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Immunological, clinical, haematological and oxidative responses to long distance transportation in horses

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ABSTRACT

Horses are transported frequently and often over long distances. Transportation may represent a physiological stressor with consequential health and welfare implications. This study reports the effects of a long distance journey on immunological, clinical, haematological, inflammatory and oxidative parameters in an Experimental Group (EG) of ten horses, comparing them with six horses of similar age and breed used as a non-transported Control Group (CG). Clinical examination and blood sampling were performed twice on all horses: immediately after unloading for the EG, and at rest on the same day for the CG (day 1); at rest on the same day one week later for both groups (day 7). On day 1 EG horses showed increased heart and respiratory rates ($P < 0.01$), rectal temperature ($P < 0.05$), capillary refilling time ($P < 0.01$), neutrophil numbers ($P < 0.01$), serum albumin ($P < 0.01$), plasma total antioxidant status ($P < 0.01$), and a lower rate of mitogen induced proliferation of lymphocytes ($P < 0.05$), in comparison with CG. On day 7 only an increase in total serum protein ($P < 0.05$) and serum globulins ($P < 0.001$) was seen in the EG. No difference in serum cortisol concentration was found. Long distance transportation induced an acute phase response impairing the cell-mediated immune response. Clinical examinations, including assessing CRT and body weight loss, and the monitoring of redox balance may be useful in evaluating the impact of extensive transport events on horses. A better understanding of the link between transportation stress, the immune system and the acute phase response is likely to inform strategies for enhancing the welfare of transported horses.

Keywords:

Transportation
Acute phase response
Redox balance
Horse
Welfare

1. Introduction

Transportation is generally regarded as an exceptionally stressful episode in the life of the animal (Knowles and Warriss, 2000), and there is an increasing public interest in and concern for the welfare of livestock during transportation (Grandin and Shivley, 2015). A number of important diseases have been associated with animal transportation (Knowles and Warriss, 2000; Padalino et al., 2016b), and links between transport and health are well documented in human medicine (Cohen et al., 2014). Horses are transported for a variety of reasons, including shows, competitions, slaughter, breeding, hospitalization and leisure. Consequently they travel frequently and over long distances, and are

estimated to be the second most-travelled species after humans (Leadon et al., 1989). Journey duration has been identified as a risk factor for the development of severe transport-related diseases, such as pleuropneumonia and enterocolitis, because the risk of their occurrence increases in journeys longer than 20 h (Padalino et al., 2015). Hence, the need for research on the implications of long distance transportation for horse health and welfare has been raised (Messori et al., 2016; Padalino et al., 2016a).

Transport-induced immune-suppression has been identified as a possible cause for the development of disease during and after long journeys (Hines, 2000; Marlin, 2004; Stull et al., 2004). In horses transported for 12 h, peripheral blood neutrophilia and a reduction in neutrophil phagocytic function were evident for at least 36 h (Raidal et al., 1997). A decrease in both B and T lymphocyte numbers was found after 38 h of road transport, with a greater effect on T cells (Oikawa and Jones, 2000). Decreased lymphocyte numbers and variation in the distribution of lymphocyte subpopulations were also

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observed after a 24 h road journey by [Stull et al. \(2004, 2008\)](#), who attributed the decline in the CD8 α^+ , CD21 $^+$, CD3 $^+$, CD4 $^+$, CD8 β^+ populations to a cortisol mediated stress response ([Stull et al., 2004, 2008](#)).

Impaired cell-mediated immunity and release of cortisol have been identified as two components of the acute phase response ([Kushner, 1982](#)), which is an immune based reaction to non-specific stimuli characterized by systemic, metabolic and physiological alterations including oxidative stress and the release of acute phase proteins ([Cray et al., 2009; Fallon et al., 2001; Kushner, 1982](#)). The nexus between transportation and acute phase responses has been investigated in animals, including pigs ([Murata, 2007](#)), camels ([Baghshani et al., 2010](#)), and horses ([Casella et al., 2012](#)). In performance horses, increased fibrinogen was documented after long journeys (37 h of transportation by plane), and a plasma concentration greater than 3.2 g/L was proposed as a reliable marker for horses at risk of pleuropneumonia (a.k.a. shipping fever) ([Leadon, 2000](#)). In slaughter horses, 6 h of road transportation induced an acute phase response, characterized by increased fibrinogen and oxidative products ([Wessely-Szponder et al., 2014, 2015](#)). Increased plasma total antioxidant status (PTAS) was detected in slaughter mares after an 8 h journey ([Niedźwiedz et al., 2013](#)) and this finding was suggested to be a homeostatic mechanism to balance the production of free radicals and the acute phase response induced by transport stress ([Ishida et al., 1999; Niedźwiedz et al., 2013](#)). Transport associated alterations of oxidative balance may induce oxidative stress with cellular damage ([Kirschvink et al., 2008](#)) and increase susceptibility to disease ([McCord, 2000](#)), if not adequately mitigated by anti-oxidant responses. Thus, oxidative stress might be involved in the development of transport-related diseases, and monitoring of the redox balance by assessment of reactive oxygen metabolites (ROMs) and PTAS could be a useful tool to assess stress and disease-susceptibility, and consequently the welfare of transported horses, as already proposed for transported ewes ([Piccione et al., 2013](#)).

A holistic, multidisciplinary approach has been advocated for research on animal welfare ([Veissier and Miele, 2014](#)). The current multidisciplinary study was conducted to assess immunological, clinical, haematological, inflammatory and oxidative responses and recovery in transported horses by comparison with a group of similar horses that had not undergone transportation and to explore potential diagnostic relationships between observed responses. It was hypothesised that clinical and haematological responses reported in previous studies of transportation in horses would be recognized in transported horses in this study, but not in control horses. It was further hypothesized that transportation would be a physiological stressor able to activate an acute phase response and decreasing transported horses' immunological capacity to react to a mitogen, and that the severity of such changes might be related to clinical examination or other laboratory findings.

2. Materials and methods

2.1. Animals

Sixteen show jumping horses of different breeds (Thoroughbred, Warm blood, Australian Stock Horse) were selected for this study. The Experimental Group (EG) comprised ten horses (7 geldings, 3 mares), aged from 5 to 15 years (10.3 ± 3.2), with body condition score of 3.0 ± 0.1 ([Carroll and Huntington, 1988](#)). These horses had travelled from Perth to Glossodia (New South Wales, Australia), a distance of 4000 km, as described below. Six horses (5 geldings, 1 mare), aged from 6 to 15 years (9.7 ± 3.6), with body condition score of 3.4 ± 0.1 ([Carroll and Huntington, 1988](#)) formed the Control Group (CG). They were resident at the horse stable in Glossodia for more than two years and had not travelled in the previous three months. They were at a similar stage of fitness and competition as the transported group and there were no statistical differences between groups for age or body condition score. The health (and hence fitness for travel) of the EG horses was

assessed by an experienced equine veterinarian (not a member of the study group) before the journey in their private stalls following criteria listed in the Australian code of animal transportation (<http://www.animalwelfarestandards.net.au/land-transport/>). The health of the CG horses was assessed by another experienced equine veterinarian (BP) in the horse stall in Glossodia (New South Wales, Australia), following the same criteria. Clinical variables for EG horses prior to transportation and CG horses were within normal ranges ([Reed et al., 2003](#)).

2.2. Journey

The EG animals left Perth at 8:44 am and reached Glossodia four days later at 6:00 am. The trip consisted of four stages: Perth–Kalgoorlie (6 h), Kalgoorlie–Adelaide (24 h), Adelaide–Melbourne (9 h) and Melbourne–Glossodia (12 h). Horses were given 12 h rest periods both at Kalgoorlie and Adelaide, and a 19 h rest stop in Melbourne. The total duration was approximately 94 h with approximately 51 h in transit and 43 h for rest stops. Horses were fed and watered on route every 6 h, during the travel section from Kalgoorlie to Adelaide. In the other travel sections, water and food were offered at the rest stops. At each rest stop the fitness for travel of each horse was assessed by trained personnel following the Australian code for animal transportation, including assessment of rectal temperature, heart and respiratory rate (<http://www.animalwelfarestandards.net.au/land-transport/>).

At the collection stable and rest points, horses were individually housed in walk in-walk out rubber lined stables and paddocks that were used only for horses in transit. The animals travelled on a semi-trailer (Mega Ark Trailers, MAN®, Munich, Germany). The ventilation system comprised venturi vents, louvres and electric fans generating an airflow which the manufacturer verified was compliant with the Australian code of animal transportation (<http://www.animalwelfarestandards.net.au/land-transport/>) throughout the trailer. When the vehicle was moving, fresh air entered through the louvres and was extracted by the venturi vents. The fans were used when the truck was stationary (e.g. feeding and watering times, fuel stops) to ensure constant air flow. The horses travelled in individual stalls, restrained by rubber cords which would break under extreme pressure. The two biggest horses were allocated 1 1/2 stall spaces to allow them a more comfortable journey. Two drivers were used for the journey and both were licensed to drive heavy combination vehicles and were experienced horse handlers with many years' experience in commercial horse enterprises. At the start of the journey the temperature in Perth was 14.0 °C with humidity at 69%. The temperature and humidity in Glossodia at the end of the journey were 17 °C and 42% respectively. The journey complied with the standards and the guidelines for the transport of horses required by the Australian code and all horses passed the assessment of fitness for travel at each rest stop before continuing the journey (<http://www.animalwelfarestandards.net.au/land-transport/>).

2.3. Experimental protocol

A case-control study design was selected to allow comparison between transported horses and a similar cohort of horses that had not been travelled. Each animal was assessed at 6:00 am within 5 min of unloading for the EG and at rest for the CG (day 1), and one week later at the same time of day (day 7), at rest conditions for both groups. Clinical assessment was conducted by an experienced equine veterinarian (BP) according to the methodology suggested by [Reed et al. \(2003\)](#) and consisted of the following parameters: demeanour, mucous membrane (colour, status), capillary refilling time (CRT), heart rate (HR), respiratory rate (RR), rectal temperature (RT) and pulmonary auscultation. CRT was assessed three times in different parts of the oral mucosa (right, middle and left side of the upper jaw, above the incisors). Gastrointestinal tract motility was assessed by auscultation of gut sounds as described previously ([Sundra et al., 2012](#)). Subjective

gastrointestinal motility scores were assigned for each quadrant: score 2 indicated regular and ongoing peristaltic activity, score 1 was assigned when the period of no borborygmi was longer than the period of peristaltic sound and a score 0 was recorded when there were no gut sounds. Results from each quadrant were summed to give a total score. A score of 7–8 was deemed normal, 1–6 was classified as reduced and 0 was absent. Body weight (BW) was assessed by the same veterinarian using a horse weight tape (Strategy, Virbac, New South Wales, Australia) positioned around the horse's girth just behind the wither (Carroll and Huntington, 1988). The EG and CG horses were clinically examined daily in this manner for a further five days (day 2–day 6) after the journey. None developed clinical signs of disease.

Blood was taken from the jugular vein of all horses into five Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), three with heparin, one with EDTA and one without an anticoagulant on day 1 and day 7. Blood samples were collected while horses were at rest and standing in a tethered stall, restrained only by lead rope, at 6:00 am on day 1 and day 7. After collection, blood samples were kept at 4 °C and analyses started within 4 h.

During the study (day 1 to day 7), all horses (EG and CG) were kept on pasture during the day and stabled overnight. They were fed at the ground level with lucerne hay and commercial horse feed (EasiRide, Prydes®, Gunnedah, New South Wales, Australia) twice daily (7:00 am, 6:00 pm) and had access to water *ad libitum*. The diet was calculated individually to meet maintenance requirements (Waldron, 2012). During the trial all horses were on the same training plan consisting of three days of complete rest, followed by four days of easy work (20 min walk and 20 min trot/day).

2.4. Haematological and biochemical parameters

Haematology was performed using the Sysmex, XT-2000i cell counter analyzer. The following parameters were recorded: red blood cells (RBC) ($\times 10^{12}/L$), haemoglobin (Hb) (g/L), hematocrit (Hct) (%), platelets (PLT) ($\times 10^9/L$), white blood cells (WBC) ($\times 10^9/L$), neutrophils (N) ($\times 10^9/L$), lymphocytes (L) ($\times 10^9/L$), monocytes (M) ($\times 10^9/L$), eosinophils (E) ($\times 10^9/L$) and basophils (B) ($\times 10^9/L$). Fibrinogen was calculated by heat precipitation (Millar et al., 1971). Serum biochemistry parameters (chlorine (Cl, mmol/L), potassium (K, mmol/L), sodium (Na, mmol/L), creatine kinase (CK, U/L), total calcium (Ca, mmol/L), albumin (Alb, g/L), aminotransferase (AST, U/L) and total serum proteins (TP, g/L)) were assessed with Thermo Scientific reagents and the Konelab 20XT photometer (Thermo Fisher Scientific, Finland) with interferential filters.

2.5. Oxidative stress parameters

Plasma was obtained by centrifugation of heparin blood tubes at $1600 \times g$ for 15 min. Reactive oxygen metabolites (ROMs) and plasma total antioxidant status (PTAS) were determined in plasma by commercial kits (d-ROMs test and PAT test, respectively, H&D srl, Parma, Italy) following manufacturer's instructions using a dedicated photometer (Free Radical Analytical System 4 Evolve, H&D srl, Parma, Italy). The intra-assay co-efficient of variations (CVs) were 3.7% and 6.4% for the d-ROMs and PAT tests respectively, while the inter-assay CVs were 1.9% and 8.1% for d-ROMs and PAT tests respectively. The concentration of ROMs was expressed as U. Carr, where $1 \text{ U. Carr} = 0.08 \text{ mg H}^2\text{O}_2/\text{dl}$, PTAS was calculated using the ferric reducibility ability of plasma (FRAP) method (Benzie and Strain, 1996), with results expressed as U. Cor, where $1 \text{ U. Cor} = 1.4 \mu\text{Mol/L}$ of ascorbic acid. The degree of oxidative stress (oxidative stress index, OSI) was estimated using the ratio of ROMs/PTAS multiplied by 100 (Crowley et al., 2013).

2.6. Immunological parameters

2.6.1. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. Briefly, lithium heparin blood tubes were centrifuged at $1455 \times g$ for 20 min, buffy coats were harvested, diluted 1:3 in phosphate buffered saline (PBS), layered over Ficoll-Paque Plus (GE Healthcare) (2:4) and centrifuged at $754 \times g$ for 30 min. Harvested PBMC were washed twice in PBS ($255 \times g$, 10 min) and resuspended in warm culture medium (RPMI 1640/10% FBS/penicillin streptomycin). Cells were counted on a flow cytometer (0500-4008 Guava® easyCyte 8HT, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) using a viability dye (4000-0040 Guava ViaCount Reagent for Flow Cytometry, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA).

2.6.2. Lymphocyte proliferation

A non-radioactive method was used to assess lymphocyte proliferation (Parish, 1999). PBMC were labelled by incubating 1×10^6 cells in warm buffer (PBS/5% newborn calf serum) for 5 min at 37 °C with the fluorescent tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (5 μM) (de Silva et al., 2010) followed by two washes with cold buffer, and resuspended in warm culture medium. The cells were then plated into a 96-well plate (final concentration of $2.5 \times 10^6/\text{mL}$) and cultured in medium alone, 10 $\mu\text{g}/\text{mL}$ concanavalin A (Con A) or 5 $\mu\text{g}/\text{mL}$ pokeweed mitogen (PWM) for 4 days at 37 °C in 5% CO_2 . At the end of the culture period, samples were acquired on a flow cytometer (Guava EasyCyte 8HT, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) to determine total cell proliferation. The lipophilic dye, CFSE, is membrane permeable but is trapped within cells following cleavage into an impermeable form which binds covalently to intracellular amine groups thus increasing its intracellular retention. Fluorescence intensity is halved with each cell division of labeled cells. Thus proliferation is detected as the loss of fluorescence intensity. Lymphocyte proliferation was measured based on the lower fluorescence of cells labelled with CFSE (using GuavaSoft™ 3.1.1, Merck Millipore, MilliporeSigma, Billerica, Massachusetts, USA) and data are presented as a percentage (% CFSE^{dim}).

2.6.3. Interferon gamma (IFN γ)

Whole blood collected into lithium heparin (500 μL) was plated into 48-well flat-bottom plates containing an equal volume of either culture medium alone or 5 $\mu\text{g}/\text{mL}$ PWM, and incubated for 2 days at 37 °C and 5% CO_2 . At the end of the culture period supernatants were harvested and stored at $-80 \text{ }^\circ\text{C}$. The Equine IFN- γ VetSet Elisa kit (Kingfisher, Biotech, Inc, St. Paul, Minnesota, USA) was used to measure IFN- γ concentration in culture supernatants following manufacturer's instructions. The absorbance was read on a plate reader (Multiskan, Thermo Electron Corporation, ThermoFisher Scientific, Waltham, Massachusetts, USA) at 450 nm. Intra- and inter assay coefficients of variation were 4.2% and 5.5%, respectively. IFN- γ was expressed in ng/mL and was calculated by subtracting the concentration in medium alone from the concentration in PWM.

2.6.4. Cortisol

Cortisol concentration was assessed in serum samples by radioimmunoassay (RIA) using the ImmunChem™ Cortisol 125 kit following manufacturer's instructions (MP Biomedicals, LLC, Orangeburg, New York, USA). Sensitivity was 0.17 $\mu\text{g}/\text{dL}$ and intra- and inter assay coefficients of variation were 5.3% and 7.5% respectively. Cortisol concentration was expressed in $\mu\text{g}/\text{dL}$.

2.7. Statistical analysis

Descriptive statistics of the data were obtained using Statulator^{beta} (<http://statulator.com/descriptive.html>). Normal distribution of all

quantitative data was checked using the Anderson-Darling test, and all data were normally distributed. All data were analysed by mixed linear model using PROC mixed procedure (SAS, version 9, 1999); in the model, horse was used as random factor, to account for multiple observations. Group (EG, CG), day (day 1, day 7) and their interaction (group × day) were specified as fixed factors. When the interaction was significant, the Tukey-Kramer post hoc test was used for multiple pairwise comparisons. Results are presented as least square mean ± standard error (SE). Pearson correlations were calculated for dependent variables using PROC Corr (SAS, version 9, 1999). Significance was defined as $P < 0.05$.

3. Results

3.1. Clinical variables

Table 1 shows the summary statistics of the clinical variables of the CG and EG horses at day 1. Soon after unloading EG horses were quiet and less responsive than normal (Reed et al., 2003). Heart rates, respiratory rates and CRT were higher than normal (Reed et al., 2003). Six of ten horses evidenced decreased borborygmi (score 1 and 0) in one or more quadrants on abdominal auscultation. Three of the horses coughed during the examinations and coarse airway sounds were audible during thoracic auscultation of these horses. Coughing resolved within 24 h after the journey and none of the three affected horses showed hyperthermia during the study.

On day 7 in the EG horses, BW was significantly increased relative to results obtained immediately following transportation (day 1: 508.9 ± 24.8 kg vs day 7: 519.6 ± 24.8 kg, $P < 0.001$) and all physiological and clinical variables were within normal ranges. Clinical variables from CG horses were in the normal range on both day 1 and day 7. None of them showed clinical signs during the trial and no variation in BW was observed (day1: 622.1 ± 32.1 kg vs day7: 622.1 ± 32.1 kg, $P = 1.000$). Comparison of RT, HR, RR and CRT between groups and on days 1 and 7 is shown in Table 2. Significant differences were observed between EC and CG horses for HR, RR, RT and CRT on day 1, but not on day 7, and significant differences were observed in EG horses between day 1 and day 7.

3.1.1. Haematological and biochemical parameters

Haematological and biochemical parameters for CG and EG on day 1 and day 7 are shown in Table 3. CG haematological parameters were within the reference range (Reed et al., 2003) and there was no variation between day 1 and day 7. There was no significant interaction

(group × day) for red blood cell count, haemoglobin, or hematocrit. There was no effect of transportation on total leukocyte counts, but there was significant variation related to transportation in leukocyte sub-populations. Specifically, EG horses demonstrated increased neutrophil counts, with lower lymphocytes and eosinophil counts at day 1, and a higher number of neutrophils and basophils at day 7, relative to CG horses.

A significant effect of the interaction (day × group) was observed for total protein ($P = 0.013$), albumin ($P = 0.001$) and globulins ($P < 0.001$). At day 7 the mean total protein concentration observed in EG horses was significantly greater than that seen in these horses on unloading (day 1) or in CG horses (day 1 and 7). At day 1 EG horses showed a higher mean serum concentration of albumin than that was observed at this time in CG horses, or in the EG group at day 7. At day 7 EG horses showed a higher mean serum concentration of globulins than that was observed at both times in CG horses, or in the EG group at day 1.

A significant interaction (group × day) was observed for potassium ($P = 0.038$), with EG horses demonstrating a significantly lower serum potassium concentration following transportation (day 1), relative to values obtained for this group on day 7 or values obtained from CG horses at either time. EG horses demonstrated a significant increase in AST activity ($P = 0.008$) in samples obtained immediately after arrival (day 1) relative to results obtained on day 7 or from CG horses at either times. There were no significant effects of the interaction (group × day) on Cl ($P = 0.441$), Na ($P = 0.969$), CK ($P = 0.648$), Ca ($P = 0.600$) or fibrinogen ($P = 0.494$).

3.1.2. Oxidative stress parameters

The effect of interaction (group × day) was not significant for ROMs (CG day 1: 167.1 ± 13.9 ; CG day 7: 161.3 ± 12.0 ; EG day1: 186.8 ± 9.3 ; EG day 7: 171.4 ± 9.3 U.Carr; $P = 0.558$) or OSI (CG day 1: 4.2 ± 0.5 ; CG day 7: 4.1 ± 0.4 ; EG day 1: 4.0 ± 0.3 ; EG day 7: 4.8 ± 0.3 ; $P = 0.111$). However there was a significant effect of the interaction (group × day) on PTAS, with an increased value registered at unloading (Figure 1).

3.1.3. Immunological parameters

The effect of interaction (group × day) was significant for lymphocyte proliferation in medium alone ($P = 0.038$), PWM ($P = 0.014$) and ConA ($P = 0.029$) (Fig. 2). In unstimulated cultures, lymphocyte proliferation was significantly increased in EG horses on day 7 in comparison to day 1. Following stimulation with either PWM or ConA, proliferation was significantly lower on day 1 in relation to day 7 and CG at

Table 1

Summary statistics for the clinical parameters in Control Group (non-transported horses) and in Experimental Group (transported horses) at day1 (soon after unloading for EG and at rest CG).

Variable	Control Group (CG)				Experimental Group (EG)				Normal range ^b
	Mean	Median	Standard deviation	Range (min-max)	Mean	Median	Standard deviation	Range (min-max)	
RT (°C)	37.3	37.4	0.2	37.1–37.6	37.9	37.8	0.2	14–24	37.0–38.5
HR (bpm)	34	36	3.3	28–36	45.2	45	4.6	40–54	30–40
RR (bpm)	10.6	12	2.0	8–12	19.4	18	3.4	14–24	8–12
CRT (sec)	2	2	0	2	3.5	4	0.6	2.5–4	1–2
GITM ^a left dorsal q	2	2	2	2	1	1	1.0	0–2	2
GITM ^a left ventral q	2	2	2	2	2	2	0.8	0–2	2
GITM ^a right dorsal q	2	2	2	2	1	1	0.5	1–2	2
GITM ^a right ventral q	2	2	2	2	1.5	1.5	0.5	1–2	2
GITM ^a total score	8	8	8	8	5.5	5.5	2.3	2–8	7/8
	Summary of non-quantitative variables				Summary of non-quantitative variables				
Membrane colour	All horses had pink membranes				7 pink/2 pale pink/1 dark				Pink
Lung sound	All horses had normal lung sounds				3 abnormal/7 normal				Normal
Membrane status	All horses had moist mucous membranes				All horses had dry mucous membranes				Moist
Demeanour	All horses were alert				All horses were quiet				Alert

H: horse; RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time; GIT: gastrointestinal tract.

^a Gastrointestinal tract motility (GITM) was assessed by auscultation of all four quadrants (q) and scored as described previously (0 = no intestinal sounds, 1 = decreased borborygmi, 2 = normal borborygmi) (Sundra et al., 2012).

^b Reed et al. (2003).

Table 2 Physiological parameters in Control Group (non-transported horses) and Experimental Group (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). Data are expressed as least square mean \pm standard error (SE). Differing superscripts within rows indicate significant difference (A,B: $P < 0.01$; a,b: $P < 0.05$) (Tukey-Kramer test).

Parameter	Control Group (n = 6)		Experimental Group (n = 10)		P values			Normal range*
	Day 1	Day 7	Day 1	Day 7	Group	Day	Group \times day	
RT ($^{\circ}$ C)	37.4 \pm 0.1 ^a	37.5 \pm 0.1 ^a	37.9 \pm 0.1 ^{bb}	37.5 \pm 0.1 ^{Aa}	0.144	0.039	0.0109	37.0–38.5
HR (bpm)	34.0 \pm 1.4 ^A	34.6 \pm 1.4 ^A	45.2 \pm 1.1 ^B	35.0 \pm 1.1 ^A	0.002	0.002	<0.001	30–40
RR (bpm)	10.6 \pm 0.9 ^A	10.6 \pm 0.9 ^A	19.4 \pm 0.7 ^B	11.8 \pm 0.7 ^A	<0.001	0.004	0.004	8–12
CRT (sec)	2.0 \pm 0.1 ^A	2.0 \pm 0.2 ^A	3.5 \pm 0.1 ^B	2.2 \pm 0.1 ^A	<0.001	<0.001	<0.001	1–2

RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time.

* (Reed et al., 2003).

either time point. IFN γ production was significantly increased in EG horses on day 1 relative to day 7 (Fig. 3). In the CG, there were no significant differences between lymphocyte proliferation responses (Fig. 2) or IFN γ (Fig. 3).

3.1.4. Cortisol

There was no significant interaction (group \times day) for cortisol (CG day 1: 4.8 \pm 0.6; CG day 7: 4.2 \pm 0.6; EG day 1: 3.9 \pm 0.4; EG day 7: 4.7 \pm 0.4 μ g/dl; $P = 0.0753$).

3.1.5. Pearson correlations

Table 4 shows the results of significant Pearson correlations. Clinical variables (RT, HR, RR, CRT) were relatively strongly correlated (r from 0.5 to 0.73) and were also positively correlated with PTAS, neutrophil count and AST. Lymphocyte count and the proliferation of lymphocytes both in ConA and PWM were negatively correlated with the clinical variables, neutrophil count and AST.

4. Discussion

This multidisciplinary case-control study assessed immunological, clinical, haematological, inflammatory, and oxidative parameters in performance horses, immediately after transport and after a 7-day recovery period, and by comparison with a non-transported group of horses at the same time points. The long distance journey was associated with an acute phase response, characterized by abnormal clinical variables, neutrophilia, impaired lymphocyte responsiveness and increased PTAS

and IFN γ . In particular, the decreased lymphocyte proliferative response at unloading supports the hypothesis that a horse's immunological capacity might be decreased after a long journey. A number of clinical examination findings (RT, HR, RR, CRT) were above the normal range, as has been previously reported (Padalino, 2015; Padalino et al., 2012; Stull and Rodiek, 2000). As evident in Table 4, these parameters were positively correlated with each other, and negatively correlated with lymphocyte proliferation suggesting that the degree of insult to homeostatic mechanisms might influence the magnitude of clinical response and severity of immunological impairment. Although correlation is a weak measure of association, these preliminary findings emphasize the importance of clinical examination in assessing an individual horse's response to transportation. Thus, in agreement with the available literature, a clinical examination after arrival is recommended as a best practice to identify horses at increased risk for disease and to plan a recovery period (Leadon and Hodgson, 2014).

In this study, the decreased proliferative response to mitogen suggests that long distance transportation impairs the ability of lymphocytes to react to a stimulus (de Silva et al., 2010; Julia and Felipe, 2016). Lymphocyte proliferation is an essential feature of the adaptive immune response to antigenic stimulation as the host responds to an infectious challenge by clonal expansion of pathogen-specific cells (Felipe, 2016). ConA and PWM are plant-based mitogens that are commonly used to assess lymphoproliferative capacity (Crotty et al., 2004; Dwyer and Johnson, 1981; Kruisbeek et al., 2004). In many species, ConA is essentially a T cell mitogen and PWM stimulates proliferation of both T and B cells. While both mitogens induce a proliferative

Table 3 Haematology and serum biochemistry parameters in Control Group (non-transported horses) and Experimental Group (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). Data are expressed as least square mean \pm standard error (SE). Differing superscripts within rows indicate significant difference (A,B: $P < 0.01$; a,b: $P < 0.05$) (Tukey-Kramer test).

Parameters	Control Group (n = 6)		Transported Group (n = 10)		P values			Normal range*
	Day 1	Day 7	Day 1	Day 7	Group	Day	Group \times day	
RBC (10^{12} /L)	6.9 \pm 0.3	7.4 \pm 0.3	7.6 \pm 0.2	7.6 \pm 0.2	0.137	0.225	0.163	6.5–12.5
Hb (g/L)	118.3 \pm 5.2	126.1 \pm 5.2	127.5 \pm 4.0	127.3 \pm 4.0	0.402	0.189	0.148	110–190
HCT (%)	32.5 \pm 1.3	34.4 \pm 1.3	34.2 \pm 1.0	34.2 \pm 1.0	0.625	0.160	0.155	32–52
WBC (10^9 /L)	6.6 \pm 0.5	6.3 \pm 0.5	7.2 \pm 0.4	7.3 \pm 0.4	0.188	0.658	0.453	6.0–13.0
Neutrophils (10^9 /L)	3.7 \pm 0.4 ^{Aa}	3.4 \pm 0.4 ^A	5.4 \pm 0.3 ^B	4.8 \pm 0.3 ^{bb}	0.002	0.074	0.0483	2.4–6.9
Lymphocytes (10^9 /L)	2.2 \pm 0.2 ^A	2.3 \pm 0.2 ^A	1.3 \pm 0.1 ^B	1.8 \pm 0.1 ^A	0.006	0.042	0.028	1.6–3.4
Monocytes (10^9 /L)	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0	0.4 \pm 0.0	0.496	0.246	0.178	0.0–0.7
Eosinophils (10^9 /L)	0.3 \pm 0.0 ^{Aa}	0.3 \pm 0.0 ^{Aa}	0.0 \pm 0.0 ^B	0.1 \pm 0.0 ^{AcB}	0.008	0.005	0.015	0.0–0.9
Basophils (10^9 /L)	0.01 \pm 0.0 ^A	0.01 \pm 0.0 ^A	0.01 \pm 0.0 ^A	0.03 \pm 0.0 ^B	0.300	0.007	0.027	0.0–0.3
PLT (10^9 /L)	179.5 \pm 19.8	174.5 \pm 19.8	158.9 \pm 15.4	153.0 \pm 15.4	0.403	0.347	0.937	80.0–300.0
Fibrinogen (g/L)	2.7 \pm 0.3	2.5 \pm 0.3	3.4 \pm 0.2	2.9 \pm 0.2	0.111	0.182	0.494	2.0–4.0
Total protein (g/L)	62.0 \pm 1.6 ^a	62.0 \pm 1.6 ^a	65.8 \pm 1.3 ^a	67.9 \pm 1.3 ^b	0.026	0.166	0.013	60.0–76.0
Albumin (g/L)	35.0 \pm 1.0 ^A	35.3 \pm 1.0 ^A	37.1 \pm 0.8 ^B	32.7 \pm 0.8 ^A	0.821	0.004	0.001	29.0–38.0
Globulins (g/L)	27.0 \pm 1.5 ^A	26.6 \pm 1.5 ^A	26.6 \pm 1.2 ^A	35.2 \pm 1.2 ^B	0.017	0.000	<0.001	26.0–40.0
Cl (mmol/L)	99.8 \pm 0.8	101.5 \pm 0.8	100.6 \pm 0.6	103.2 \pm 0.6	0.146	0.002	0.444	99–110
K (mmol/L)	3.8 \pm 0.1 ^a	3.7 \pm 0.1 ^a	3.4 \pm 0.1 ^b	3.7 \pm 0.1 ^a	0.105	0.474	0.038	2.8–5.0
Na (mmol/L)	141.1 \pm 0.5	142.0 \pm 0.5	142.0 \pm 0.4	142.8 \pm 0.4	0.117	0.077	0.969	132.0–150.0
Ca (mmol/L)	3.0 \pm 0.0	3.0 \pm 0.0	3.0 \pm 0.0	3.0 \pm 0.0	0.684	0.121	0.600	2.78–3.32
CK (U/L)	176.2 \pm 16.2	193.7 \pm 16.2	175.1 \pm 12.5	205.5 \pm 12.5	0.737	0.108	0.648	<400.0
AST (U/L)	227.1 \pm 27.4 ^a	246.8 \pm 27.4 ^a	322.8 \pm 21.2 ^{bb}	283.7 \pm 21.5 ^A	0.068	0.325	0.008	<400

RBC: Erythrocytes, Hb: hemoglobin, HCT: hematocrit, WBC: white blood cells; Cl: chlorine, K: potassium, Na: sodium, CK: creatine kinase, Ca: total calcium, AST: aminotransferase.

* Normal range of the laboratory that performed the analysis.

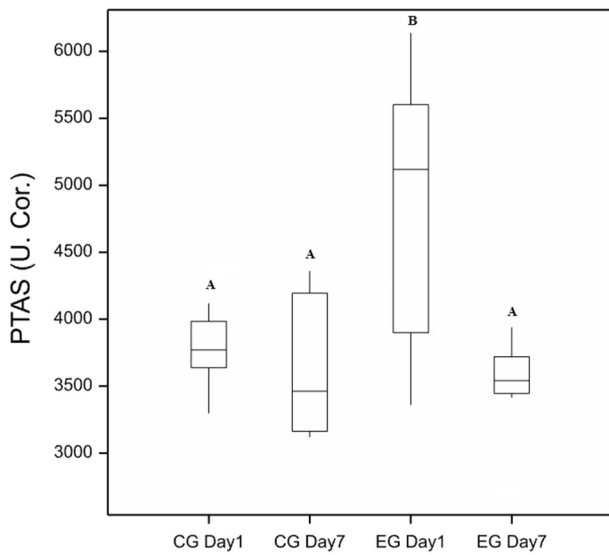


Fig. 1. PTAS in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). (A,B: $P < 0.01$)

response in equine lymphocytes (Robbin et al., 2011; Sanada et al., 1992), the specific lymphocyte subtypes that respond are not known in this species. Therefore the findings relate to the overall lymphocyte response.

As was observed in the current study, impairment of mitogen induced lymphocyte proliferation has been reported in calves after a 4-hour journey (Murata et al., 1987), in steers after a 10-hour journey (Blecha et al., 1984), in pigs transported to a fattening farm (Artursson et al., 1989), in horses transported for 38 h (Oikawa and Jones, 2000), and only in the more stress susceptible horses after 24 h journey (Stull et al., 2004). The observed reduction was transient, with complete restoration of the proliferative response observed following a 7-day recovery period. Interestingly and as previously observed by Oikawa and Jones (2000), lymphocytes from the transported horses demonstrated increased spontaneous proliferation on day 7 (Artursson et al., 1989; Oikawa and Jones, 2000). The increased mitogen-induced IFN γ response observed in the EG group on day 1 compared to day 7, was

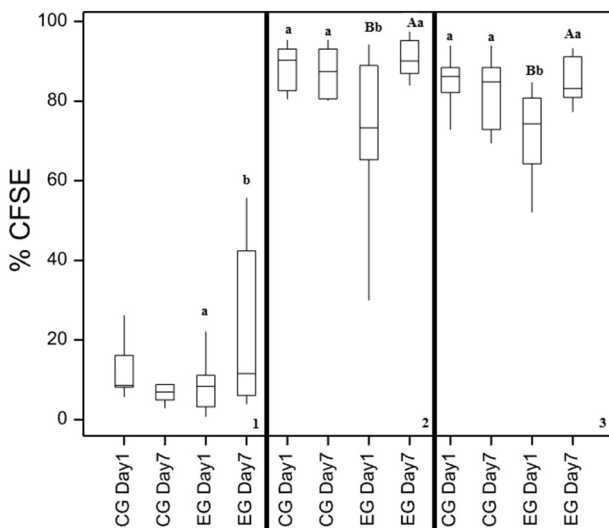


Fig. 2. Proliferation of lymphocytes in culture medium alone (1), ConA (2), PWM (3) in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey at rest for EG and at rest for CG). Different subscript shows statistical differences (A,B: $P < 0.01$; a,b: $P < 0.05$)

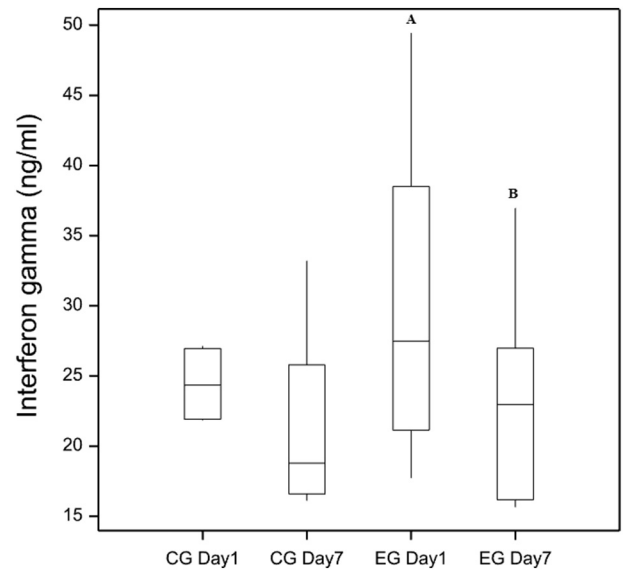


Fig. 3. PWM-induced IFN γ response in Control Group (CG) (non-transported) and Experimental Group (EG) (transported horses) at day 1 (soon after unloading for EG and at rest CG) and day 7 (7 days after the journey for EG and at rest for CG). (A,B: $P < 0.01$)

unexpected and might be related to the immunosuppressive effect of this cytokine. IFN γ can have a suppressive effect on lymphocyte proliferative responses via activation of other T cell subsets (Gajewski and Fitch, 1988; Sheng et al., 2008) or antigen presenting cells (Shimabukuro et al., 1992). As IFN γ is produced by both innate and adaptive immune cells, the cell type responsible for the observed increase warrants further investigation.

The changes in leucocyte counts observed in the current study are in line with previous studies. In horses that did not develop disease, transportation induced neutrophilia and lymphopenia without increasing the total leucocyte count (Oikawa et al., 2005; Stull et al., 2008). Such changes in leucocyte populations, and impaired leucocyte function, have been related to the transport-induced increase in cortisol (Hines, 2000; McGlone et al., 1993; Stull et al., 2004). However, no change in cortisol concentrations was found in the current study. This finding is in disagreement with the literature, as increased cortisol has been reported in many previous studies including after short journeys (Fazio et al., 2013; Tateo et al., 2012) or transportation for 12-hours (Baucus et al., 1990) or 24-hours (Stull and Rodiek, 2000; Stull et al., 2004, 2008). However limited data are available for transportation lasting several days. Schmidt et al. (2010) evaluated salivary cortisol in response to road transport over 1370 km and 2 day transport eight days later, and showed that cortisol peaked at mid-transport and then tended to decrease with each day of transport. The authors suggested that this response was likely due to a degree of adaptation. In our study, cortisol was not determined prior to or during the journey, so it is not possible to know whether the normal values observed on arrival represent an adaptive response (due to negative feedback inhibition after prolonged stress, or due to habituation of the animal to the stressor (Moberg and Mench, 2000)) or whether there was no observable increase in cortisol attributable to transportation in this study, as has been reported in some previous studies (Söder et al., 2012). In pigs transported for 24 and 48 h, no differences in cortisol were found at unloading, and the authors commented that cortisol concentrations may return to baseline levels before the end of the journey if the transport is of long duration (Pineiro et al., 2007).

In contrast with previous studies (Stull and Rodiek, 2000; Tadich et al., 2015), no variations in RBC, hematocrit or haemoglobin were observed in the current study. Increased red cell mass following journeys of shorter duration has been attributed to transport-induced catecholamine release and/or dehydration (Stull and Rodiek, 2000; Tadich et

Table 4
Pearson correlation (expressed as value of r) between the dependant variables which reported at least one significant correlation ($P < 0.05$)

	RT	HR	RR	CRT	PTAS	N	L	TP	K	ALB	Glo	AST	IFN γ	Media	PWM	ConA
RT	*															
HR	0.503***	*														
RR	0.630***	0.733***	*													
CRT	0.622***	0.742***	0.767***	*												
PTAS	0.344*	0.527***	0.679***	0.420**	*											
N	0.213ns	0.566***	0.542***	0.468***	0.277ns	*										
L	-0.352*	-0.370*	-0.486***	-0.602***	-0.300ns	-0.352*	*									
TP	-0.039ns	0.238ns	0.142ns	0.105ns	0.047ns	0.649***	-0.207ns	*								
K	-0.243ns	-0.316ns	-0.440**	-0.431**	-0.460***	-0.269ns	0.403*	-0.232ns	*							
Alb	0.235ns	0.439**	0.408*	0.346*	0.408*	0.174ns	-0.030ns	0.124ns	-0.440*	*						
Glo	-0.171ns	-0.041ns	-0.109ns	-0.287ns	-0.199ns	0.478***	-0.030ns	0.819***	0.047ns	-0.466***	*					
AST	0.564***	0.487***	0.669***	0.508***	0.615***	0.397*	-0.333ns	-0.036ns	-0.287ns	-0.021ns	-0.019ns	*				
IFN γ	0.217ns	0.303ns	0.284ns	0.146ns	0.373*	0.109ns	-0.034ns	-0.489**	-0.027ns	-0.037ns	-0.228ns	0.404*	*			
Media	-0.417*	-0.166ns	-0.249ns	-0.169ns	-0.135ns	0.077ns	0.020ns	0.223ns	0.201ns	-0.198ns	0.313ns	-0.262ns	-0.155ns	*		
PWM	-0.577**	-0.546***	-0.615***	-0.571***	-0.241ns	-0.371*	0.239ns	-0.090ns	0.411*	-0.223ns	0.048ns	-0.442*	-0.108ns	0.4935**	*	
ConA	-0.466**	-0.524**	-0.615***	-0.551***	-0.193ns	-0.525**	0.285ns	-0.138ns	0.449**	-0.350*	0.079ns	-0.364*	-0.042ns	0.423*	0.876***	*

RT: rectal temperature; HR: heart rate; RR: respiratory rate; CRT: capillary refilling time; PTAS: plasma total antioxidant status; N: Neutrophils; L: Lymphocytes; TP: total proteins; K: potassium; Alb: albumin; Glo: globulins; IFN γ : PWM-induced IFN γ concentration; Media: proliferation of lymphocytes in culture medium alone; ConA: proliferation of lymphocytes in culture with ConA; PWM: proliferation of lymphocytes in culture with PWM.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

et al., 2015). At unloading, our horses were very quiet and less responsive, suggesting that the acute catecholamine response to transportation had waned, due to depletion of catecholamine or habituation to external stimuli for coping with the long journey (Alexander and Irvine, 1998; Moberg and Mench, 2000).

Changes on clinical examination and serum biochemistry parameters were expected based on previous studies. It is reported that transportation causes increased rectal temperature, heart rate and respiratory rate, decreased intestinal peristalsis (Oikawa et al., 2005), and variation in plasma concentrations of AST and potassium (Codazza et al., 1974). These changes have been interpreted as an inevitable response to transportation associated with catecholamine release, muscle work and thermoregulation (Leadon et al., 1991; Padalino et al., 2012). In EG horses, CRT, which has previously been suggested as the most reliable welfare indicator for adequate water intake or absence of prolonged thirst (Dalla Costa et al., 2014), was much higher at unloading than in CG. This suggests that animals suffered from thirst, thus watering on route every 4.5 h might be recommended in Australia as already applied in Europe (Anon, 2014). The EG animals increased their body weight (BW) at day 7 by approximately 2%. Dehydration and weight loss have been considered as the most common consequences of transportation, with transport-related weight loss ranging from 0.10 to 0.63% BW per hour of journey and recovery time usually within three days of arrival (Marlin, 2004). As serum total proteins and albumin were still within their normal range, a severe degree of dehydration was not present (Reed et al., 2003). Thus, the observed changes in CRT and body weight in this study might be considered as common and mild reactions to transit stress and limitations to water intake. The monitoring of CRT and assessment of body weight by tape following transportation are easy practices which all owners and agents could perform before and after journey, and are therefore recommended as readily implemented measures to assess the recovery of horses following transportation.

Our preliminary results on the effects of a long journey on the immune system are interesting because transport-related diseases usually become clinically evident in the week after the transport. In our study, transport-induced changes reversed by day 7 and no animals became unwell. Our data suggest that a rest period is fundamental for the immune system to recover and to react to the pathogens to which animals may be exposed during the journey (Raidal et al., 1997) and in the new environment after arrival (Herholz et al., 2008). It is therefore better to avoid adding further stress such as a strenuous exercise (e.g. racing or competition), soon after unloading. The ideal rest duration after long hauling warrants future research, as interim data were not collected between day 1 and day 7 in the current study.

The reported changes in albumin, fibrinogen and globulins might be interpreted as a transport induced inflammatory response (Cray et al., 2009; Crisman et al., 2008). The higher value of albumin recorded at unloading might be associated with dehydration or haemoconcentration (Stull and Rodiek, 2000). The lower value registered at day 7 might be explained because homeostatic mechanisms have recovered post-transportation hydration indices, or because albumin is considered a negative acute phase response (APR) protein (Crisman et al., 2008). Globulin concentration was increased on day 7 in the EG group, consistent with increased positive APPs (Cray et al., 2009; Crisman et al., 2008). In our study, there was no significant variation in fibrinogen, but it is worth noting that the average value for EG horses at unloading was higher than 3.2 g/L, which was proposed by Leadon as an indicator of shipping fever (Leadon et al., 1991); in particular the three horses with abnormal pulmonary auscultation at unloading had a fibrinogen concentration of 4 g/L. Pre-transportation fibrinogen values were not determined in the current study. Further studies including serum protein electrophoresis and repeated determination of specific APPs might enhance the understanding of the link between transit stress and APPs in horses.

The reported changes in the oxidative/antioxidative parameters, PTAS and ROMs, should also be interpreted as components of the acute phase response induced by transport stress (Onmaz et al., 2011; Wessely-Szponder et al., 2015). We did not find a significant increase in ROMs, as reported in transported ewes (Piccione et al., 2013), however, peaks in oxidative products may be observed one or two days after transportation (Nazifi et al., 2009; Piccione et al., 2013; Wessely-Szponder et al., 2015), suggesting serial sampling during recovery might be considered in future studies. The highest ROMs values were observed on day 1 in EG horses in the current study, with a simultaneous and marked increase of PTAS, which was almost double the normal range (Kusano et al., 2016). The latter could be interpreted as an antioxidant response to the increased production of free radicals for avoiding oxidative stress (Celi, 2011; Niedźwiedź et al., 2013). This extremely high value of PTAS could be related also to the marked increase in serum albumin concentrations on day 1 in EG horses. Albumin is indeed assessed within PTAS as the sulfhydryl (SH) groups of albumin are considered a significant element of extracellular antioxidant defense system (Celi, 2011). However, it has been estimated that proteins contribute only 10% of PTAS values, while uric acid, ascorbic acid and α -tocopherol contribute for 60, 5 and 5%, respectively (Benzie and Strain, 1996). Thus, our data are in agreement with the literature suggesting that transportation induces an increase in PTAS, likely due to mobilization of antioxidants (Niedźwiedź et al., 2013). It has been reported that animals under stress mobilize antioxidants to balance the increased production of free radicals (Sconberg et al., 1993), thereby avoiding oxidative damage to the cells (Ralston and Stives, 2012). Oxidative stress has been implicated in numerous disease processes in human and veterinary medicine and oxidative parameters have been proposed as biomarkers to identify animals at risk of diseases (Kirschvink et al., 2002, 2008; Soffler, 2007). In particular, PTAS was proposed as useful indicator of stress in transported calves, since it may be more sensitive and reliable than the measure of single antioxidant parameters (Pregel et al., 2005). Consequently, monitoring of PTAS and ROMs pre and after journey might be recommended as best practices to identify animals at risk of oxidative stress, and consequently poor health and welfare outcomes. In further studies, it would be interesting to determine if depletion of antioxidants might be a cause of the development of oxidative stress and transport-related diseases.

Contrary to previous reports (Leadon, 2000; Marlin et al., 2011; Messori et al., 2016; Oikawa et al., 2005), no horse in the current study developed pyrexia or other clinical complications, demonstrating that transportation over long distances without adverse health complications is possible when management is consistent with the Australian code of animal transportation (<http://www.animalwelfarestandards.net.au/land-transport/>) and current best practice recommendations (Kohn, 2000; Padalino, 2015). Since we did not have any sick horses, our results need to be confirmed in future studies comparing healthy and unhealthy horses after being transported for long distance, with all parameters measured before and after transportation.

Our results are preliminary and should be interpreted with caution because this study has a number of limitations. As in other studies (Pineiro et al., 2007), the lack of sampling prior to and during the journey means that the effects of transportation on measured variables can only be inferred. However, as animals are often not assessed prior to transport in a 'real-world' setting, reliable welfare indicators were recently proposed only at unloading (Messori et al., 2016), and the current study was designed to allow comparison with a non-transported control population and to assess recovery following arrival. Determinations of other important APPs, such as C-reactive protein, haptoglobin or serum amyloid A, or other stress hormones, such as catecholamine, were also not included in the current study. Assessing a greater number of horses would also be beneficial. Notwithstanding these limitations, this study has enhanced the understanding of the implications of long transportation for horse health and welfare using a multidisciplinary approach.

5. Conclusions

Overall, this multidisciplinary case-control study suggests that long distance transportation was associated with an acute phase response characterized by neutrophilia, hyperglobulinemia, increased IFN γ and PTAS and an impairment of the immune system evidenced by reduced lymphocyte responsiveness. Recovery was evident by 7 days after arrival, by which time most values were not different to non-transported control horses. Transport was also associated with clinical changes, including prolonged CRT and mild weight loss. Clinical examination, including assessing dehydration by CRT and body weight by tape, and the monitoring of redox balance are proposed as useful means to evaluate the effect of transport on horse health and welfare and to ensure optimal recovery of horses following transportation. Further studies are needed to investigate the duration of transport-induced immunosuppression and to ascertain whether this impairment of the immune system and antioxidant depletion after transportation may be linked with the development of transport-related diseases.

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Author Contributions

Barbara Padalino, Nicole Carter and Kumudika de Silva conceived the experiment; Barbara Padalino collected the data; Barbara Padalino and Nicole Carter analysed the samples, Barbara Padalino and Sharanne Raidal analyzed the data and wrote the paper; Pietro Celi, Gary Muscatello, Nicole Carter, Leo Jeffcott and Kumudika de Silva edited the paper.

Conflicts of Interest

The authors declare no conflict of interest.

Ethical considerations

The ethical aspects of this study have been approved by the University of Sydney Animal Ethics Committee (AEC) (Project Number 2015/950).

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