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ORIGINAL ARTICLE

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Differences in bone turnover markers and injury risks between local and international horses: A Victorian Spring Racing Carnival study

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Abstract

Background: Musculoskeletal injuries (MSI) are common in racehorses and have been of increasing concern in horses travelling internationally to compete. Understanding the differences in bone turnover between local horses and international horses following long-distance air transportation may inform MSI prevention strategies.

Objectives: To understand the differences in bone turnover markers and risk of MSI between local horses and international horses following long-distance air transportation.

Study design: Prospective cohort.

Methods: The concentrations of bone turnover markers (OCN and CTXI), markers of stress (cortisol), inflammation (serum amyloid A) and circadian rhythm (melatonin), and bisphosphonates were determined in blood samples collected twice (14–17 days apart), from horses following international travel ($n = 69$), and from local horses ($n = 79$). The associations between markers, long-distance travel and MSI were determined using multivariable generalised linear regression models.

Results: Within 3–5 days post-transport, concentrations of cortisol in international horses were higher than those of local horses (main effect, Coef. 0.39; 95% CI 0.24, 0.54; $p < 0.001$) but they decreased and were not different to those of local horses at the second timepoint (interaction effect, Coef. −0.27; 95% CI −0.46, −0.07; $p = 0.007$). After adjusting for age and sex, OCN and CTXI were not significantly different between international and local horses; however, OCN was lower in international horses at timepoint 2 (interaction effect, Coef. −0.16; 95% CI −0.31, −0.01; $p = 0.043$). The prevalence of MSI was higher in the international (26%; 95% CI 16, 38%) compared with local horses (8%; 95% CI 3, 16%; $p < 0.001$), with all severe MSI sustained by the international horses. At the second timepoint compared with the first timepoint post-transport, cortisol remained high or increased (interaction effect, Coef. 0.43; 95% CI 0.24, 0.61; $p < 0.001$) and OCN increased (interaction effect, Coef. 0.26; 95% CI 0.08, 0.44; $p = 0.006$) in the horses that sustained severe MSI.

Main limitations: Horse population and racing career parameters differed between groups. Bone turnover markers have low sensitivity to detect local bone changes.
Conclusions: Most horses showed minimal effects of long-distance air transport within 2 weeks relative to local horses as assessed by stress and bone turnover markers. Screening for persistent high cortisol and evidence of net bone formation after long-distance air transportation may help to identify racehorses at high risk of catastrophic MSI.

KEYWORDS
athlete, bone turnover, cortisol, horse, long-distance transportation

1 | INTRODUCTION

Musculoskeletal injuries (MSI) are common in racehorses and represent about 70% of the reason for equine racing fatalities.[1–5] Many of these MSI are bone fatigue-related fractures that occur because of repetitive cycles of high magnitude loading of bone sites during high-speed galloping exercise.[6–8] Such bone loading results in densification of the subchondral bone through bone modelling and the suppression of bone remodelling at known focal sites subjected to the highest loads. This allows bone microdamage, microscale cracks that may progress to fatigue fractures, to accumulate.[9–12] From epidemiological evidence, MSI in racehorses are the product of a complex interaction between various biological, environmental, and horse management risk factors.[13–15] For instance, the risk of MSI is higher in older horses, entire males, those that start their racing careers at an older age and those with longer racing careers.[15–17] Most MSI are difficult to detect early using routine clinical imaging techniques. For this reason, predictive tools that are based on the race history and physiological biomarkers of a racehorse have been investigated to better identify horses at greater risk of potentially catastrophic MSI.[18,19]

Both the frequency and distances horses are transported for participation in racing carnivals have increased progressively over recent decades, with the internationalisation of racing resulting in the greater use of long-distance air transport.[20] Physiological stress, indicated by increased cortisol in body fluids, is commonly observed in horses during transportation over both short and long distances.[21,22] In humans, increased cortisol is associated with reduced bone formation and increased bone resorption that drives bone loss and risk of stress fracture with osteoporosis.[23] The stress experienced by horses during long-distance air transportation has the potential to disrupt bone modelling and remodelling through its effect on bone cells and therefore impact the risk of MSI. The adjustment of the circadian rhythm of horses following long-distance air transportation is also unknown. Understanding how long-distance transportation of racehorses affects bone turnover will inform horse management practices that are aimed at reducing the risk of MSI, including better prediction of severe MSI following air transportation. In this study, we aimed to identify differences in markers of bone turnover, stress, circadian rhythm and inflammation between local racehorses and racehorses that underwent long-distance air transportation, to investigate whether these differences in biomarkers were also associated with increased risk of MSI, and to investigate if MSI or severe MSI can be predicted based on biomarker levels. We hypothesised that following international travel, racehorses would show high levels of the biomarkers for bone resorption and stress, and that larger increases in these biomarkers would be associated with MSI.

2 | MATERIALS AND METHODS

We used the statistical software Stata/SE 14.2 package 'power repeated' to conduct a power analysis for repeated-measures analysis of variance to estimate power and effect sizes.[24] To inform our study design we consulted previous studies that had observed changes in markers of bone turnover between injured ($n = 8$) and uninjured ($n = 18$) Thoroughbreds: the concentration of a marker of bone resorption was higher in injured horses compared with the uninjured horses [Crosslinked C-telopeptide of type I collagen (CTXI; ng/mL): 0.6285 ± 0.08 vs. 0.4152 ± 0.13], and the concentration of a marker of bone formation was lower in the injured horses compared with the control horses [Osteocalcin (OCN; ng/mL): 43.92 ± 5.73 vs. 59.53 ± 10.07].[18] Based on numbers from previous years of international horses imported to race for the VSRC, we anticipated that over 3 years we could sample 60 international horses, with 1:1 local control matched only on their eligibility for the Victorian Spring Racing Carnival (VSRC) ($n = 120$). We estimated we could detect small effect sizes across groups at two timepoints (delta = 0.25) based on within horse correlation between timepoint samples of 0.7, at a power of 80% and statistical level of $p < 0.05$.

2.1 | Horse population

Elite Thoroughbred racehorses trained in Melbourne, Australia (local horses; $n = 79$), and those trained overseas and transported by air to Melbourne for participation in races during the VSRC (international horses; $n = 69$), were sampled in September to November over 3 years (2017–2019). Local horses that were aimed at races during

the VSRC and had been randomly selected for drug testing by the principal racing authority were voluntarily enrolled in the study. All international horses arriving at the Werribee International Horse Centre during the study period were invited to voluntarily participate (2017 $n = 25$; 2018 $n = 48$; 2019 $n = 36$). The international horses sampled for the study had been transported by air over 1–10 time zones eastward or westward: from Japan (one time zone eastward, $n = 4$), United Kingdom, Ireland and France (9–10 time zones eastward, $n = 59$), or USA (7–10 time zones westward, $n = 6$). The international horses passed pre-export veterinary inspections which excluded clinically detectible injuries before departure. The horses spent 2 weeks pre-departure and 2 weeks post-arrival in quarantine facilities with access to racetracks to maintain their training. International horses were released from quarantine after 14 days post-arrival and were cleared to race if regulatory requirements were satisfied. Both local and international horses were sampled at their stables on a non-race day.

2.2 | Sample collection

Two blood samples (timepoints 1 and 2) were collected at 14–17 days interval from all recruited horses (local and international). For the local horses, timepoint 1 samples were collected from horses enrolled in the study at the trainers' stables in Melbourne during random regulatory sampling by the racing authority. Samples were collected from the local horses, in September to November around the time of arrival of the international horses and timepoint 2 samples were collected 14–17 days later. For the international horses, timepoint 1 samples were collected at 3–5 days post-arrival in Melbourne and timepoint 2 samples were collected 14–17 days later. This sampling interval is the same as the length of the quarantine period and the routine sample collection for regulatory testing by the principal regulatory authority and not controlled by the research investigators for this study. Blood collection was performed by an official regulatory veterinarian via jugular venepuncture, between 5:39 AM and 10:32 AM for local horses and between 7:25 AM and 12:44 PM for international horses. Blood was collected in BD vacutainer® 10 mL plain tubes, allowed to clot at room temperature then transported to the laboratory to be processed immediately or kept at 4°C during transport for a maximum of 3 hour. Blood tubes were centrifuged at 3500 rpm for 10 min and serum was separated into Eppendorf tubes in 0.5 mL aliquots and stored at −80°C. Frozen samples for biomarker measurement were thawed in batches at room temperature immediately prior to assay.

2.3 | Biomarker assays

Crosslinked C-telopeptide of type I collagen (CTXI) using Crosslap® CTXI ELISA kits (Immunodiagnostics System, Boldon, UK), which had been previously validated for use in horses, was used as a measure of bone resorption.[25] Undiluted serum samples were run in duplicate following the manufacturer's instructions over seven assay batches run on seven different days. The mean coefficient of variation (CV) for the assays was 8% (inter-assay) and 2.9% (intra-assay).

Osteocalcin (OCN) was determined as a measure of bone formation using MicroVue® osteocalcin enzyme immunoassay kits (Quidel), which had been validated for use in horses.[26] Serum samples were diluted 1:2 with each sample run in duplicate over seven assay batches run on seven different days. The mean CV was 9% (inter-assay) and 2.5% (intra-assay).

Mean CV were within the acceptable limits specified by assay kit manufacturers: Inter-assay CV of 10% for OCN and CTXI, and intra-assay CV of 10% for OCN and 3% for CTXI.

A ratio of bone formation to bone resorption was calculated as OCN divided by the CTXI at each timepoint (OCN/CTXI ratio) using raw data before log transformation.

Cortisol, a biomarker of stress, was measured in serum using a solid-phase, competitive chemiluminescent enzyme immunoassay using Vet Cortisol kits (Siemens Healthineers, Australia) on the IMMULITE® 1000 immunoassay analyser.

A biomarker used to determine inflammation in horses, serum amyloid A (SAA) was measured in undiluted serum using a latex agglutination turbidinometric reaction with the LZ-SAA assay (Eiken Chemical Co. Ltd.) on a Cobas Integra 400 Plus analyser.

The presence of antiresorptive drugs, bisphosphonates, in the serum samples was also measured to determine possible effects on bone turnover markers. Sample preparation was adapted from a previous study[27] and followed the accredited method for the qualitative screening of equine serum for bisphosphonates by National Association of Testing Authorities (NATA), Australia. Analysis was carried out by liquid chromatography mass spectrometry (LCMS) using a Shimadzu 8060 mass spectrometer (Shimadzu Corp.) coupled to a Nexera LC-30AD (Shimadzu Corp.) liquid chromatograph. Chromatographic separation was achieved using a Restek Raptor Biphenyl column (2.1 mm × 100 mm, 1.8 µm particle size) (Restek). Bisphosphonates were measured in all horses at the first timepoint. For horses with samples containing bisphosphonate at the first timepoint, the second timepoint samples were also tested for confirmation.

Melatonin was measured to determine the status of the circadian rhythm by reverse-phase C-18 column extraction of serum, followed by double antibody radioimmunoassay (RKMEL-2, Buhlmann Laboratories AG). This assay is based on the Kennaway G280 anti-melatonin antibody,[28] with 2-[^{125}I]-iodomelatonin as the radioligand following the protocol provided by Buhlmann Laboratories. Where serum volume permitted, 500 µL of serum was extracted and reconstituted to 1000 µL volume using supplied assay buffer. The lowest limit of quantitation of the assay using 500 µL of extracted plasma was 1.0 pg/mL. In cases where the available serum volume was less than 500 µL, 250 µL serum was extracted and reconstituted to 1000 µL using the supplied buffer. For these samples, the lowest limit of quantitation of the assay was 2.0 pg/mL. Samples were assayed in duplicate and the mean CV of the assays was 2.9% (inter-assay) and 10.0% (intra-assay), for low concentration quality control (mean value 3.9 pg/mL) and, 6.0% (inter-assay) for high concentration quality control (mean value 27.8 pg/mL). The inter-assay

TABLE 1 Horse population data showing the distribution of age, sex, musculoskeletal injury (MSI) and racing history between the local and international horses aimed at the Victorian Spring Racing Carnival, 2017–2019.

	Horse groups			
	All racehorses	Local	International	*p* values (Int vs. Loc)
Age (years)				
Median, min to max	4.7, 2 to 8	4.1, 2 to 8	4.8, 3 to 8	<0.001[A]
Sex				<0.001[B]
Females	33 (22.4%)	31 (39.2%)	2 (2.9%)	
Geldings	76 (51.7%)	41 (51.9%)	35 (51.5%)	
Entire males	38 (25.9%)	7 (8.9%)	31 (45.6%)	
Injury[a]				
No Injury	124 (83.8%)	73 (92.4%)	51 (73.9%)	
Injured	24 (16.2%)	6 (7.6%)	18 (26.1%)	0.002[B]
Mild injury	18 (12.2%)	6 (7.6%)	12 (17.4%)	0.04[B]
Severe injury	6 (4.1%)	0 (0%)	6 (8.7%)	0.005[B]
Racing history (median, min to max)				
Career race starts	15, 0 to 49	10, 0 to 49	17, 2 to 41	<0.001[A]
Career length (days)	749, −61 to 2261	457, −61 to 1804	848, 164 to 2261	<0.001[A]
Active career length (days)	292, −61 to 962	142, −61 to 767	375, 39 to 962	<0.001[A]
Mean rest period (days)	147, 62.52 to 355	140, 69 to 274	158, 64 to 355	<0.001[A]
Time since last rest (days)	51, 0 to 356	37, 0–356	129, 0 to 277	<0.001[A]
Time since last race (days)	15, 0 to 402	10, 0 to 402	45, 2 to 355	<0.001[A]

Note: *p* value for comparison between local and international horses using [A]Wilcoxon rank-sum test, [B]Pearson's chi squared.
[a]Mild and severe injuries are subsets of injured.

CV of an internal quality control comprising pooled, extracted plasma (mean value 12.4 pg/mL) was 19.5%.

3 | HORSE RACING AND INJURY HISTORY

Racing data for all the horses participating in the study were extracted from the Australian official racing repository which includes racing histories for imported international horses. From the race result data, race career history variables were generated including number of career race starts, career earnings, number of rest periods (spell or layup; >60 days between race starts), mean rest period duration, time since last rest period, total resting time, career length (days between first and last race prior to sampling), active career length (career length minus total rest period time), change of trainer, and time with current trainer. All horses recruited for this study were examined by regulatory veterinarians before and after participation in races, trials and official gallops and routinely by their veterinarians for injury at their stables, and trainers were required to notify the racing authority of any injury. In addition, all international horses were monitored by regulatory veterinarians in the quarantine centre daily. Records of MSI or lameness reported by veterinarians after the first sample collection were reviewed by an equine surgeon (RCW) and classified as mild, severe or catastrophic MSI. Mild MSI were defined as those where training could continue or non-specific lameness where the cause was not identified. Severe MSI were defined as bone fracture or any injury to bone, flexor tendons, or suspensory ligaments that required, surgery or euthanasia. Catastrophic MSI are a subset of severe MSI defined as MSI for which a horse was euthanised. Occurrence of injury was followed up for until the end of the VSRC in the last week of November in each year (up to 74 days after the collection of the first timepoint sample).

4 | STATISTICAL ANALYSES

Continuous variables were assessed for normality using the Shapiro–Wilk test. Median, and minimum to maximum values or numbers (%) for each horse population and race history variables are reported stratified by local and international horse groups and compared using Wilcoxon rank sum tests if continuous and not normally distributed or Pearson's chi-squared if categorical (Table 1). Biomarker values were non-normally distributed and therefore log transformed for analysis. For SAA, undetectable levels were replaced with half the minimum detectable value (SAA = 0.01 μg/mL). Pairwise correlation was used to assess correlations between biomarker levels and days between sampling and MSI, overall and for each timepoint. Three analyses were conducted using (1) generalised linear mixed models for each biomarker outcome (OCN, CTX1, SAA, Cortisol, Melatonin; log transformed); (2) generalised linear mixed models with a Bernoulli family

and logit link for MSI as an outcome for all horses; and (3) subset generalised linear mixed models with a Bernoulli family and logit link for MSI and severe MSI as an outcome for international horses only. Coefficients or Odds Ratios (OR) and their 95% confidence intervals (95% CI) are reported. All models were adjusted for random-effects at the horse-level to account for measures of biomarkers taken at two timepoints for each horse. Bone turnover marker values were adjusted for fixed effects of the bone marker assay batch. Horse age, horse sex, racing career history, timepoint, year of sampling and time of day for sample collection were investigated as predictors of biomarker values and MSI. Multivariable models were generated to predict MSI risk and to determine area under the ROC curve (AUC), sensitivity, and specificity. Variables $p < 0.20$ in univariable analysis were included in multivariable models and retained using a backwards stepwise process if $p < 0.05$ or if they were effect modifiers. Horse-level and race history variables that were significantly different between the international and local horses were investigated as confounders of the final multivariable models. Two-way interactions between timepoints and biologically plausible study factors were assessed. The empirical cut-off points of the biomarkers, for predicting MSI or severe MSI, were based on Hosmer & Lemeshow's discrimination criteria.[29] Model diagnostics included the Hosmer–Lemeshow's goodness-of-fit test, the link test to identify model specification error, examination of tolerance (>0.1), and the variance inflation factor (VIF <10). Best fitting models were determined by assessing Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC) and Area Under the ROC Curve (AUC). All statistical analyses were conducted using Stata version 15.1 (StataCorp).

5 | RESULTS

5.1 | Horse population

Distributions of age, sex, MSI, and racing history in the local and international horses are presented in Table 1. The international horses were on average 1 year older, had markedly longer racing careers, including longer time since last rest and longer time since last race, and had a greater proportion of entire males, and lower proportion of females, compared with the local horses ($p < 0.001$). Prevalence of all MSI was higher among the international horses (26.09%; 95% CI 16.25, 38.06) compared with the local horses (7.59%; 95% CI 2.84, 15.80). Of the 18 mild MSI, two were suspected suspensory apparatus injuries, one superficial digital flexor tendon injury, one unspecified pastern injury, and the remaining were unspecified lameness. Of the six severe MSI, two were complete condylar fractures (one medial and one lateral), one incomplete lateral condylar fracture of the third metacarpus, one pelvic fracture, one catastrophic humeral fracture, and one catastrophic radial fracture. Days between sample collection at timepoint 1 and MSI (mean ± SD; range: 22.88 ± 10.79; 1 to 40) or timepoint 1 and the end of follow-up (52.52 ± 8.20; 39 to 74) was not correlated with biomarker levels ($p > 0.05$).

5.2 | Differences in biomarkers between international and local horses

Univariable analyses of the serum biomarker outcomes including differences between international and local horses are presented in Table S1. Bone turnover markers (OCN and CTX1) were lower in the international horses compared with the local horses at both timepoints, (OCN: Coef. −0.15; 95% CI −0.27, −0.02; $p = 0.022$, CTXI: −0.30; −0.44, −0.15; $p < 0.001$); however, there was no overall difference after adjusting for age and sex. OCN was lower at timepoint 2 in the international horses compared with the local horses after adjusting for horse age and sex, and assay batch (main effect, Coef. 0.06; 95% CI −0.07, 0.18; $p = 0.389$; interaction effect, Coef. −0.16; 95% CI −0.31, −0.01; $p = 0.043$; Figure 1A). Osteocalcin and CTX1 decreased with measures of career progression such as age, number of race starts and career length. Entire males and geldings had lower levels of bone turnover markers than females. A bisphosphonate (tiludronic acid) was detected in 6 horses (2 local, 4 international) but no differences in CTX1 and OCN levels were observed between these horses and those in which bisphosphonates were not detected. Cortisol was highest in entire males, followed by geldings then females ($p = 0.0008$). Serum cortisol, adjusted for sex, was higher in the international horses at arrival

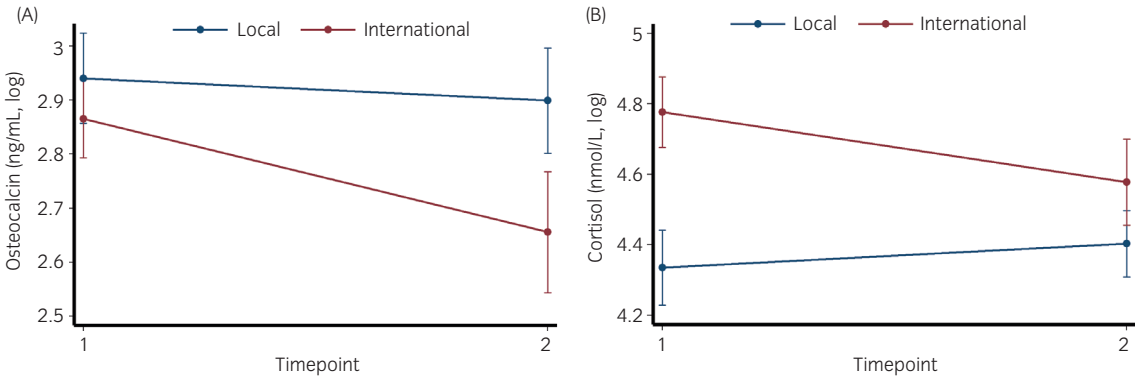

FIGURE 1 Predictive margins plot showing differences in log OCN (A) and log cortisol (B) between the local (blue) and the international (red) horses at timepoints 1 and 2. Error bars represent 95% confidence intervals.

TABLE 2 The level of blood biomarkers between the injured and uninjured horses and the international horses that had severe MSI and those with no injury.

		OCN (ng/mL)[a]		CTXI (ng/mL)[b]		Cortisol (nmol/L)[c]		Melatonin (pg/mL)[d]		SAA (μg/mL)	
	N	Timepoint 1 (mean ± SD)	Timepoint 2 (mean ± SD)	Timepoint 1 (mean ± SD)	Timepoint 2 (mean ± SD)	Timepoint 1 (mean ± SD)	Timepoint 2 (mean ± SD)	Timepoint 1 (mean ± SD)	Timepoint 2 (mean ± SD)	Timepoint 1 (mean ± SD)	Timepoint 2 (mean ± SD)
All horses											
Injury											
Uninjured	124	19.53 ± 7.77^{A}	18.10 ± 8.13^{B}	0.20 ± 0.17^{A}	0.23 ± 0.18^{A}	108.00 ± 127.19^{A}	95.77 ± 37.58^{B}	1.68 ± 3.54	1.29 ± 0.92	13.77 ± 76.01	13.95 ± 73.64
Injured	24	19.94 ± 5.97^{AC}	17.93 ± 6.33^{BD}	0.16 ± 0.08^{B}	0.28 ± 0.39^{A}	120.02 ± 39.97^{C}	108.66 ± 44.47^{D}	1.14 ± 0.47	1.15 ± 0.44	0.14 ± 0.27	0.09 ± 0.29
Severe injury											
No	124	19.63 ± 7.62	18.05 ± 7.99	0.20 ± 0.16	0.23 ± 0.17	109.93 ± 119.54	96.14 ± 38.43	1.61 ± 3.30	1.27 ± 0.88	11.96 ± 70.90	12.19 ± 68.94
Yes	6	18.86 ± 2.28	18.72 ± 3.42	0.12 ± 0.04	0.45 ± 0.79	112.13 ± 19.21	136.48 ± 29.25	1.01 ± 0.02	1.13 ± 0.24	0.19 ± 0.31	0.06 ± 0.12
International horses											
Injury											
Uninjured	51	17.77 ± 5.88^{E}	15.31 ± 7.42^{F}	0.15 ± 0.06	0.19 ± 0.10	141.28 ± 189.10^{E}	108.10 ± 42.85^{F}	1.10 ± 0.28	1.42 ± 1.26	9.85 ± 61.92^{A}	15.40 ± 76.18^{C}
Injured	18	20.42 ± 5.88^{G}	17.37 ± 6.13^{E}	0.14 ± 0.06	0.29 ± 0.45	130.92 ± 36.93^{G}	110.94 ± 47.03^{H}	1.17 ± 0.54	1.10 ± 0.27	0.08 ± 0.19^{B}	0.12 ± 0.34^{B}
Severe injury											
No	63	18.45 ± 6.2^{I}	15.57 ± 7.34^{K}	0.15 ± 0.06	0.19 ± 0.09	140.93 ± 169.38^{I}	106.14 ± 43.98^{JK}	1.13 ± 0.38	1.36 ± 1.15	7.86 ± 55.30	12.50 ± 68.68
Yes	6	18.86 ± 2.28^{I}	18.72 ± 3.42^{I}	0.12 ± 0.04	0.45 ± 0.79	112.13 ± 19.21^{J}	136.48 ± 29.25^{IL}	1.01 ± 0.02	1.13 ± 0.24	0.19 ± 0.31	0.06 ± 0.12
Local horses											
Injury											
Uninjured	73	20.76 ± 8.68	20.06 ± 8.08	0.24 ± 0.21	0.26 ± 0.22	84.75 ± 38.37	87.15 ± 30.90	1.19 ± 0.56	1.02 ± 0.05	16.51 ± 84.79	12.93 ± 72.33
Injured	6	18.43 ± 6.53	19.60 ± 7.22	0.19 ± 0.11	0.25 ± 0.08	85.53 ± 29.70	102.20 ± 39.43	1.32 ± 0.78	1.10 ± 0.28	0.32 ± 0.41	0.02 ± 0.02

Note: Values within the same biomarker with different letter superscript are statistically different. For groups with no superscript, no differences were observed.

[a]Adjusted for age, sex and assay batch.

[b]Adjusted for age, sex and assay batch and time of the day.

[c]Adjusted for sex.

[d]Adjusted for time of the day.

(timepoint 1) compared with the local horses (main effect, Coef. 0.39; 95% CI 0.24, 0.54; $p < 0.001$), but this level decreased and was no longer different at timepoint 2 (interaction effect, Coef. −0.27; 95% CI −0.46, −0.07; $p = 0.007$; Figure 1B). Melatonin adjusted for sampling time, and SAA were not different between international and local horses.

5.3 | Differences in biomarkers between injured and uninjured in all horses

Univariable analyses investigating associations between serum biomarker outcomes and MSI are presented in Table S2. The biomarker levels stratified by local and international, and injured and uninjured horses at each timepoint are presented in Table 2. OCN, adjusted for assay batch, horse age and sex and time of the day were higher at timepoint 1 than timepoint 2 (Coef. −0.10; 95% CI −0.20, −0.01; $p = 0.034$) but not different between injured and uninjured horses (Coef. 0.12; 95% CI −0.01, 0.25; $p = 0.078$; Figure 2A). Injured horses had lower CTX1 at timepoint 1 compared with uninjured horses, when adjusted for assay batch, horse age and sex, and time of day (main effect, Coef. −0.14; 95% CI −0.33, 0.06; $p = 0.162$; interaction effect, Coef. 0.30; 95% CI 0.04, 0.55; $p = 0.022$; Figure 2B). Cortisol levels, adjusted for horse sex, were greater in injured horses regardless of timepoint (Coef. 0.18; 95% CI 0.04, 0.33; $p = 0.015$; Figure 2C). Neither SAA nor melatonin were associated with MSI in adjusted analysis. Two of the six horses with detectible bisphosphonates were injured (mild MSI; 1 each of local and international) but there was no association between bisphosphonates and MSI.

5.4 | Differences in biomarkers between injured and uninjured international horses only

Because all severe MSI ($n = 6$) were sustained by international horses, a subset analysis was performed to determine differences between serum biomarkers (outcome) and MSI categories for the international horses only (Table S2). The level of biomarkers measured at each timepoint between international horses with severe injury and those with no injury is presented in Table 2. The international horses with severe MSI had greater OCN after adjustment for assay batch, and horse age and sex, at the second timepoint compared with the uninjured international horses (main effect, Coef. 0.15; 95% CI −0.01, 0.31; $p = 0.072$; interaction effect, Coef. 0.20; 95% CI 0.05, 0.35; $p = 0.007$; Figure 3A). Cortisol was greater in international horses with severe MSI at timepoint 2, after adjustment for sex (main effect, Coef. −0.07; 95% CI −0.24, 0.10; $p = 0.441$; interaction effect, Coef. 0.43; 95% CI 0.24, 0.61; $p < 0.001$; Figure 3B). SAA was lower in international horses with mild MSI compared with those without injury (Coef. −1.13; 95% CI −1.8, −0.5; $p = 0.001$). CTX1 and

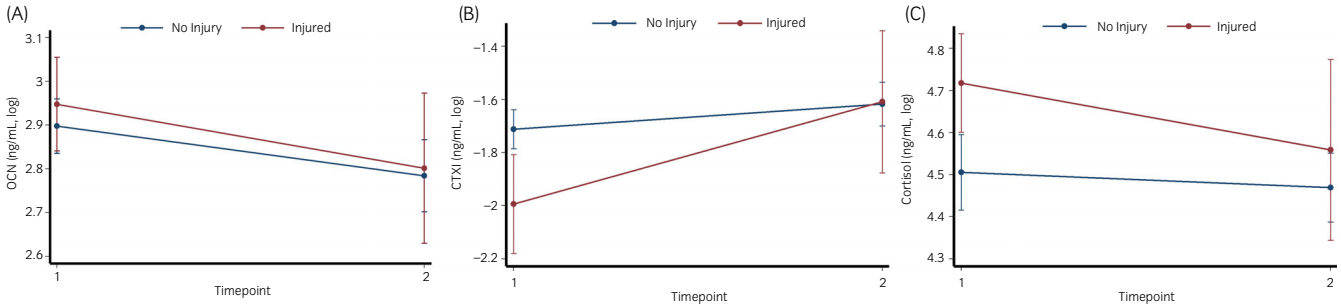

FIGURE 2 Predictive margins plot showing in all horses combined (local and international) the differences in log OCN (A), log CTXI (B) and log cortisol (C) between uninjured (blue) and injured (red) at timepoints 1 and 2. Error bars represent 95% confidence intervals.

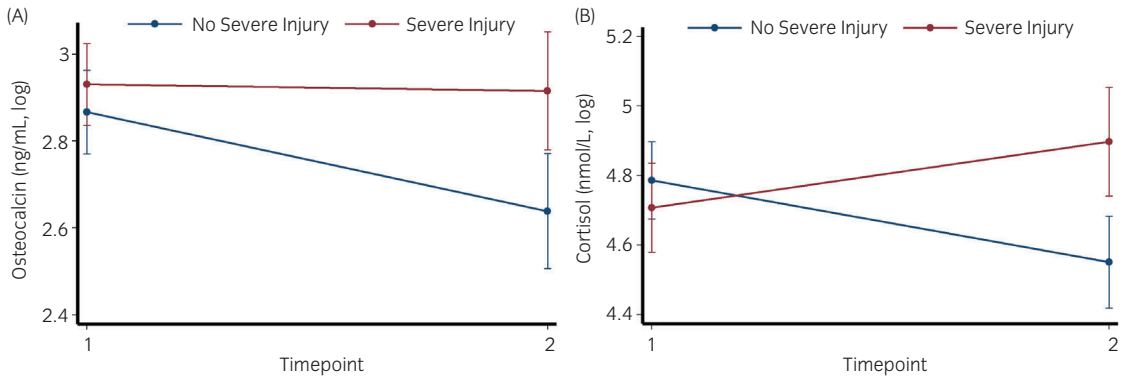

FIGURE 3 Predictive margins plot showing differences in log OCN (A) and log cortisol (B) between the international horses without injury (blue) and those with severe injury (red) during timepoints 1 and 2. Error bars represent 95% confidence intervals.

TABLE 3 Multivariable logistic regression model showing the association of musculoskeletal injury (MSI) with biomarker levels and differences across timepoints 1 and 2 in all horses (local and international), aimed at the Victorian Spring Racing Carnival, 2017–2019.

		MSI		
All horses	**Coef.**	**Odds ratio**	**95% CI**	***p* value**
OCN (ng/mL; log)	2.01	7.46	1.77, 31.46	0.006
Timepoint	7.71	2229.65	7.28, 682 445	0.008
Timepoint X OCN (ng/mL; log)	−1.52	0.22	0.05, 0.93	0.04
CTX1 (ng/mL; log)	−1.27	0.28	0.08, 0.96	0.044
Timepoint X CTX1 (ng/mL; log)	1.80	6.08	1.97, 18.77	0.002
Horse age (years)	0.1	1.10	0.73, 1.64	0.657
Sex				
Female	Ref	1		
Gelding	−0.81	2.24	0.53, 9.49	0.273
Entire males	0.11	2.51	0.56, 11.31	0.23
Area under ROC		0.7371		
Positive predictive value[a]		31.25%		
Negative predictive value[a]		92.43%		
Sensitivity[a]		71.43%		
Specificity[a]		68.95%		

Note: Model adjusted for assay batch; X represents interaction effect.
[a]Values at optimum cut-off.

melatonin were not associated with severe MSI and none of the horses with severe MSI had detectable bisphosphonates.

5.5 | Prediction of MSI by biomarkers in all horses

Univariable analyses investigating associations between MSI, serum biomarkers and other predictors for all horses are presented in Table S3. In multivariable analysis, greater odds of MSI were associated with higher levels of OCN, and lower levels of CTX1 at timepoint 1, increasing at timepoint 2 (Table 3).

5.6 | Prediction of MSI by biomarkers in international horses only

For international horses only, subset analyses were conducted to investigate associations between severe MSI, serum biomarkers and other predictors. Univariable analyses are presented in Table S3. International horses had greater odds of sustaining a severe MSI with older horse age, greater OCN at either timepoint, and if cortisol remained high or increased between timepoint 1 and 2 (Table 4, Figure 4). All severe injuries ($n = 6$) were sustained by male horses that originated from Great Britain or Ireland.

5.7 | Biomarker thresholds for prediction of MSI and severe MSI

The optimal predictive cutpoint and the sensitivity and specificity of each biomarker for predicting MSI and severe MSI in racehorses are presented in Table S4. None of the biomarkers had an AUC at the empirical optimal cutpoint, for all timepoints combined or either timepoint separately, that was acceptable for prediction of MSI. For prediction of severe MSI, OCN and CTX1 on their own were not acceptable; however, the OCN/CTX1 ratio had acceptable discrimination, for timepoint 1 and all timepoints combined (Figure 5A). The best predictor of severe MSI was cortisol at timepoint 2, having acceptable to excellent discrimination (Figure 5B; AUC = 0.79).

6 | DISCUSSION

The differences in bone turnover and risk of MSI between local racehorses and international racehorses, after long-distance transportation were investigated in this study. Differences in the biomarker levels between the international and local horses were observed, even after adjustment for inherent differences between the two groups: international horses were older, with a greater proportion of entire males, longer racing careers, longer mean rest periods, and more time since their last rest period compared with the local horses. Cortisol, a stress marker, was higher in international horses immediately after long-distance air transportation but decreased and was not different to that of the local horses after approximately 14 days. Contrary to our hypothesis, a marker of bone resorption (CTXI), and the marker of bone formation (OCN), were lower in the international horses than in the local horses. After adjustment for horse age and sex, OCN was lower in the international horses than in the local horses only at the second timepoint. CTXI, rather than being higher as we hypothesised, was lower in injured horses at the first timepoint compared with uninjured horses. The prevalence of MSI was greater among international horses and those that sustained severe MSI were all international horses,

TABLE 4 Multivariable logistic regression model showing the association of severe musculoskeletal injury (severe MSI) with biomarkers and their differences across the two timepoints in the international horses aimed at the Victorian Spring Racing Carnival, 2017–2019.

		Severe MSI		
	Coef.	Odds ratio	95% CI	*p* value
OCN (ng/mL; log)	5.78	322.42	6.91, 15308.09	0.003
Cortisol (nmol/L; log)	−6.12	0.0022	0.00002, 0.25	0.01
Timepoint	−25.71	$6.8e^{-12}$	$1.18e^{-18}$, 0.00004	0.001
Timepoint X Cortisol	5.48	239.08	8.30, 6887.67	0.001
Horse age (years)	1.01	2.75	1.04, 7.28	0.04
Sex				
Gelding	Ref	1		
Female	1.91	6.75	0.77, 59.00	0.08
Entire males	0.76	2.14	0.21, 21.92	0.5
Area under ROC		0.9259		
Positive predictive value[a]		32.26%		
Negative predictive value[a]		98.13%		
Sensitivity[a]		83.33%		
Specificity[a]		83.33%		

Note: Model adjusted for assay batch; X represents interaction effect.
[a]Values at optimum cut-off.

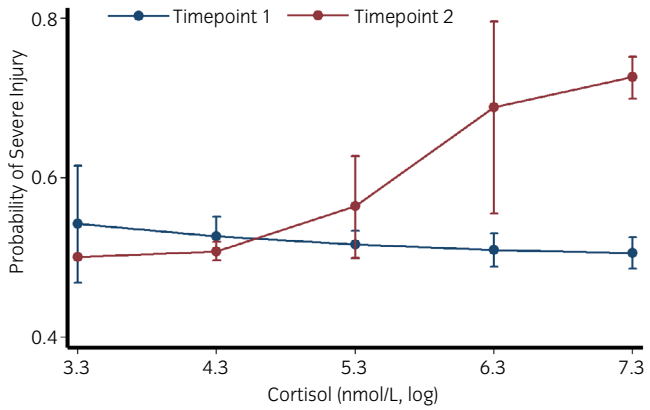

FIGURE 4 Margins plot showing probability of severe injury in the international horses by the serum concentration of cortisol (log) at timepoint 1 (blue) and timepoint 2 (red). Error bars represent 95% confidence intervals constricted at 0 to 1.

males, older in age, and had persistently high or increasing cortisol together with levels of OCN that were higher than the international horses that were not injured.

Increased cortisol is reported in horses after long-distance transport by road and in humans following air transportation.[30,31] In the international horses that had no or mild MSI, cortisol decreased over approximately 14 days indicating recovery from transportation stress. Similarly, in horses following long-distance road transport, cortisol returned to baseline level 1–7 days after travel.[22,32,33] Also consistent with our findings, basal cortisol levels are higher in males than females, in both horses and humans.[34]

Serum melatonin, adjusted for sample collection time, was not different between the international and local horses suggesting that synchronisation of the circadian rhythm of the international horses to the time zone at the destination occurred during the 3–5 days post-arrival prior to collection of the first sample. The majority of the international horses originated from Great Britain, Ireland and Europe and had travelled eastward across 9–10 time zones. Following similar travel by humans, re-establishment of the circadian rhythm takes approximately 5 days.[35,36] Taken together, the cortisol and melatonin patterns in the uninjured international horses support recovery from transportation stress by the second sample collection timepoint.

Serum amyloid A, an acute phase marker of inflammation was not different between local horses and horses undergoing long-distance air transportation in this study suggesting an absence of inflammation at 3–5 days post-transportation. Others have observed increases in SAA in horses with clinical signs of illness 1 day after transportation.[37] The half-life of SAA in horse is short, its concentration rises within 6 h after stimulation and returns to normal level within 12 h following the withdrawal of the stimulation.[38,39] Hence, travel induced inflammation may be completely absent or resolved prior to sample collection for this study. Lower SAA observed as univariable association with longer time since last race suggests the horses recover progressively from the inflammation associated with high intensity racing exercise.

Compared with the local horses, lower levels of bone turnover markers were observed in the international horses, which could mostly be explained by being older and predominantly male. Bone turnover decreases with increasing age in horses,[40,41] and we found that in this population of elite athletic horses, entire males and geldings had lower OCN and CTXI compared with females. The sex difference in bone turnover markers we observed is in contrast to a study of two-year-olds that found OCN and carboxyterminal cross-linked telopeptide (ICTP; a marker of bone resorption) were higher in male horses compared with females.[42] In humans, OCN and CTXI

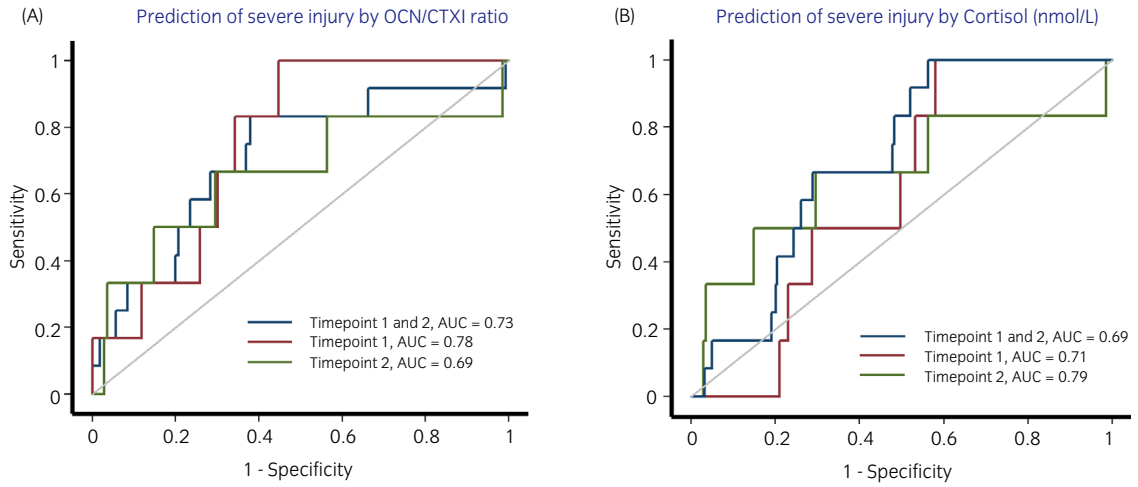

FIGURE 5 Receiver operating characteristic (ROC) curve for predicting severe injury in the international horses using the OCN/CTX1 ratio (A), and cortisol levels (B), at individual timepoints (1 and 2) or the combined timepoints.

were higher in younger (<50 years old) males relative to females but became lower in males when compared with females at an older age (>50 years old).[43,44] Age-related differences in bone turnover between the human sexes is associated with a decline in oestrogen with the onset of menopause in females.[45] All horses in the current study were elite athletes and therefore comparatively young so it is not clear whether hormonal changes associated with ageing produced the differences in bone turnover between entire male and female horses observed. We speculate that other factors such as sex related differences in training management, or higher skeletal muscle mass in males leading to greater magnitude of bone loading, may also contribute to the differences in bone turnover between male and female horses.

Osteocalcin was lower at the second timepoint in the international horses than the local horses following age and sex adjustment. This indication of decreased bone formation could be a response to higher cortisol observed in the international horses. Exposure to glucocorticoids suppresses osteoblast differentiation and bone formation which, depending on the species, can be prolonged.[46] For example, glucocorticoids suppress bone formation for 1–8 weeks in mice with the effects still present 3 months after withdrawal.[47–49] In humans, a glucocorticoid dose starts to suppress serum OCN level within 4–7 h after administration.[50] The overall effect of glucocorticoid treatment in humans is seen during an initial phase of rapid bone loss within the first 6 months followed by a later phase of reduced bone formation for up to 3 years.[51] Bone loss in the appendages was still present 1 year following glucocorticoids withdrawal in kidney transplant patients.[52] Although we have noted the association between high cortisol after long distance transportation and decreased level of the bone formation marker 14 days later in horses, the entire duration of this suppression and actual changes occurring in the bone is unknown.

Bisphosphonates are antiresorptive drugs that have been shown to suppress CTXI in horses.[53–55] We did not observe an effect of tiludronic acid on CTXI in this study probably due to the low number of horses detected with bisphosphonates, four of which were international horses, a group in which CTXI levels were already low.

Our findings that horses with MSI had a lower level of bone resorption marker compared with uninjured horses and that international horses with severe MSI had a higher level of bone formation marker is in contrast to 2-year-old horses that sustained MSI, where a higher level of the marker of bone resorption and a lower level of the marker of bone formation were observed compared with those with no MSI.[18] In two other studies of 2- and 3-year-old horses, no differences in bone turnover markers were observed between horses with fatigue bone fracture and uninjured horses.[26,56] The horses in those studies were younger and had undergone less training and racing compared with the horse population in our study which are older, have advanced racing careers and have different patterns of bone turnover.[40,57] Similarly, the sampling intervals and duration between this study and others are different. These differences in demographic, racing profiles and sampling may contribute to the different pattern of bone turnover markers observed in our study compared with elsewhere.

To our knowledge, this is the first study to find an association between cortisol, a biomarker of stress, and MSI. Higher cortisol levels are associated with increased risk of injury in female athletes and military recruits and is thought to be an indicator of training strain and psychological stress.[58–60] Cortisol alone, measured at approximately 14 days interval after long distance air transportation predicted the risk of severe MSI 79% of the time. Of the 42 horses flagged as at-risk at the second timepoint (cortisol threshold >120 nmol/L), five of those sustained a severe injury; or approximately 1 in every 8 horses flagged as at-risk based on cortisol levels. However, the test had good sensitivity, correctly classifying 5/6 (83%) of the severe injury cases, and specificity was 74%. Consistent with other studies in athletic humans and horses,[18,26,61] individual bone turnover markers were less accurate at predicting the risk of MSI, although we observed that the OCN/CTXI ratio at both timepoints predicted severe MSI 73% of

the time in the international horses that had undertaken long distance air transportation when bone turnover markers only were used to predict the risk of MSI. The sensitivity of OCN/CTXI ratio is good (83%) but specificity is low (62%). A statistical model combining bone and cartilage markers to predict the risk of MSI in 2–3-year-old horses had similar accuracy.[26]

Moreover, these statistical models combining biomarkers of bone turnover are still limited in diagnostic power. Combining multiple biomarkers together with the horse-level variables, that is, measuring both serum level of cortisol and OCN at two timepoints, and adjusting for horse age and sex, in international horses after long distance air transportation, the predictive capability of the risk of severe MSI was increased to 92%, the highest of any statistical model for predicting MSI in racehorses. However, practically, diagnostic tests would likely be implemented separately to screen at-risk horses.

The marker of inflammation (SAA) was lower in the international horses with MSI compared with other international horses, but this was not the case for horses with severe MSI. SAA has been observed to increase in racehorses after sustaining tendon/muscle injury during exercise, but no changes were observed following bone injuries consistent with our findings.[62–65]

Horses in this study that trained and raced following international air transport to Australia had higher MSI rates than local horses also competing during the VSRC. The risk of catastrophic MSI is similar between Australia and Great Britain and Ireland where the international horses that were injured originated, but they tended to be older with longer race careers and included more entire males than local horses, all of which are risk factors for catastrophic MSI.[15] Although this may explain some of the difference in injury rates, and also explained the lower markers for bone resorption, it did not explain the lower levels of a marker for bone formation in international horses 2 weeks following transport. Previous training workloads were likely different between the local and the international horses; however, we did not have access to this information. As these horses were in an unfamiliar training and racing environment it was expected that they would show some evidence of skeletal adaptation via higher OCN levels after 2 weeks especially with change of training exercise and increased exercise intensity. Adaptative bone formation tends to increase at the onset of increased exercise intensity. We speculate that the higher cortisol levels in international horses at the first time point contributed to residual suppression of bone formation up to the 2-week period.

In contrast, the international horses that developed severe MSI had higher levels of OCN as well as persistence of high cortisol. The combination of persistent stress and net bone formation suggests these horses were responding to an increase in training level and/or the presence of underlying injury. In human athletes, strenuous exercise, over exertion or the presence of bone pain are sources of stress.[66–68] In athletic horses, training or racing on unfamiliar track surfaces, change in rider, and increased exercise intensity can also elicit a stress response.[69,70] Bone modelling is consistently associated with the presence of microdamage.[12] Therefore, although the high cortisol levels may have suppressed bone formation in the absence of injury, in horses where microdamage was accumulating rapidly, regional acceleratory phenomena may override this effect.[71]

The limitations of this study should be considered. There were inherent differences in the age, sex and racing histories between the local and international horses. Training programmes were likely also different between the two groups. However, records of training were not available and therefore changes in training regimen and its effect on cortisol and other biomarkers could not be considered in the statistical analyses. Participation in the study was voluntary and the local horses were randomly selected by the principal racing authority based on their nomination for races held during the VSRC. Samples were obtained at convenience and not controlled for this study. Consequently, it was not possible to match local horses with similar age, sex and racing and training histories to the international horses. However, statistical models were adjusted for these differences; and additional international only models were generated to further explain effects. Age, sex and race career matched controls should also be considered in future studies. Though impactful, severe MSI are low in prevalence. Six cases of severe MSI, all in international horses, were recorded during the study period, therefore these findings should be interpreted with caution. The international horses had more veterinary oversight by the regulatory body than the local horses; however, there were strict reporting requirements for all the horses (local and international), therefore it is unlikely that any injury in the local horses were overlooked. Obtaining baseline blood sample(s) prior to departure from the international horse's origin country, and/or more frequent longitudinal sampling and including the horses' training records may be necessary to obtain a greater understanding of the relationship between international travel, biomarkers, MSI as well as actual changes that occur in bone. Blood samples were collected from racehorses after some may have completed their morning training routine. Samples were collected from both groups in a similar manner and in the same time frame such that any effect of pre-sampling exercise was random. Likewise, cortisol is not increased by routine exercise in more experienced horses of similar racing career to the horses sampled in this study.[72]

In conclusion, despite the inherent differences between the local horses and international horses undertaking long distance air transportation to participate in the VSRC, it was possible to identify differences in biomarker levels between the two groups that persisted after adjustment for the differences. Monitoring of markers of bone turnover, stress and inflammation in racehorses training in quarantine following long distance air transportation demonstrated that the majority of horses recovered from the effects of transport within 2 weeks apart from a lower level of a bone formation marker than local horses at 2 weeks post transport. Although this study reports associations only without implication of causation, the international horses that went on to develop severe MSI had evidence of ongoing stress and bone formation levels that did not decrease during the first 2 weeks, consistent with a response to a more intense training stimulus and/or the accumulation of microdamage. Screening athletic horses for elevated levels of cortisol and OCN may help to identify high risk of MSI among elite athletic horses.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Babatunde A. Ayodele: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; validation; visualization; writing – original draft; writing – review and editing. **Charles N. Pagel:** Supervision; writing – review and editing. **Eleanor J. Mackie:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; visualization; writing – review and editing. **Fiona Armour:** Methodology; writing – review and editing. **Sean Yamada:** Methodology; writing – review and editing. **Paul Zahra:** Methodology; writing – review and editing. **Natalie Courtman:** Methodology; writing – review and editing. **R. Chris Whitton:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review and editing. **Peta L. Hitchens:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review and editing.

DATA INTEGRITY STATEMENT

B. Ayodele had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

The study was approved by the University of Melbourne Animal Ethics Committee (Reference 1614001).

INFORMED CONSENT

Consent for sample collection was given by horses' trainers.

DATA AVAILABILITY STATEMENT

The data that support findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by the editor due to lack of provision in the owner informed consent process.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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