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EXERCISE PHYSIOLOGY

Effects of aquatic conditioning on cartilage and bone metabolism in young horses

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Abstract

While beneficial in rehabilitation, aquatic exercise effects on cartilage and bone metabolism in young, healthy horses has not been well described. Therefore, 30 Quarter Horse yearlings (343 ± 28 kg; 496 ± 12 d of age) were stratified by age, body weight (**BW**), and sex and randomly assigned to 1 of 3 treatments for 140-d to evaluate effects of aquatic, dry, or no exercise on bone and cartilage metabolism in young horses transitioning to an advanced workload. Treatments included nonexercise control (CON; $n = 10$), dry treadmill (DRY; $n = 10$), or aquatic treadmill exercise (H2O; $n = 10$; water: 60% wither height, WH). Horses were housed individually (3.6 × 3.6 m) from 0600 to 1800 hours, allowed turnout (74 × 70 m) from 1800 to 0600 hours, and fed to meet or exceed requirements. During phase I (days 0 to 112), DRY and H2O walked on treadmills 30 min/d, 5 d/wk. Phase II (days 113 to 140) transitioned to an advanced workload 5 d/wk. Every 14-d, WH, hip height (**HH**), and BW were recorded. Left third metacarpal radiographs on days 0, 112, and 140 were analyzed for radiographic bone aluminum equivalence (**RBAE**). Every 28-d, serum samples were analyzed for osteocalcin and C-telopeptide crosslaps of type I collagen (**CTX-1**), and synovial fluid samples were analyzed for prostaglandin E_2, collagenase cleavage neopeptide (C2C), collagenase of type I and type II collagen, and carboxypeptide of type II collagen using ELISAs. All data were analyzed using PROC MIXED of SAS, including random effect of horse within treatment, and repeated effect of day. Baseline treatment differences were accounted for using a covariate. There were treatment × day interactions ($P < 0.01$) where OC and CTX-1 remained consistent in both exercise groups while inconsistently increasing in CON. There were no treatment differences ($P > 0.30$) in RBAE, BW, or HH, but all increased over time ($P < 0.01$). There were no treatment × day interactions of synovial inflammation or markers of cartilage metabolism; however, there was an effect of day for each marker ($P<0.03$). Changes in biomarkers of cartilage turnover in horses exercised at the walk, whether dry or aquatic, could not be distinguished from horses with access to turnout alone. This study indicates that early forced exercise supports consistent bone metabolism necessary for uniform growth and bone development, and that there are no negative effects of buoyancy on cartilage metabolism in yearlings transitioned from aquatic exercise to a 28-d advanced workload.

Key words: aquatic conditioning, bone, cartilage, equine, exercise

Abbreviations

BW	body weight
C1,2C	collagenase of type I and type II collagen
C2C	collagenase cleavage neopeptide
CPII	carboxypeptide of type II collagen
CTX-1	C-telopeptide crosslaps of type I collagen
HH	hip height
PGE_2	prostaglandin E_2
RBAE	radiographic bone aluminum equivalence
WH	wither height

Introduction

To achieve a level of competitiveness in a young animal's performance career, it is a common practice in the equine industry to enroll horses in an exercise-training program at a young age (12 to 18 mo). This period of growth is characterized by adaptation of both bone and soft tissue structures. Early career starts have been associated with career longevity, which is likely due to strengthening by early stimulation of the musculoskeletal system (Rogers et al., 2012). Cartilage adaptation early in life is essential for a well-structured articular cartilage collagen network, as cartilage remains fairly static once mature (Brama et al., 2000). In contrast, bone undergoes remodeling throughout the life of the animal but is only capable of modeling during early stages of life. During modeling, osteoclasts resorb bone from areas under minimal stress while osteoblasts regulate bone deposition in areas subjected to greater force, allowing for net bone formation (Langdahl et al., 2016). This period of growth may be used to maximize musculoskeletal adaptation and development, which may improve athletic career and decrease the incidence of musculoskeletal-related injury (Barneveld and van Weeren, 1999).

Recently, the equine industry has seen a rise in popularity of aquatic treading. Buoyancy in aquatic therapy lifts and reduces axial loading on articular joints by minimizing vertical ground forces (King et al., 2013). Mice exposed to partial weight bearing have shown a decrease in bone mineral density with a 20% reduction in weight bearing (Ellman et al., 2013). While decreased load on bones may be beneficial in rehabilitation cases to reduce stress on damaged bone, this technology is also being used to prepare young horses for sales and future performance. Currently, there is limited data available investigating the use of aquatic conditioning programs and the effect on bone and cartilage when transitioned into a high intensity exercise program on dry land.

Conditioning and training of performance horses involves many factors such as behavior modification, cardiovascular fitness and skeletal strength. Of these factors, it is most difficult to assess strength of the skeletal framework until a physical symptom is observed when an injury arises (Nielsen et al., 1997). Biomarkers in serum and synovial fluid have potential to detect subtle or early changes to tissues before clinical signs are evident (McIlwraith, 2005). Biomarkers relative to bone and cartilage turnover may provide valuable insight to the effect of exercise in the juvenile horse. Such metabolic biomarkers include: osteocalcin, a serum biochemical marker of bone formation; C-telopeptide crosslaps of type I collagen (**CTX-1**), a serum biochemical marker of bone degradation; synovial prostaglandin E_2 (**PGE_2**), a marker of joint inflammation; catabolic collagenase cleavage neopeptide (**C2C**) and collagenase of type I and type II collagen (**C1,2C**) indicative of cartilage degradation; and anabolic carboxypeptide of type II collagen (**CPII**) which increases in an effort to repair articular cartilage (LePage et al., 2001). Therefore, the objectives of this study were to determine the influence of early forced exercise and type of exercise on serum and synovial fluid biomarkers of young horses and to provide insight into the effects of conditioning on joint inflammation and cartilage/bone turnover. Furthermore, to investigate the effects of differing conditioning programs on joint inflammation, cartilage metabolism, and bone mineralization when transitioned from a moderate to an advanced workload on a dry surface.

Materials and Methods

All procedures and handling of horses were approved by the Texas A&M University Institutional Animal Care and Use Committee (2017-0376).

Horses and treatments

Thirty yearling Quarter horses (343 ± 28 kg; 496 ± 12 d of age; 15 geldings and 15 fillies) of similar breeding were stratified by body weight (**BW**), age, and sex and randomly assigned to 1 of 3 treatment groups during the 140-d trial (Table 1). Treatments consisted of nonexercised controls (**CON**; n = 10), treadmill-exercised horses (**DRY**; n = 10), and aquatic treadmill-exercised horses (**H2O**; n = 10). Prior to the start of the trial, radiographs of both radial carpal joints were performed by Texas A&M University Equine Field Services (College Station, TX). All horses determined to be radiologically normal were included in the study and fed 1.25% BW (as fed) commercial concentrate feed (SafeChoice Mare and Foal, Nutrena, Minneapolis, MN); intake was adjusted for BW every 14 d. Concentrate was offered in 2 equal feedings, individually at 0600 and 1800 hours, and all horses were allowed ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*). Diets were formulated to meet or slightly exceed nutrient requirements for young, exercising horses undergoing rapid growth (NRC, 2007). Throughout the trial, horses were housed in individual stalls (3.6 m × 3.6 m) and allowed turnout (74 m × 70 m) in dry-lot pens with access to coastal bermudagrass hay for ~10 hr/d to mimic housing conditions for young horses entering into training programs.

Exercise protocol

Nonexercised CON received no forced exercise for the duration of the study. Prior to day 0, exercise groups were familiarized (5 d) with each treadmill apparatus (HorseGym USA, Wellington, FL) until they were accustomed to loading onto and walking on the treadmill. The acclimation period included each H2O horse walking in and out of the treadmill, as well as walking into the unit, and adding and draining water in order to acclimate each horse to the sound of the unit (Adair, 2011). Exercise protocol for horses assigned to DRY and H2O was

Table 1. Average BW, average age, number of mares, and number of geldings for each treatment group at allocation on day 0

	BW, kg	Age, d	Number of mares	Number of geldings
CON	342	494	5	5
DRY	343	494	4	6
H2O	345	500	6	4

divided into 2 phases. Phase I (days 0 to 112) represented a long-term submaximal exercise program, where DRY and H2O groups were exercised 5 d/wk for 21 min at the walk, increasing 5 min/wk until a total time of 30 min was reached and maintained throughout the remainder of the study. Phase I began with horses walking 1.2 and 0.2 m/s was added every 28 d until the end of phase I to reach a maximum speed of 1.8 m/s. The water treadmill took 8 min to fill and 8 min to drain, and water level was maintained at 60% of wither height (**WH**) throughout treadmill exercise. Water temperature averaged 27 °C throughout the project to maximize comfort (Adair, 2011). The transition to phase II began on day 113 in which treadmill exercise ended and both DRY and H2O groups exercised in a circular, free stall exerciser (Priefert Manufacturing, Mt. Pleasant, TX) 5 d/wk to evaluate bone and cartilage responses to patterns of strain following their respective conditioning programs to completion of the study on day 140. During phase II (Table 2), a 30-min bout of exercise was divided equally in the clockwise and counterclockwise directions. During the first week of phase II, horses alternated between walking and trotting, and speed progressed each week of phase II until horses alternated between walking and extended cantering in the 4th week.

Sample collection

Growth measurements were recorded every 14 d and included BW, WH, and hip height (**HH**) prior to morning feeding. Measures, including HH and WH, were obtained by the same individual at every timepoint using an altitude stick (Sullivan Supply, Inc., Hillsboro, TX). Synovial fluid and serum samples were obtained every 28 d for the entire 140-d trial. Sampling occurred Fridays after all horses had completed exercise, which allowed 2 d to recover before exercise began again the following Monday. Samples were collected at the same time every sample day to account for potential diurnal variation. Blood was collected via jugular venipuncture into 10-mL additive-free sterile blood collection tubes (BD Vacutainer, Franklin Lakes, NJ). Blood samples remained at room temperature for ~1 hr prior to centrifugation at 2,000 × *g* at 10 °C for 20 min (ALC, PM140R, Thermo Fisher Sci., Waltham, MA) to harvest serum. Synovial fluid was collected via sterile arthrocentesis of the right radial carpal joint by a board-certified veterinary surgeon from the Texas A&M University Large Animal Clinic (College Station, TX). Horses were sedated using xylazine HCl, which was administered intravenously at recommended dosages. The carpal joint was aseptically aspirated from a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone, to a depth of ~12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996). Synovial fluid was transferred to 10-mL additive-free tubes, and immediately placed on ice. All samples were stored at –80 °C for later analysis. On days 0, 112, and 140, dorsal-palmar radiographs of the third metacarpal bone were performed by Texas A&M Equine Field Services for later analysis. Digital radiographs were obtained using a Minxray T90 generator and a VetRocket DR Processor. Images were taken at a focal distance of 71 cm and exposure of 70 kVp and 0.08 ms. Radiographs were standardized by using a cassette holder and taking each radiograph equidistant from the bone. An aluminum (Al) stepwedge penetrometer of 11 steps ranging from 5 to 35 mm in 3 mm increments was placed in the same plane as the bone.

Sample analysis

Synovial fluid samples were used to evaluate markers of joint inflammation, cartilage metabolism, and bone turnover. Synovial fluid collected throughout the trial was analyzed for PGE_2, C1,2C, C2C, and CPII using commercial ELISA kits previously validated for use in the horse (Bertone et al., 2001; de Grauw et al., 2006). Synovial fluid was plated directly for analysis of PGE_2 and C1,2C, and diluted 1:2 and 1:4, respectively, for C2C and CPII. Final concentrations of these markers were read using a microplate reader with optical density set at 450 nm (Bio-Rad 680 Microplate Reader, Bio-Rad Laboratories, Hercules, CA). Mean minimum detectable levels of PGE_2, C2C, C1,2C, and CPII were 30.9 pg/mL, 10 ng/mL, 50 ng/mL, and 0.03 µg/mL, respectively.

Serum concentrations of osteocalcin were determined using a commercial competitive immunoassay (MicroVue Osteocalcin; Quidel Corporation, San Diego, CA). Serum samples were diluted 1:5 and final concentrations were read using a microplate reader with optical density set at 405 nm. Mean minimum detectable level of osteocalcin was 0.45 ng/mL. Serum concentrations of CTX-1 were determined using an enzyme immunological test (Serum CrossLaps (CTX-1) ELISA; Immunodiagnostic Systems, Ltd., Gaithersburg, MD). Serum samples were plated directly, and final concentrations were read using a microplate reader with optical density set at 450 nm. Mean minimum detectable level of CTX-1 was 0.020 ng/mL. Intra-assay coefficients of variability (CVs) were established as acceptable at ≤15% for PGE_2, ≤12% for CTX-1, ≤10% for cartilage markers, and ≤7% for osteocalcin. Dorsal-palmar radiographs of the left third metacarpal were analyzed for radiographic bone aluminum equivalence (**RBAE**) using Quantity One (Bio-Rad Laboratories) as described by O'Connor-Robison and Nielsen (2013).

Statistical analysis

Data were analyzed using the PROC UNIVARIATE method of SAS (SAS Inst. Inc., Cary, NC) to determine normality. All non-normal data (PGE_2) were log transformed to establish normality. Subsequently, data were analyzed using the PROC MIXED method of SAS. The model contained effects for treatment, day, and

Table 2. Exercise protocol for DRY and H2O horses during phase II (days 112 to 140)

	Counterclockwise[2], min					Clockwise				
Week[1]	2 min	5 min	2 min	5 min	1 min	1 min	5 min	2 min	5 min	2 min
1	1.75	4.0	1.75	4.0	1.75	1.75	4.0	1.75	4.0	1.75
2	1.75	4.0	1.75	6.0	1.75	1.75	4.0	1.75	6.0	1.75
3	1.75	6.0	1.75	6.0	1.75	1.75	6.0	1.75	6.0	1.75
4	1.75	6.0	1.75	6.5	1.75	1.75	6.0	1.75	6.5	1.75

[1]Week of phase II exercise (days 112 to 140).
[2]Velocity of free-stall exerciser (Priefert Manufacturing, Mt. Pleasant, TX) in meter per second.

treatment × day interactions to determine the effects of various exercise treatments on cartilage and bone metabolism markers. The model contained a random effect of horse within treatment and a repeated effect of day. There were baseline treatment differences in CPII, osteocalcin, and CTX-1, therefore all cartilage and bone biomarkers were run with day 0 as a covariate to account for baseline treatment differences in biomarker concentrations and to maintain consistency in statistical model across all biomarkers. Significance was defined for all variables when $P \leq 0.05$, and a trend toward significance was established at $P \leq 0.10$.

Results

Growth parameters

There was no influence of exercise treatment on BW ($P > 0.67$; Figure 1). However, there was an influence of time ($P < 0.01$) as all horses, regardless of treatment, gained BW over the 140-d trial. WH tended to have a treatment × day interaction ($P < 0.08$; Figure 2) characterized by a difference in response in the first 28 d of the study and HH was not affected by treatment ($P > 0.12$; Figure 3), but all measurements increased over time ($P < 0.01$).

Markers of inflammation and cartilage metabolism

There were no treatment × day interactions on synovial inflammation and markers of cartilage metabolism ($P > 0.21$); however, there was an effect of day for each of the selected biomarkers ($P < 0.03$). Synovial PGE_2 concentrations were not influenced by treatment; nevertheless, concentrations in all treatment groups tended to be lower on day 56 ($P < 0.08$; Figure 4) when compared with other days throughout the project. Catabolic C2C decreased from days 28 to 56 ($P < 0.01$; Figure 5), then increased to completion of the study at day 140 ($P < 0.01$). Synovial fluid concentrations of CPII decreased from days 28 to 56 ($P < 0.01$; Figure 6) and increased at each time point from days 56 to 140 ($P < 0.05$). Synovial fluid C1,2C concentrations decreased from days 28 to 56 ($P = 0.01$; Figure 7) and plateaued throughout the remainder of phase I exercise, before increasing in response to phase II ($P < 0.01$). The ratio of CPII to C2C was investigated; however, there were no main effects of treatment, day, or treatment × day and therefore the results were not included in the study.

Biomarkers of bone metabolism

A treatment × day interaction was observed for osteocalcin and CTX-1 where concentrations remained consistent in both DRY and H2O treatment groups throughout the 140-d project compared with CON which increased in an incongruent fashion ($P < 0.01$; Figures 8 and 9). While a molecular response was observed through osteocalcin and CTX-1, RBAE analysis of radiographic images were not influenced by treatment ($P > 0.50$; Figures 10 and 11); although, there was an effect of day in which both medial and lateral aspects increased in bone optical density throughout the trial ($P < 0.01$). The ratio of osteocalcin to CTX-1 was investigated; however, there were no main effects of treatment or treatment × day and therefore the results were not included in the study.

Discussion

This study assessed the effects of aquatic and dry treadmill exercise on bone and joint metabolism in young horses. Growth measurements including WH, HH, and BW of the yearling horses increased throughout the 140-d study. This was expected as horses have not met their mature size at one year of age and were fed to meet or exceed requirements of growing horses on a rapid plane of growth, in light exercise (NRC, 2007).

Serum osteocalcin is a previously established marker of equine bone formation produced by osteoblasts, which are responsible for deposition of bone (Nielsen et al., 1998: Billinghurst et al., 2003). In the current study, osteocalcin concentrations remained consistent in both exercise groups throughout the study. However, the CON group had an incongruent increase in osteocalcin throughout the study. Serum CTX-1 has been previously identified as a reliable indicator of the effects of exercise on the developing skeletal system (McIlwraith, 2005). Concentrations of CTX-1 followed the same trend as osteocalcin, remaining consistent in DRY and H2O while increasing incongruently in CON. Together, these markers indicate that early forced exercise is beneficial for producing consistent bone turnover in young horses, while lack of forced exercise in young horses may lead to inconsistent bone turnover. An increased risk of fracture has been documented in humans during times of increased skeletal growth and increased

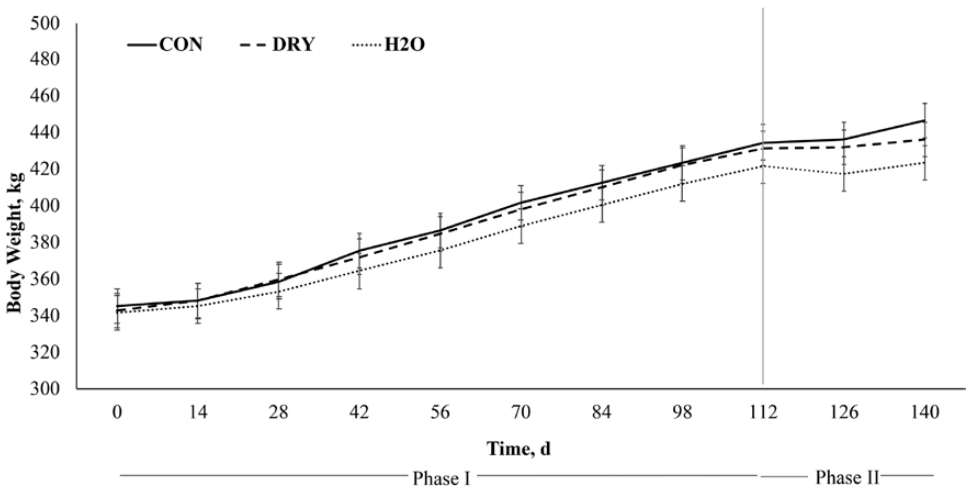

Figure 1. Mean BW in kg (least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (P = 0.67), day (P < 0.01), and treatment × day (P = 0.12).

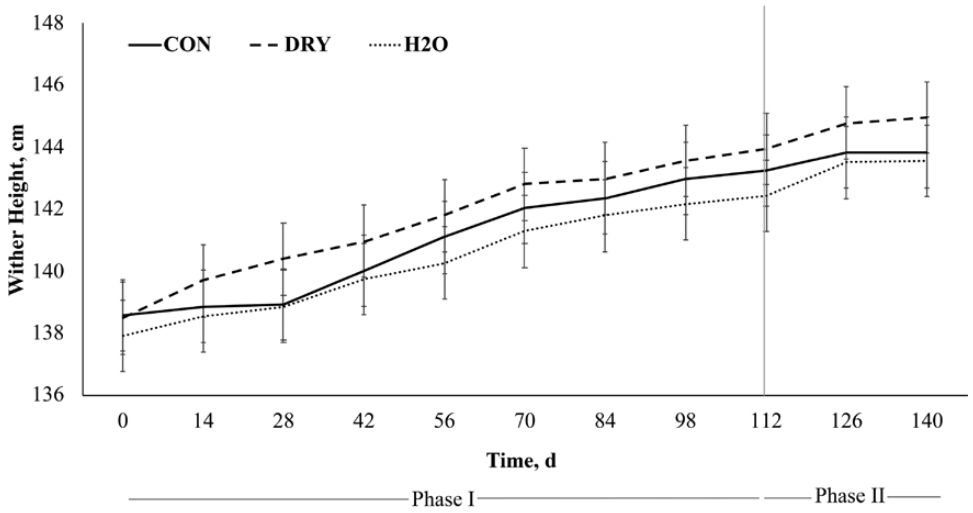

Figure 2. Mean WH (cm; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment ($P = 0.72$), day ($P < 0.01$), and treatment × day ($P = 0.07$).

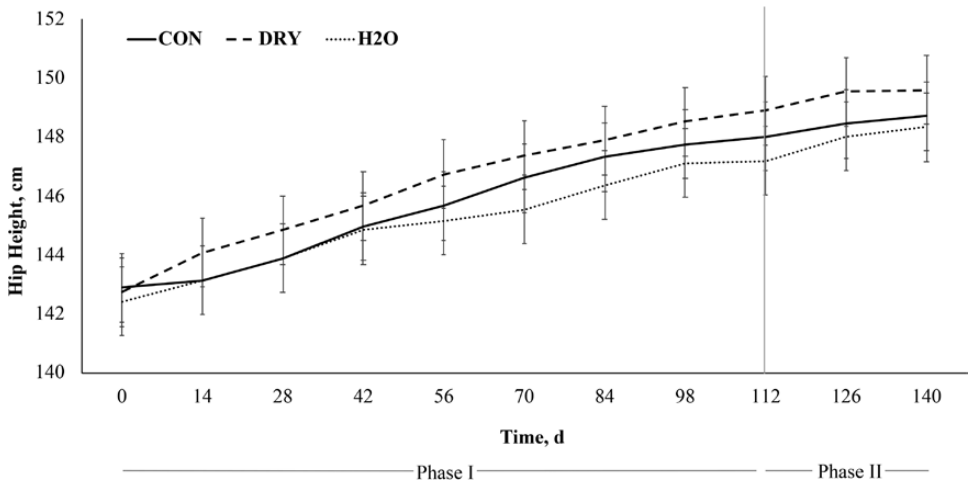

Figure 3. Mean HH (cm; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment ($P = 0.73$), day ($P < 0.01$), and treatment × day ($P = 0.12$).

bone turnover (Parfitt, 1994). Additionally, Lepeule et al. (2013) found that young horses with irregular exercise patterns had an increased risk of osteoarthritic status.

Metacarpal RBAE analysis is a non-invasive method to assess relative bone optical density in individual animals. No treatment or treatment × day effects were observed in the current study, but there was an effect of day. Both the medial and lateral RBAE increased in all horses throughout the study, which was to be expected as horses were still growing. These results agree with the study by Spooner et al. (2008) which reported that there was no significant change in RBAE of horses that underwent endurance training compared with pasture rearing. This indicates that walking alone may be insufficient to induce phenotypic changes to bone optical density in young horses in only 140 d, which may be attributed in part to the long bone turnover period (Frost, 1990).

The effect of time on cartilage inflammation and metabolism was driven by a decrease in PGE_2, C2C, C1,2C, and CPII concentrations from days 28 to 56. This decrease occurred concurrently with increase in bone formation as measured by osteocalcin. The subchondral region of bone and cartilage is related both physiologically and pathophysiologically, working together as one functional unit (Imhof et al., 1999); therefore, there may be a relationship between an increase in bone metabolism and a decrease in response of articular cartilage. However, in the current study, markers of cartilage inflammation and metabolism were measured in synovial fluid, while markers of bone metabolism were measured in serum which may represent systemic changes rather than changes in a specific region of bone.

Synovial fluid PGE_2 is well established as an early indicator of joint inflammation and predictor of joint disease (Bertone et al., 2001). However, a lack of change in synovial fluid PGE_2 concentrations in young horses undergoing strenuous exercise has been previously documented (Frisbie et al., 2008), and agrees with the present study, as there were no significant treatment or treatment × day effects on PGE_2 concentrations in synovial fluid. This indicates that the transition from a submaximal

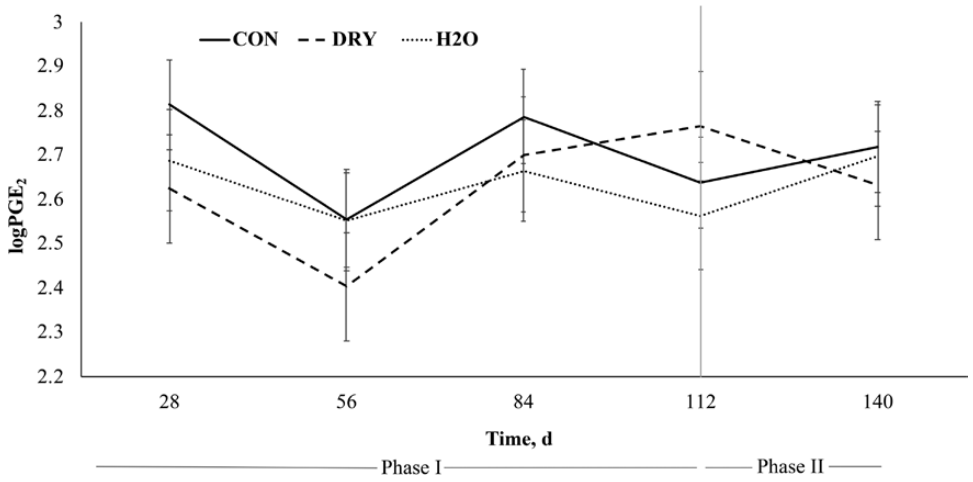

Figure 4. Log of synovial fluid PGE_2 concentrations (least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.69), day (*P* < 0.03), and treatment × day (*P* = 0.78).

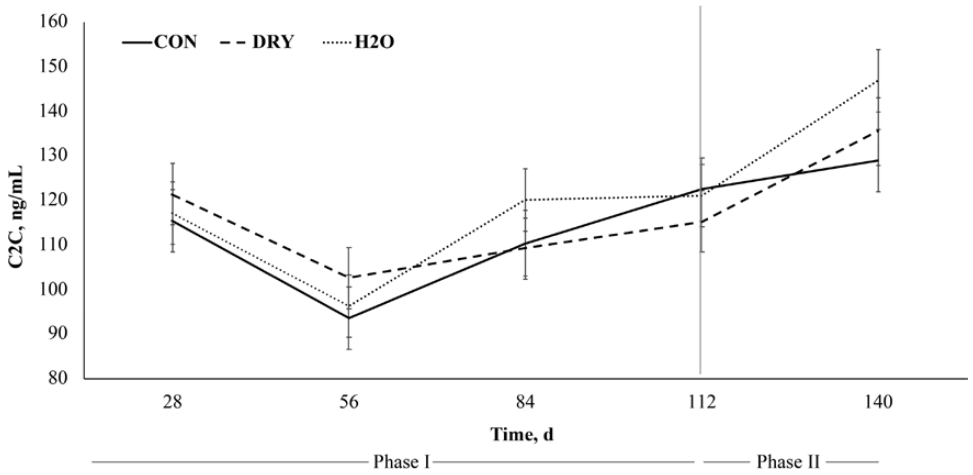

Figure 5. Synovial collagenase cleavage neopeptide (C2C) concentrations (ng/mL; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.65), day (*P* < 0.01), and treatment × day (*P* = 0.59).

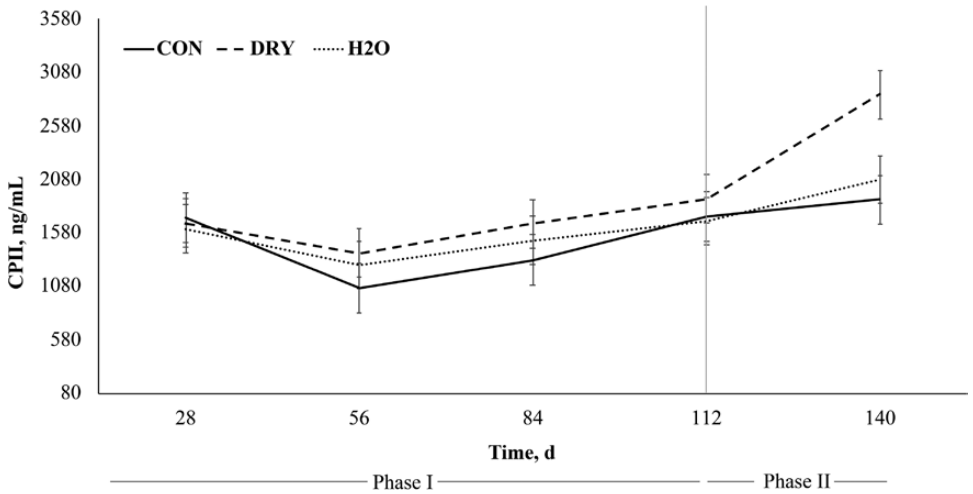

Figure 6. Synovial CPII concentrations (ng/mL; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.30), day (*P* < 0.01), and treatment × day (*P* = 0.21).

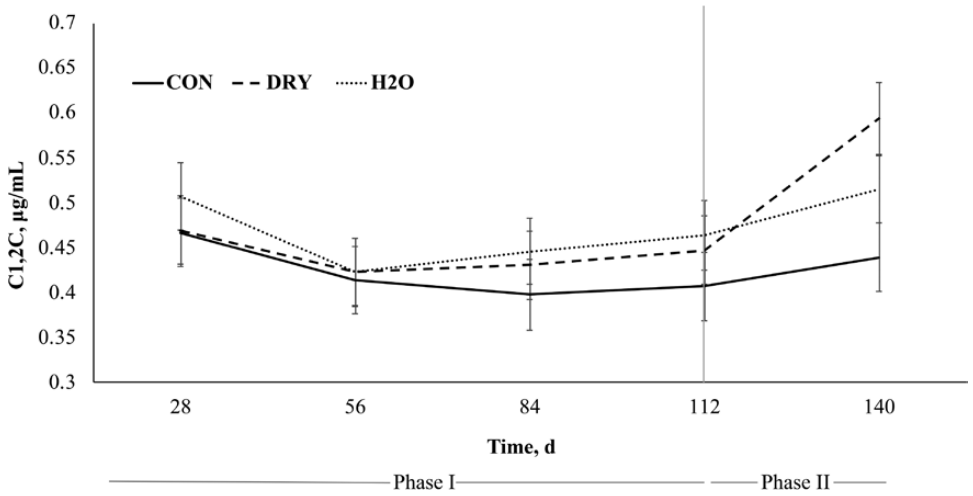

Figure 7. Synovial C1,2C concentrations (µg/mL; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.35), day (*P* < 0.01), and treatment × day (*P* = 0.45).

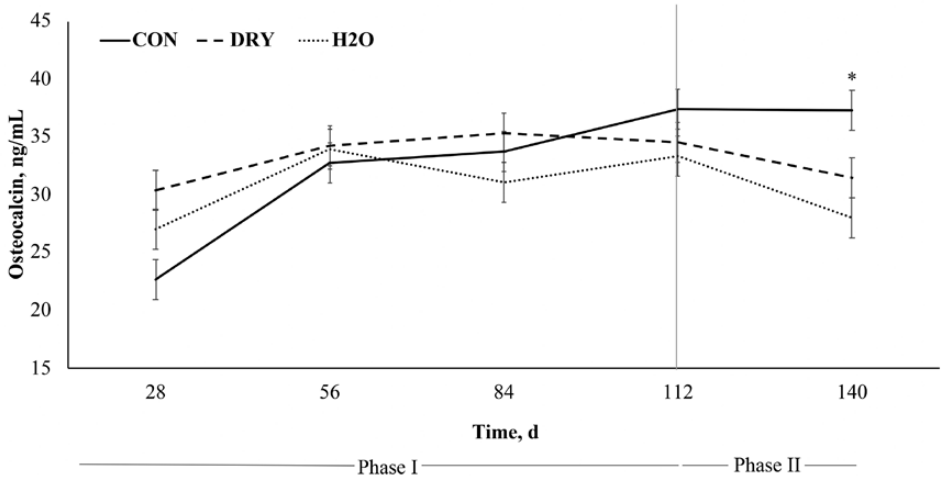

Figure 8. Serum osteocalcin concentrations (ng/mL; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.33), day (*P* < 0.01), and treatment × day (*P* < 0.01). *Within day, CON differs from DRY and H2O (*P* < 0.05). ǂWithin day, CON tends to differ from DRY and H2O (*P* < 0.10).

exercise program in phase I to a high-intensity exercise program in phase II does not cause excess inflammation or damage to articular cartilage, even for horses exposed to a reduced load due to buoyancy in an aquatic environment. Furthermore, this study utilized healthy yearling horses, so it was expected that they would not display any indicators of joint disease.

In regard to articular cartilage, C2C is an indicator of degradation of type II collagen. Following an insult, type II collagen unwinds, exposing epitopes including C2C, which are then released into synovial fluid where they may be detected as a biomarker of collagen metabolism. While there was an effect of time from days 56 to 140 where C2C concentrations increased in all groups, the highest values reported (136.95 ± 4.11 ng/mL) are still within range of normal yearling joints reported by Leatherwood et al. (2016)(211.80 ± 10.17 ng/mL). A more comprehensive indicator of overall cartilage degradation, C1,2C, takes into account both type I and type II cartilage degradation. In the current study, no effects of treatment or treatment × day were observed. Frisbie et al. (2008) describe an increase in C1,2C with exercise; however, their animals underwent a strenuous exercise program including trotting and galloping, unlike the submaximal exercise program of the present study, which would not have placed the same degree of force on joints.

Contrastingly, CPII is utilized as a marker of type II collagen formation. It is counter to C2C and is released when an attempt is made to repair type II collagen. There was no effect of treatment or treatment × day, similar to C2C, which was expected as there was no significant degradation to stimulate subsequent regeneration. In response to changes in C2C over time, CPII followed the same trend, decreasing from days 28 to 56, then increasing from day 56 to completion of the study. This demonstrates the relationship between cartilage formation and cartilage degradation indicative of cartilage turnover. It is important to note that horses of this age are still undergoing significant cartilage turnover, which decreases with

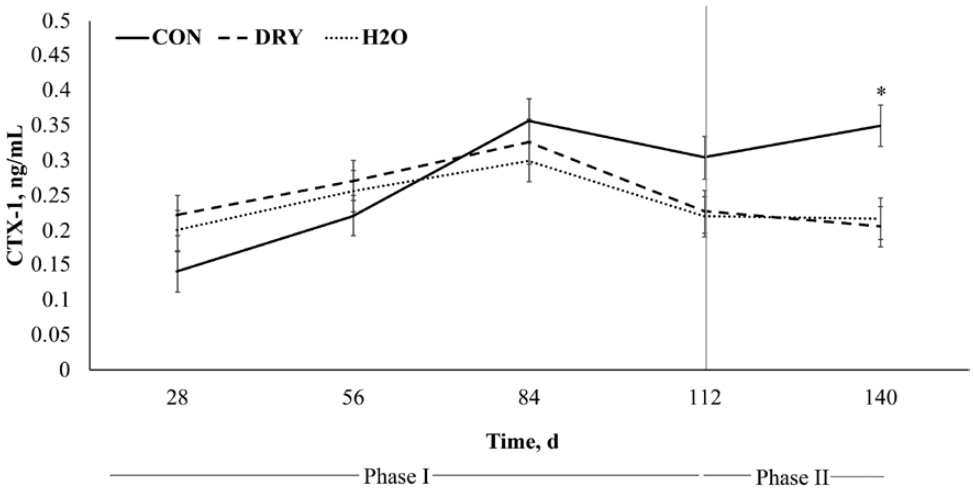

Figure 9. Serum CTX-1 concentrations (ng/mL; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.37), day (*P* < 0.01), and treatment × day (*P* < 0.01). *Within day, CON differs from DRY and H2O (*P* < 0.05). ‡Within day, CON tends to differ from DRY and H2O (*P* < 0.10).

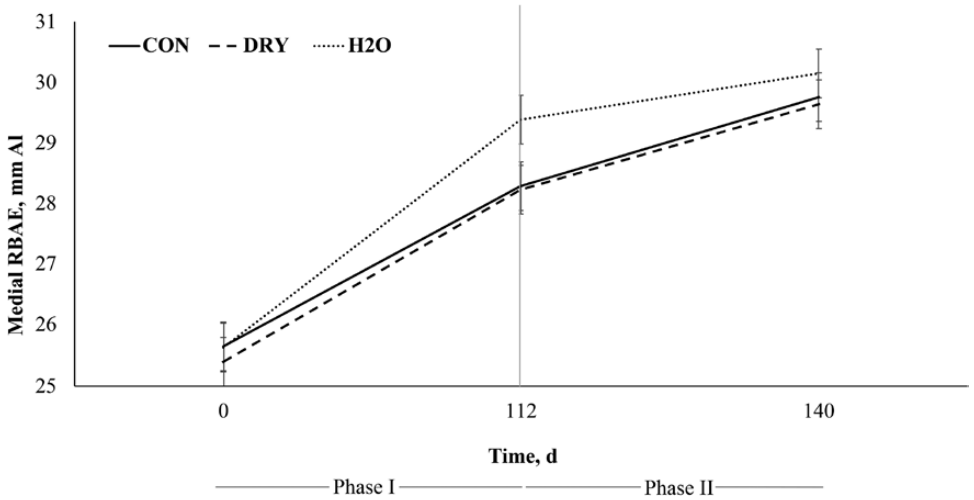

Figure 10. RBAE of the medial aspect of the left third metacarpal (mm AI; least squares means ± SEM) over time (d) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.77), day (*P* < 0.01), and treatment × day (*P* = 0.86).

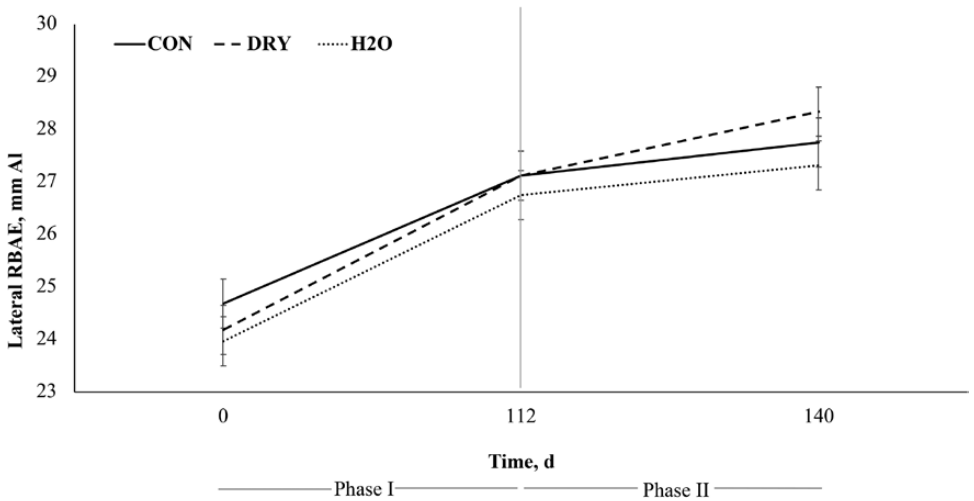

Figure 11. RBAE of the lateral aspect of the left third metacarpal (mm AI; least squares means ± SEM) over time (*d*) in horses undergoing no forced exercise (CON; *n* = 10), treadmill exercise (DRY; *n* = 10), or aquatic treadmill exercise (H2O; *n* = 10). Main effects include treatment (*P* = 0.30), day (*P* < 0.01), and treatment × day (*P* = 0.21).

age, solidifying the importance of early training for cartilage adaptation to future performance levels.

Additional extraneous factors involved in the introduction of phase II exercise include change in exercise surface, trajectory of motion, and progressing workload. During phase I, horses were exercised on treadmills equipped with a synthetic running belt surface. This differed from the natural sand surface of the free-stall exerciser in phase II; however, the treatment of both exercising groups was consistent throughout. During phase I exercise, horses tracked in a straight line, then were introduced to a circular track of the free-stall exerciser in phase II. Similar to the exercise surface, this variable was kept consistent across treatment groups, and exercise was divided evenly in either direction in order to equally distribute joint forces. The introduction of phase II exercise was gradual, with speeds increasing weekly.

All horses had access to turnout for ~10 hr/d, where voluntary exercise was allowed. Evidence suggests that voluntary sprinting improves bone optical density and cartilage tissue in weanlings when compared to those housed in stalls (Bell et al., 2001; Billinghurst et al., 2003). While an effort was made to limit size of turnout to prevent the occasional sprint, a sprint of only 50 m, 5 d/wk may be sufficient to increase bone metabolism in young calves used as a model for horses (Hiney et al., 2004). The results observed across all treatment groups during phase I suggest that turnout and voluntary exercise may be sufficient to support joint health in young horses, similar to walking on a treadmill. Quantification of voluntary exercise would be ideal; however, there are challenges to doing so in horses as the number of steps, gait, speed, and distance is important factors that influence force on bones and joints. Accelerometers are validated using video to determine thresholds for different gaits (Morrison et al., 2015), which may be challenging when measuring multiple individuals in a pasture setting. Additionally, it is difficult to account for high activity levels in young horses, and they are gregarious in nature which may result in loss of device (DuBois et al., 2015). Furthermore, agreement has not been reached on device placement to obtain the most accurate data (Dubois et al., 2015; Morrison et al., 2015).

The objective of this study was to mimic industry standards of conditioning programs, which typically require 3 to 4 mo of slow to moderate work followed by 1 to 2 mo of speed and discipline-specific skill training. Phase I exercise was developed to be comparable to aquatic treadmill protocols utilized in the industry, where exercising horses at speeds higher than a walk is uncommon. If the high-intensity exercise program had been extended beyond 28 d, changes in articular cartilage metabolism may have been observed; in addition, extension of the high-intensity exercise program beyond 28 d may elicit further changes in bone metabolism, as the bone turnover period due to increased mechanical loading is not complete in 28 d (Frost, 1990).

In conclusion, the results from this study indicate that walking, whether in a dry or aquatic environment, is likely insufficient force to alter molecular regulation of joint metabolism. Early forced exercise, whether in a dry or aquatic environment, produced consistent bone metabolism, while nonexercised horses exhibited incongruent bone turnover in order to maintain a similar bone mineral density. However, in the current study, there were no effects of buoyancy on joint development in yearling horses transitioned to an advanced workload, when water was set at 60% of WH. As water height increases, buoyancy increases and forces on bones and joints decrease (King et al., 2013). Various water heights may be utilized to achieve different objectives for individual animals, but that was not investigated in the current study. Effects of various water heights on bone and joint development may be explored further in the future. Further research is necessary to determine the effects of aquatic conditioning on parameters of athletic capability, such as heart rate, respiration rate, and musculature.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

References

Adair, H. S. 2011. Aquatic therapy for conditioning and treatment of tendon and ligament injuries. *AAEP Proc.* **57**:181–185.

Barneveld, A., and P. R. van Weeren. 1999. Conclusions regarding the influence of exercise on the development of the equine musculoskeletal system with special reference to osteochondrosis. *Equine Vet J Suppl.* **31**:112–119. doi:10.1111/j.2042–3306.1999.tb05323.x

Bell, R. A., B. D. Nielsen, K. Waite, D. Rosenstein, and M. Orth. 2001. Daily access to pasture turnout prevents loss of mineral in the third metacarpus of Arabian weanlings. *J. Anim. Sci.* **79**:1142–1150. doi:10.2527/2001.7951142x

Bertone, A. L., J. L. Palmer, and J. Jones. 2001. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet. Surg.* **30**:528–538. doi:10.1053/jvet.2001.28430

Billinghurst, R. C., P. A. Brama, P. R. van Weeren, M. S. Knowlton, and C. W. McIlwraith. 2003. Significant exercise-related changes in the serum levels of two biomarkers of collagen metabolism in young horses. *Osteoarthr. Cartilage* **11**:760–769. doi:10.1016/s1063-4584(03)00152-3

Brama, P. A. J., J. M. TeKoppele, R. A. Bank, A. Barneveld, and P. R. van Weeren. 2000. Functional adaptation of equine articular cartilage: the formation of regional biochemical characteristics up to age one year. *Equine Vet. J.* **41**(6):564–571. doi:10.2746/042516400 776563626

DuBois, C., E. Zakrajsek, D. B. Haley, and K. Merkies. 2015. Validation of triaxial accelerometers to measure the lying behaviour of adult domestic horses. *Animal* **9**:110–114. doi:10.1017/S175173111400247X

Ellman, R., J. Spatz, A. Cloutier, R. Palme, B. A. Christiansen, and M. L. Bouxsein. 2013. Partial reductions in mechanical loading yield proportional changes in bone density, bone architecture, and muscle mass. *J. Bone Miner. Res.* **28**:875–885. doi:10.1002/jbmr.1814

Frisbie, D. D., F. Al-Sobayil, R. C. Billinghurst, C. E. Kawcak, and C. W. McIlwraith. 2008. Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthr. Cartilage* **16**:1196–1204. doi:10.1016/j.joca.2008.03.008

Frost, H. M. 1990. Skeletal structural adaptations to mechanical usage (SATMU): 2. Redefining Wolff's law: the remodeling problem. *Anat. Rec.* **226**:414–422. doi:10.1002/ar.1092260403

de Grauw, J. C., C. H. van de Lest, R. van Weeren, H. Brommer, and P. A. Brama. 2006. Arthrogenic lameness of the fetlock: synovial fluid markers of inflammation and cartilage turnover in relation to clinical joint pain. *Equine Vet. J.* **38**: 305–311. doi:10.2746/042516406777749236

Hiney, K. M., B. D. Nielsen, and D. Rosenstein. 2004. Short-duration exercise and confinement alters bone mineral content and shape in weanling horses. *J. Anim. Sci.* **82**: 2313–2320. doi:10.2527/2004.8282313x

Imhof, H., M. Breitenseher, F. Kainberger, T. Rand, and S. Trattnig. 1999. Importance of subchondral bone to articular cartilage

in health and disease. *Top. Magn. Reson. Imaging* **10**:180–192. doi:10.1097/00002142-199906000-00002

King, M. R., K. K. Haussler, C. E. Kawcak, C. W. McIlwraith, and R. F. Reiser. 2013. Mechanisms of aquatic therapy and its potential use in managing equine osteoarthritis. *Equine Vet. Educ.* **25**(4):2002–209. doi:10.1111/j.2042-3292.2012.00389.x

Langdahl, B., S. Ferrari, and D. W. Dempster. 2016. Bone modeling and remodeling: potential as therapeutic targets for the treatment of osteoporosis. *Ther. Adv. Muskuloskel. Dis.* **8**(6):225–235. doi:10.1177/1759720X16670154

Leatherwood, J. L., K. L. Gehl, J. A. Coverdale, C. E. Arnold, R. A. Dabareiner, K. N. Walter, and E. D. Lamprecht. 2016. Influence of oral glucosamine supplementation in young horses challenged with intra-articular lipopolysaccharide. *J. Anim. Sci.* **94**:3294–3302. doi:10.2527/jas.2016-0343

Lepage, O. M., B. Carstanjen, and D. Uebelhart. 2001. Non-invasive assessment of equine bone: an update. *Vet. J.* **161**: 10–22. doi:10.1053/tvjl.2000.0541

Lepeule, J., N. Bareille, C. Robert, J. P. Vallette, S. Jacquet, G. Blanchard, J. M. Denoix, and H. Seegers. 2013. Association of growth, feeding practices and exercise conditions with severity for the osteoarticular status of limbs in French foals. *Vet J.* **197**:65–71. doi:10.1016/j.tvjl.2013.03.043

McIlwraith, C. W. 2005. Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. *Equine Vet. J.* **37**:473–482. doi:10.2746/042516405774480102

McIlwraith, C. W., and G. W. Trotter. 1996. *Joint disease in the horse*. Philadelphia, PA: W.B. Saunders.

Morrison, R., D. G. Sutton, C. Ramsoy, N. Hunter-Blair, J. Carnwath, E. Horsfield, and P. S. Yam. 2015. Validity and practical utility of accelerometry for the measurement of in-hand physical activity in horses. *BMC Vet. Res.* **11**:233. doi:10.1186/s12917-015-0550-2

Nielsen, B. D., G. D. Potter, L. W. Green, E. L. Morris, M. Murray-Gerzik, W. B. Smith, and M. T. Martin. 1998. Characterization of changes related to mineral balance and bone metabolism in the young racing quarter horse. *J. Equine Vet. Sci.* **18**(3): 190–200. doi:10.1016/S0737-0806(98)80374-2

Nielsen, B. D., G. D. Potter, E. L. Morris, T. W. Odom, D. M. Senor, J. A. Reynolds, W. B. Smith, and M. T. Martin. 1997. Changes in the third metacarpal bone and frequency of bone injuries in young quarter horses during race training – observations and theoretical considerations. *J. Equine Vet Sci.* **17**(10):541–549. doi:10.1016/S0737-0806(97)80227-4

NRC. 2007. *Nutrient requirements of horses*. 6th rev. ed. Washington, DC: National Academies Press.

O'Connor-Robison, C. I., and B. D. Nielsen. 2013. Comparison of two software packages for determining radiographic bone aluminum equivalent values. *Compar. Ex. Phys.* **9**(3/4):210–222. doi:10.3920/CEP13024

Parfitt, A. M. 1994. The two faces of growth: benefits and risks to bone integrity. *Osteoporos. Int.* **4**:382–398. doi:10.1007/BF01622201

Rogers, C. W., C. F. Bolwell, and E. K. Gee. 2012. Proactive Management of the Equine Athlete. *Animals (Basel)*. **2**: 640–655. doi:10.3390/ani2040640

Spooner, H. S., B. D. Nielsen, A. D. Woodward, D. S. Rosenstein, and P. A. Harris. 2008. Endurance training has little impact on mineral content of the third metacarpus in two-year-old Arabian horses. *J. Equine Vet. Sci.* **28**(6):359–362. doi:10.1016/j.jevs.2008.04.0 12