Immunophysiological responses of horses to a 12-hour rest during 24 hours of road transport

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Thirty-eight mature horses were assigned to one of two equal groups to evaluate two treatments consisting of either 24 hours of continuous road transport (24T) or two 12-hour periods of transport separated by off-loading, resting and feeding the horses for 12 hours (12/12T). A subset of six horses from each group served as controls for the other group. The horses were loaded into a commercial straight-deck trailer and travelled loose in one of two standard-sized compartments. After the journeys the horses were put back into their paddocks for a 24-hour recovery period. Venous blood samples were collected before loading, after unloading and after the 24-hour recovery period. Transport significantly increased the horses' cortisol concentrations, neutrophil counts and neutrophil:lymphocyte (NL) ratios, and decreased the numbers of all the lymphocyte subpopulation cell types. Collectively, no significant differences were observed between the two treatments in the horses' cortisol concentrations, total leucocyte counts, neutrophil and lymphocyte counts, NL ratios, and the CD8a+ and CD21+ lymphocyte subpopulations, but there were differences in the numbers of CD3+, CD4+, and CD8b+ subpopulations. The inclusion of a 12-hour rest-stop interrupted the transport-related decline in the lymphocyte subpopulations and allowed them to recover towards their resting levels.

HORSES often travel long distances by road for breeding, competitions, sales and for processing as food for human consumption. There have been few studies of the effects of a rest-stop during a long journey by road, and it is possible that the immunophysiological responses observed during long journeys may be affected by a mid-journey rest. The aim of this study was to examine the effect of a 12-hour stop for rest and feeding on the immunophysiological responses of horses transported by road for 24 hours.

MATERIALS AND METHODS

Animals and study design

Thirty-eight mature, light horses with a mean (sd) weight of 452 (45 kg) and of various breeds and unknown histories were purchased by a commercial dealer who specialised in slaughter-type horses; they were transported to a facility near Lubbock, Texas, approximately six days before the study. There were 25 mares and 13 geldings whose dentition-estimated ages ranged from three to 25 (11·9 [1·0]) years and whose body condition scores ranged between 3 and 6 (4·9 [0·1]; Henneke and others 1983). They were identified by numbered collars, divided by sex and ranked by age, and randomly allocated into two equal groups (1 and 2) based on sex and age ranking. A subset of six horses from each group was selected by similar randomisation criteria to serve as controls for the other group. Before and during the study, the groups were kept in separate dirt paddocks (50 x 50 m) and fed grass hay ad libitum and a grain concentrate mix daily. Water was freely available. The horses' diets were not analysed.

Two transport treatments were evaluated. They consisted of either 24 hours of continuous transport (24T) or two 12-hour periods separated by offloading, resting and feeding for 12 hours (12/12T). Four trips were scheduled, each trip consisting of transporting one of the two groups of 19 horses by one of the two treatments, so that each horse experienced each treatment. Group 1 was transported by 24T on April 1, followed by 12/12T on April 22; group 2 was transported by 12/12T on April 8, followed by 24T on May 1. The control horses remained in their paddock with their group during the transport of the other group and all the horses remained in their paddocks between trips. The mean maximum and minimum temperatures for Lubbock during the sampling days were 24 (0·5)°C and 8 (0·5)°C (National Weather Service 2004).

Transport protocol

A commercial straight-deck trailer (2003, Wilson, straight-deck, slat-sided livestock) pulled by a semi-tractor (2003, Peterbilt, three-axle, conventional, extended head) was used for all the journeys. Plywood panels lined the inside walls of the trailer to a height of 2·5 m. Two compartments were used in the trailer, with the front compartment (4·5 x 2·4 m) holding nine horses allowing 1·2 m^2 per horse, and the back compartment (5·3 x 2·4 m) containing 10 horses with 1·3 m^2 per horse. The horses travelled in the same compartment on each trip. They were not fed or watered while in transit, and no bedding was provided on the floor of the trailer.

The horses were loaded into the trailer at approximately 08.30. During the 24-hour trips the horses in group 1 travelled 1705 km and those in group 2 travelled 1808 km. After the journeys the horses were unloaded and put back in their paddocks for a 24-hour recovery period. During the 12/12T trips the horses in group 1 travelled 1655 km and those in group 2 travelled 1671 km, equal distances being travelled during each 12-hour period. Between the two parts of the journey the horses were unloaded at 20.30 at the Texas Tech University cattle feedlot facility and put into large paddocks for the 12-hour rest period, during which they were provided with hay and water ad libitum. For the second part of the journey they were loaded into the trailer at approximately 08.30 the next day. At the end of this journey, at 20.30, the horses were unloaded and put in their paddocks for a 24-hour recovery period. Each horse was observed regularly for changes in appetite and signs of disease throughout the journeys and during the rest and recovery periods.

Collection of physiological data

The horses were gathered in a chute system for the collection of blood samples and for loading. Blood samples were taken from the 24T horses before they were loaded, immediately after they were unloaded, and 24 hours after the journey. Blood samples were taken from the 12/12T horses before they were loaded, immediately after they were unloaded at the Texas Tech facility, before they were reloaded for the second 12-hour trip, immediately after they were unloaded, and 24

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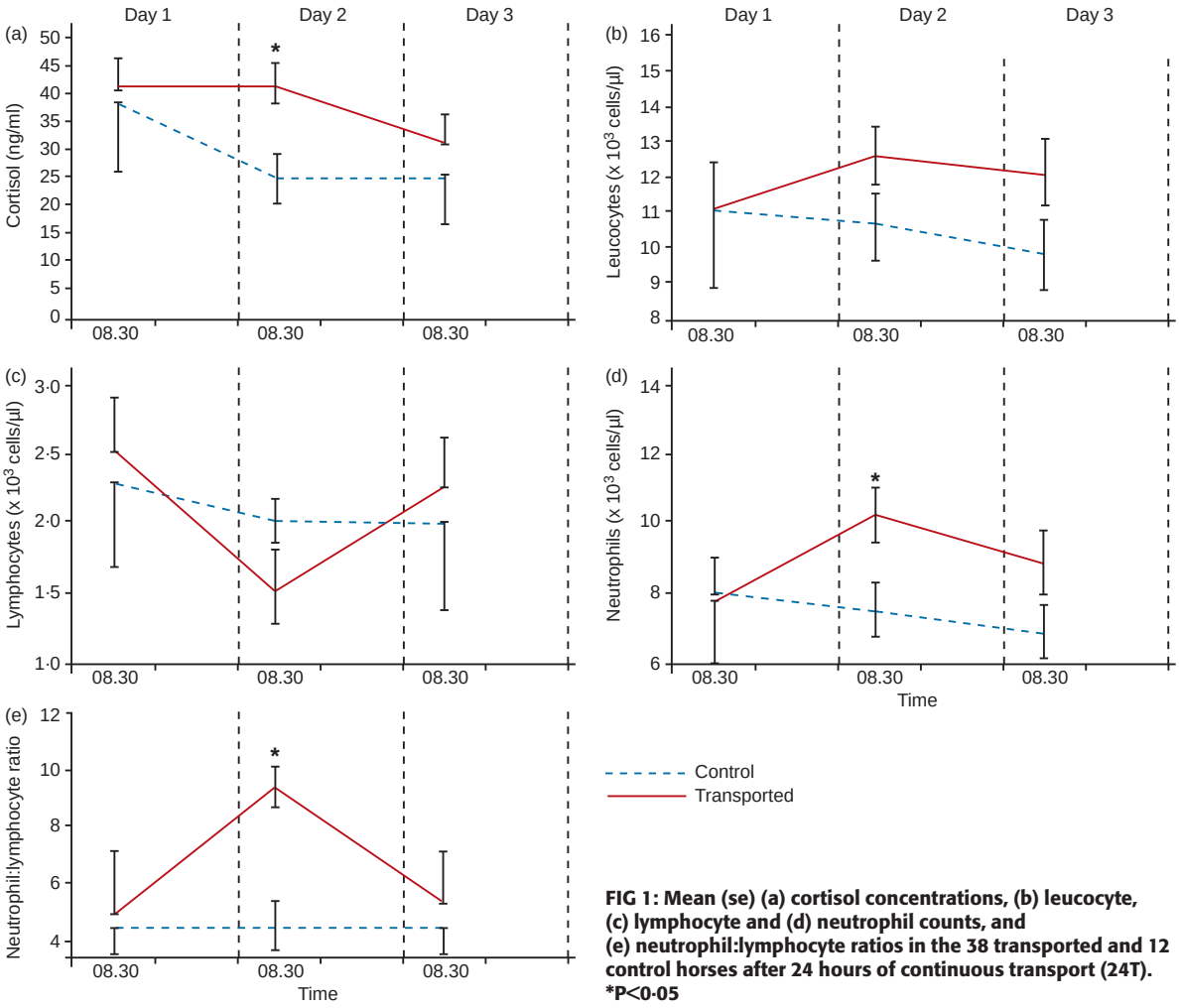

FIG 1: Mean (se) (a) cortisol concentrations, (b) leucocyte, (c) lymphocyte and (d) neutrophil counts, and (e) neutrophil:lymphocyte ratios in the 38 transported and 12 control horses after 24 hours of continuous transport (24T). *P<0·05

hours later. The six control horses remained in their paddocks and blood samples were collected at the same times as from the horses undergoing the 12/12T or 24T treatments. Blood samples (20 ml) for the measurement of serum cortisol were collected by jugular venepuncture into evacuated glass tubes, immediately placed on ice and allowed to clot; the serum was separated and stored at –56°C. Blood for differential white blood cell (WBC) counts was collected into tubes containing EDTA (Vacutainer). Blood for immunophenotyping was collected into acid citrate dextrose tubes (Vacutainer), and stored overnight at 4°C before analysis.

Laboratory analyses

The serum cortisol concentration was determined in duplicate by a microplate enzyme technique (Munro and Stabenfeldt 1984, Munro and Lasley 1988), the intra- and intercoefficients of variation of which were less than 15 per cent. Complete blood cell counts and white blood cell differential counts were determined by an automated cell counter (Cell-Dyne), from which the neutrophil:lymphocyte (NL) ratios were calculated.

Immunocytometry was used to quantify the lymphocyte subpopulations in the peripheral blood as described by Stull and others (2004). Briefly, unconjugated, murine monoclonal antibodies specific for equine leucocyte antigens CD3 (UC F6G-3; Blanchard-Channell and others 1994), CD4 (CVS-4; Lunn and others 1991), and CD8 using two distinct monoclonal antibodies to differentiate CD8a (Lunn and others 1991) and CD8b (Workshop Designation 101; Lunn and others 1998) were used to characterise T cells. In addition, a phycoerythrin-conjugated cross-reactive anti-human CD21 mAb (Clone #B-ly4; Pharmingen) was used to identify B cells.

Leucocyte preparations from the refrigerated blood samples were stained and analysed by flow cytometry (FACSCalibur; Becton Dickinson), by the method described by Blanchard-Channell and others (1994). Absolute cell counts were derived from the leucocyte count, and the immunophenotypic analysis was performed by using Winlist software (Verity Software House). The results are expressed as the mean (se) number of cells/µl of whole blood.

Statistical analyses

The data were analysed by a repeated measures (over time) analysis of variance (PROC GLM) (Anon 1989). Owing to the numbers and complexity of the parameters and to minimise the number of horses, the experimental design used each horse as an independent experimental unit. During the 12/12T protocol five blood samples were collected, whereas only three samples were collected during the 24T protocol. As a result, three sampling points (pre and post journey and recovery) were used to compare the treatments by the repeated measures analysis. The 'pre' sample was collected at 08.30 for both the 12/12T and 24T journeys. The 'post' sample was collected at the end of the journeys, that is at 08.30 and 20.30 on day 2 for the 24T and 12/12T journeys, respectively. The 'recovery' sample was collected after the horses had had 24 hours of rest on day 3 at 08.30 and 20.30 for the 24T and 12/12T journeys, respectively. The difference between the treatments in the time of collection of the samples on days 2 and 3 was unavoidable owing to the 12-hour rest period in the 12/12T protocol. However, the samples taken

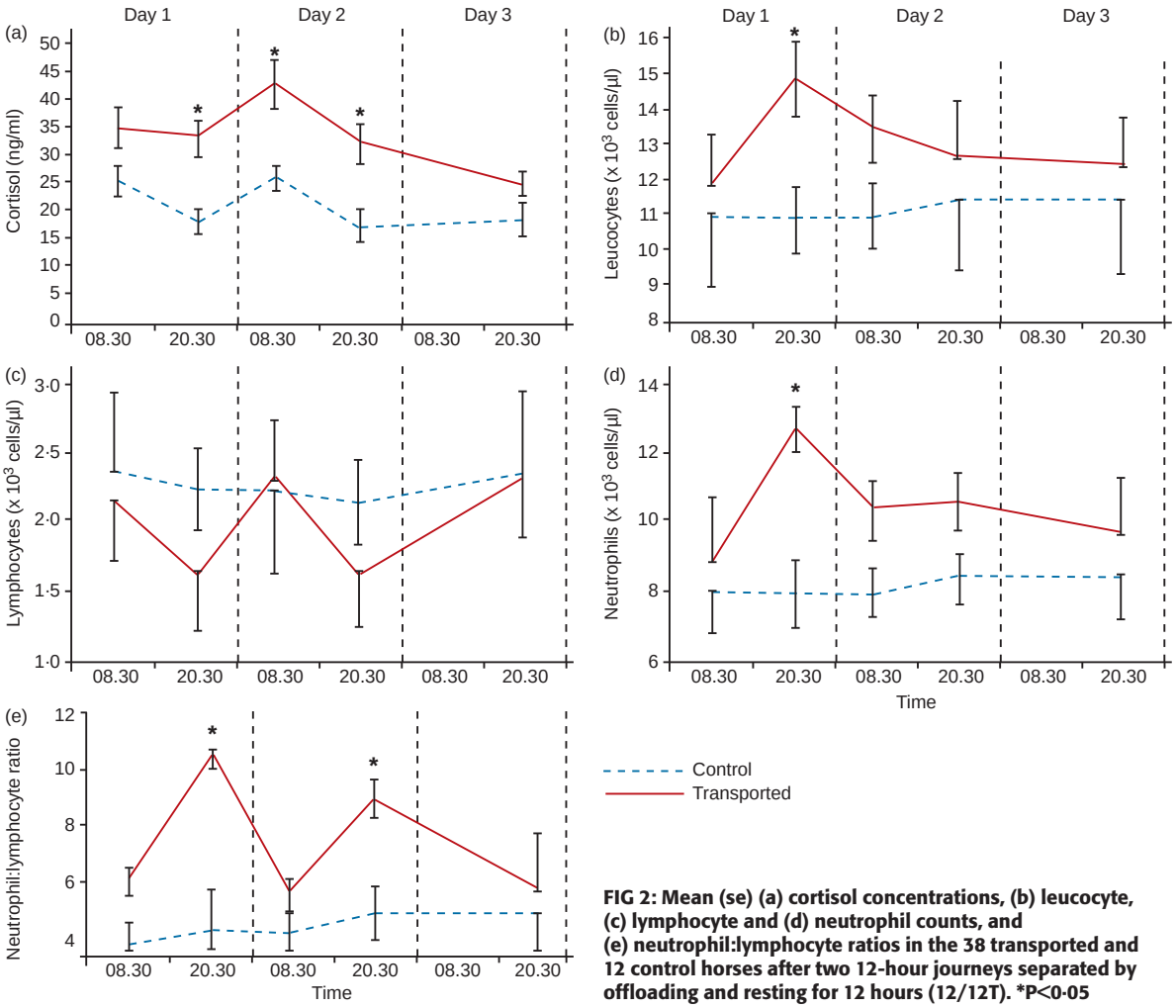

FIG 2: Mean (se) (a) cortisol concentrations, (b) leucocyte, (c) lymphocyte and (d) neutrophil counts, and (e) neutrophil:lymphocyte ratios in the 38 transported and 12 control horses after two 12-hour journeys separated by offloading and resting for 12 hours (12/12T). *P<0·05

from the control horses made it possible to distinguish between a transport effect and potential diurnal effects.

An early repeated-measures model included effects for the order of the treatments within groups, transport (transported and control horses), time, treatments (12/12T and 24T) and their interactions. The analysis showed that the treatment order within groups was not statistically significant and it was therefore removed from the model. To compare the horses' responses to the 12/12T and 24T protocols, a repeated-measures analysis of variance was used, based on the responses of the transported horses relative to their control samples and relative to each other. Fisher's least-significant difference test was used to compare the treatment means of the transported and control horses' physiological measurements (12/12T and 24T) (Steel and Torrie 1960). Similar analyses were used for the lymphocyte subpopulations, except that no samples from the control horses were available. Post hoc comparisons among the sampling points within a treatment were made, based on a contrast of the temporal effect against the initial measurements in the samples collected before the journeys of either kind. Pearson correlation coefficients were calculated to test the associations between changes in cortisol concentration and selected variables. Significance was accepted when P was less than 0·05.

RESULTS

General transport and treatment effects

The study's design minimised any factors related to the order in which the two treatments were carried out. These factors would include influences such as the horses becoming accustomed to the transport vehicle, sampling procedures, handling routines, changes in the horses' health, and social factors associated with their being enclosed together. None of the major physiological indices showed an effect of the order of the treatments, with the possible exception of cortisol (P=0·07). The data from groups 1 and 2 were therefore combined for further statistical analyses of the transport or treatment effects (Table 1).

There were significant (P<0·05) differences common to both treatments in the mean cortisol concentration, neutrophil and lymphocyte counts and NL ratio between the transported horses and the control horses (Table 1), but there was no transport-associated change in total leucocyte counts. However, there were no significant differences between the two treatments in the cortisol concentration, total leucocyte, lymphocyte and neutrophil counts, and NL ratio of the horses (Table 1).

Immunophysiological measurements

Cortisol The mean (se) cortisol concentration in resting horses has been reported to be 45 (1) ng/ml, with a daily peak concentration in the morning (65 [2] ng/ml) and a low concentration in the evening (25 [2] ng/ml) (Stull and Rodiek 1988). The mean cortisol concentrations at the different sampling times for the control horses ranged from 17 (1) ng/ml to 26 (2) ng/ml for the 12/12T horses and from 25 (2) ng/ml to 39 (3) ng/ml for the 24T horses (Figs 1, 2). There was a daily rhythm in cortisol concentration in the control horses of the 12/12T group, from which both morning and evening samples were collected (Fig 2). All

TABLE 1: Levels of significance (P values) for the differences between the transported and control horses with respect to the transport x time effect, and between the two groups of horses (24T and 12/12T; treatment x time effect) for cortisol concentration, total leucocyte count, neutrophil count, lymphocyte count, and neutrophil:lymphocyte ratio

Measurement	Transport x time	Treatment x time
Cortisol (ng/ml)	0·0004	0·29
Total leucocyte count (x 10^3 cells/µl)	0·14	0·21
Neutrophil count (cells/µl)	0·01	0·15
Lymphocyte count (cells/µl)	0·01	0·45
Neutrophil:lymphocyte ratio	0·01	0·47

the samples from the control horses of the 24T group were collected in the morning. At the end of their journey the cortisol concentration of the horses of the 24T group (43 [3] ng/ml) was significantly higher (P<0·05) than that of the control horses (25 [4] ng/ml). After their first 12-hour journey the mean cortisol concentration of the 12/12T horses was higher (P<0·05) than that of the control horses, and remained higher in the samples collected before they were reloaded for the second 12-hour journey and at the conclusion of transport (Figs 1, 2).

Total leucocyte counts (TLC) The ranges in the mean TLC of the control horses for both the 12/12T (10·9 [1·1] to 11·4 [0·9] x 10^3/µl) and 24T horses (9·8 [0·9] to 11·0 [1·0] x 10^3/µl) remained within the reference range (5·5 to 14·3 x 10^3/µl) for healthy horses (Jain 1993) (Figs 1, 2). The peak TLC in the 24T horses at the end of their journey (12·5 [0·7] x 10^3/µl) was not significantly different from that of the control horses (10·5 [0·8] x 10^3/µl). The peak mean TLC (14·8 [0·9] x 10^3/µl) in the 12/12T horses was observed at the end of their first 12-hour journey and was significantly (P<0·02) above that of the control horses (10·9 [0·8] x 10^3/µl) at the same time. No other significant differences in mean TLC were observed between the control and transported horses of either group.

Lymphocyte and neutrophil counts and NL ratio The ranges in the mean total lymphocyte counts in the control horses for the 12/12T group (2·1 [0·3] to 2·3 [0·3] x 10^3/µl) and 24T group (2·0 [0·3] to 2·3 [0·3] x 10^3/µl) were within the normal range (1·5 to 7·7 x 10^3/µl) established for healthy horses (Jain 1993) (Figs 1, 2). There was a significant decrease (P=0·01) in the mean lymphocyte counts of the horses during the journeys. However, the lymphocyte counts of the transported horses were not significantly different from those of the control horses collected at the same times in either the 12/12T or 24T group.

The ranges in the mean neutrophil counts in the control horses of both the 24T group (7·0 [0·8] to 8·2 [0·8] x 10^3/µl) and the 12/12T group (7·9 [0·9] to 8·4 [0·8] x 10^3/µl) remained within the reference range for healthy horses (2·3 to 8·6 x 10^3/µl) (Jain 1993) (Figs 1, 2). In both groups there were increases in neutrophil counts in parallel with those in the TLC. In the 24T group, the neutrophil count peaked at 10·3 (0·7) x 10^3/µl at the end of the journey and in the 12/12T group it peaked at 12·5 (0·9) x 10^3/µl at the end of the first 12-hour journey; in both groups the neutrophil counts at these times were significantly greater (P<0·05) in the transported horses than in the control horses.

The ranges in the mean NL ratio in control horses of the 12/12T group (3·8 [0·4] to 5·0 [0·7] and 24T group (4·4 [0·9] to 4·5 [0·8]) were slightly higher than that established for healthy horses (0·8 to 2·8; Morris and Large 1990) (Figs 1, 2). The peak NL ratio (9·4 [1·1]) in the 24T group observed at the end of the journey was higher (P=0·02) than that of the control horses (4·5 [0·7]). The NL ratio of the 12/12T group had distinctive peaks after both the first (10·5 [1·4]) and second (9·0 [1·0]) 12-hour journeys, significantly higher (P<0·03) than in the control horses at the same times (4·3 [0·5] and 5·0 [0·7], respectively).

Lymphocyte subpopulations CD lymphocyte typing assays were not performed on samples collected from the control horses owing to the cost of the assays. However, in both groups of horses, the repeated measures analysis showed significant (P<0·0001) transport-associated responses in CD3+, CD4+, CD8a+, CD8b+ and CD21+ lymphocyte subpopulations. The responses were different (P<0·05) in the two groups for some lymphocyte subpopulations (CD3+, CD4+ and CD8b+) but not for others (CD8s+ and CD21+) (Table 2). Table 2 shows the mean values relative to the pretransport sample for each group.

In general, the patterns of change were similar for each lymphocyte subpopulation, with declines in cell numbers after the journeys of 20 per cent in the 12/12T group, and 40 per cent in the 24T group (Table 2). After either the 12-hour period for rest and feeding or the 24-hour recovery period, most of the subpopulations had increased to pretransport levels (Table 2). An exception to this pattern was observed in the 12/12T horses in which the number of CD21+ cells had significantly increased (P=0·01) above the pretransport levels (294 [43]/µl) to 362 [48]/µl. In addition, in the 24T horses, the mean numbers of CD8a+ and CD8b+ cells had not recovered completely (P<0·03) after 24 hours rest.

Streptococcus equi infection

No clinical abnormalities were observed before the study, but seven of the horses in the 24T group, and 12 of the horses in the 12/12T group, including two control horses in each group, showed clinical signs of *Streptococcus equi* infection after the first two transport trials on April 1 and April 8. All the horses maintained full appetites, and the horses showing clinical signs were therefore not treated medically. In addition, because both groups of horses responded similarly to

TABLE 2: Mean (se) populations of the different subtypes of lymphocytes in groups of 19 horses transported either for 12 hours followed by a 12-hour rest-stop and then a further 12-hour journey (12/12T) or for 24 hours (24T) measured at the times indicated

	12/12T					24T			
	Day 1		Day 2		Day 3	Day 1	Day 2	Day 3	
Cell type	08.30 Pre	20.30 Unload	08.30 Reload	20.30 Post	20.30 Recovery	08.30 Pre	08.30 Post	08.30 Recovery	P
CD3+ (cells/µl)	1290 (153)	959 (108)*	1436 (179)	1064 (140)*	1525 (194)	1942 (191)	1229 (146)*	1814 (207)	0·002
CD4+ (cells/µl)	832 (88)	618 (65)*	891 (102)	659 (77)*	929 (113)	1325 (127)	836 (100)*	1278 (141)	0·020
CD8a+ (cells/µl)	424 (72)	343 (52)*	436 (83)	358 (69)*	442 (81)	602 (70)	382 (51)*	503 (70)*	0·070
CD8b+ (cells/µl)	381 (64)	289 (40)*	390 (76)	297 (58)*	400 (72)	543 (61)	313 (39)*	455 (65)*	0·004
CD21+ (cells/µl)	294 (43)	212 (32)*	362 (48)*	206 (40)*	347 (65)	301 (34)	184 (26)*	313 (43)	0·20

* P<0·05 relative to the 'pre' measurement

each of the transport treatments in spite of the signs of the clinical disease (Table 1), it was concluded that exposure to *S equi* did not affect the magnitude or direction of their transport-associated immunophysiological responses.

DISCUSSION

Previous studies in horses have observed increases in cortisol concentration, total leucocyte counts, and NL ratios after 24-hour journeys by road, with the levels usually returning to normal within 12 to 24 hours (Stull 1999, Stull and Rodiek 2000, Stull and others 2004). Cortisol has been implicated as a mediator of stress-associated immune suppression, and it was therefore important to expand the examination of the immune system beyond differential white blood cell counts to include the constitution of specific lymphocyte subpopulations. These data showed that there was a transport-associated decrease in the numbers of all the subpopulation cell types. The cell numbers recovered within 24 hours of the end of the journeys with the exception of the CD8a+ and CD8b+ cell types of the 24T horses (Table 2). These broad changes are similar to those observed in a previous study of horses in which it was proposed that the stress of transport altered the distribution of lymphocytes, probably by changes in their trafficking properties from the vascular system into the lymphatic circulation or regional lymphoid tissues (Stull and others 2004). The proposed mechanism together with the data from this study indicate a more universal transported-associated redistribution of lymphocyte subpopulations, rather than specific changes in one particular subpopulation.

The primary aim of this study was to determine whether there would be any immunophysiological benefits from including a 12-hour stop for rest and feeding in the middle of a 24-hour journey. Collectively, no significant differences between the treatments were observed in cortisol concentration, TLC, neutrophil and lymphocyte counts, NL ratios, and the CD8a+ and CD21+ lymphocyte subpopulations. There were, however, treatment differences in the response of the CD3+, CD4+ and CD8b+ subpopulations. Since the spectrum of monoclonal antibodies was selected to differentiate B cells (CD21+), T cells (CD8b+), and natural killer cells (CD8a+) and the changes were restricted to the CD3+, CD4+, and CD8b+, it would appear that the T cells were the principal cell group affected by the treatment. Each of the subpopulations were below the pretransport levels after either the first 12-hour or the 24-hour journey, but the relative reduction in cell numbers was greater in the horses transported for 24 hours (Table 2). The data suggest that there was a gradual decline in cell numbers within these subpopulations during the journeys and that the inclusion of a 12-hour rest period not only interrupted this decline, but allowed the subpopulations to recover towards resting levels. The effect of transport was also evident in the relative delay in the post-transport recovery to pretransport levels of the CD8a+ and CD8b+ cell numbers in the 24T horses compared with their 12/12T counterparts. In view of the fact that transport is associated with increased concentrations of cortisol, and that the immunoregulatory functions of glucocorticoids are well established, it is possible that many of the changes in immune cell populations were cortisol-mediated. Previous studies have shown that changes in the distance travelled, the time allowed for rest, stocking density, the use of crossties, or other management factors induce cortisol-associated differences in physiological response (Smith and others 1994, Friend and others 1998, Stull and Rodiek 2002). In this study the horses of the 12/12T group were located during the 12-hour rest-stop in an unfamiliar environment, rather than in their 'home' paddocks, which may have increased the levels of certain stress-related variables or exposed them to unfamiliar pathogens. The increased cortisol concentrations observed after their 12-hour rest-stop may have been partially due to an increase in the circadian rhythm, as observed in the control horses, and also to their unfamiliar environment during the blood collection procedures. However, their NL ratio was not increased at the same time, and the increase in cortisol was probably due to the short-term stress experienced at the blood collection site.

The significant immunological differences between the two groups of horses were observed in spite of the likely immunophysiological variability between different days in the same individual, and between different individuals on the same day. Furthermore, the *S equi* infection could have affected this inter- and intra-individual variability. In view of the natural history of this pathogen, the outbreak was not unexpected (Sweeney 1990). The disease was enzootic to the facility where the horses were maintained during the study, and one or more of the study horses may have been exposed to the organism during the purchasing process. The disease outbreak could have confounded the results of the study, but a detailed analysis showed that the immunophysiological effects of the journeys were independent of the *S equi* infection. Furthermore, since the disease would not be uncommon in horses mixed together at sales, breeding, or competition facilities and subsequently transported, the outbreak could be viewed as a relevant and practical aspect of the study.

The results of this investigation show that horses undergoing a 24-hour journey by road experience a cortisol-mediated stress response that induces a universal redistribution of lymphocyte subpopulations. The inclusion of a 12-hour stop for rest and feeding interrupted the decline in the lymphocyte subpopulations.

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