

# Exercising sheep as a preclinical model for musculoskeletal research

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## OBJECTIVE

To establish an orthopedic, preclinical, ovine model of controlled exercise using an equine walker.

## ANIMALS

20 Dorset-Polypay sheep.

## PROCEDURES

Sheep underwent 11 weeks of exercise, 4 days per week. Exercise duration and intensity increased until sheep performed 25 minutes at 1.3 m/s and 5 min at 2.0 m/s. Physical/lameness examinations were conducted every 14 days. Blood was collected every 28 days for analysis of serum bone biomarkers (SBB): bone alkaline phosphatase (BALP), procollagen type I amino-terminal propeptide (PINP), carboxy-telopeptide of type I collagen cross-links (CTX-I), tartrate-resistant acid phosphatase 5b (TRAP5b), and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL).

## RESULTS

Sheep adapted easily to group exercise. Animals grew taller ( $P = .006$ ) but had a 4% weight loss ( $P = .003$ ). RANKL was reduced on days 28 and 84 compared to day 56 ( $P < .05$ ), CTX-1 was reduced on days 28 and 84 compared to days 0 and 56 ( $P < .05$ ), and TRAP5b was greater on day 28 compared to day 0 ( $P = .009$ ). BALP and PINP did not change.

## CLINICAL RELEVANCE

The described preclinical model of exercising sheep has distinct advantages including ease of handling, an established lameness scale, commercially available ovine SBB assays, and the ability to alter footing characteristics and complete circular exercise. Decreasing CTX-I and RANKL with no change in BALP and PINP suggests reduced bone resorption over the study period. Future studies may include a sedentary group or utilize adult animals to alleviate any influence of growth on SBB.

**Keywords:** preclinical model, sheep, large animal model, orthopedic model, grouped exercise.

**P**reclinical models of orthopedic disease range from small animal models (eg, mouse, rat, rabbit) to larger animal models (eg, dog, sheep, horse). Exercise is a critical component of many musculoskeletal disease processes, making exercise models particularly important. By controlling exercise, musculoskeletal disease processes and therapeutic interventions can be evaluated in a more clinically relevant and precise way.

Ovine and caprine models of musculoskeletal disease are common<sup>1-3</sup> and offer distinct

advantages. Previous studies have utilized sheep as a model for exercising horses<sup>4</sup> and to determine the effects of musculoskeletal interventions such as bisphosphonates.<sup>5</sup> Despite anatomical differences between horses and sheep (eg, hooves, body size, digestive system), sheep exhibit similar kinetic and kinematic characteristics to horses when scaled for size.<sup>6</sup> Additionally, during walking, sheep have a similar percentage distribution of body weight on their limbs as horses.<sup>6</sup> This makes sheep a valuable orthopedic animal model for studying musculoskeletal diseases in horses. Moreover, sheep and goats may be acquired from well-managed breeding sources (eg, university farms) with relatively uniform genetics and are easy to handle. Nevertheless,

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ovine exercise models have remained time consuming, requiring several weeks of individual treadmill training and acclimation.<sup>7-9</sup> In addition, sheep may refuse to exercise on a treadmill.<sup>7-9</sup> Moreover, treadmill exercise may result in a different gait pattern than in overground outdoor conditions.<sup>10,11</sup> This may affect results, especially in studies assessing musculoskeletal injury and therapeutics. Both the animal model as well as the method of exercise are important factors to consider when performing musculoskeletal research.

Given the limitations of existing ovine exercise models, this study sought to assess the feasibility of exercising a group of juvenile sheep using an equine walker in an exercise program of increasing duration and intensity over 84 days. Outcome measures included physical examinations, lameness evaluations, and serum bone biomarkers. The study hypothesized that sheep would complete an incremental exercise protocol with limited evidence of morbidity on physical or lameness examinations and serum bone markers would reflect an overall increase in bone mineral content as a result of exercise.

## Methods

### Animals and management

The animal use experimental protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (202000264). All sheep were obtained from an established flock at the MSU Sheep Teaching and Research Center (Lansing, MI).

Ten castrated male (wethers, 81 ± 5 kg) and 10 female (ewes, 67 ± 5 kg) juvenile Dorset X Polypay sheep (254 ± 5 days of age) were used. Juvenile sheep were defined as an animal that had reached approximately 80% of their mature weight.<sup>12</sup> Sheep weight was recorded every 14 days, starting 20 weeks before the study began, with the goal of achieving 80% of their mature weight by the start of the study. Sheep were sheared 9 weeks before starting the exercise protocol. Two weeks before starting the study, animals were moved to the MSU Bennett Road Farm, where they were housed in an indoor pen of 21.6 m<sup>2</sup> with straw bedding, in similar housing conditions and managed by the same personnel as in their previous location. Sheep were fed a diet consisting of 90% dry matter total mixed ration (TMR) containing chopped hay (82%), a corn/soybean blend (16.5%), and a mineral blend (1.5%, Marvo Mineral Company). Based on the average daily amount of TMR provided (33 to 37 kg), and sheep's weights, animals consumed approximately 2.0% to 2.3% of their body weight (BW) in dry matter per day. The sheep had continuous access to water.

### Study design

Sheep were acclimated to the holding facility, sampling crate, and circular 20-m diameter walker

(Q-Line Horse Exerciser) over a 2-week period, between 07:00 and 10:00 h, before starting the study. Sheep were moved through the chute system twice a week to acclimate them to sample collections and physical examination. Thirty-two meters of fencing connected the outside walker to the interior pens so that sheep could be moved from the barn to the walker with minimal handling. The walker used in the study consisted of 4 bays, each separated by safety flex push panels equipped with electric shock (8.0 kV), and a 2NS sand surface with a depth of 5 cm on an 18-cm depth base of crushed asphalt. The exercise training commenced by setting the walker to a walking speed of 1.3 m/s. Under the guidance of one of the researchers, the 20 sheep were led to the walker with minimal encouragement, 4 days a week. During the initial acclimation period, each sheep walked clockwise and counterclockwise on alternating days for 5 minutes per day, respecting the designated divisions of each bay. The electric shock stimulus was employed to train the animals to remain in 1 bay of the walker at a time with no complications observed. Sheep predominantly positioned themselves in the middle and back of 1 bay.

### Exercise protocol

The study took place between June 7 and August 30, 2021. Sheep were subjected to an exercise protocol that was created based on previous treadmill studies.<sup>4,13</sup> The animals were exercised between 07:00 and 10:00 h under the supervision of a veterinarian (FBV). Sheep initially walked briskly for 10 min/day. The duration of exercise was increased by 5 min each week, until reaching a maximum of 30 min of exercise per day at which time a strenuous pace (2.0 m/s<sup>13</sup>; **Supplemental Video S1**) was included in the middle of the workout (**Table 1**). Sheep were exercised 4 times per week, alternating between exercising clockwise and counterclockwise each day.

**Table 1**—Exercise protocol and estimated distance traveled for juvenile sheep on a circular 20-m diameter walker.

Week	Exercise duration	Estimated distance traveled
1	10 min (WS)	780 m
2	15 min (WS)	1,170 m
3	20 min (WS)	1,560 m
4	25 min (WS)	1,950 m
5	30 min (WS)	2,340 m
6-8	13.75 min (WS) → 2.50 min (TS) → 13.75 min (WS)	2,445 m
9-11	12.50 min (WS) → 5 min (TS) → 12.50 min (WS)	2,550 m

Sheep were exercised 4 times per week alternating between clockwise and counterclockwise directions each day. The TS bout was incorporated in the middle of the 30-minute exercise protocol, starting on week 6.

TS = Trotting speed at 2.0 m/s. WS = Walking speed at 1.3 m/s.

## Physical examinations and lameness evaluation

A physical examination was performed between 07:00 and 09:00 h on day 0 and every 14 days for the duration of the study before exercise. The height, BW, resting heart rate (HR), respiratory rate (RR), and rectal temperature (RT) of the animals were recorded during the physical examinations, before exercising on that day. All physical examinations were conducted with minimal handling and minimal restraint using the indoor chute system and crate scale previously described during the acclimation period to reduce stress. BW was measured using a manual weigh crate (Prattley), height was measured from the ground to the withers with a ruler; HR and RR measurements were assessed via auscultation, positioning a stethoscope posterior to the animal's elbow on the left thoracic wall for the HR and over the trachea for the RR; and RT was measured using a digital thermometer. Animals were observed by a veterinarian (FBV) as they walked to the walker, during the entire exercise period, and following exercise as they walked back to the holding pens. In addition, every 2 weeks for the duration of the study, all sheep were graded for lameness by 2 veterinarians (ACC and FBV) using a sheep subjective lameness scoring system.<sup>14</sup> Each veterinarian assigned each sheep a score from 0 to 6. A score of 0 indicated no lameness, a 1 indicated an irregular posture without stride shortening, and a 2 was assigned to a noticeable head nod with a shortened stride. A score of 3 was assigned to an animal that displayed significant discomfort while moving indicated by excessive head flicking and stride shortening, a 4 indicated a reluctance to bear weight during movement, a 5 indicated inability to stand up and reluctance to move, and a 6 indicated an animal was unable to stand or move.<sup>14</sup>

## Blood collection

On days 0, 28, 56, and 84, 20-mL blood samples were collected from each sheep through jugular venipuncture using nonheparinized serum-separator blood between 7:00 and 9:00 h. Blood was stored on ice and allowed to coagulate for 1 hour before centrifugation at 2,000 X *g* for 15 min. Serum was aliquoted into microcentrifuge tubes and frozen at -80 °C for 8 to 12 months until analysis.

## Serum analysis

Serum samples were thawed before testing. Ovine-specific enzyme-linked immunoassays (Kendall Scientific) were performed following the manufacturer's recommendations for serum concentrations of amino-terminal bone-specific alkaline phosphatase (BALP), procollagen type I amino-terminal propeptide (PINP), receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), cross-linked C-terminal telopeptides of type I collagen (CTX-I), and tartrate-resistant acid phosphatase isoenzyme 5b (TRAP5b) with a SpectraMax ABS Microplate Reader (Molecular Devices, LLC) at 450 nm (**Table 2**).

**Table 2**—Summary of ovine-specific serum bone biomarkers and functions.<sup>15</sup>

Biomarker	Abbreviation	Function <sup>15</sup>
Bone-specific alkaline phosphatase	BALP	Bone formation and mineralization
Procollagen type I amino-terminal propeptide	PINP	Bone formation and mineralization
Receptor activator of nuclear factor- $\kappa$ B ligand	RANKL	Bone resorption; osteoclast activity
Tartrate-resistant acid phosphatase isoenzyme 5b	TRAP5b	Bone resorption; osteoclast activity
Carboxy-telopeptide of type I collagen cross-links	CTX-I	Bone resorption

## Statistical analysis

Sheep physical parameters and serum bone biomarkers were analyzed using a mixed-effects model that included the fixed effects of time, sex, and the interaction between time and sex, as well as repeated measures of time with individual sheep as the subject effect. The mixed-effects model was applied using the MIXED procedure of SAS 9.4. The normality of data was assessed by diagnostic plots of residuals for each independent variable. All data, except for serum biomarkers and lameness evaluations, were normally distributed. Serum bone biomarker data were log transformed and followed a normal distribution after transformation. Height and BW were included as potential covariates in the model, but no significant correlations were detected between them and the independent variables. Therefore, they were removed from the final model. Post hoc multiple comparisons of least-squares mean between different levels of sex and weeks were conducted using the Tukey test. Results are reported as means  $\pm$  SD.

Lameness evaluations were analyzed as ordinal data. Each sheep had 2 sets of data, 1 from each veterinarian, and the lameness score for each individual was averaged and rounded to the nearest integer. Due to the low prevalence of lameness with only 6 out of 120 evaluations scoring 1 or 2, the data were transformed into binary format: 0 represented the absence of lameness and 1 represented the presence of lameness (average score of 1 or 2). A logistic regression model was applied to the binary data using RStudio v.2022.07.2. Post hoc comparisons of the estimated marginal means between weeks were conducted using the Tukey method and the R package 'emmeans.' The significance level was set at  $P \leq .05$  for all analyses.

## Results

### Physical examinations and lameness evaluations

All sheep were able to use the walker without any issues and all individuals completed the exercise protocol as outlined with no alterations. Interactions

**Table 3**—Initial (d 0) and final (d 84) body weight (BW) and height (H) of 20 juvenile sheep.

Sex	BW (d 0)	BW (d 84)	P value
Wethers (kg)	81 ± 5	78 ± 5	.34 <sup>†</sup>
Ewes (kg)	67 ± 5	64 ± 6	
Average BW (kg)	74 ± 9	71 ± 9	< .001 <sup>‡</sup>
Sex	H (d 0)	H (d 84)	P value
Wethers (cm)	69 ± 3	73 ± 3	.44 <sup>†</sup>
Ewes (cm)	67 ± 3	69 ± 3	
Average H (cm)	68 ± 3	71 ± 3	< .001 <sup>‡</sup>

Values are expressed as mean ± SD.

<sup>†</sup>P value represents the interaction between time and sex.

<sup>‡</sup>P value represents the fixed effect of time.

**Table 4**—Physical parameters in 20 juvenile sheep evaluated every 14 days for 84 days.

Day	HR (BPM)	RR (bpm)	RT (°C)
0	138 ± 27 <sup>a</sup>	68 ± 10 <sup>a,b,c</sup>	39.2 ± 0.5 <sup>a,b</sup>
14	136 ± 16 <sup>a</sup>	61 ± 12 <sup>b,c</sup>	39.3 ± 0.4 <sup>a</sup>
28	98 ± 11 <sup>c,d</sup>	58 ± 11 <sup>c</sup>	39.4 ± 0.3 <sup>a</sup>
42	112 ± 15 <sup>b,c</sup>	73 ± 10 <sup>a</sup>	39.0 ± 0.3 <sup>b,c</sup>
56	89 ± 15 <sup>d</sup>	68 ± 7 <sup>a,b,c</sup>	39.0 ± 0.1 <sup>b,c</sup>
70	114 ± 13 <sup>b</sup>	70 ± 12 <sup>a,b</sup>	38.7 ± 0.3 <sup>c</sup>
84	83 ± 10 <sup>d</sup>	76 ± 15 <sup>a</sup>	39.1 ± 0.4 <sup>a,b</sup>
Reference values	65–80 <sup>16</sup>	20–38 <sup>17</sup>	38.3–39.9 <sup>18</sup>

Values are expressed as mean ± SD.

HR = Resting heart rate in beats per minute (BPM). RR = Respiratory rate in breaths per minute (bpm). RT = Rectal temperature in Celsius (°C).

<sup>a–d</sup>Values with different superscripts are significantly different by the Tukey test ( $P \leq .05$ ).

**Table 5**—The number of animals with and without lameness at each time point based on a subjective grading scale.<sup>14</sup>

Day	n	Frequency	
		Animals without lameness	Animals with lameness
0	20	20	0
16	20	19	1
30	20	18	2
44	20	18	2
58	20	20	0
80 <sup>1</sup>	20	19	1
Total observations	120	114	6

<sup>1</sup>Lameness evaluation was assessed on the last day of exercise.

**Table 6**—Blood serum bone biomarkers in 20 juvenile sheep evaluated every 4 weeks for 84 days.

Day	Bone formation markers		Bone resorption markers		
	BALP (ng/mL)	PINP (ng/mL)	RANKL (ng/mL)	CTX-I (ng/mL)	TRAP5b (ng/mL)
0	7.7 ± 4.3	1.7 ± 1.3	31.8 ± 11.9 <sup>a,b</sup>	9.8 ± 2.9 <sup>a</sup>	22.4 ± 14.1 <sup>b</sup>
28	9.1 ± 6.4	1.9 ± 1.2	25.9 ± 9.4 <sup>b</sup>	7.7 ± 2.0 <sup>b</sup>	30.7 ± 28.5 <sup>a</sup>
56	10.7 ± 9.6	— <sup>1</sup>	32.6 ± 7.0 <sup>a</sup>	9.5 ± 2.5 <sup>a</sup>	30.4 ± 31.7 <sup>a,b</sup>
84	8.0 ± 5.2	1.6 ± 1.0	24.8 ± 11.9 <sup>b</sup>	7.3 ± 1.6 <sup>b</sup>	27.3 ± 31.8 <sup>a,b</sup>

Values are expressed as mean ± SD.

See Table 2 for remainder of key.

<sup>a,b</sup>Values with different superscripts are significantly different by the Tukey test ( $P \leq .05$ ).

<sup>1</sup>Day 56 PINP data were excluded from the analysis due to laboratory error and lack of sufficient serum samples.

between sex and time were only detected for height and BW. Thus, males and females were grouped for the analysis of HR, RR, RT, lameness evaluation, and serum bone biomarkers. No significant differences were found between the initial and final BW of males or females, and this similarity was kept until the end of the study. However, the overall average BW for both sexes decreased over time ( $P < .001$ ) (**Table 3**). No significant differences were found in the initial and final height of males or females. However, the average height for both sexes increased over time ( $P < .001$ ). At the beginning of the study, males and females had no differences in height and this similarity was kept until the end of the study.

Resting HR (measured in beats per minute) was higher on days 0 and 14 in comparison to days 28, 42, 56, 70, and 84 ( $P < .001$ ). Resting RR was lower on day 28 in comparison to days 42, 70, and 84 ( $P \leq .2$ ). RT was mildly but significantly decreased ( $P < .001$ ) on day 70 when compared to days 0, 14, 28, and 84 (**Table 4**).

Veterinarians reported lameness in only 5 animals throughout the study, and all lamenesses were considered a grade 2 or below. Only 1 animal was observed to be lame twice during the study. The prevalence of lameness on different study days did not change throughout the study (**Table 5**). Due to the mild, transient lameness no animals were withdrawn from the study.

### Serum bone biomarkers

ELISA kits were validated; all kits showed percentage recoveries between 80% and 120% as previously described.<sup>19</sup> PINP data was not included in the analysis for day 56 due to laboratory error and the lack of additional serum samples. No differences were detected in bone formation and mineralization markers (BALP or PINP) throughout the study. Bone resorption markers, RANK-L, TRAP5b, and CTX-1 varied throughout the study. TRAP5b was increased on day 28 (30.7 ± 28.5 ng/mL) compared to day 0 (22.4 ± 14.1 ng/mL,  $P = .009$ ). In contrast, CTX-I was higher ( $P \leq .05$ ) on days 0 (9.8 ± 2.9 ng/mL) and 56 (9.5 ± 2.5 ng/mL) compared to days 28 (7.7 ± 2.0 ng/mL) and 84 (7.3 ± 1.6 ng/mL). Furthermore, RANKL was increased on day 56 (32.6 ± 7.0 ng/mL) compared to days 28 (25.9 ± 9.4 ng/mL;  $P = .04$ ) and 84 (24.8 ± 11.9 ng/mL;  $P = .007$ ; **Table 6**).

## Discussion

Multiple large animal models are available for the investigation of musculoskeletal disease and therapeutic interventions.<sup>1-4,20-23</sup> Previous ovine models have incorporated treadmill exercise,<sup>7-9</sup> which is labor intensive, may not reflect normal movement and can only be performed in a straight line on a specific surface. This study aimed to evaluate the feasibility of an ovine group exercise model utilizing a commercially available walker system. Specifically, the study aimed to evaluate the animals' ability to be trained as a group using a walker and incremental distance and speed increases. Outcome measures included physical parameters, lameness, and serum bone biomarkers.

After a 2-week adaptation period, the juvenile sheep easily learned their routine, facilitating ease of handling during the study period. Each exercise session required approximately a total of 5 minutes for guiding the animals to the walker and back to their pen, and only 1 person was needed to handle all animals. This contrasts with previous exercise studies<sup>7-9</sup> involving sheep, where the animals were individually trained to use a treadmill for several weeks, requiring a significant amount of time, and the potential that animals may refuse to cooperate with the exercise protocol. No sheep refused the exercise protocol in the present study and no traumatic injuries were observed due to the use of the exerciser. Group exercise has many advantages over individual treadmill exercise. Sheep can be highly stressed by isolation.<sup>24</sup> Some researchers have attempted to mitigate this stress by exercising the animals in pairs<sup>25</sup> or in the company of other sheep next to the treadmill.<sup>26</sup> In contrast, our method of exercise utilizes the flock dynamic to facilitate exercise and minimize handling stress.

There are multiple advantages to using sheep for musculoskeletal research; sheep tend to be a more uniform population than other large research animals like horses. In our study, the 20 sheep were of relatively similar size according to their sex (Table 3) and close in age ( $254 \pm 5$  days of age). This is especially important when studying younger populations as juveniles undergo both bone modeling and remodeling. Bone modeling involves the shaping and growth of bone during development and in response to mechanical forces, while bone remodeling is the coordinated process of resorption and formation by osteoclasts and osteoblasts, respectively, allowing them to adapt to changing mechanical loads and repair damage throughout life.<sup>27</sup> Understanding bone modeling and remodeling is essential for the investigation of pharmacological and/or surgical interventions in juvenile animals, as these interventions may have different outcomes in juveniles than in adults. Additionally, the availability of both male and female sheep is important in preclinical and translational research because it allows for the study of sex-specific differences and responses to interventions. This is particularly relevant to current public funding entity requirements such as NIH, which

encourage the inclusion of both sexes in preclinical research.<sup>28</sup> In contrast, when using other large animal models such as dairy calves, male animals may be easier to acquire.<sup>29</sup> In summary, the use of sheep has multiple noteworthy advantages including the relative genetic and physical uniformity, the availability of both sexes, and the willingness to exercise in large groups.

The use of a walker as a mechanism for exercising sheep offers multiple advantages. Circular exercise is a common training method for species like horses, which may be ridden or worked on the ground in round pens or by lunging.<sup>27</sup> The walker allows researchers to assess the effects of circular exercise at different speeds and different diameters. Previous studies<sup>29</sup> have used calves as an animal model for horses to investigate the effects of circular exercise. However, calves are resistant to exercising for greater than 30 minutes, requiring constant verbal encouragement to move, and sustain lower speeds (1.1 to 1.5 m/s).<sup>29</sup> Sheep in this study were able to easily sustain 30 min of exercise and reach faster speeds (2.0 m/s) with minimal morbidity including only mild, transient lameness in a few animals. No sheep was observed to experience significant discomfort due to lameness (grade 3). Further increases in speed and duration of exercise are possible in this model, making it potentially suitable to investigate more challenging exercise protocols.

The walker may result in a more natural gait than treadmill exercise. Prior research in horses and humans suggests treadmill exercise significantly alters gait,<sup>30,31</sup> including stride length and stance duration.<sup>11</sup> Furthermore, cases where animals are required to be tethered with a halter to the treadmill may result in significant alterations in gait, leading to differences in bone characteristics between left and right limbs, including cortical area and fracture force.<sup>29</sup> In contrast, using a walker enables natural gait and bidirectional exercise, which may prevent significant confounding factors when studying bone characteristics in musculoskeletal research. The walker allows for variability in gait among individual animals. This was observed especially at 2.0 m/s, where individual animals varied their gait between a fast walk, a trot, or a gait similar to a horse's canter (Supplemental Video S1). Additionally, the walker exposes the animals to a footing experience that can be similar to that of common overground surfaces and provides the possibility of testing different overground surface materials. No substantial lameness was observed during the study period, despite exercising 4 days per week. The maximum lameness score observed was "2" on a scale from "0" to "6."<sup>14</sup> In total, 5 animals were identified as having mild lameness during the 84-day period, suggesting that the exercise protocol did not cause substantial lameness in the sheep.<sup>32</sup> All lamenesses were transient. Only 1 individual showed lameness in 2 consecutive lameness examinations.

Although significant lameness was not observed, sheep lost approximately 4% of their BW over the course of the study, and this may have been due

to a combination of factors, including exercise and heat stress. According to a previous study<sup>33</sup> using Merino sheep, the exercise group (1 hour of exercise per day at speeds up to 2.5 m/s) had a lower carcass weight (0.5 to 0.9 kg) than the sedentary group, suggesting that the lower weight gain was attributed to their exercise protocol. Weather may have also been a contributing factor in weight loss.<sup>18</sup> Average maximum temperatures ranged from 25.6 °C (19.6 to 32.7 °C) in June to 28.8 °C (22.7 to 32.1 °C) in August.<sup>34</sup> Inclusion of air humidity would have provided a more accurate assessment of the risk of heat stress in the exercised sheep. Feed consumption and weight gain are negatively impacted by heat stress.<sup>18</sup> The animals were exercised in the morning, and their pen was located indoors to mitigate heat stress. However, the increase in average maximum temperatures, as well as exercise, may have played a role in the sheep's weight loss during the study. Although the mean body weight of females was lower than males throughout the study, the time by sex interaction of weight was nonsignificant. The lack of difference was likely due to the small sample size of 10 animals per sex. A post hoc power analysis comparing the weight means and SD by sex (10 animals per sex) results in a power of 0.24 and 0.21 for males and females, respectively. Despite a 4% loss in BW, sheep were able to complete the exercise protocol during the warmer days, without the presence of other comorbidities detected during the physical examinations and daily monitoring.

Physical examination parameters may also reflect environmental factors and exercise acclimation throughout the study. Resting HR decreased from baseline (day 0), which may indicate a modest improvement in fitness. However, HR and RR remained above the normal resting range for sheep.<sup>16,17</sup> This can likely be attributed to the stress of handling<sup>35</sup> coupled with environmental factors.<sup>18</sup> Future research could use telemetric monitoring methods<sup>36</sup> to accurately measure resting HR without stressing animals and provide a better real-time measurement of HR response to handling and exercise,<sup>37</sup> allowing researchers to adjust the exercise protocol intensity and better assess the response to exercise.

In addition to physical measurements, serum markers of bone metabolism can provide a minimally invasive and specific way to assess the dynamic changes in bone metabolism (bone formation and resorption).<sup>15</sup> Decreases in bone resorption markers (CTX-I and RANKL) were observed on days 28 and 84 in our study. CTX-I is indicative of osteoclast activity,<sup>38</sup> while RANKL is the ligand that induces maturation of osteoclasts.<sup>39</sup> The reduction of CTX-I would indicate an overall reduction in bone resorption. However, a contradictory increase in TRAP5b was observed on day 28. TRAP5b is a marker for osteoclast numbers<sup>38</sup> and has been associated with increased bone resorption in some conditions.<sup>40</sup> TRAP5b is released from both mature and immature/prefusion osteoclasts<sup>38</sup> and may reflect overall osteoclast numbers instead of an active resorption process underway. To further understand the seemingly

contradictory findings, future studies could include cathepsin K, which is only produced in mature resorbing osteoclasts.<sup>38</sup> In contrast to our findings, CTX-1 has been found to increase in foals<sup>41</sup> or remain the same in yearlings<sup>42</sup> under exercise; however, our animals were considerably more mature entering the study period at 80% of their adult body weight. Bone formation markers (BALP and PINP) did not change during the present study. This is consistent with previous studies where no changes in bone formation markers were found when juvenile horses were exercised 3 days per week.<sup>42,43</sup> Bone serum markers may also reflect growth-related changes.<sup>44</sup> Sheep were approximately 254 ± 5 days of age (8.5 months), and animals grew taller. Future studies should include an aged-matched, nonexercising control group to determine the effect of exercise versus growth.

Whenever utilizing an animal model, differences between species must be recognized and results interpreted within the limitations of the model. Sheep have been widely used in orthopedic research,<sup>1-3</sup> despite their clear physiological and anatomical differences from humans and other species. When comparing sheep and horses, there are noticeable differences in their anatomy and physiology. Horses are perissodactyls with 1 toe per hoof, while sheep are artiodactyls with 2 cloven toes per hoof.<sup>45</sup> These anatomical differences can make it challenging to study diseases involving structures distal to the metacarpo/metatarsophalangeal (fetlock) joints. However, sheep may be useful for addressing pathologies originating proximal to the fetlock joint or systemic responses to intravenous or intramuscular drugs like bisphosphonates. Bone formation is consistently favored in response to mechanical strain across various animal species<sup>27</sup> including horses,<sup>46</sup> calves,<sup>47</sup> gilts,<sup>48</sup> and even roosters.<sup>49</sup> Furthermore, sheep and horses are quadrupeds with comparable kinetics and kinematics when adjusted for body size.<sup>6</sup> That stated, all results must be interpreted while recognizing the limitations associated with using sheep to model horses. An additional limitation of the study was infrequent sampling for serum bone biomarker analysis. Serum bone biomarkers were measured at 4 different time points during the study. It is well established that serum bone biomarkers can respond differently to acute and chronic exercise<sup>50</sup>; thus, more frequent sampling, particularly following physical activity, may have provided a more accurate representation of the acute effects of physical activity.<sup>50</sup> Furthermore, the study lacks complementary measurements of bone structural changes, such as bone microarchitecture imaging analysis, which could have provided further insight into the relationship between serum bone biomarker responses and bone structural changes during the study period.

In summary, this study shows that using a walker to exercise juvenile sheep in groups can be an effective and practical way of studying different orthopedic interventions without causing significant morbidity. The sheep readily acclimated to the walker. The animals were easily trained, and each exercise session required only 1 person to handle

all animals. No animals in this study refused to exercise. Additionally, exercising the sheep in groups mitigated the stress and welfare concerns caused by individual training on a treadmill, providing a more natural footing surface than treadmills. Despite the observed mild weight loss, the sheep were able to exercise without significant comorbidities. Further investigation using this translational model should include a sedentary group and focus on evaluating additional exercise protocols. Overall, this ovine, exercising model has multiple advantages including ease of handling, availability of serum biomarkers, and the ability to test multiple exercise characteristics including duration and intensity in an efficient and practical manner.

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## Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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## Supplementary Materials

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