P*-values in each of the factors affecting live weight at birth (LW0) and weaning (LWW), and the average daily growth rate (g/d) (AGR) and age at weaning (AW) in Rasa Aragonesa lambs.

	LW0	LWW	AGR	AW
Farm	0.046	<0.001	<0.001	<0.001
Sex	0.002	0.002	0.169	0.638
Type of parturition	<0.001	<0.001	<0.001	<0.001
Treatment	0.049	0.029	0.181	0.681
Farm x Sex	0.787	0.259	0.092	0.240
Farm x Type of parturition	0.579	0.198	<0.001	0.130
Farm x Treatment	0.733	0.565	0.092	0.806
Sex x Type of parturition	0.946	0.637	0.356	0.130
Sex x Treatment	0.918	0.100	0.050	0.956
Type of parturition x Treatment	0.010	0.175	0.397	0.217

Table 2. Mean (±S.E.) live weight at birth (LW0) and at weaning (LWW) (kg), average growth rate (AGR) (g/d), and age at weaning (AW) (d) of male and female Rasa Aragonesa lambs that were born of ewes that either did (MEL) or did not (CTR) receive a melatonin implant in the last third of pregnancy.

Sex	Group	LW0 (kg)	LWW (kg)	AGR (g/d)	AW (d)
Male	CTR (128)	4.15±0.05	12.13±0.12[a]	174.31±3.33[a]	46.9±0.6
	MEL (166)	4.20±0.07	12.62±0.15[b]	188.59±4.12[b]	46.2±0.8
Female	CTR (120)	3.93±0.06	11.87±0.11	173.82±3.45	47.0±0.7
	MEL (161)	3.94±0.07	11.87±0.12	170.29±3.25	47.4±0.6

Means within an effect with no common superscript are different $P<0.05$.

Table 3. Mean (±S.E.) live weight at birth (LW0) and at weaning (LWW) (kg), average growth rate (AGR) (g/d), and age at weaning (AW) (d) of singleton and multiple Rasa Aragonesa lambs born of ewes that either did (MEL) or did not (CTR) receive a melatonin implant in the last third of pregnancy.

		Singleton				Multiple			
Sex	Group	LW0 (kg)	LWW (kg)	AGR (g/d)	AW (d)	LW0 (kg)	LWW (kg)	AGR (g/d)	AW (d)
Male	CTR (128)	4.49±0.07[a]	12.36±0.19[a]	184.82±5.91[a]	43.7±0.9[a]	3.93±0.07	11.98±0.14	167.38±3.78	49.1±0.8
	MEL (166)	4.79±0.11[b]	13.19±0.25[b]	211.83±6.69[b]	40.6±0.8[b]	3.87±0.08	12.29±0.18	175.55±4.66	49.3±0.9
	Total	4.61±0.06[a]	12.70±0.16[a]	195.91±4.59[a]	42.4±0.6	3.90±0.0[a]	12.12±0.1[a]	171.06±2.9[a]	49.2±0.6
Female	CTR (120)	4.38±0.09	12.12±0.22	188.97±7.25	42.6±1.2	3.73±0.06	11.75±0.13	166.32±3.28	49.2±0.7
	MEL (161)	4.53±0.10	12.22±0.27	173.82±6.09	44.9±1.0	3.71±0.05	11.73±0.13	168.92±3.85	48.4±0.8
	Total	4.43±0.07[b]	12.12±0.11[b]	183.22±5.10b	43.5±0.8	3.72±0.0[b]	11.74±0.0[b]	167.46±2.4[b]	48.9±0.5
	CTR (327)	4.44±0.06[a]	12.25±0.15	186.68±4.59	43.2±0.7	3.82±0.05	11.86±1.00	166.83±2.48	49.2±0.5
	MEL (248)	4.68±0.08[b]	12.78±0.19	195.95±5.09	42.4±0.7	3.79±0.05	12.01±0.11	172.18±3.02	48.8±0.6

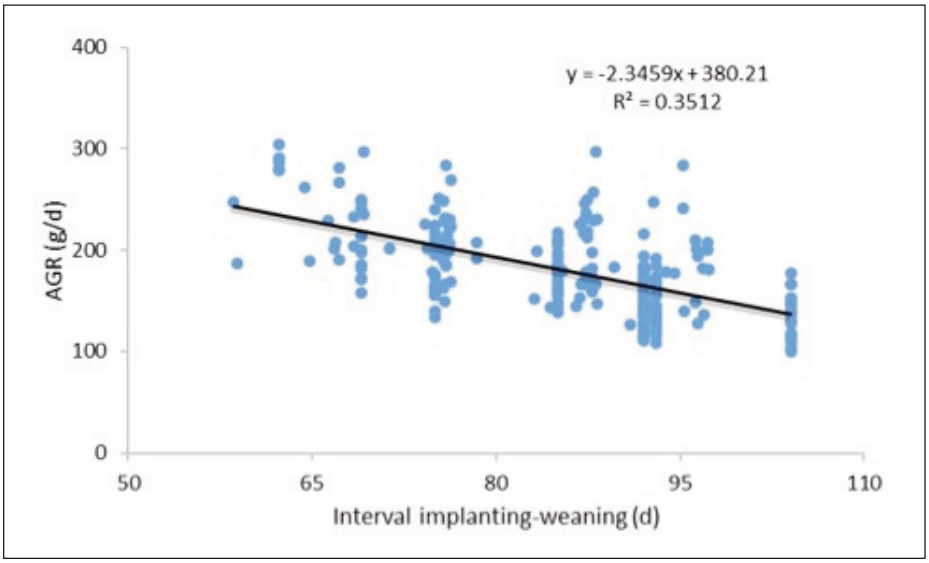
Means within an effect with no common superscript are different $P<0.05$.

strongest in male lambs. Similarly, Abecia et al. (2021) reported that the effects of melatonin implants in the mothers was significant in male lambs, only; specifically, male lambs reared by melatonin-treated ewes had significantly higher LW at weeks 2, 3, and 4 than did male lambs that had been reared by untreated ewes. In goats, melatonin implants in the dry period increased milk yield and the weight gain of male offspring, only (Avilés et al., 2019). Wallace et al. (2014) reported that, in early postnatal life, lamb sex had a significant effect on adipose tissue gene expression in favor of male lambs because female lambs had lower IGF1, IGF2, IGF1R, IGF2R, and hormone-sensitive lipase mRNA expression levels, which are associated with growth and reflect the sexual dimorphism in body composition.

Table 4. Matrix of correlations between the interval between the insertion of a melatonin implant in Rasa Aragonesa ewes in the last third of pregnancy and weaning (IIW), live weight at birth (LW0) and at weaning (LWW) (kg), average growth rate (AGR) (g/d), and age at weaning (AW) (d) of lambs (*$P<0.01$; ** $P<0.001$).

	IIW	LW0	LWW	AGR	AW
IIW		-0.240**	-0.162*	-0.593**	0.763**
LW0	-0.240**		0.293**	0.183**	-0.492**
LWW	-0.162*	0-293**		0.727**	-0.102*
AGR	-0.593**	0.183**	0.727**		-0.627**
AW	0.763**	-0.492**	-0.102*	0.627**	

Figure 1. Linear regression between the interval between the insertion of a melatonin implant in Rasa Aragonesa ewes in the last third of pregnancy and weaning (d) and the average growth rate of their lambs (AGR) (g/d).



The effects of melatonin implants in the mothers on the growth of male lambs might have been because they consumed the most colostrum, or the colostrum had the best quality. Elsewhere (Canto et al., 2022), we showed that ewes that received a melatonin implant 40 d before lambing produced colostrum that had higher IgG concentrations than did the colostrum from non-implanted ewes, and that ewes that had singleton male lambs had higher colostrum IgG concentrations (54.57 ± 5.37 mg IgG mg/mL) than ewes that had singleton female lambs (34.66 ± 4.30 mg/mL) (Abecia et al., 2020). In sheep, colostrum is important in the development of the immune system, post-natal growth, and thermoregulation, and mediates the formation of the ewe-lamb bond (Agenbag et al., 2021). In addition to increasing neonate survival, access to colostrum in the neonatal period can have a positive effect on future production, development, and reproductive efficiency of lambs through growth factors that facilitate neonatal growth and development. Öztabak and Özpinar (2006) reported that, from the

Table 5. Mean (±S.E.) live weight at birth (LW0) and at weaning (LWW) (kg), average growth rate (AGR) (g/d), and age at weaning (AW) (d) of Rasa Aragonesa lambs born of ewes that either did (MEL) or did not (CTR) receive a melatonin implant in the last third of pregnancy, and the interval between implantation and lambing (IIL) (IIW: interval between implantation with melatonin and weaning).

Group	IIL (d)	AW (d)	IIW (d)	LW0 (kg)	LWW (kg)	AGR (g/d)
CTR (n=329)	--	47.0±0.5[a]	--	4.05±0.04	12.00±0.08[a]	174.07±2.36[a]
MEL 1 (n=63)	30.34±0.34	44.1±1.3[b]	74.48±1.23[a]	3.94±0.11[a]	12.49±0.20[b]	200.37±5.92[b]
MEL 2 (n=61)	36.77±0.12	47.4±1.1[a]	84.19±1.11	4.04±0.09	12.24±0.21	177.64±5.07[a]
MEL 3 (n=62)	40.31±0.17	48.3±0.7[a]	88.64±0.65	4.09±0.09	12.13±0.19	169.71±4.64[a]
MEL 4 (n=60)	47.61±0.68	47.2±0.8[a]	94.83±0.86[a]	4.22±0.10[b]	12.16±0.21	170.87±5.09[a]

Means within an effect with no common superscript are different $P<0.05$.

second week onward, rearing method has an effect on body weight gain; specifically, lambs that were reared with their mothers and received colostrum had a higher mean body weight in lactation than did lambs that were reared without colostrum or artificially.

In our study, the correlations between implantation-weaning interval and the LW and AGR of lambs, indicated that the efficacy of the melatonin implants in improving lamb performance was greatest for individuals in which the melatonin implant was inserted closest to parturition. However, the later in pregnancy that the ewe was implanted, the smaller the effect on the development of mammary tissue because milk fat and total solid content were higher in ewes that had been implanted immediately after parturition than they were in control ewes at day 45 of lactation, only, which was close to weaning, and had no effect on the amount of milk produced. Although milk production and quality was not assessed, apparently, a single melatonin implant can affect the mammary gland of the ewes until the implant is exhausted. The implants can release melatonin for up to 100 d (Forcada et al., 2002), therefore, probably, the poorer performances of the lambs of mothers that had been implanted > 30 d before lambing was due to the earlier absorption of the implant such that the beneficial effects of melatonin on milk production diminished earlier in lactation.

Conclusions

Melatonin treatment of pregnant ewes before lambing increased lamb performance until weaning and, in particular, the effects were observed in singleton male lambs, who had the highest LW at birth and weaning, and the highest growth rate. These results, and our previous findings on the effect of melatonin treatment at the end of pregnancy, open new possibilities to optimize lamb performances during lactation. It remains to be elucidated what is the minimal interval between implantation in the pregnant ewes and parturition that maximizes the growth of the lambs during lactation.

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