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

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My hobbies

- Horse rider/driver
- Race horse owner

My Jobs

- Horse breeder
- Equine veterinarian

My passions

- Research on horse welfare
- Transportation stress
The effects of transportation on equine behaviour, health status and welfare

- Transportation causes physical and mental stress
- Transportation may lead to the development of health problems
- Transportation is one of the major causes of economical loss in the equine industry
- Transportation is a matter of welfare concern
Transport-related health problems

› Dehydration
› Fatigue and poor performance
› Diarrhoea
› Heat stroke
› Muscular problems
› Laminitis
› Colic
› Enterocolitis
› Inflammation of airways
› Transport pneumonia
Risk factors

- Journey duration
- Season
- Pre-existing subclinical and clinical diseases
- Breed
- Type of vehicle
- Management
✓ Management of transport is a key factor for reducing transport stress
✓ A number of codes for animal transportation have been developed worldwide in the last decade to minimize transport stress and suffering of travelling horses
SB8.1 A person in charge must ensure time off water does not exceed the time periods given below for each class of horse:

<table>
<thead>
<tr>
<th>Class</th>
<th>Maximum time off water (hours)</th>
<th>Minimum Spell duration (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horses over 6 months old</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>Lactating mares</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Foals less than 6 months old</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Mares known to be more than 7.5 months pregnant</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Food and water

GB8.8 Adult horses should be fed and watered at floor level every five hours and as soon as possible after unloading, with a suitable quality and quantity of feed and water to minimise colic risk.
Gap of knowledges

Why am I sick and my journey mates are fine??
Effect of transport on the immune system

- **Transport-induced immune-suppression** is a possible cause for the development of disease (Hines, 2000; Marlin, 2004; Stull et al., 2004).

- 12 hour journey causes peripheral blood neutrophilia and a reduction in neutrophil phagocytic function (Raidal et al., 1997).

- 38 hours of road transport causes a decrease in both B and T lymphocyte numbers (Oikawa and Jones, 2000).

- 24 hour road journey decreases lymphocyte numbers with a decline in CD8α⁺, CD21⁺, CD3⁺, CD4⁺, CD8β⁺ (Stull et al., 2004, 2008).

The long journey affected my immune system!
The acute phase response 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).

Impaired cell-mediated immunity and release of cortisol have been identified as two components of the acute phase response (Kushner, 1982).

Transportation induces an acute phase response in animals (pigs (Murata, 2007), camels (Baghshani et al., 2010), and horses (Casella et al., 2012)).

What does “acute phase response” mean?
Useful biomarkers

- **Fibrinogen** increases after long journeys, 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).

- **Plasma total antioxidant status (PTAS)** increases after journey as a homeostatic mechanism to balance the production of free radicals and the acute phase response induced by transport stress (Ishida et al., 1999; Niedźwiedź et al., 2013).

- Oxidative stress might be involved in the development of transport-related diseases

- **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).
It was 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.

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.
Materials and methods: Animals

› **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 formed **the experimental group (EG)** and travelled from Perth to Glossodia (New South Wales, Australia), a distance of 4,000 km.

› **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 formed **the control group (CG)**. They were resident at the horse stable in Glossodia and had not travelled in the previous three months.

› The health (and hence fitness for travel) of the EG and CG horses was assessed following the criteria of the Australian Code
Materials and methods: Journey

- The trip consisted of four stages: Perth-Kalgoorlie (six hours), Kalgoorlie-Adelaide (24 hours), Adelaide-Melbourne (nine hours) and Melbourne-Glossodia (12 hours).
- Horses were given 12 hour rest periods both at Kalgoorlie and Adelaide, and a 19 hour rest stop in Melbourne.
- The total duration was approximately 94 hours with approximately 51 hours in transit and 43 hours for rest stops.
- Horses were fed and watered on route every 6 hours, during the travel section from Kalgoorlie to Adelaide. In the other travel sections, water and food were offered at the rest stops.
- At the collection stable and rest points, horses were individually housed in walk in-walk out rubber lined stables and paddocks.
Materials and methods: Journey

» The animals travelled on a semi-trailer (Mega Ark Trailers, MAN®).
» The ventilation system comprised venturi vents, louvres and electric fans.
» The two biggest horses were allocated 1½ stall spaces.
» Two drivers with good horse handling and driving skills.
» The journey complied with the Australian Code
» All horses passed the assessment of fitness for travel at each rest stop before continuing the journey
» At departure in Perth, the temperature was 14.0°C with humidity at 69%. At arrival in Glossodia the temperature was 17°C with humidity at 42%.
Materials and methods: experimental protocol

- Each animal was assessed and blood was taken at 6:00 am within 5 minutes 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 and consisted of the following parameters: demeanour, mucous membrane (colour, status), capillary refilling time (CRT), heart rate (HR), respiratory rate (RR), rectal temperature (RT), gut and lung sounds auscultation.

- Body weight (BW) was assessed using a horse weight tape.
Materials and methods: experimental protocol

- 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®, Australia) twice and **water ad libitum**.
- 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 minute walk and 20 minute trot/day).
- 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.**
Materials and methods: haematological and biochemical parameters

✓ **Blood Cells Count** (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) \(\times 10^9/L\)) was performed using the Sysmex, XT-2000i cell counter analyzer.

✓ **Serum biochemistry** parameters (chlorine (Cl, mmol/L), potassium (K, mmol/L), sodium (Na, mmol/L), total calcium (Ca, mmol/L), creatine kinase (CK, U/L), aminotransferase (AST, U/L), albumin (Alb, g/L) and total serum proteins (TP, g/L)) were assessed with Thermo Scientific reagents and the Konelab 20XT photometer.

✓ **Fibrinogen** was calculated by heat precipitation (Millar et al., 1971).
Reactive oxygen metabolites (d-ROMs) and plasma total antioxidant status (PTAS) were determined in plasma by commercial kits (d-ROMs test and PAT test) following manufacturer’s instructions using a dedicated photometer (Free Radical Analytical System 4 Evolvo).

- The concentration of d-ROMs was expressed as $\text{U. Carr}$, where $1 \text{ U. Carr} = 0.08 \text{ mg H}_2\text{O}_2/\text{dl}$,
- The concentration PTAS was expressed as $\text{U.Cor}$, where $1 \text{ U.Cor} = 1.4 \text{ µMol/L of ascorbic acid}$.
- The degree of oxidative stress (oxidative stress index, OSI) = $\frac{\text{d-ROMs}}{\text{PTAS}} \times 100$
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation.

Labelled with the fluorescent tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (5 μM) (de Silva et al., 2010).

Cultured in medium alone, 10 μg/mL concanavalin A (Con A) or 5 μg/mL pokeweed mitogen (PWM) for 4 days.

Samples were acquired on a flow cytometer (Guava EasyCyte 8HT) to determine total cell proliferation.

Fluorescence intensity is halved with each cell division of labeled cells. Thus proliferation is detected as the loss of fluorescence intensity.

Lymphocyte proliferation is presented as a percentage (% CFSE\text{dm}).
Materials and methods: *Interferon gamma (IFNγ) and cortisol*

› Whole blood was cultured with an equal volume of either culture medium alone or 5 μg/mL PWM, and incubated for 2 days.

› The **Equine IFN-γ VetSet Elisa kit** (Kingfisher, Biotech, USA) was used to measure IFN-γ concentration in culture supernatants.

› IFN-γ was calculated by subtracting the concentration in medium alone from the concentration in PWM (ng/mL).

› 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) (µg/dL).
Materials and Methods: statistical analysis

- **Descriptive statistics** of the data were obtained using Statulator\textsuperscript{beta}.

- 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 (day1, day7) and their interaction (group×day) were specified as fixed factors.

- 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.
## Results: clinical variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (CG)</th>
<th>Experimental group (EG)</th>
<th>Normal range**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>RT (°C)</td>
<td>37.3</td>
<td>37.4</td>
<td>0.2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>34</td>
<td>36</td>
<td>3.3</td>
</tr>
<tr>
<td>RR (bpm)</td>
<td>10.6</td>
<td>12</td>
<td>2.0</td>
</tr>
<tr>
<td>CRT (sec)</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>GITM* left dorsal q</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>GITM* left ventral q</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>GITM* right dorsal q</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>GITM* right ventral q</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>GITM* total score</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

### Summary of non-quantitative variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (CG)</th>
<th>Experimental group (EG)</th>
<th>Normal range**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane colour</td>
<td>All horses had pink membranes</td>
<td>7 pink/ 2 pale pink/1 dark</td>
<td>pink</td>
</tr>
<tr>
<td>Membrane status</td>
<td>All horses had moist mucous membranes</td>
<td>All horses had dry mucous membranes</td>
<td>moist</td>
</tr>
<tr>
<td>Lung sounds</td>
<td>All horses had normal lung sounds</td>
<td>3 horses had abnormal lung sounds/ 7 had normal lung sounds</td>
<td>moist</td>
</tr>
<tr>
<td>Demeanour</td>
<td>All horses were alert</td>
<td>All horses were quiet</td>
<td>alert</td>
</tr>
</tbody>
</table>
## Results: Haematological Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group (n=6)</th>
<th>Transported Group (n=10)</th>
<th>P Value</th>
<th>Normal range*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 1</td>
<td>Day 7</td>
</tr>
<tr>
<td>RBC (10^{12}/L)</td>
<td>6.9±0.3</td>
<td>7.4±0.3</td>
<td>7.6±0.2</td>
<td>7.6±0.2</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>32.5±1.3</td>
<td>34.4±1.3</td>
<td>34.2±1.0</td>
<td>34.2±1.0</td>
</tr>
<tr>
<td>WBC(10^9/L)</td>
<td>6.6±0.5</td>
<td>6.3±0.5</td>
<td>7.2±0.4</td>
<td>7.3±0.4</td>
</tr>
<tr>
<td>Neutrophils (10^9/L)</td>
<td>3.7±0.4^{Aa}</td>
<td>3.4±0.4^{A}</td>
<td>5.4±0.3^{B}</td>
<td>4.8±0.3^{Bb}</td>
</tr>
<tr>
<td>Lymphocytes(10^9/L)</td>
<td>2.2±0.2^{A}</td>
<td>2.3±0.2^{A}</td>
<td>1.3±0.1^{B}</td>
<td>1.8±0.1^{A}</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.7±0.3</td>
<td>2.5±0.3</td>
<td>3.4±0.2</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>62.0±1.6^{a}</td>
<td>62.0±1.6^{a}</td>
<td>65.8±1.3^{a}</td>
<td>67.9±1.3^{b}</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35.0±1.0^{A}</td>
<td>35.3±1.0^{A}</td>
<td>37.1±0.8^{B}</td>
<td>32.7±0.8^{A}</td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>622.1±32.1</td>
<td>622.1±32.1</td>
<td>508.9±24.8^{A}</td>
<td>519.6±24.8^{B}</td>
</tr>
<tr>
<td>Cl (mmol/L)</td>
<td>99.8±0.8</td>
<td>101.5±0.8</td>
<td>100.6±0.8</td>
<td>103.2±0.6</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>3.8±0.1^{a}</td>
<td>3.7±0.1^{a}</td>
<td>3.4±0.1^{b}</td>
<td>3.7±0.1^{a}</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>141.1±0.5</td>
<td>142.0±0.5</td>
<td>142.0±0.4</td>
<td>142.6±0.4</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>3.0±0.0</td>
<td>3.0±0.0</td>
<td>3.0±0.0</td>
<td>3.0±0.0</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>176.2±16.2</td>
<td>193.7±16.2</td>
<td>175.1±12.5</td>
<td>205.5±12.5</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>227.1±27.4^{a}</td>
<td>246.8±27.4^{a}</td>
<td>322.8±21.2^{Bb}</td>
<td>283.7±21.5^{A}</td>
</tr>
<tr>
<td>Cortisol (µg/dL)</td>
<td>4.8 ± 0.6</td>
<td>4.2 ± 0.6</td>
<td>3.9 ± 0.4</td>
<td>4.7 ± 0.4</td>
</tr>
</tbody>
</table>
The Lymphocyte proliferation rate was significantly lower at unloading.
Results: IFNγ

IFNγ was significantly higher at unloading
No variations for d-ROMs and OSI were found, while a marked increased of PTAS was registered at unloading.
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.

Clinical variables might predict the status of the immune system.
Conclusions

› This multidisciplinary case-control study suggests that long distance transportation was associated with an acute phase response characterized by neutrophilia, hyperglobulinemia, increased IFNγ and PTAS and an impairment of the immune system evidenced by reduced lymphocyte responsiveness.

› The decreased lymphocyte proliferative response at unloading supports the hypothesis that a horse’s immunological capacity might be decreased after a long journey.

› Transport was also associated with clinical changes, including prolonged CRT and mild weight loss.

› Recovery was evident by 7 days after arrival.
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 whether the administration of immuno-modulators or antioxidants prior to journey might decrease the incidence of transport-related diseases.
Clinical examinations before and after journey are recommended as best practices!
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https://authors.elsevier.com/a/1UUYT7sTTuyOo
Acknowledgments

› Animal Transport Association
› Combined Horse Transport
› Goldners Horse Transport
› Owners and horses
May we organise a similar experiment on the effects of transportation by air?
Thanks for listening!!!

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