END OF PROJECT REPORT 5230

Animal Transport: Developing optimum animal handling procedures and effective transport strategies in the food production chain to improve animal welfare and food quality.

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Teagasc acknowledges with gratitude the support of European Union Structural Funds (EAGGF) in financing this research project

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RMIS No. 5230 Report date: 2007

Beef Production Series No.83

ISBN 1 84170 494 6

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1. Overall summary

A series of studies were performed to investigate the effect of transport on liveweight, physiological and haematological responses of cattle. The first study was carried out over a 6 week period in the Spring of 2004. Eighty-four continental x bulls (mean weight (s.d.) 367 (35) kg), naïve to transport, were randomly assigned to one of six journey (J) times of 0, 6, 9, 12, 18 and 24h transport at a stocking density of 1.02m^2/bull. Blood samples were collected by jugular venipuncture before, immediately after and at 1, 2, 4, 6, 8, 12 and 24h and bulls were weighed before, immediately after, and at 4, 12 and 24h. Bulls travelling for 6h (280 km), 9h (435 km), 12h (582 km), 18h (902 km) and 24h (1192 km) lost 4.7, 4.5, 5.7 ($P \leq 0.05$), 6.6 ($P \leq 0.05$) and 7.5 ($P \leq 0.05$) percentage liveweight compared with baseline. During the 24h recovery period liveweight was regained to pre-transport levels. Lymphocyte percentages were lower ($P \leq 0.001$) and neutrophil percentages were higher ($P \leq 0.05$) in all animals. Blood protein and creatine kinase, glucose and NEFA concentrations were higher ($P \leq 0.05$) in the bulls following transport and returned to baseline within 24h. In conclusion, liveweight and some physiological and haematological responses of bulls returned to pre-transport levels within 24h having had access to feed and water. Transport of bulls from 6 – 24hours did not impact negatively on animal welfare.

In another study, forty continental × weanling heifers (245, s.d 32.2kg) were transported (T) by truck from Ireland to France on a roll-on roll-off ferry at a stocking density of 0.93m^2/animal and then by road for 9-h to a French lairage. Twenty T heifers were unloaded (ULT) and rested for 12h in the lairage and the remainder rested (RT) on the transporter. All heifers had access to hay and water. After the rest period, the heifers were re-loaded. The subsequent journey by road from France to Spain was 9h travel, 7h rest (on the transporter) and a further 7h travel. All T heifers were blood sampled prior to transport (day (d) 0), on arrival in the French lairage (d 4) after 12h rest in the French lairage, on arrival at the feedlot in Spain (d 6) and on d 8, 10, 12 and 36). Twenty continental × weanling heifers (247, s.d. 36.0 kg) remained in Ireland as controls (C) and were blood sampled at the same times as T heifers. Heifers were weighed on d 0, 4, 6, 12 and 36 of the study. Heifers transported to France lost 6.2% of their live weight while C heifers lost 2.1%. Both ULT and RT heifers had lower ($P<0.05$) live weight than C heifers on d 6. During the sea crossing (22h) from Ireland to France, heifers spent 39% of time lying. Neutrophil number was greater ($P<0.05$) at d 6 in RT heifers remaining on the transporter (in France) than ULT heifers. In conclusion, there was no welfare advantage in resting animals on a transporter during the rest period (in France). Transport had no adverse effect on the welfare of weanling heifers transported from Ireland to Spain.

The practice of transporting cattle results in a stress response that is associated with increased disease susceptibility, especially to opportunistic respiratory pathogens, that is thought to be due in part to an alteration in immune function. Neutrophils are phagocytic immune cells important in lung defense and also targets of stress responses. The objective of this study was to investigate changes in the expression of candidate genes known to be important for neutrophil-mediated immunity, to gain a greater understanding of neutrophil function, and, to develop a signature of changes associated with the risk of disease following transportation stress. These proinflammatory neutrophil genes encompassed functions of apoptosis (A1 and Fas), tissue remodeling (MMP-9), margination (L-selectin), bacterial killing (BPI), and wound healing (betaglycan), as well as the glucocorticoid receptor, GRα. To explore these functions, blood was collected, plasma harvested, and neutrophils isolated from 6 Belgian Blue x Friesian bulls (231 ± 7.0 kg in weight; 282 ± 4 days of age) at -24, 0, 4.5, 9.75, 14.25, 24, and 48 h relative to commencement of a 9h transportation by truck. Plasma cortisol concentrations were elevated at 4.5 and 9.75 h, peaking at 50.64 ± 4.46 ($P < 0.0001$), confirming that the animals were stressed by transportation. Blood neutrophil counts were elevated between 4.5 and 14.25 h ($P < 0.0001$), reaching a peak over 3-fold higher than -24h. Fas expression was profoundly down-regulated ($P = 0.02$) by transportation stress, while MMP-9, BPI, and L-selectin were up-regulated ($P = 0.003$, < 0.001, and 0.002, respectively). Changes in betaglycan, GRα, and A1 could not be detected, and no change in the housekeeping gene β-actin was observed. Taken together, these gene expression changes and massively increased neutrophil numbers indicate that the transportation stress scenario may enable these proinflammatory cells to create excessive tissue damage with their longer life, increased proteolytic and bactericidal potential, and increased ability to marginate and migrate to sites of infection, causing a greater risk for severe respiratory disease.

2. Effect of transport for up to 24hours followed by twenty-four hours recovery on liveweight, physiological and haematological responses of bulls

2.1 Introduction

Transportation of livestock is perceived as an acute stressor and involves several potential stressors that result in increased cortisol (Kenny and Tarrant, 1987a,b and Earley et al., 2006a; 2006b; Tennessen et al., 1984), altered products of energy and protein metabolism (Todd et al*.,* 2000), with associated changes in appetite and growth rate and a challenged immune system (Blecha et al., 1984; 1986 and Murata et al., 1987; 1987 and Swanson and Morrow-Tesch, 2001 and Buckham et al., 2007) resulting in increased disease susceptibility. Grandin (1997) reported that efforts to decrease stress during handling and transport of pigs and cattle improves animal welfare and production. Studies have been carried out to determine the optimum stocking density, the maximum duration of transportation, the timing of rest stops and which components of the transport process are the most stressful to cattle (Gupta et al., 2007, and Earley and O'Riordan, 2006a; 2006b, and von Borell, 2001 and Kenny and Tarrant, 1987a; 1987b; and Knowles, 1999 and Tarrant et al., 1992). Physical factors such as noise or vibrations; psychological/emotional factors, such as unfamiliar environment or social regrouping; and climatic factors, such as temperature, humidity, or oxygen concentration, are also involved in the transport process. The transport of livestock can have major implications for their welfare, and there is strong public interest and scientific endeavour aimed at ensuring that the welfare of transported animals is optimal (SCAHAW, 2002). Warriss et al. (1995) transported steers (12-18mo old) by road for 5, 10 and 15 h and they lost 4.6, 6.5 and 7.0% of their liveweight; and recovery to pre-transport liveweight took 5 d. However, there is limited scientific data on the physiological and haematological recovery of animals after long durations of transport and in particular the physiological recovery of animals in the 24h post-transport.

The objective of the present study was to investigate the effect of transport on liveweight, physiological and haematological responses of bulls after road transport of 0, 6, 9, 12, 18 and 24h and on their physiological recovery in the 24h period post-transport.

2.2 Materials and methods

Care of animals

This study was conducted in Teagasc, Grange Research Centre, County Meath, Ireland. All procedures were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876, and the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994.

Animal diets and composition

Bulls had *ad libitum* access to grass silage (in vitro DM digestibility = 762 g/kg), supplemented with 2.0 kg (as fed) barley/soybean concentrate (crude protein = 114.5 g/kg DM) per animal per day at Grange, Beef Research Centre. There was free access to water.

Transport vehicle and environmental conditions

The study was conducted in Spring 2004 over a 6 week period. A total of 5 separate transport journeys were made of duration 6, 9, 12, 18 and 24h. All animals were naïve to transport. On the morning of the journey, animals were loaded into two fan-ventilated pens on a transporter at a stocking density of 1.02 m^2 per animal, and transported by road on an air suspension articulated transporter. Bulls travelled for 6h (280 km), 9h (435 km), 12h (582 km), 18h (902 km) and 24h (1192 km) and during each journey, transport was by primary and secondary roads and different road types and surfaces were encountered.

The transporter was fitted with sensors for measuring ambient temperature (^oC), relative humidity (RH; %), carbon dioxide (CO_2; ppm), hydrogen sulphide (H_2S; ppm), ammonia (NH_3; ppm) air velocity (m/s) and vapour density (g/m^3) continuously during transport. The ambient temperature and relative humidity during transport were recorded continuously using TinyTalk dataloggers (Radionics, Dublin, Ireland). Environmental measurements on the transporter including gases (NH_3, H_2S, CO_2), relative humidity (RH) and temperature were recorded using QRae (Shawcity Ltd., UK), Testo 175 and Testo 445 portable multifunction probes (Testo UK, Ltd).

Treatment groups

Eighty-four continental × bulls (mean weight (s.d.) 367 (35) kg) were randomly assigned by weight to one of six journey (J) times of 0 (no transport), 6, 9, 12, 18 and 24h transport at a stocking density of 1.02m^2/bull. On the morning of the journey, animals were blood sampled pre-transport by jugular venipuncture to provide baseline physiological levels and again after the journey. Blood samples were collected by jugular venipuncture before ((-0.25h) (bleed 1)), immediately after ((0h) (bleed 2)) and at 1h (bleed 3), 2h (bleed 4), 4h (bleed 5), 6h (bleed 6), 8h (bleed 7), 12h (bleed 8) and 24h (bleed 9).

Bulls were weighed and rectal temperature taken before, -24h, -0.25h, immediately after, and at 4, 12 and 24h relative to transport. Non-transported animals (J0) were moved to a novel pen (n=2) with 6 animals/pen in the housing environment, silage and meals were withdrawn and animals had access to hay and water for 24h, followed by a 24h period with *ad libitum* access to grass silage supplemented with 2.0 kg barley/soybean concentrate. Control animals were blood sampled at times corresponding to transported animals. On completion of each of the transport journeys (J6 – J24), animals were returned for a 24h rest period to novel pens (n=2) with 6 animals/pen in the

housing environment and had *ad libitum* access to grass silage supplemented with 2.0 kg barley/soybean concentrate.

Water intake

Water consumption (litres) was recorded during transport and in the 24h period post-transport. Flow metres were attached to the animal drinking containers and consumption was recorded on a pen basis in the housing environment and on the transporter.

Body (rectal) temperature

The rectal body temperature was monitored before and after transport using a digital electronic thermometer (Jorgen Kruuse A/S; Model VT-801BWC Lot No 0701).

Assay procedures for physiological and haematological variables.

Heparinised blood samples were collected by jugular venipuncture and the plasma was separated by centrifugation at 1,600 x *g* at 8 ^{o}C for 15, and subsequently stored at –20 ^{o}C. Albumin, urea, globulin, total protein, β-hydroxybutyrate (βHB), and creatine kinase (CK) were measured on an automatic analyser (Olympus AU 400, Japan) using the reagents supplied by Olympus.

Plasma haptoglobin concentrations were measured using an assay kit (Tridelta Development Ltd., Wicklow, Ireland) on an automated analyzer (spACE; Alfa Wassermann, Inc., West Caldwell, NJ) according to the manufacturer's procedure. Plasma fibrinogen concentrations were measured according to the method described by Becker et al*.,* (1984) on an automated analyzer (spACE).

Concentrations were determined using a commercial kit (Randox Laboratories Ltd., Crumlin, Antrim, N. Ireland; catalogue No. GL2623) on an automated analyzer (Hitachi 705, Boehringer Mannheim, Lewes, East Sussex, UK).

Red blood cell (RBC) number, white blood cell (WBC) number, differential WBC (percentage lymphocyte and neutrophil), packed cell volume (PCV), haemoglobin (Hb) concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet numbers were determined for unclotted (EDTA-treated) whole-blood samples with an automated cell counter (Celltac MEK-6108K; Nihon-Kohdon, Tokyo, Japan) within 1h of blood sampling. Thin blood smears were also prepared on glass slides and stained using the haematology 3-step stain for differential WBC counts (Accralab, Biochemical Sciences; Fisher Scientific Company, Middletown, VA).

Statistics

Data were analysed using SAS/STAT. Physiological and haematological measurements were tested in a one-way ANOVA by PROC MIX, using a means statement with a Tukey option to detect treatment differences. A matched-pair t-test was used to detect differences pre- and post-transport for each treatment. Physiological, haematological and liveweight data were analysed using the repeated measures procedure in PROC MIX to detect differences in treatments while controlling for time effects. A Wilcoxan signed rank test was used to detect differences pre- and post-transport for each treatment (Snedecor and Cochran, 1989). A probability of $P < 0.05$ was chosen as the level of significance for the statistical tests.

2.3 Results

Environment

The mean temperature of the shed environment where the animals were housed was 15.6 ^{o}C (min 6.0 and max 19.3). The environmental data recorded during each transport journey are reported in Table 1. CO_2 levels tended to increase with the longer journeys and values were higher during J24 compared with other journeys. H_2S and NH_3 levels remained low throughout the series of transport journeys. Vapour density and ambient temperature during the 9h transport journey (J9) was higher compared with the other journey times.

Water intake

Table 2 shows the water intake of animals during and after transport. Animals travelling for 12h did not consume water during the journey.

Liveweight

There was no significant difference in liveweight among treatments on days 0 (pre-transport). Liveweight was altered by transportation in the current study, and results are shown in Table 3. Bulls travelling for 6, 9, 12, 18 and 24h lost 4.7, 4.5, 5.7, 6.6 and 7.5 ($P \leq 0.05$) percentage liveweight compared with baseline while control animals lost. Animals transported for 18 and 24hours had lower liveweight *($P \leq 0.01$)* compared with baseline. During the 24h recovery period liveweight was regained to pre-transport levels (Table 3).

Temperature data

No effect ($P > 0.05$) of transport was observed in the rectal temperature data (data not shown).

Physiological and metabolic variables

In non-transported control animals (J0), there was no statistical significant (P>0.05) change in albumin concentrations (Table 4). Animals transported for 6h (h) (J6) had greater albumin concentrations at bleed 2 (0h post-transport) *(P< 0.05)* compared with baseline (pre-transport). Animals transported for 9h (J9) had greater *(P< 0.05)* albumin concentrations 0h and 1h post-transport) compared with pre-transport baseline. Animals transported for 12h (J12) had lower *(P< 0.05)* albumin concentrations at 0h, 1h and 2h post-transport compared with baseline. Following transportation of animals for 18h (J18) albumin concentrations were greater *(P< 0.05)* at 0h and 1h post-transport compared with pre-transport values. Following the 24h transport journey, albumin concentrations were greater (*P< 0.05)* at 0h, 1h and 2h post-transport.

In non-transported control animals (J0), there was no statistical significant (P>0.05) change in total protein concentrations (Table 4). Animals transported for 6h (J6) and 9h (J9) had greater (*P< 0.05*) total protein concentrations at 0h and 1h post-transport) compared with pre-transport (*P< 0.05*). Animals transported for 12h (J12) had greater (*P < 0.05*) total protein concentrations at 0h, 1h and 2h post-transport compared with baseline. Following transportation of animals for 18h (J18) total protein concentrations were greater (*P< 0.05*) at 0h and 1h post-transport compared with pre-transport values. Following a 24h transport journey, total protein concentrations were greater than baseline (*P< 0.05*) at 0, 1, 2, 4 and 8h post transport .

Non-transported control animals (J0) had greater (*P*<0.05) urea concentrations than baseline at 0h to 12h and were not different at 24h (Table 5). Animals transported for 6h (J6) had greater (*P*<0.05) urea concentrations at bleeds 0h to 12h post-transport than pre-transport values. Animals transported for 9h (J9) had greater (*P < 0.05*) urea concentrations at 0h and 1h post-transport compared with bleed 1 (pre-transport). Animals transported for 12h (J12) had greater (*P < 0.05*) urea concentrations at 0h, 1h, 2h, 4h, 6h and 8h post-transport. Following transportation of animals for 18 (J18) and 24h (J24) urea concentrations were greater (*P < 0.05*) at 0h to 12h post-transport and were not different (P>0.01) at 24h post-transport, compared with pre-transport.

βHB concentrations were greater (*P < 0.05*) at 6h to 24h following the end of the 24h experimental period in non-transported control animals (J0), when compared with baseline concentrations (Table 5). Animals transported for 6h (J6) and 9h (J9) had greater (*P*<0.05) βHB concentrations than baseline at 2 h to 24h post transport. While, animals transported for 12h (J12) had greater (*P < 0.05*) βHB concentrations than baseline at 4h to 24h post-transport. Following transportation of animals for 18h (J18) βHB concentrations were greater (*P < 0.05*) than baseline at 6h to 24h and animals transported for 24h had greater (*P < 0.05*) βHB concentrations than baseline at 8 h, 12h and 24h post-transport.

Globulin concentrations were unchanged (*P>0.01*) (Table 6) in non-transported control animals (J0), and in animals transported for 6 (J6), 9 (J9) and 18h (J18). Animals transported for 12h (J6) had greater (*P*<0.05) globulin concentrations than baseline at 0h and 1h post-transport, compared with bleed 1 (pre-transport) (*P < 0.05*), while animals transported for 24h (J24) had greater (*P < 0.05*) globulin concentrations than baseline 0h, 1 h, 2 h and 4h post-transport.

CK activities were greater (*P>0.05*) than baseline in non-transported control animals (J0) at 0h to 8 h post the 24h experimental rest period (Table 6). Animals transported for 6h (J6) had greater CK activities than baseline at 4 h to 12h post-transport (*P < 0.05*). Animals transported for 9h (J9) had greater (*P<0.05*) CK activities at 1h post-transport while animals transported for 12 (J12) h had greater levels than baseline at 2 h to 8 h post transport. Animals transported for 18h (J18) had greater CK activities than baseline (*P < 0.05*) at 2 h to 12h post-transport. Following a 24h transport journey, CK activities were greater than baseline (*P < 0.05*) at 0h to 12h post-transport.

Haptoglobin concentrations were greater (*P < 0.05*) than baseline in non-transported control animals at 24h following the end of the experimental 24h rest period, (Table 7), and were unchanged in animals transported for 6 (J6) and 9h (J9). Animals transported for 12h (J12) had greater (*P < 0.05*) haptoglobin concentrations than baseline 1h, 12 and 24h post-transport. Following transportation of animals for 18h (J18) haptoglobin concentrations were greater than baseline (*P < 0.05*) at 24h post-transport whereas animals transported for 24h had greater haptoglobin concentrations than baseline (*P < 0.05*) at 6 to 24h post-transport.

In non-transported control animals (J0), glucose concentrations were lower (*P < 0.05*) than baseline at 0h, 4h, 6h, 8 h and 12 h relative to the end of the initial 24h experimental period (Table 7). Animals transported for 6h (J6) had greater (*P < 0.05*) glucose concentrations than baseline at 1h and 2 h post-transport. Following transportation of animals for 9h (J9) and 12h (J12) and 24h (J24) glucose concentrations were greater (*P < 0.05*) at 0h, 1 h and 2 h post-transport compared with baseline, whereas animals transported for 18h (J18) had greater glucose concentrations (*P < 0.05*) at 0h and 1 h post-transport.

NEFA concentrations were greater in non-transported control animals (J0) at 0h, 1h, 2, h and 24h compared with baseline (Table 7). Animals transported for 6h (J6) had greater (*P < 0.05*) NEFA concentrations than baseline at 12h post-transport. Following transportation of animals for 9 (J9) and 12h (J12), NEFA concentrations were lower (*P < 0.05*) at 0h to 6h post-transport. Following transportation of animals for 18h (J18), NEFA concentrations were greater (*P < 0.05*) at 0h and 24h post-transport compared with baseline. Following a 24h transport journey, NEFA concentrations were greater than baseline at 0h, 1h and 24h post-transport compared with pre-transport).

Haematological variables

In non-transported control animals (J0), WBC numbers were greater ($P < 0.05$) than baseline at 4 h to 24h (Table 8). Animals transported for 6h (J6) had greater ($P < 0.05$) WBC numbers at 0h to 24h post transport. Similarly, animals transported for 9h (J9) and 18h had greater ($P < 0.05$) WBC numbers than baseline at 0h to 12h post-transport, whereas animals transported for 12h (J12) had greater ($P < 0.05$) WBC numbers than baseline at 0h to 24h post-transport. Following a 24h transport journey, WBC numbers were greater than baseline ($P < 0.05$) at 0h to 24h post-transport.

In non-transported control animals, lymphocyte percentage was lower and neutrophil percentage was greater than baseline at 6h to 24h following the end of the 24h experimental period (Table 8). Animals transported for 6 (J6), 9 (J9), 12 (J12), 18 (J18) and 24h (J24) had lower ($P < 0.05$) lymphocyte percentage and greater ($P < 0.05$) neutrophil percentage than baseline at 0h to 24h post-transport.

RBC numbers were lower ($P < 0.05$) than baseline in non-transported control animals at 0h to 24h relative to the end of the first 24h experimental period (Table 9). There were no differences in RBC numbers in J6, J9, J12, J18 and J24 treatment groups following transport.

Haemoglobin (Hb) concentrations were lower ($P < 0.05$) at 0h to 24h in non-transported control animals compared with baseline (Table 9). There were no differences ($P > 0.05$) in Hb concentrations in J6, J9, J18 and J24 treatment groups following transport while animals transported for 12h had lower Hb concentrations ($P < 0.05$) than baseline at 24h post-transport.

PCV % was lower ($P<0.05$) at 0h to 24h post-transport (Table 9) compared with baseline in non-transported control animals. Animals transported for 6h (J6) and 12h (J12) had lower ($P<0.05$) PCV % than baseline at 24h post-transport. There were no differences ($P>0.05$) in PCV % in J9, J18 and J24 treatments following transport.

There were no differences in MCV and MCHC concentrations following transport (Data not shown). Platelet numbers were lower ($P<0.05$) at 0h (424.33 mean ± 140.4 s.d.) in non-transported control animals compared with 4 h (536.7 mean ±135.4 s.d), 6h (561.3 mean ± 155.8 s.d.) and 12h (539.9 mean ±124.0 s.d.) and values returned to baseline at 24h. Platelet numbers were not different ($P <0.05$) post-transport for J6 to J24 treatments (data not shown).

2.4 Discussion

The results of the present series of transport journeys by roads showed that transportation of bulls affected liveweight, haematological and physiological measurements of metabolism, immune function and inflammation. The changes in liveweight post-transport support previous published findings (up to 11% loss) that may be attributed to a loss of gutfill over the journeys and possibly due to dehydration, urination and fasting during the longer journeys (Arthington et al., 2003 and Knowles, 1999 and Tarrant, 1990 and Tarrant et al., 1992). It is of interest to note that non-transported control animal also showed a loss in liveweight. The loss in liveweight in control animals in the present study may be attributed to the change in diet, as silage and meals were withdrawn and animals had access to hay and water only for 24h. Rectal temperature was not changed during the respective transport journeys indicating that there was no clinical infection induced by transport. The development of electrolyte and acid-base imbalances has been reported in extended transport journeys where fasting has exceeded 2 days or more (Schaefer et al., 1988). In the present study, transportation had effects on metabolism as demonstrated by significant changes in the plasma concentrations of albumin, globulin, total protein, urea, βHB, NEFA, glucose and creatine kinase.

In the present study, albumin and protein concentrations increased with journey duration in transported animals. However, by 24h post-transport concentrations had returned to pre-transport levels. Transport stress has been reported to cause dehydration and may manifest itself as a hyperproteinemia (Atkinson, 1992 and Schaefer et al., 1992). The changes in albumin and protein levels seen post-transport are more likely the result of metabolic compensation for a mild metabolic acidosis secondary to water loss and feed deprivation.

Increases in plasma glucose concentrations are mainly due to glycogenolysis associated with the increase in circulating catecholamines and glucocorticoids which are released during the stress of transport (Shaw and Tume, 1992). Glucose levels returned to baseline in all animals within 4 hour of transport. Urea, NEFA and βHB concentrations were elevated in control and all transported animals and concentration remained higher than baseline for animals transported on journey durations ranging from from 0h to 24h. Urea concentrations had returned to pre-transport values by 12h in non-transported control animals, while animals transported from 6 to 24hours had high plasma concentrations compared with baseline by 24h post-transport.

These findings suggest that pre-transport mixing and transportation alters protein metabolism. Increased energy metabolism is a hallmark of the stress response as the body prepares to react to a potentially stressful situation. We have previously reported increases in several of these protein metabolites in response to transportation (Earley and O'Riordan, 2006a; 2006b and Tarrant et al., 1992). These differences may be due to a number of factors including the duration of the journey

and the fact that animals had not access to feed during transportation. Additionally, circulating CK is often measured in transported cattle as a measure of bruising (Tarrant, 1990), indicating that the bulls in the current study may have experienced some degree of muscular activity and physical stress. Increases in the plasma activity of CK after different transport journeys has been described by different authors (Warriss et al., 1995 and Knowles, 1999 and Tadich et al., 2002). Indeed, Warriss et al. (1995), reported a direct relationship between the duration of transport and the rise in the activity of the enzyme. While Holmes et al. (1973) reported that fasting can also increase the activity of the enzyme, and the rise could be masked by the high values obtained after transport. In the present study, CK activities had returned to pre-treatment baseline values within 12h for all transported animals with the exception of animals transported for 24h.

Changes in acute phase protein concentrations during transportation have been reported but the results are variable. Acute phase proteins are present in very low concentrations in plasma and increase in concentration following tissue injury and inflammation (Cheville, 1999; Skinner et al., 1994). In the present study haptoglobin concentrations were increased in control and transported animals up to 24h post-transport, with the exception of the animals transported for 6h. Murata and Miyamoto (1993) reported that serum haptoglobin was elevated in calves transported for 2 days and levels were negatively correlated with lymphocyte function. In a separate experiment transporting bulls at different stocking densities, plasma haptoglobin concentrations were unchanged, while plasma fibrinogen levels were reduced (Earley and O'Riordan, 2006a; 2006b). Fibrinogen, ceruloplasmin, serum amyloid-A, and α-acid glycoprotein were assayed in the plasma of transported and commingled calves and found to be increased post-transportation; however, haptoglobin concentrations were higher in non-transported versus transported calves (Arthington et al., 2003).

Leach (1981) reported changes in the haematological responses of cattle to transport while Tarrant et al. (1992) reported increased RBC, PCV and Hb following transportation of steers. In the present study, control animals and all transported animals had higher lymphocyte and lower neutrophil numbers pre-transport. In the present study, RBC numbers and haemoglobin were within the normal blood referenced ranges (Schalm, 1961; 1984 and Jain, 1986 and Kaneko, 1989). The lymphopaenia and neutrophilia observed in control animals is most likely due to the effect of stress related to the mixing and the handling procedures. Blood lymphocytes contain concentrations of glucocorticoid and adrenergic receptors (Preisler et al., 2000), which are down-regulated in response to stress (Burton et al., 2005), suggests that alterations in the blood cell composition of lymphocytes may have an important role in the responsiveness of the immune system when stress challenged. There was no major change in PCV% in animas transported for 9, 18 and or 24hours while PCV% was higher pre-transport in control animals, and animals transported for 6 and 12h respectively. Elevated PCV% has been reported following transport in association with higher erythrocyte counts in the circulation (Kent and Ewbank, 1983 and Tarrant et al., 1992) and a

significant increase in PCV values indicates mainly dehydration. This is in agreement with Tarrant et al. (1992) who reported an increase in red blood cell count, haemoglobin, total protein and PCV values in steers transported by road for 24h using different stocking densities. Tadich et al. (2002) found an increase in PCV values in cattle transported by road for 12 and 24h. Measures of immunological changes relate to immune cell numbers in the blood and immune cell function. A number of studies have reported leukocytosis that is marked by neutrophilia, and which may be present with a decrease in the number of other cells (lymphopaenia, eosinopaenia) (Blecha et al., 1984 and Murata et al., 1987 and Tarrant et al., 1992). The normal referenced ranges for differential counts, neutrophils are in the range 15-45 (Schalm, 1961 and Radostits et al., 1994). Within the range of transport times analysed, there were no significant changes in MCV, MCHC and platelet numbers. In the present study, the changes in the composition of the blood cell variables reflect the physiological or pathophysiological response of the body to the stress of mixing, fasting and/or transportation. The neutrophilia and lymphopenia following transportation observed in this study are in agreement with previously reported findings following a variety of stressors, including transport stress (Blecha et al., 1984 and Murata et al., 1987 and Tarrant et al., 1992).

Control animals showed similar changes in physiological and haematological responses to transported animals. Most of the physiological and haematological variables which changed as a consequence of transport had recovered to baseline values within 24h of the completion of the journey. Transport of bulls from 6 – 24hours did not impact negatively on animal welfare. More studies are needed in the area of pre-transport handling of animals and focus should be on correlating behaviour to physiological indicators of stress. An increased understanding of the mechanism of stress and physiological adaptation induced in animals by mixing, handling and transport will lead to a greater understanding of transportation stress. It is concluded that non-transported control animals showed similar biological effects on the physiological and haematological variables to transported animals, however, future transport studies will need to determine how much deviation from normal values is unacceptable from an animal welfare point of view.

Table 1: Environmental conditions recorded during transport. The values are expressed as mean with minimum and maximum values.

Transport Journeys		Ambient	During Transport						
		Temp °C	**CO_2 ppm**	**Relative humidity %**	**Temp °C**	**Wind Speed m/s**	**Vapour density td °C**	**H_2S ppm**	**NH_3 ppm**
J6	**Mean**	8.36	453.79	89.54	7.58	0.85	5.93	0.01	1.26
	Minimum	6.10	356.00	72.50	4.70	0.09	4.30	0.00	0.00
	Maximum	10.30	897.00	99.90	10.80	2.46	10.80	0.10	5.70
J9	**Mean**	15.29	531.59	93.79	14.41	0.60	13.38	0.00	0.39
	Minimum	11.30	388.00	81.10	10.40	0.00	10.40	0.00	0.00
	Maximum	17.70	922.00	99.90	17.70	2.27	15.90	0.00	2.90
J12	**Mean**	7.84	668.78	74.74	8.59	0.67	4.30	0.01	0.93
	Minimum	0.70	447.00	61.50	3.50	0.00	1.20	0.00	0.00
	Maximum	14.20	1903.00	89.20	12.60	3.01	8.70	0.20	7.70
J18	**Mean**	7.13	545.50	78.09	8.26	0.76	4.64	0.04	0.00
	Minimum	3.50	399.00	65.00	4.70	0.00	0.50	0.00	0.00
	Maximum	9.90	858.00	91.00	10.50	3.34	8.50	0.20	0.00
J 24	**Mean**	4.90	770.94	86.24	6.44	0.64	4.17	0.00	0.08
	Minimum	-3.10	401.00	64.30	-0.10	0.00	-1.10	0.00	0.00
	Maximum	18.10	2373.00	99.90	16.00	3.08	12.20	0.00	2.90

Table 2. Water intake (litres) per animal during and after different transport journey times (J)

J duration (h)	Water intake (L) during transport	Distance (km)	Post - Transport (water intake (l)		
			0 - 4h	5 - 12h	13 - 24h
J0	4.1	0.0	20.2	4.5	0.0
J6	5.0	280	8.0	3.5	0.5
J9	5.0	435	11.5	1.5	6.0
J12	0.0	582	8.5	6.5	4.0
J18	1.8	902	5.5	7.0	3.0
J24	3.9	1192	9.0	7.0	1.0

Table 3. Changes in liveweight (kg ± s.d.) in control and transported bulls for the respective journey (J) times.

	Pre-transport			Post-transport		
	-24h	-0.25h	0h	4h	12h	24h
J0	370.5	373.2	352.8	360.1	366.8	356.9
	±49.84	±48.47	±45.81	±48.35	±48.94	±46.39
J6	370.8	367.1	349.8	362.2	363.2	362.2
	±32.61	±31.80	±29.61	±30.08	±32.55	±33.36
J9	369.3	362.4	346.2	359.2	355.2	361.2
	±50.11	±50.88	±48.15	±48.03	±48.91	±49.97
J12	367.7	367.0	346.1	360.8	359.7	365.8
	±26.23	±26.64	±26.11	±27.10	±27.09	±27.45
J18	365.2^{b}	363.8^{b}	339.5^{a}	345.6^{ab}	350.1^{ab}	349.3^{ab}
	±27.00	±26.28	±22.65	±23.96	±24.88	±25.79
J24	362.8^{b}	359.7^{b}	332.6^{a}	346.4^{ab}	354.7^{ab}	352.7^{ab}
	±21.05	±20.71	±19.60	±19.53	±22.20	±21.24

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24h at a stocking density of 1.02m^2 per animal; ^{a,b,} Means with different superscripts within rows differ $P < 0.05$.

Table 4. Treatment means (± s.d.) for plasma albumin and total protein pre (-0.25h) and post transport (1, 2, 4, 6, 8, 12 and 24 h).

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
Albumin (g/L)									
J0	26.4 ±2.15	27.1 ±2.08	27.3 ±2.04	26.8 ±1.82	27.3 ±1.97	27.3 ±1.72	27.1 ±1.82	27.6 ±1.97	26.9 ±2.12
J6	27.7 ±2.13 [a]	29.6 ±2.35 [b]	29.4 ±2.53 [ab]	29.2 ±2.29 [ab]	29.1 ±2.16 [ab]	28.5 ±2.29 [ab]	27.6 ±1.97 [ab]	27.8 ±2.08 [ab]	27.5 ±2.13 [ab]
J9	27.1 ±2.29 [a]	29.3 ±2.48 [b]	29.11 ±2.6 [b]	28.4 ±2.41 [ab]	27.7 ±2.56 [ab]	27.6 ±2.38 [ab]	27.7 ±2.18 [ab]	27.5 ±2.18 [ab]	27.6 ±2.18 [ab]
J12	27.8 ±1.32 [a]	29.8 ±1.80 [b]	30.0 ±1.80 [b]	29.5 ±1.50 [b]	28.8 ±1.37 [ab]	28.3 ±1.42 [ab]	28.6 ±1.31 [ab]	28.3 ±1.49 [ab]	28.5 ±1.36 [ab]
J18	27.3 ±2.31 [a]	29.7 ±2.22 [b]	29.3 ±2.31 [b]	28.7 ±1.75 [ab]	28.9 ±1.77 [ab]	28.7 ±1.83 [ab]	28.5 ±1.76 [ab]	28.3 ±1.93 [ab]	27.4 ±2.21 [ab]
J24	27.8 ±1.68 [b]	29.7 ±1.92 [b]	29.9 ±2.18 [b]	30.2 ±2.31 [b]	28.9 ±2.22 [ab]	28.8 ±2.20 [ab]	28.9 ±2.15 [ab]	28.4 ±2.05 [ab]	28.3 ±2.14 [ab]
Total protein (g/L)									
J0	66.4 ±4.07	68.5 ±4.34	67.5 ±3.53	67.5 ±3.89	67.9 ±4.12	68.4 ±3.10	67.1 ±3.86	69.1 ±2.81	68.2 ±3.57
J6	66.9 ±3.07 [a]	70.6 ±2.35 [b]	70.4 ±3.75 [b]	69.2 ±2.06 [ab]	68.3 ±3.09 [ab]	67.1 ±3.87 [ab]	66.4 ±2.97 [ab]	66.2 ±2.96 [ab]	65.3 ±2.80 [ab]
J9	67.4 ±5.30 [a]	72.5 ±4.52 [b]	71.7 ±4.19 [b]	70.3 ±3.77 [ab]	67.9 ±3.10 [ab]	67.3 ±3.48 [ab]	67.8 ±3.44 [ab]	68.2 ±4.12 [ab]	68.6 ±4.47 [ab]
J12	65.4 ±3.6 [a]	71.1 ±3.94 [b]	70.9 ±4.04 [b]	69.5 ±3.74 [b]	67.7 ±3.73 [ab]	67.0 ±3.38 [ab]	67.4 ±2.78 [ab]	67.2 ±2.88 [ab]	67.8 ±2.85 [ab]
J18	67.14 ±4.8 [a]	73.0 ±5.19 [b]	71.9 ±5.42 [b]	71.4 ±5.60 [ab]	71.0 ±5.07 [ab]	70. 2 ±6.58 [ab]	70.1 ±5.54 [ab]	70.1 ±5.28 [ab]	68.2 ±6.56 [ab]
J24	67.1 ±3.55 [a]	72.8 ±3.35 [b]	73.0 ±3.57 [b]	73.4 ±3.27 [b]	70.6 ±2.87 [b]	69.3 ±2.92 [ab]	70.0 ±3.15 [b]	69.3 ±3.05 [ab]	69.0 ±3.31 [ab]

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly (*P < 0.05).*

Table 5. Treatment means (± s.d.) for plasma urea and βHB pre (-0.25h) and post transport (1, 2, 4, 6, 8, 12 and 24 h).

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
Urea (mmol/L)									
J0	2.3 ±0.48[a]	4.1 ±1.00[b]	4.1 ±0.98[b]	4.3 ±0.98[b]	4.6 ±0.96[b]	4.6 ±1.09[b]	4.3 ±1.28[b]	3.5 ±1.27[b]	2.9 ±0.72[ab]
J6	2.4 ±0.39[a]	2.9 ±0.37[b]	3.0 ±0.42[b]	3.2 ±0.37[b]	3.7 ±0.45[b]	4.2 ±0.49[b]	4.5 ±0.55[b]	4.4 ±0.69[b]	3.6 ±0.56[ab]
J9	3.2 ±0.77[a]	3.5 ±0.79[b]	3.6 ±0.77[b]	3.9 ±0.85[ab]	4.1 ±0.85[ab]	4.4 ±0.92[ab]	4.3 ±1.03[ab]	3.7 ±0.95[ab]	2.9 ±0.88[ab]
J12	3.3 ±0.75[a]	4.1 ±0.53[b]	4.3 ±0.48[b]	4.5 ±0.51[b]	4.8 ±0.57[b]	4.7 ±0.68[b]	4.2 ±0.83[b]	3.5 ±0.79[ab]	2.6 ±0.59[ab]
J18	2.2 ±0.49[a]	4.3 ±0.47[b]	4.5 ±0.52[b]	4.6 ±0.54[b]	4.8 ±0.62[b]	4.5 ±0.71[b]	4.0 ±0.71[b]	3.2 ±0.74[b]	2.3 ±0.62[ab]
J24	2.4 ±0.59[a]	5.4 ±1.01[b]	5.4 ±0.98[b]	5.5 ±1.00[b]	5.7 ±0.94[b]	5.7 ±0.96[b]	5.4 ±1.10[b]	4.3 ±1.15[b]	2.8 ±0.66[ab]
βHB (mmol/L)									
J0	0.18 ±0.05[a]	0.22 ±0.04[ab]	0.20 ±0.05[ab]	0.19 ±0.08[ab]	0.25 ±0.09[ab]	0.29 ±0.11[b]	0.36 ±0.19[b]	0.28 ±0.07[b]	0.29 ±0.08[b]
J6	0.17 ±0.05[a]	0.16 ±0.04[ab]	0.17 ±0.04[ab]	0.22 ±0.04[b]	0.25 ±0.05[b]	0.25 ±0.04[b]	0.25 ±0.04[b]	0.24 ±0.03[b]	0.26 ±0.04[b]
J9	0.18 ±0.05[a]	0.16 ±0.05[ab]	0.19 ±0.05[ab]	0.25 ±0.06[b]	0.31 ±0.06[b]	0.32 ±0.07[b]	0.30 ±0.06[b]	0.28 ±0.13[b]	0.30 ±0.06[b]
J12	0.20 ±0.06[a]	0.16 ±0.04[ab]	0.17 ±0.03[ab]	0.23 ±0.03[ab]	0.28 ±0.05[b]	0.30 ±0.06[b]	0.29 ±0.05[b]	0.25 ±0.05[b]	0.32 ±0.09[b]
J18	0.18 ±0.03[b]	0.20 ±0.07[ab]	0.14 ±0.05[ab]	0.18 ±0.04[ab]	0.20 ±0.05[ab]	0.28 ±0.07[b]	0.27 ±0.07[b]	0.28 ±0.06[b]	0.31 ±0.05[b]
J24	0.20 ±0.03[a]	0.18 ±0.07[ab]	0.14 ±0.05[ab]	0.17 ±0.05[ab]	0.22 ±0.04[ab]	0.27 ±0.04[ab]	0.36 ±0.08[b]	0.39 ±0.19[b]	0.34 ±0.15[b]

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly (*P < 0.05).*

Table 6. Treatment means (± s.d.) for plasma globulin and creatine kinase pre (-0.25h) and post transport (1, 2, 4, 6, 8, 12 and 24 h).

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
Globulin (g/L)									
J0	40.0	41.4	40.2	40.6	40.6	41.0	40.0	41.6	41.3
	±2.94	±3.34	±2.86	±3.01	±3.24	±2.83	±3.17	±2.66	±2.56
J6	39.2	41.1	41.0	39.9	39.0	38.6	38.8	38.4	37.8
	±3.13	±2.52	±3.18	±2.76	±3.56	±3.70	±3.40	±3.18	±3.29
J9	40.3	43.2	42.6	41.9	40.3	39.7	40.1	40.7	41.0
	±5.76	±5.13	±4.90	±5.13	±4.16	±4.97	±4.66	±5.00	±4.91
J12	37.6	41.3	41.0	40.1	39.0	38.7	38.9	38.9	39.4
	±3.89 [a]	±3.58 [b]	±3.72[b]	±3.68 [ab]	±3.72 [ab]	±3.32 [ab]	±2.80 [ab]	±2.96 [ab]	±2.86 [ab]
J18	39.9	43.4	42.6	42.6	42.2	41.5	41.6	41.8	40.7
	±5.62	±5.91	±6.25	±6.29	±5.96	±7.21	±6.10	±5.82	±6.60
J24	39.3	43.1	43.1	43.2	41.7	40.5	41.1	40.9	40.7
	±3.43 [a]	±3.14[b]	±3.50[b]	±2.50 [b]	±2.30 [b]	±2.32 [ab]	±2.19 [ab]	±2.53 [ab]	±2.88 [ab]
CK (U/L)									
J0	159.6	437.1	511.4	551.4	552.4	524.8	483.6	430.4	381.0
	±32.49 [a]	±372.0 [b]	±345.3 [b]	±348.0 [b]	±305.4 [b]	±254.0 [b]	±223.1 [b]	±183.7 [ab]	±153.6 [ab]
J6	160.6	377.9	443.9	521.1	627.0	691.4	605.6	487.42	427.2
	±42.55 [a]	±194.0 [ab]	±226.3 [ab]	±286.0 [ab]	±316.1[b]	±375.4[b]	±275.4 [b]	±252.0 [b]	±355.2 [ab]
J9	242.2	428.3	522.7	469.7	511.1	469.0	357.4	364.5	319.6
	±189.5[a]	±293.6[ab]	±373.8[b]	±264.1 [ab]	±353.5[ab]	±381.8 [ab]	±181.0 [ab]	±256.3 [ab]	±272.8 [ab]
J12	187.7	368.5	398.2	548.3	545.0	497.3	494.8	431.7	404.6
	±62.73 [a]	±174.4 [ab]	±170.36 [ab]	±303.16[b]	±317.79 [b]	±337.20[b]	±305.24 [b]	±269.67 [b]	±337.13 [ab]
J18	159.6	472.9	497.1	467.1	445.3	477.9	475.7	437.5	393.4
	±55.52 [a]	±238.9 [ab]	±298.7 [ab]	±224.0[b]	±198.8 [b]	±213.1 [b]	±187.5[b]	±173.9[b]	±314.57 [ab]
J24	162.3	496.20	545.7	598.6	598.7	600.0	631.6	552.0	340.3
	±33.74 [a]	±245.50[b]	±253.80 [b]	±291.46 [b]	±297.77 [b]	±321.22 [b]	±365.33 [b]	±370.85 [b]	±153.0 [ab]

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly ($P < 0.05$).

Table 7. Treatment means (± s.d.) for plasma haptoglobin, glucose and NEFA pre (-0.25h) and post transport (1, 2, 4, 6, 8, 12 and 24 h).

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
Haptoglobin (Hb binding capacity/L)									
J0	0.16 ±0.07 [a]	0.34 ±0.32 [ab]	0.34 ±0.35 [ab]	0.32 ±0.33 [ab]	0.34 ±0.35 [ab]	0.32 ±0.35 [ab]	0.33 ±0.37 [ab]	0.36 ±0.37 [ab]	0.60 ±0.36 [b]
J6	0.10 ±0.02	0.08 ±0.02	0.09 ±0.02	0.10 ±0.03	0.12 ±0.08	0.11 ±0.03	0.09 ±0.02	0.11 ±0.03	0.16 ±0.06
J9	0.15 ±0.31	0.16 ±0.28	0.16 ±0.26	0.15 ±0.24	0.15 ±0.22	0.18 ±0.26	0.19 ±0.26	0.22 ±0.28	0.29 ±0.34
J12	0.10 ±0.03 [a]	0.14 ±0.07 [ab]	0.17 ±0.08 [b]	0.14 ±0.06 [ab]	0.13 ±0.05 [ab]	0.13 ±0.06 [ab]	0.14 ±0.06 [ab]	0.17 ±0.09 [b]	0.23 ±0.14 [b]
J18	0.09 ±0.03 [a]	0.16 ±0.07 [ab]	0.15 ±0.07 [ab]	0.13 ±0.05 [ab]	0.14 ±0.06 [ab]	0.14 ±0.07 [ab]	0.14 ±0.05 [ab]	0.14 ±0.08 [ab]	0.26 ±0.25 [b]
J24	0.05 ±0.01 [a]	0.12 ±0.05 [ab]	0.13 ±0.08 [ab]	0.12 ±0.05 [ab]	0.11 ±0.05 [ab]	0.14 ±0.09 [b]	0.24 ±0.10 [b]	0.25 ±0.10 [b]	0.41 ±0.21 [b]
Glucose (mmol/L)									
J0	4.7 ±0.53 [a]	4.1 ±0.47 [b]	4.3 ±0.58 [ab]	4.3 ±0.72 [ab]	3.8 ±0.34 [b]	4.0 ±0.43 [b]	4.0 ±0.43 [b]	4.1 ±0.44 [b]	4.4 ±0.38 [ab]
J6	4.7 ±0.44 [a]	4.9 ±1.68 [ab]	5.4 ±0.65 [b]	5.4 ±0.85 [b]	4.9 ±0.42 [ab]	4.9 ±0.41 [ab]	4.9 ±0.36 [ab]	4.9 ±0.50 [ab]	4.2 ±0.32 [ab]
J9	4.4 ±0.39 [a]	5.7 ±0.79 [b]	5.5 ±0.71 [b]	5.2 ±0.55 [b]	4.7 ±0.42 [ab]	4.6 ±0.39 [ab]	4.4 ±0.44 [ab]	4.3 ±0.55 [ab]	4.3 ±0.23 [ab]
J12	4.8 ±0.52 [a]	6.3 ±0.85 [b]	6.0 ±0.92 [b]	5.5 ±0.82 [b]	5.3 ±0.72 [ab]	4.9 ±0.70 [ab]	4.8 ±0.68 [ab]	4.6 ±0.53 [ab]	4.7 ±0.48 [ab]
J18	4.7 ±0.75 [a]	5.7 ±0.96 [b]	5.9 ±1.54 [b]	5.1 ±0.72 [ab]	4.7 ±0.41 [ab]	4.5 ±0.46 [ab]	4.5 ±0.36 [ab]	4.2 ±0.32 [ab]	4.4 ±0.34 [ab]
J24	4.3 ±1.41 [a]	5.8 ±0.53 [b]	5.4 ±0.44 [b]	5.1 ±0.42 [b]	4.8 ±0.56 [ab]	4.6 ±0.76 [ab]	4.2 ±0.48 [ab]	4.1 ±0.43 [ab]	4.2 ±0.38 [ab]
NEFA (µmol/L)									
J0	0.25 ±0.10 [a]	0.88 ±0.25 [b]	0.69 ±0.28 [b]	0.42 ±0.20 [b]	0.34 ±0.14 [ab]	0.32 ±0.14 [ab]	0.29 ±0.14 [ab]	0.22 ±0.11 [ab]	0.56 ±0.20 [b]
J6	0.26 ±0.12 [a]	0.35 ±0.13 [ab]	0.19 ±0.06 [ab]	0.14 ±0.05 [ab]	0.15 ±0.09 [ab]	0.18 ±0.08 [ab]	0.25 ±0.15 [ab]	0.42 ±0.22 [b]	0.23 ±0.09 [ab]
J9	0.24 ±0.10 [a]	0.32 ±0.20 [ab]	0.12 ±0.07 [b]	0.09 ±0.05 [b]	0.10 ±0.04 [b]	0.13 ±0.09 [b]	0.15 ±0.16 [ab]	0.22 ±0.15 [ab]	0.22 ±0.11 [ab]
J12	0.28 ±0.10 [a]	0.32 ±0.12 [ab]	0.12 ±0.03 [b]	0.11 ±0.07 [b]	0.12 ±0.09 [b]	0.15 ±0.12 [b]	0.21 ±0.15 [ab]	0.21 ±0.10 [ab]	0.35 ±0.11 [ab]
J18	0.19 ±0.11 [a]	0.40 ±0.18 [b]	0.17 ±0.13 [ab]	0.16 ±0.10 [ab]	0.23 ±0.23 [ab]	0.30 ±0.31 [ab]	0.25 ±0.19 [ab]	0.30 ±0.25 [ab]	0.55 ±0.17 [b]
J24	0.27 ±0.15 [a]	0.44 ±0.16 [b]	0.40 ±0.11 [b]	0.25 ±0.12 [ab]	0.22 ±0.07 [ab]	0.22 ±0.15 [ab]	0.15 ±0.14 [ab]	0.13 ±0.07 [ab]	0.48 ±0.22 [b]

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly ($P < 0.05$).

Table 8. Treatment means (± s.d.) for various blood cell types prior to transport and after transport

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
WBC ($x10^3/\mu L$)									
J0	9.2 ±3.59 [a]	9.1 ±1.86 [ab]	8.9 ±2.00 [ab]	9.8 ±2.39 [ab]	11.5 ±2.66 [b]	11.3 ±2.29 [b]	11.9 ±2.34 [b]	12.6 ±2.50 [b]	12.2 ±2.35 [b]
J6	8.9 ±0.82 [a]	14.7 ±2.16 [b]	15.2±2. 36 [b]	15.1 ±3.14 [b]	14.4 ±2.80 [b]	14.5 ±2.18 [b]	14.2 ±2.71 [b]	13.5 ±2.26 [b]	11.8 ±1.87 [b]
J9	9.7 ±1.47 [a]	15.3 ±3.29 [b]	15.7 ±3.15 [b]	15.5 ±3.12 [b]	15.2 ±2.79 [b]	15.1 ±2.80 [b]	15.1 ±2.89 [b]	13.8 ±2.31 [b]	11.7 ±2.16 [ab]
J12	9.3 ±1.22 [a]	12.7 ±2.83 [ab]	13.2 ±2.98 [b]	13.3 ±3.23 [b]	13.2 ±2.69 [b]	13.3 ±2.71 [b]	13.7 ±2.77 [b]	12.5 ±2.32 [b]	11.6 ±2.47 [b]
J18	10.4 ±2.99 [a]	13.1 ±2.21 [b]	13.1 ±2.15 [b]	13.4 ±1.96 [b]	13.1 ±2.27[b]	14.0 ±2.25 [b]	14.1 ±2.17 [b]	13.9 ±2.33 [b]	12.2 ±1.98 [ab]
J24	10.4 ±2.72 [a]	11.9 ±2.33 [b]	12.7 ±2.64 [b]	12.2 ±2.40 [b]	12.6 ±2.14 [b]	13.3 ±2.30 [b]	14.1 ±2.59 [b]	13.4 ±2.69 [b]	14.0 ±6.77 [b]
Lymphocyte (%)									
J0	73.2 ±10.57 [a]	77.8 ±10.60[ab]	74.5 ±8.43 [ab]	71.9 ±10.67[ab]	68.3 ±12.45[ab]	62.0 ±13.48[b]	58.3 ±13.01[b]	44.8 ±12.20[b]	63.8 ±8.76[b]
J6	65.2 ±14.65 [a]	43.6 ±5.58 [b]	46.7 ±11.15 [b]	43.1 ±6.49 [b]	42.9 ±6.99 [b]	46.3 ±7.37 [b]	47.2 ±7.95 [b]	56.0 ±7.72 [b]	57.3± 5.48 [b]
J9	65.9 ±15.52 [a]	39.8 ±10.07 [b]	41.4 ±12.09 [b]	41.8 ±9.96 [b]	47.7 ±6.75 [b]	48.4 ±7.39 [b]	47.8± 8.36 [b]	52.0 ±11.92 [b]	53.7 ±9.20 [b]
J12	74.6 ±7.48 [a]	49.5 ±15.29 [b]	46.5 ±7.09 [b]	49.0 ± 10.79 [b]	59.5 ±11.78 [b]	60.0 ±11.80 [b]	51.0 ±10.09 [b]	51.6 ±7.19 [b]	63.6 ±9.29 [b]
J18	24.1 ±10.05 [a]	19.8 ±10.81[b]	23.1 ±8.55 [b]	26.6 ±10.81[b]	29.9 ±13.11[b]	36.1 ±13.87[b]	40.3 ±13.22 [b]	54.1 ±12.77 [b]	34.8 ±8.61[b]
J24	69.4 ±7.74 [a]	50.8 ±6.66 [b]	56.6 ±11.33 [b]	49.2 ±11.11 [b]	47.2 ±7.43 [b]	56.1 ±9.06 [b]	53.8 ±9.49 [b]	52.2 ±10.70 [b]	56.3 ±12.14 [b]
Neutrophil (%)									
J0	30.8 ±15.06 [a]	55.2 ±6.73 [ab]	55.5 ±8.74 [ab]	55.6 ±6.49 [ab]	56.1 ±6.99 [ab]	52.8 ±8.37[b]	51.3 ±9.73 [b]	42.3 ±8.47 [b]	42.8 ±5.48[b]
J6	25.6 ±4.94 [a]	60.1 ±10.19 [b]	59.1 ±12.74 [b]	57.9 ±10.05 [b]	51.8 ±6.74 [b]	51.0 ±7.62 [b]	50.5 ±8.27 [b]	45.8 ±11.72 [b]	42.8 ±10.74 [b]
J9	22.9 ±8.11 [a]	48.6 ±16.81 [b]	52.8 ±7.49 [b]	50.8 ±10.77 [b]	39.9 ±11.86 [b]	38.7 ±10.37 [b]	47.5 ±9.76 [b]	46.4 ±7.94 [b]	34.7 ±9.34 [b]
J12	68.4 ±11.02 [a]	52.8 ±10.99 [b]	48.9 ±6.84 [b]	50.3 ±11.80 [b]	50.8 ±8.08 [b]	51.8 ±9.59 [b]	43.5 ±9.96 [b]	49.6 ±10.03 [b]	59.8 ±12.27 [b]
J18	28.2 ±10.15 [a]	46.1 ±10.97 [b]	50.4 ±6.52 [b]	48.7 ±11.27[b]	48.7 ±8.71[b]	44.8 ±10.31[b]	54.5 ±9.90 [b]	46.7 ±11.42[b]	36.8 ±12.11 [b]
J24	28.9 ±7.98 [a]	48.7 ±6.97 [b]	42.7 ±11.94 [b]	49.0 ±11.00 [b]	50.2 ±7.28 [b]	41.3 ±9.66 [b]	42.3 ±8.13 [b]	43.6 ±11.37 [b]	41.8 ±13.93 [b]

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly ($P < 0.05$).

Table 9. Treatment means (± s.d.) for various red blood cell (RNB) number, whole blood haemoglobin concentrations, and packed cell volume (PCV) prior to transport and after transport

Transport	-0.25h pre	0h	1 h post	2h post	4h post	6h post	8h post	12h post	24h post
RBC (x 10^6/µL)									
J0	10.0 ±0.80 [a]	9.3 ±1.09 [b]	8.8 ±0.91 [b]	8.8 ±0.88 [b]	9.1 ±0.85 [b]	9.3 ±0.90 [b]	8.8 ±0.82 [b]	9.1 ±0.75 [b]	9.0 ±0.69 [b]
J6	9.4 ±1.11	9.5 ±1.31	9.5 ±1.19	9.4 ±1.06	9.2 ±1.33	9.2 ±0.80	9.0 ±0.85	9.1 ±0.79	8.7 ±0.75
J9	9.3 ±1.59	9.6 ±1.49	9.6 ±1.33	9.5 ±1.34	9.3 ±1.45	9.2 ±1.28	9.3 ±1.27	9.4 ±1.45	9.1 ±1.31
J12	9.6 ±1.14	9.5 ±0.94	9.7 ±1.14	9.8 ±1.06	9.6 ±1.07	9.4 ±1.04	9.4 ±1.05	9.4 ±0.97	9.0 ±0.99
J18	9.9 ±1.43	9.8 ±1.04	9.8 ±0.85	9.8 ±1.02	10.1 ±0.97	9.8 ±1.04	9.9 ±1.01	10.0 ±0.87	9.2 ±0.98
J24	8.8 ±0.98	8.9 ±0.67	9.1 ±0.89	9.2 ±0.74	9.0 ±0.85	8.7 ±0.73	8.8 ±0.75	8.9 ±0.87	8.7 ±0.81
Hb (g /dL)									
J0	11.2 ±1.10 [a]	10.4 ±1.12 [b]	10.0 ±1.05 [b]	9.8 ±0.88 [b]	10.1 ±0.89 [b]	10.2 ±0.90 [b]	9.7 ±0.97 [b]	9.9 ±0.76 [b]	9.9 ±0.81 [b]
J6	10.0 ±1.04	10.0 ±0.98	10.0 ±0.96	10.0 ±0.73	9.7 ±1.06	9.6 ±0.51	9.5 ±0.71	9.6 ±0.61	9.3 ±0.70
J9	9.5 ±1.07	9.5 ±0.90	9.7 ±0.80	9.5 ±0.79	9.3 ±0.98	9.3 ±0.85	9.2 ±0.95	9.6 ±0.97	9.2 ±0.90
J12	9.9 ±0.73 [a]	9.8 ±0.66 [ab]	10.1 ±0.68 [ab]	10.1 ±0.64 [ab]	9.9 ±0.61 [ab]	9.7 ±0.56 [ab]	9.9 ±0.66 [ab]	9.8 ±0.62 [ab]	9.3 ±0.55 [b]
J18	10.1 ±1.13	10.0 ±0.93	10.1 ±0.80	10.0 ±0.81	10.1 ±0.76	9.9 ±0.98	9.8 ±0.70	10.1 ±0.86	9.1 ±1.06
J24	9.0 ±0.92	9.1 ±0.48	9.4 ±0.74	9.5 ±0.68	9.2 ±0.68	9.2 ±0.78	9.3 ±0.86	9.3 ±0.82	9.3 ±0.76
PCV (%)									
J0	33.9 ±3.45 [a]	31.7 ±3.73 [b]	29.8 ±3.44 [b]	29.5 ±2.99 [b]	30.3 ±3.25 [b]	30.3 ±3.05 [b]	29.1 ±3.42 [b]	30.2 ±2.89 [b]	29.7 ±2.92 [b]
J6	30.4 ±3.05 [a]	31.2 ±3.22 [ab]	31.1 ±3.19 [ab]	30.8 ±2.52 [ab]	29.9 ±3.06 [ab]	29.7 ±2.00 [ab]	29.2 ±2.78 [ab]	29.5 ±2.15 [ab]	27.6 ±2.05 [b]
J9	28.5 ±3.72	29.7 ±3.35	29.8 ±2.98	29.2 ±3.02	28.7 ±3.37	28.3 ±3.02	28.5 ±3.23	28.9 ±3.53	27.7 ±3.08
J12	29.9 ±2.74 [a]	30.0 ±2.38 [ab]	30.4 ±2.31 [ab]	30.4 ±2.39 [ab]	29.7 ±2.14 [ab]	29.1 ±2.12 [ab]	29.1 ±2.36 [ab]	29.0 ±2.21 [ab]	27.9 ±1.83 [b]
J18	30.1 ±3.57	29.9 ±3.19	30.1 ±2.85	29.5 ±2.44	30.6 ±2.70	29.8 ±3.30	29.8 ±2.49	30.5 ±3.01	28.0 ±3.75
J24	27.2 ±3.01	27.4 ±1.75	27.8 ±2.15	28.3 ±2.14	27.3 ±1.84	27.1 ±2.07	27.1 ±2.16	27.6 ±2.40	27.0 ±2.39

Control = J0 not transported; J6, J9, J12, J18 and J24 = transported for 6, 9, 12, 18 and 24 h at a stocking density of 1.02m^2 per animal; [a,b,] within row means not having a common superscript differ significantly ($P < 0.05$).

3. The welfare of weanling heifers transported by road and sea.

3.1 Introduction

The protection of animals during transport is an important concern of the European Commission. The first Community legislation on the protection of animals during transport, Council Directive 77/489/EC, reflected the relevant 1968 Convention of the Council of Europe. It has since been replaced by the more detailed Council Directive 91/628/EC as amended by Directive 95/29/EC which introduced changes such as the approval of transporters, route plan, as well as loading densities and travelling times limit. Additional legislation reinforcing Directive 91/628/EEC will be adopted in 1995, 1997 and 1998. More recently the EU commission proposed a new regulation relating to the transport of animals (Appendix I COM2003). Additional legislation reinforcing Directive 91/628/EEC was adopted in 1995, 1997, 1998 and 2005.

In a review of cattle transport by road it was reported that approximately 3 to 11% loss of liveweight occurs and that losses increase with increased journey times (Knowles et al., 1999a; 1999b). Knowles (1999b) investigated the physiological and behavioural effects on cattle of transporting them for periods of 14, 21, 26 and 31hours, including a stop for a rest and drink on the lorry after 14 hours. The authors concluded that the physiological changes that were found after a 31h journey indicated that transport was not detrimental to the animals. Eicher, (2001) summarised the behavioural, physiological and immunological consequences of animal transport research with relevance to the dairy industry and concluded that the duration of the journey has a great impact than the distance on young calves and after long transport, most animals drink and then lie down. Studies also showed that young calves habituate to transport, unlike cows. Marahrens et al. (2003) studied the physiological and behavioural consequences of the transport of heifers, bulls and steers by road from northern Germany to Mediterranean ports and concluded that animals should be prepared carefully pre-transport, i.e. with reference to energy and fluid balance, and to be fed at sufficient time intervals during the journey to maintain physiological homeostasis and normal expression of behaviour.

Grigor et al. (2001) investigated the effects of space allowance during transportation and duration of a mid-journey lairage period on measures of stress, injury, dehydration, food restriction and rest in young calves. The authors concluded that the duration of the mid-journey lairage was not an important factor and while there was little evidence that transport affected immunological variables, there was evidence to indicate the health of the calves was adversely affected post transport. Kenny and Tarrant (1987a; 1987b) have observed that confining animals on a moving vehicle is the most stressful component of transportation, while others reported that loading and unloading cause the most stress to cattle (Tennessen et al., 1984). Physiological and haematological responses associated with transport of animals are well studied. Decreased neutrophil and lymphocyte numbers following transportation have been documented in previous

work (Earley et al., 2006a; 2006b; Blecha et al., 1984; Kent and Ewbank, 1986). During transport, Grandin (1997) reported that animals become stressed by either psychological stress (restraint, handling, novelty) or physical stress (fasting, fatigue, injury or thermal extremes). During long distance transport animals are subjected to fasting periods and exhaustion (Honkovaara et al., 1999; Murata and Hirose, 1991) and exhibit lower stress profiles than animals travelling over shorter journey periods. This adaptation appears to take place during long distance transport as animals adapt to the novelty of transport while during short distance transport they don't habituate and may express acute (psychological) stress. Increased heart rate and plasma stress hormones (cortisol, adrenaline) during transport are a consequence of a non-specific stress reaction to the novelty of the transport process and the environment, which has been reported to elicit a wide array of physiological changes in blood constituents (Dantzer and Mormède, 1983).

In July 2003, the Commission adopted a proposal for a Regulation on animal transport which sought to radically overhaul the rules governing the transport of animals in Europe for journeys of more than 9hours, including domestic transport in Ireland and long duration journeys to the Continent. There have been few studies that have investigated the effects of transport of weanling animals from Ireland to the Continent of Europe in a roll-on roll-off transporter by road, by sea, followed by road, on the physiology, immunology and behaviour of animals during the journey and post-transport. In addition no studies have investigated the effects on the animals of allowing the animals to rest on a roll-on roll-off transporter without unloading following sea transport (22h) and land transport (9h).

The study hypothesis was that the welfare of animals transported from Ireland to Spain by sea and road will be compromised in transit if after completion of the sea and land part of the journey that they are allowed to remain on the transporter for a 12-hour period without unloading and lairaging with full access to feed and water, prior to completion of the remaining part of the journey by road to the feedlot. In order to test this hypothesis, appropriate physiological, haematological, immunological and behavioural measurements were made on the animals to quantify the effect of the transport stages on the degree of stress imposed and the ability of the animals to cope with that stress.

3.2 Material and methods

Care of animals

All procedures were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876, and the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994.

Animal diets and composition

In Ireland, heifers had *ad libitum* access to grass silage (*in vitro* DM digestibility = 755 g/kg), supplemented with 2.0 kg (as fed) barley/soybean concentrate (DM 843g/kg; crude protein = 115.0g/kg DM) per animal per day at Grange, Beef Research Centre. The animals in Spain were fed an *ad libitum* finishing diet, consisting of concentrate diet (DM 875g/kg; Crude protein 153 g/kg) and straw (DM 899g/kg; Crude protein 45.0 g/kg). All animals had free access to water.

Transport vehicle and environmental conditions

The transporter was fitted with sensors for measuring ambient temperature (^{o}C), relative humidity (RH; %), carbon dioxide (CO_2; ppm), hydrogen sulphide (H_2S; ppm), ammonia (NH_3; ppm) air velocity (m/s) and vapour density (g/m^3) continuously during transport. The ambient temperature and relative humidity during transport were recorded continuously using TinyTalk dataloggers (Radionics, Dublin, Ireland). Environmental measurements on the transporter including gases (NH_3, H_2S, CO_2), relative humidity (RH) and temperature were recorded using QRae (Shawcity Ltd., UK), Testo 175 and Testo 445 portable multifunction probes (Testo UK, Ltd).

Treatment groups

The study was conducted in November 2003. On the morning of the journey, 60 continental × weanling heifers (mean live weight 245, s.d 33.2kg) that had been weaned two weeks previously on farm of origin were blood sampled pre-transport by jugular venipuncture to provide baseline physiological levels and again after the journey. Blood samples were collected by jugular venipuncture before ((-0.25h) (bleed 1)), immediately after ((0h) (bleed 2)) and at 1h (bleed 3), 2h (bleed 4), 4h (bleed 5), 6h (bleed 6), 8h (bleed 7), 12h (bleed 8). Non-transported control animals (J0) were moved to a novel pen (n=2) with 10 animals/pen in the housing environment, and animals had access to *ad libitum* silage and 2kg of 2.0 kg barley/soybean concentrate and were blood sampled at times corresponding to transported animals.

The average speed during the sailing ranged from 11 to 13.5 knots/hr. The transported animals were assigned to one of four pens (n=10 animals/pen) on the lower deck of fan ventilated double deck articulated transporter. Twenty T heifers were unloaded (ULT) and rested for 12h in the lairage and the remainder rested (RT) on the transporter. All heifers had access to hay and water. After the rest period, the heifers were re-loaded. The subsequent journey by road from France to Spain was 9h travel, 7 h rest (on the transporter) and a further 7 h travel. The journey from the lairage at Fougeres in France to the feedlot in Spain (1300km) involved different road surfaces ranging from motorways to country lanes. All T heifers were blood sampled prior to transport (day (d) 0), on arrival in the French lairage (d 4), on arrival at the farm in Spain (d 6) and on d 8, 10, 12

and 36. Twenty continental × weanling heifers (247, s.d. 36.0 kg) remained in Ireland as controls (C) and were blood sampled at the same times as T heifers. Heifers were weighed on d 0, 4, 6, 12 and 36 of the study.

Body (rectal) temperature

Rectal body temperature was taken prior to transport (day (d) 0) (bleed 1), on arrival in the French lairage (d 4; bleed 2), after 12h rest in the lairage (bleed 3), on arrival at the feedlot in Spain (d 6;bleed 4) and on d 8 , 10, 12 and 36 of the study (bleeds 5, 6, 7 and 8). The rectal body temperature was monitored using a digital electronic thermometer (Jorgen Kruuse A/S; Model VT-801BWC Lot No 0701).

Behaviour

Lying and standing behaviour of the heifers on the transporter were monitored and video-recorded using 460 lines high resolution black-white cameras (Eneo, Germany) with built in 12 watt infra red lighting positioned in the 4 individual pens of the transporter. The cameras recorded 19-25 frames /sec and images were transferred to a personal computer using a multiplex card manufactured by CCTV (UK).

Assay procedures for physiological and haematological variables.

Heparinised blood samples were collected by jugular venipuncture and the plasma was separated by centrifugation at 1,600 x *g* at 8 ^{o}C for 15, and subsequently stored at –20 ^{o}C. Albumin, urea, globulin, total protein, β-hydroxybutyrate (βHB), and creatine kinase (CK) were measured on an automatic analyser (Olympus AU 400, Japan) using the reagents supplied by Olympus.

Plasma haptoglobin concentrations were measured using an assay kit (Tridelta Development Ltd., Wicklow, Ireland) on an automated analyzer (spACE; Alfa Wassermann, Inc., West Caldwell, NJ) according to the manufacturer's procedure. Plasma fibrinogen concentrations were measured according to the method described by Becker et al. (1984) on an automated analyzer (spACE).

Concentrations were determined using a commercial kit (Randox Laboratories Ltd., Crumlin, Antrim, N. Ireland; catalogue No. GL2623) on an automated analyzer (Hitachi 705, Boehringer Mannheim, Lewes, East Sussex, UK).

Red blood cell (RBC) number, white blood cell (WBC) number, differential WBC (percentage lymphocyte and neutrophil), packed cell volume (PCV), haemoglobin (Hb) concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet numbers were determined for unclotted (EDTA-treated) whole-blood samples with an automated

cell counter (Celltac MEK-6108K; Nihon-Kohdon, Tokyo, Japan) within 1h of blood sampling. Thin blood smears were also prepared on glass slides and stained using the haematology 3-step stain for differential WBC numbers (Accralab, Biochemical Sciences; Fisher Scientific Company, Middletown, VA).

Blood samples for interferon-γ (IFN-γ) determination were collected by jugular venipuncture into aseptic vacutainer tubes containing lithium heparin and the stimulated lymphocyte production of IFN-γ was determined following whole blood culture. The lymphocyte production of IFN-γ was determined (Earley and Crowe, 2002) following stimulation *in vitro* with either phosphate buffered saline alone, keyhole limpet hemocyanin (KLH; 20 μ g/1.5 mL blood), or concanavalin A (Con-A; 20 μg/1.5 mL blood) in whole blood culture; the IFN-γ concentration in the harvested plasma samples was measured using a specific ELISA procedure (CSL, Biosciences, Parkville, Victoria, Australia).

Plasma was harvested from heparin anti-coagulated blood following its centrifugation at 1600 x *g* at 4°C for 15 minutes and stored at -80°C until subsequent cortisol analysis. Cortisol concentration was measured using a commercially available RIA kit (Corti-cote, ICN Pharmaceuticals, Orangeburg, NY) as described in Fisher et al. (1997).

Statistics

Data were analysed using SAS/STAT. Physiological and haematological measurements were tested in a one-way ANOVA by PROC MIX, using a means statement with a Tukey option to detect treatment differences. A matched-pair t-test was used to detect differences pre- and post-transport for each treatment. Physiological, haematological, metabolic and liveweight data were analysed using the repeated measures procedure in PROC MIX to detect differences in treatments while controlling for time effects. A Wilcoxon signed rank test was used to evaluate differences between pre- and post-transport for each treatment (Snedecor and Cochran, 1989). A probability of $P < 0.05$ was chosen as the level of significance for the statistical tests. Measurements for WBC numbers, platelet numbers, CON-A induced IFN-γ, PHA-induced IFN-γ, albumin, glucose, NEFA, urea, haptoglobin, fibrinogen and CK concentrations were not normally distributed and were analysed by PROC GLM, using ranked data in a Kruskall-Wallis test with a Tukey option to evaluate treatment differences. A count of the total number of occurrences of each behaviour (lying and standing) was made for each scan time point. The PROC FREQ, chi-square statistic was used to determine the differences. Percentage time values were calculated from the total count data and since the animals were subjected to continuous recordings, the count data was expressed as percentage time.

3.3 Results

Environment

The mean temperature of the shed environment where the animals were housed was 12.9 ^{o}C (min 1.8 and max 19.6). The environmental data recorded during the total transport journey were as follows; the mean CO_2 levels (ppm) recorded was 819.7 (± 298.6 s.d.) min 364 and max 1700; mean relative humidity (%) 70.2 (± 9.9 s.d); min 44.4% and max 99.9%; mean temperature 15.0^{o}C (± 4.3 s.d.) min 2.7 and max 21.3; mean wind velocity 0.30m/s (± 0.37 s.d.) min 0.00 to 2.37 m/s; mean vapour density 9.4 g/m^3 (± 3.7 s.d.). The environmental data recorded while animals remained on the transporter in France for 12h was; mean CO_2 levels (ppm) recorded was 874.8 (± 322.1 s.d.) min 506 and max 1870 (ppm); mean relative humidity (%) 75.7 (± 6.2 s.d); min 64.1% and max 93.1%; mean temperature 13.9^{o}C (± 2.5 s.d.) min 9.5 ^{o}C and max 18.9 ^{o}C; mean wind velocity 0.14m/s (± 0.21 s.d.) min 0.00 m/s to 0.76 m/s; mean vapour density 9.6 g/m^3 (± 1.9 s.d.) min 6.4 g/m^3 and max 14.7 g/m^3 .

Liveweight and behaviour

There was no significant difference in liveweight among treatments on days 0 (pre-transport). Liveweight was altered by transportation in the current study. RT animals lost 7.6% liveweight and ULT animals lost 7.8% at the time of arrival in Spain (day 6), and control animals lost 2.44% liveweight during that time. On day 6, all RT animals and ULT animals had greater liveweight loss (RT mean % -7.61± 2.81 s.d. and ULT mean % -7.89 ± 2.65 s.d) than control (mean % -1.33± 1.78 s.d.) and values were not different from control on days 12 and 36. At d 36, there was no difference in liveweight among treatments. During the sea crossing (22 h) from Ireland to France, heifers spent 61% of time lying and 39% of the time standing; from France to Spain by road (9h travel, 7 h rest (on the transporter) and a further 7 h travel), animals spent 51% of the time standing and 49% of the time lying.

Temperature data

Rectal body temperature were taken prior to transport (day (d) 0) (bleed 1), on arrival in the French lairage (d 4; bleed 2) after 12h rest in the French lairage (d 5; bleed 3), on arrival at the feedlot in Spain (d 6; bleed 4) and on d 8, 10, 12 and 36 of the study (bleeds 5, 6, 7 and 8) (Table 10). Control animals had increased body temperature at bleed 4. RT animals remaining on the transporter in France for 12h, had elevated body temperature at the end of that period, and again had raised body temperature on days 10 (bleed 6), 12 (bleed 7), and 36 (bleed 8) of the study. ULT animals that were unloaded at the lairage in France had elevated body temperature at the end of the 12h rest period. There was a significant overall effect of bleed and treatment x bleed interaction *($P<0.001$)* while treatment was not significant. One of the RT animal that remained on the transporter in

France developed clinical signs of bovine respiratory disease and died at arrival in the Spanish feedlot.

Haematological variables

WBC numbers were lower (*P<0.05*) in control animals on d 12 and greater on d 36 compared with baseline values (Table 11). RT and ULT animals had greater (*P<0.05*) WBC numbers than baseline on d 3 (arrival in France) to d 36. There was no difference in WBC numbers between RT and ULT animals and numbers were greater (*P<0.05*) than control values from d 3 to d 36. There was a significant overall effect of treatment and treatment × bleed interaction *(P<0.001).*

Neutrophil % was lower (*P<0.05*) in control animals on d 3, d 6 and d 36 compared with d 12 and was not different from baseline values (Table 11). RT animals had greater (*P<0.05*) neutrophil % on d 6 and d 10 than d 12. ULT animals had lower neutrophil % than control on d 12. There was no significant overall effect of treatment, bleed or treatment × bleed interaction *(P>0.05).*

Lymphocyte % was lower (*P<0.05*) in control animals at d 12 compared with d 5 and were not different from baseline values (Table 11). RT animals had lower (*P<0.05*) lymphocyte % on d 6 and d 10 compared with d 12. ULT animals had greater lymphocyte % than controls on d 12. There was no significant overall effect of treatment, bleed or treatment × bleed interaction *(P>0.05).*

PCV% was lower (*P<0.05*) over time (d3 to d 36) compared with baseline values in control, T and ULT animals (Table 11). At d 0, RT animals had lower (*P<0.05*) PCV% than ULT animals. RT and ULT animals had lower PCV% (*P<0.05*) than control on d 3 to d 36. There was a significant overall effect (*P<0.05*) of treatment, bleed and treatment × bleed interaction *(P<0.05)*.

Hb concentrations were lower (*P<0.05*) in control animals compared with baseline on d 6 to d 36, while levels were lower (*P<0.05*) on d 5 to d 36 in RT animals than baseline (Table 12). ULT animals had lower (*P<0.05*) concentrations on d 3, d 6, d 10 and d 36 than baseline. HB concentrations were lower (*P<0.05*) in RT and ULT animals on d 5, d 8, d d10, d 12 and d 36 compared with control. There was a significant overall effect of treatment, bleed and treatment × bleed interaction *(P<0.05).*

Control animals had lower (*P<0.05*) RBC number on d 5 to d 36 than the pre-transport baseline (Table 12). RT animals had greater (*P<0.05*) RBC number on d 3 and lower (*P<0.05*) RBC number on d 10 to 36. ULT animals had lower (*P<0.05*) RBC number on d 10 to d 36. RT animals had greater (*P<0.05*) RBC numbers on d 3, d 6, d 8 and lower (*P<0.05*) on d 36 compared with control. ULT animals had greater (*P<0.05*) RBC number on d 0 to d 36 compared with controls.

There was a significant overall effect of treatment, bleed and treatment × bleed interaction *(P<0.001).*

MCHC levels were greater (*P<0.05*) in control animals on d 36 than baseline. RT and ULT animals had lower (*P<0.05*) MCHC levels on d 3 to 36 than baseline values (Table 12). There was no difference between T and ULT animals in MCHC levels, however, values were greater (*P<0.05*) than control on d 3 to d 36. There was a significant overall effect (*P<0.05*) of treatment and treatment × bleed interaction *(P<0.001).*

There was no change in platelet numbers (Table 12). On d 36 platelet numbers were lower (*P<0.05*) in RT and ULT animals compared with control and baseline levels on d 0.

Immunological variables

There was no significant *(P>0.001)* effect of treatment, bleed or treatment × bleed interaction in Con-A induced Interferon-(IFN)γ production (Table 13). IFN-γ levels were greater (*P<0.05*) in control animals on d 3, d 12 and d 36 than baseline values. In RT animals IFN-γ levels were lower (*P<0.05*) on d 3, d 5 and d 6 and were greater (*P<0.05*) than baseline on d 36. In ULT animals IFN-γ levels were lower (*P<0.05*) on d 6 and were greater (*P<0.05*) than baseline on d 36. Both RT and ULT animals had lower (*P<0.05*) IFN-γ levels than control on d 3, d 5 and d 6 and levels had returned to control values on d 36.

There was no significant effect of treatment, bleed or treatment x bleed interaction *(P>0.001) in* phytohaemagglutin-A induced interferon-γ production (Table 13). IFN-γ levels were lower (*P<0.05*) in control animals on d 5, d 6 and d 8 and were greater (*P<0.05*) than baseline on d 36. In RT animals IFN-γ levels were lower (*P<0.05*) on d 6 and were greater (*P<0.05*) than baseline on d 10 and d 12. In ULT animals IFN-γ levels were lower on d 6 and d 36 and were greater than baseline on d 10 and d 12. RT and ULT animals had lower IFN-γ levels than control on d 6 and d 8, and had higher levels at d 12.

Physiological variables

There was no significant overall effect of treatment, bleed and treatment × bleed interaction (P>0.05) on plasma cortisol concentrations. Cortisol concentrations were greater (*P<0.05*) in control animals on d 36, lower on d 12 than on d 3 and were not different from baseline values (Table 13). RT animals had greater (*P<0.05*) cortisol concentrations on d 3, d 5 and d 36 than on d 12 and were not different from baseline. In ULT animals cortisol concentrations were greater (*P<0.05*) on d 36 than on d 5, d 6, and d 8.

Control animals had lower ($P<0.05$) albumin concentrations on d 6, d 8 and d 36 than baseline values (Table 14). RT animals had greater ($P<0.05$) albumin concentrations on d 3 and d 5, and lower ($P<0.05$) concentrations on d 8, d 10 and d 12 than baseline (Table 14). ULT animals had greater ($P<0.05$) albumin concentrations on d 3, and lower albumin concentrations on d 8 to d 36. RT animals had lower ($P<0.05$) albumin concentrations on d 12 compared with control. In control animals there was no change ($P>0.05$) over time in globulin concentrations (Table 14).

RT animals had greater ($P<0.05$) globulin concentrations on d 10 and d 12 compared with control, while ULT animals had greater levels on d 6, d 10, d 12 and d 36 and were not different ($P>0.05$) from control (Table 14). There was no significant change ($P>0.05$) in protein concentration over time in control and ULT animals (Table 14). RT animals had greater ($P<0.05$) protein concentrations on d 6 compared with baseline. There was no difference ($P>0.05$) in protein concentrations between RT and ULT animals from d 3 to d 36.

Glucose concentrations were lower ($P<0.05$) in control animals from d 3 to d 36 compared with baseline values (Table 14). In RT animals, glucose concentrations were lower ($P<0.05$) on d 8, d 10 and d 12 and had normalised to baseline values by d 36. ULT animals had lower ($P<0.05$) glucose concentrations on d 8, d 10 and d 12 compared with baseline. ULT animals had greater ($P<0.05$) glucose concentrations on d 3, d 5 and d6 and lower values on d 8 compared with controls.

NEFA concentrations were lower ($P<0.05$) in control animals from d 3 to d 36 compared to baseline values (Table 15). In RT and ULT animals, NEFA levels were lower ($P<0.05$) at d 12 and d 36 compared with baseline values. RT and ULT animals had greater ($P<0.05$) NEFA concentrations on d 3, d 5, and d 6 and had lower ($P<0.05$) concentrations on d 12 and d 36 compared with controls.

Control animals had lower ($P<0.05$) βHB concentrations on d 3, d 12 and d 36 compared with baseline (Table 15). RT and ULT animals had lower ($P<0.05$) βHB concentrations on d 12 and d 36. βHB concentrations in RT animals were lower ($P<0.05$) on d 36 compared with control.

Urea concentrations were lower ($P<0.05$) in control animals on d 3, d 8, d 10, d 12 and d 36 compared with baseline values (Table 15). In RT animals urea concentrations were greater on d 3, d 5, d 6 and d 36 while ULT animals had greater ($P<0.05$) urea concentrations on d 3, d 5, d 6 and d 36. Urea concentrations were higher ($P<0.05$) in RT and ULT animals from d 3 to d 38 compared with controls. There was a significant overall effect ($P<0.05$) of treatment on urea concentrations.

Haptoglobin concentrations were lower in control animals than baseline on d 12 and d 36 (Table 16). RT and ULT animals had lower haptoglobin concentrations than baseline on d 36 and haptoglobin concentrations were lower than controls in RT and ULT animals on d 36.

There was no change ($P>0.05$) in fibrinogen concentrations from d 0 to d 12, in control animals, while levels were lower ($P<0.05$) on d 36 than baseline (Table 16). RT and ULT animals had lower ($P<0.05$) levels of fibrinogen than baseline on d 12 and d 36. RT and ULT animals had lower ($P<0.05$) fibrinogen concentrations on d 3 and d12 compared with control.

CK activity was lower ($P<0.05$) on d 3 to d 36 in control animals, than baseline (Table 16) whereas, RT animals had lower ($P<0.05$) CK activity on d 10, d 12 and d 36 than baseline. ULT animals had lower ($P<0.05$) CK activity on d 3, d 6, d 10, d 12 and d 36 than baseline values. RT animals had greater ($P<0.05$) CK activity on d 8 and d 10 compared with control values and ULT animals had greater CK activity than control on d 10.

3.4 Discussion

The physiological, immunological and behavioural effects of transporting heifers from Ireland to France by ferry (by road (4 h), by sea (22 h), by road (3 h), including a rest and drink on the transporter after 12h, to Spain by road (18h)), were studied in 40 transported animals and 20 control animals. The results of the study showed that transportation of weanling heifers to Spain affected liveweight, haematological and physiological variables of metabolism, immune function and inflammation. The performance of the animals remaining on the transporter in France was not different post-transport from the transported animals that were unloaded and lairaged in France. The loss in liveweight recorded in the transported animals are in accordance with previously reported transport studies where body weight loss ranged from 3 to 11% (Arthington et al., 2003; Earley et al 2006a; 2006b; Knowles, 1999a; Tarrant et al., 1998; Tarrant et al., 1992). Marahrens et al (2003) reported that loss of body weight in steers (-6.65%) coming from pasture was higher compared to bulls (-4.6%) during long distance transport but animals recovered well during lairage. In the present study, the loss in liveweight was also apparent in control non-transported animals and this decrease may be attributed to the management of the animals as they were removed from grazing pasture and fed *ad libitum* silage and 2kg of concentrates. At the end of the study period there was no difference in liveweight among treatments.

The behavioural responses of animals during transport, particularly lying and standing behaviour, are a very useful measure of animal welfare during transport. The incidence of lying behaviour was more apparent during the latter stages of the journey and this finding concurs with the findings of Knowles et al. (1999a) where cattle were transported for 24h.

Rectal temperature of the animals remaining on the transporter in France for 12h, was elevated at the end of that period and again on days 10, 12 and 36 of the study while animals that were unloaded at the lairage in France had elevated body temperature at the end of the 12h rest period. Tarrant et al., (1988) emphasized that the most stressful aspect of the transportation process for cattle was being confined on a moving vehicle and suggested that confinement on a stationary vehicle, loading/unloading and re-penning in a new environment are less stressful. The results further suggest that confinement on a stationary vehicle may be detrimental to the subsequent health of animals and may pre-dispose animals to infections like bovine respiratory disease.

White blood cell (WBC) numbers were greater in all transported animals from bleed 2 (at arrival in France) onwards and there was a significant overall effect of treatment and treatment x bleed interaction. Control animals showed transient changes in WBC numbers. T animals had greater ($P<0.05$) neutrophil number and lower lymphocyte number at bleed 4 and 6 than at bleed 7. ULT animals had lower neutrophil numbers and greater lymphocyte number than control at bleed 7 (day 12). The neutrophilia and lymphopenia following transportation observed in this study are in agreement with previously reported findings following a variety of stressors, including transport

stress (Blecha et al., 1984 and Murata et al., 1987 and Tarrant et al., 1992). The data generated in this study from transported cattle is supportive of previously reported changes in lymphocyte sub-sets following transport. Furthermore, changes in lymphocyte and neutrophil populations may suggest some form of dysregulation during the period relating to changes in hormone profiles or other factors associated with mixing and assembly of animals pre-transport.

Inexplicably, the PCV% declined in response to transport. All transported animals had lower PCV% than control from bleeds 2 to 8 and control animals had lower PCV% than baseline. It has been reported that raised PCV% following transport in association with higher erythrocyte counts in the circulation (Kent and Ewbank, 1986a; 1986b; Tarrant et al., 1992) indicates dehydration. There was a significant overall effect of treatment, bleed and treatment x bleed interaction with respect to haemoglobin, RBC number and mean cell haemoglobin concentration.

Mormede et al. (1982) reported that cattle were more susceptible to disease challenge in the days immediately following transportation. These observations have been rendered more concrete by a large body of work indicating that both intensity and duration of stressors may be important in bringing about changes in immunological functions (Minton, 1994). In the present study, lymphocyte functional assays in terms of PHA-induced and CON-A induced IFN-γ production were used to assess immune function during and after transport. Induction of a proliferative response induced by antigen *in vitro* has been shown to be representative of cellular immunocompetence (Kristensson et al., 1994; Swanson et al., 2001). The results showed that all transported animals had lower IFN-γ levels than control at bleeds 2, 3 and 4 and levels had returned to control values at day 36. RT animals had lower IFN-γ levels than ULT animals at day 36. This latter finding indicated that RT animals had greater suppression of lymphocyte function after transport than animals that went through unloading and resting off the transporter at the lairage in France. Lymphocytes play a critical role in host immunity to infection as they respond to infectious agents through production of antibodies, cytokines, and through specific T-cell mediated immune responses (Burton et al., 2005) and play a crucial role in the control of infection (Kehrli and Goff, 1989; Kehrli and Harp, 2001). Furthermore, Murata et al. (1987) observed a decrease of T lymphocytes in the peripheral blood of calves after transportation, however, evaluation of the T lymphocyte subpopulations was not performed. More recently, Riondato et al. (2007) analysed alterations in peripheral blood lymphocyte subsets in transported calves with increased cortisol and catecholamine levels in order to better characterise the stress-induced immune response and showed that transport induced a significant reduction in peripheral blood lymphocyte subsets detected by a panel of mAbs which was no longer present at 24h after arrival.

Several lines of evidence indicate that the psychological stress associated with transport, based on changes in heart rate and plasma cortisol concentrations, is generally highest during loading and

pre-transport management of animals (Eldridge et al., 1988; Warriss et al., 1995). Physiological responses to stressors include changes in metabolic variables in which catecholamines and peptides are co-released from the sympatho-adrenal system where they act as crucial mediators. The release of cortisol from the adrenal glands in response to stress can have profound effects on the circulation and functional capacities of immune cells. During transport, animals generally habituate to the transport conditions and elevated plasma cortisol concentrations are not sustained over the entire journey. Increased plasma cortisol concentrations, an indicator of HPA axis activation, have been reported in nearly all transportation studies of cattle as compared to pre-transportation baseline concentrations or those obtained from non-transported controls (Arthington et al., 2003; Crookshank et al., 1979; Gupta et al., 2007; Kenny and Tarrant, 1987a; 1987b; Kent and Ewbank, 1983; Knowles et al., 1999b; Tarrant et al., 1992; Yagi et al., 2004). In addition to its other roles, the HPA promotes lipolysis and elevated free fatty acid (FFA) levels in blood (Carey, 1998). Calf transport has been shown to induce significant alterations in blood cortisol and catecholamine levels and in the concentrations of their specific receptors lymphocyte glucocorticoid receptor (GR) and β-adrenergic receptor (β-AR) (Odore et al., 2004) and circulating hormone levels and lymphocyte AR and GR concentrations returned to physiological values within 24h post-transport, thus suggesting a normalisation of the endocrine profile and, probably of the immune system functions.

In the present study, the changes in metabolic variables were similar to previously reported changes after transport (Earley et al., 2006a; 2006b). These observations have been rendered more concrete by a large body of research indicating that metabolic variables are useful indicators in the diagnosis and prognosis of pathological states (Ginsburg and Haga, 2006). Plasma concentrations of albumin, globulin, protein, glucose concentrations were greater in all transported animals with control animals showing no major changes. These results are in agreement with previous data in the literature indicating that the central nervous system plays an important role in the regulation of hepatic glucose and lipid metabolism *via* the sympathetic nervous system and metabolic hormones (Sapolsky et al., 2000). Changes in the circulating levels of biological variables are often used to study the impact of treatments on metabolism. Interestingly, NEFA, βHB and urea concentrations were lower than baseline in control and all transported animals. Knowles et al., (1999) investigated the effects of transport duration in cattle and reported similar findings with βHB concentrations. βHB is a key indicator of hepatic ketogenesis and is the primary ketone body found in blood. In contrast, prolonged fasting has been shown to increase lipid catabolism resulting in higher blood levels of βHB. It is possible that the changes reported in NEFA, BHB and urea concentrations in this study, may be related to the combined effects of diet change and the journey duration.

Plasma concentrations of haptoglobin were lower in control animals and were unchanged in transported animals, fibrinogen concentrations were lower in all transport treatments and levels

decreased in control animals at day 36. Acute phase proteins, such as haptoglobin and fibrinogen, are released by hepatocytes in response to tissue injury or infection (Pang et al., 2006; Gånheima et al., 2007; Murata et al., 2004). Serum haptoglobin concentrations may also be indicators of bovine respiratory disease (BRD). Plasma concentration of fibrinogen, another APP, has been shown to increase in cattle with inflammatory disorders (McSherry et al., 1970) and has been used for many years to evaluate inflammatory disease in cattle (Eckersall and Conner, 1988; Petersen et al., 2004). Yeager et al. (2004) has reported that the pro-inflammatory cytokines, including interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and interleukin-1-beta (IL-1β), act synergistically with glucocorticoids to induce or suppress the expression of liver-derived acute phase proteins. However, the mechanisms by which glucocorticoids and stress alter acute phase protein expression in the absence of concomitant cytokine production is less well understood.

In response to physical stress or exercise, the enzyme CK leaks from sarcoplasm of muscle cells into the bloods, due to increased permeability of the sarcolemma muscle cell membrane and therefore, elevated plasma CK activity is a useful indicator of muscular activity. Conversely, in the present study, CK activities were lower in all T and control animals compared to their pre-transport baseline values.

In conclusion, the results from this study show that heifers undergoing transportation by road and sea, followed by road, at a spatial allowances of $0.93m^2$ showed physiological, haematological and immunological responses that were within normal referenced ranges (Schalm, 1961, 1984; Kaneko, 1989). The confinement of animals on a stationary vehicle during the mid-journey 12h rest period may be detrimental to the subsequent health of animals and may pre-dispose animals to infections like bovine respiratory disease.

Table 10. Changes in body temperature in control and transported (RT and ULT) heifers. The values are expressed as mean (kg) ± s.d.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
Control	39.45 ±0.65 ^{ax}	39.49 ±0.58 ^{abx}	39.69 ±0.69 ^{abx}	40.32 ±0.66 ^{bx}	39.62 ±0.61 ^{abx}	39.52 ±0.67 ^{abx}	39.46 ±2.63 ^{abx}	39.00 ±0.48 ^{abx}
RT	39.20 ±0.50 ^{ax}	39.42 ±0.62 ^{abx}	38.99 ±0.87 ^{aby}	39.89 ±0.84^{bx}	39.01 ±0.45 ^{aby}	39.62 ±0.53 ^{bx}	39.62 ±0.66 ^{abx}	39.43 ±0.51 ^{aby}
ULT	39.24 ±0.56 ^{ax}	39.69 ±0.77 ^{bx}	39.13 ±0.82 ^{aby}	40.11 ±0.84 ^{bx}	39.11 ±0.62 ^{aby}	39.62 ±0.80 ^{abx}	39.71 ±0.97 ^{abx}	39.30 ±0.48 ^{abxy}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal;
^{a,b,} within row means not having a common superscript differ significantly (*P < 0.05).*
^{x,y,} within column means not having a common superscript differ significantly (*P < 0.05).*

Table 11. Treatment effects on haematological variables pre and post-transport. Values are expressed as mean ± SD except for WBC which is expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	WBC ($1x10^3$ µl)							
Control	8.85	8.10	9.00	8.25	9.40	8.00	6.90	9.35
	5.7-13.7 ^{abx}	2.7-11.0 ^{bx}	2.3-12.5 ^{abx}	3.5-13.2 ^{bx}	3.9-15.9 ^{abx}	3.9-11.2 ^{abx}	3.5-13.0 ^{ax}	7.2-14.9 ^{bx}
RT	10.10	19.06	12.85	19.61	14.11	11.51	17.41	12.30
	5.5-14.3 ^{ax}	9.5-46.6 ^{by}	5.71-41.51 ^{by}	8.7-65.01 ^{by}	6.9-29.61 ^{by}	6.9-25.31 ^{by}	5.8-33.71 ^{by}	5.1-30.2 ^{by}
ULT	9.30	16.00	12.96	16.86	15.06	14.41	16.75	12.10
	3.8-12.9 ^{ax}	6.51-23.7 ^{by}	5.1-25.7 ^{by}	6.5-30.41 ^{by}	8.01-27.91 ^{by}	5.41-32.91 ^{by}	8.91-25.91 ^{by}	5.7-16.2 ^{bxy}
	Neutrophil percentage (%)							
Control	36.25 ±14.65 ^{abx}	32.95 ±13.05 ^{bx}	38.22 ±12.46 ^{abx}	33.20 ±9.65 ^{bx}	35.95 ±12.11 ^{abx}	38.10 ±10.44 ^{abx}	42.10 ±10.42 ^{ax}	32.44 ±13.96 ^{bx}
RT	38.00 ±13.91 ^{abx}	37.16 ±11.79 ^{abx}	39.20 ±10.47 ^{abx}	43.95 ±14.53 ^{bx}	37.16 ±12.67 ^{abx}	42.67 ±9.24 ^{bx}	33.79 ±13.49 ^{ax}	40.68 ±15.48 ^{abx}
ULT	37.30 ±7.66 ^{abx}	34.35 ±12.46 ^{abx}	37.78 ±17.37 ^{abx}	38.84 ±16.55 ^{abx}	34.55 ±14.38 ^{abx}	37.53 ±9.39 ^{abx}	31.47 ±9.03 ^{aby}	36.06 ±12.42 ^{abx}
	Lymphocyte percentage (%)							
Control	62.35 ±15.11 ^{abx}	64.26 ±13.35 ^{abx}	60.11 ±11.54 ^{bx}	65.85 ±9.84 ^{abx}	62.85 ±12.18 ^{abx}	60.40 ±10.00 ^{abx}	57.30 ±10.92 ^{ax}	64.88 ±14.14 ^{abx}
RT	59.80 ±13.63 ^{abx}	58.63 ±11.53 ^{abx}	59.15 ±11.11 ^{abx}	53.89 ±14.82 ^{bx}	61.37 ±12.74 ^{abx}	54.94 ±9.08 ^{bx}	65.68 ±13.69 ^{ax}	58.42 ±15.62 ^{abx}
ULT	59.15 ±10.23 ^{abx}	62.05 ±12.20 ^{abx}	59.89 ±16.91 ^{abx}	59.00 ±15.23 ^{abx}	63.25 ±14.45 ^{abx}	60.82 ±9.15 ^{abx}	68.32 ±9.07 ^{aby}	62.82 ±12.51 ^{abx}
	PCV%							
Control	39.49 ±1.94 ^{axy}	39.09 ±3.53 ^{abx}	38.32 ±2.73 ^{bx}	36.71 ±3.40 ^{bx}	36.51 ±3.24 ^{bx}	36.54 ±2.80 ^{bx}	35.39 ±3.41 ^{bx}	33.69 ±3.21 ^{bx}
RT	39.18 ±3.01 ^{ax}	34.41 ±3.29 ^{by}	32.17 ±5.58 ^{by}	32.26 ±2.44 ^{by}	31.36 ±2.82 ^{by}	29.75 ±2.98 ^{by}	29.78 ±2.77 ^{by}	28.09 ±2.88 ^{by}
ULT	40.23 ±4.06 ^{ay}	33.63 ±3.86 ^{by}	32.06 ±2.94 ^{by}	32.98 ±3.31 ^{by}	32.01 ±3.34 ^{by}	30.97 ±2.54 ^{by}	30.74 ±3.38 ^{by}	28.97 ±2.54 ^{by}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of $0.93m^2$ per animal;

^{a,b,} within row means not having a common superscript differ significantly ($P < 0.05$).

^{x,y,} within column means not having a common superscript differ significantly ($P < 0.05$).

Table 12. Treatment effects on haematological variables pre and post-transport. Values are expressed as mean ± SD except for MCHC and platelet number which are expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	Hb (g/dl)							
Control	13.23	13.15	12.95	12.38	12.34	12.24	11.94	11.46
	±0.75 ^{ax}	±1.12 ^{abx}	±0.89 ^{abx}	±1.16 ^{bx}	±1.12 ^{bx}	±0.96 ^{bx}	±1.16 ^{bx}	±1.04 ^{bx}
RT	13.14	12.83	11.91	12.05	11.48	10.89	10.89	9.12
	±1.06 ^{ax}	±1.11 ^{abx}	±1.94 ^{by}	±0.89 ^{bx}	±0.95 ^{by}	±0.95 ^{by}	±0.93 ^{by}	±1.00 ^{by}
ULT	13.56	12.49	12.11	12.27	12.01	11.41	11.37	9.42
	±1.42 ^{ax}	±4.21 ^{bx}	±1.12 ^{bxy}	±1.16 ^{bx}	±1.15 ^{bxy}	±0.99 ^{by}	±1.14 ^{bxy}	±0.86 ^{by}
	RBC (x10^{6}µl)							
Control	10.51	10.38	10.05	9.71	9.74	9.69	9.42	8.83
	±0.79 ^{ax}	±0.82 ^{abx}	±0.64 ^{bx}	±0.72 ^{bx}	±0.66 ^{bx}	±0.70 ^{bx}	±0.84 ^{bx}	±1.07 ^{bx}
RT	10.90	11.75	10.93	11.10	10.73	10.31	10.13	7.31
	±0.96 ^{axy}	±0.97 ^{by}	±1.79 ^{abxy}	±0.83 ^{aby}	±1.04 ^{aby}	±0.99 ^{bxy}	±0.98 ^{bxy}	±0.73 ^{by}
ULT	11.28	11.31	11.10	11.35	11.01	10.76	10.49	7.55
	±1.56 ^{ay}	±1.14 ^{aby}	±1.28 ^{aby}	±1.26 ^{aby}	±1.33 ^{aby}	±1.15 ^{by}	±1.34 ^{by}	±0.66 ^{by}
	MCHC (g/dl)							
Control	33.55	33.60	33.60	33.60	33.65	33.40	33.65	34.10
	32.4-34.6 ^{ax}	32.8-34.7 ^{abx}	32.9-35.4 ^{abx}	32.4-35.0 ^{abx}	32.8-34.9 ^{abx}	32.8-34.6 ^{abx}	32.7-35.5 ^{abx}	32.8-35.1 ^{bx}
RT	33.10	37.40	37.50	37.50	36.90	36.50	36.50	32.10
	31.6-35.0 ^{ay}	35.3-38.6 ^{by}	29.8-39.1 ^{by}	35.8-38.5 ^{by}	34.3-38.1 ^{by}	35.2-38.3 ^{by}	33.3-39.4 ^{by}	30.4-33.9 ^{by}
ULT	33.80	37.50	37.80	37.50	37.45	37.05	36.90	32.40
	32.5-34.7 ^{ay}	33.7-39.9 ^{by}	35.6-40.1 ^{by}	35.4-38.7 ^{by}	35.7-39.4 ^{by}	34.6-38.2 ^{by}	34.3-39.3 ^{by}	31.1-33.8 ^{by}
	Platelet number (x10^{3}µl)							
Control	484.50	574.50	535.50	604.00	651.00	674.50	764.00	552.50
	87-1474 ^{ax}	176-1266 ^{abx}	105-1241 ^{abx}	43-1169 ^{abx}	20-1283 ^{abx}	133-1233 ^{abx}	48-1355 ^{abx}	49-1151 ^{abx}
RT	627.00	628.00	652.50	677.00	618.00	595.00	560.00	240.00
	210-1548 ^{ax}	158-1221 ^{abx}	128-1266 ^{abx}	331-1113 ^{abx}	283-1089 ^{abx}	292-1159 ^{abx}	349-1124 ^{abx}	210-287 ^{by}
ULT	480.50	442.50	440.00	570.00	548.00	545.50	554.50	253.00
	104-1830 ^{ax}	271-1492 ^{abx}	254-1440 ^{abx}	215-1468 ^{abx}	223-1344 ^{abx}	259-1109 ^{abx}	196-1242 ^{abx}	207-287 ^{by}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12 h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal; ^{a,b,} within row means not having a common superscript differ significantly ($P < 0.05$). ^{x,y,} within column means not having a common superscript differ significantly ($P < 0.05$).

Table 13. Treatment effects on immunological and hormone variables pre and post-transport. Values are expressed as mean ± SD except for cortisol which is expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	Con-A induced interferon-γ production O.D. @ 450 nm							
Control	0.08	0.15	0.09	0.08	0.08	0.14	0.12	0.16
	0.01-0.274 ^{ax}	0-0.331 ^{ax}	0-0.706 ^{abx}	0.003-0.455 ^{abx}	0.034-0.314 ^{abx}	0.018-1.047 ^{abx}	0.061-0.774 ^{bx}	0.015-0.813 ^{bx}
RT	0.08	0.02	0.02	0.02	0.14	0.14	0.09	0.24
	0.001-0.415 ^{ax}	0-0.442 ^{by}	0-0.132 ^{by}	0-0.314 ^{by}	0.015-0.278 ^{abx}	0.014-0.394 ^{abx}	0.043-0.752 ^{abx}	0.007-0.828 ^{bx}
ULT	0.07	0.04	0.03	0.01	0.04	0.14	0.08	0.20
	0.011-0.358 ^{ax}	0-2.076 ^{aby}	0-0.219 ^{aby}	0-0.069 ^{by}	0-0.643 ^{abx}	0-0.864 ^{abx}	0-0.654 ^{abx}	0.029-0.358 ^{bx}
	PHA induced interferon-γ production O.D. @ 450 nm							
Control	0.07	0.09	0.03	0.04	0.33	0.24	0.05	0.36
	0.005-1.341 ^{ax}	0-0.777 ^{abx}	0-0.441 ^{bx}	0-0.19 ^{bx}	0.037-1.029 ^{bx}	0.023-0.79 ^{abx}	0-0.406 ^{abx}	0-0.767 ^{bx}
RT	0.04	0.08	0.07	0.01	0.02	0.22	0.21	0.05
	0-0.645 ^{ax}	0-0.566 ^{abx}	0-0.579 ^{abx}	0-0.13 ^{by}	0-0.545 ^{aby}	0.065-1.831 ^{bx}	0.033-2.149 ^{by}	0-0.275 ^{abx}
ULT	0.11	0.13	0.05	0.01	0.05	0.41	0.25	0.02
	0-1.183 ^{ax}	0-0.541 ^{abx}	0-0.736 ^{abx}	0-0.083 ^{by}	0-0.394 ^{aby}	0.066-1.678 ^{bx}	0.052-2.029 ^{by}	0-0.346 ^{bx}
	Cortisol (ng/ml)							
Control	19.21	9.26	11.77	12.77	12.82	12.84	12.46	19.74
	13.92 ^{abx}	6.06 ^{bx}	7.48 ^{abx}	7.78 ^{abx}	6.28 ^{abx}	12.29 ^{abx}	6.45 ^{abx}	8.96 ^{ax}
RT	20.68	16.78	9.56	12.09	12.01	10.60	8.11	26.25
	12.12 ^{abx}	22.00 ^{bx}	5.89 ^{bx}	6.93 ^{abx}	9.32 ^{abx}	6.90 ^{abx}	3.87 ^{ax}	16.42 ^{bx}
ULT	19.49	15.36	9.41	14.64	8.98	9.79	10.44	20.63
	8.14 ^{abx}	6.60 ^{abx}	4.86 ^{bx}	9.14 ^{bx}	3.80 ^{abx}	3.31 ^{abx}	6.26 ^{ax}	10.46 ^{abx}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal;
^{a,b,} within row means not having a common superscript differ significantly (*P < 0.05).*
^{x,y,} within column means not having a common superscript differ significantly (*P < 0.05).*

Table 14. Treatment effects on metabolic variables pre and post-transport. Values are expressed as mean ± SD except for albumin and glucose which is expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	Albumin (g/L)							
Control	33.30	33.35	32.95	33.00	32.15	32.65	32.70	31.25
	26.0-37.5 ^{ax}	26.0-38.4 ^{abx}	23.8-39.0 ^{abx}	25.8-38.2 ^{bx}	25.2-37.2 ^{bx}	26.1-38.2 ^{abx}	26.1-37.3 ^{abx}	25.9-33.9 ^{bx}
RT	32.45	34.10	33.65	32.50	30.90	30.50	29.80	33.90
	29.0-37.5 ^{ax}	30.5-39.7 ^{bx}	28.2-38.2 ^{abx}	30.2-39.4 ^{abx}	25.6-34.8 ^{bx}	27.8-35.0 ^{bx}	27.8-33.7 ^{by}	26.6-34.8 ^{abx}
ULT	34.25	35.75	33.05	34.25	32.10	31.05	31.55	32.20
	26.2-39.2 ^{ax}	26.8-40.8 ^{bxy}	26.9-40.7 ^{abx}	24.9-39.8 ^{abx}	24.2-36.0 ^{bx}	24.3-35.0 ^{bx}	24.0-36.0 ^{bxy}	25.0-34.4 ^{bx}
	Globulin (g/L)							
Control	41.10	39.40	40.30	41.30	41.80	41.20	43.30	43.00
	7.35 ^{ax}	6.48 ^{abx}	6.60 ^{abx}	5.26 ^{abx}	5.46 ^{abx}	6.18 ^{abx}	5.55 ^{abx}	6.51 ^{abx}
RT	40.95	41.76	40.81	43.79	41.15	44.82	44.61	44.37
	5.57 ^{ax}	4.37 ^{abx}	4.46 ^{abx}	5.24 ^{abx}	6.39 ^{abx}	5.68 ^{bx}	5.42 ^{bx}	8.02 ^{abx}
ULT	39.28	39.28	38.56	40.99	40.32	42.85	42.50	43.71
	6.56 ^{ax}	6.09 ^{abx}	6.29 ^{abx}	6.21 ^{bx}	5.41 ^{abx}	5.98 ^{bx}	5.74 ^{bx}	7.18 ^{bx}
	Protein (g/L)							
Control	73.82	72.41	72.57	73.42	73.48	73.29	75.35	73.56
	±6.14 ^{ax}	±5.65 ^{abx}	±6.04 ^{abx}	±4.86 ^{abx}	±5.67 ^{abx}	±7.01 ^{abx}	±6.76 ^{abx}	±5.63 ^{abx}
RT	74.36	75.99	74.48	77.03	72.11	75.81	75.09	76.15
	±5.47 ^{ax}	±4.07 ^{aby}	±4.38 ^{abx}	±4.71 ^{bx}	±6.32 ^{abx}	±5.49 ^{abx}	±5.26 ^{abx}	±6.10 ^{abx}
ULT	73.09	74.24	71.56	74.38	71.88	74.17	73.60	75.18
	±6.11 ^{ax}	±5.11 ^{abxy}	±5.04 ^{abx}	±6.90 ^{abx}	±5.20 ^{abx}	±6.20 ^{abx}	±5.44 ^{abx}	±5.34 ^{abx}
	Glucose (mmol/L)							
Control	5.00	4.10	4.05	3.90	4.15	4.10	4.00	4.00
	3.9-5.9 ^{ax}	3.5-5.0 ^{bx}	3.7-5.4 ^{bx}	3.4-5.2 ^{bx}	3.6-5.3 ^{bx}	3.3-5.0 ^{bx}	3.3-4.8 ^{bx}	3.4-4.6 ^{bx}
RT	4.65	4.20	4.15	4.20	4.10	4.20	4.15	4.95
	3.4-7.5 ^{ax}	3.7-8.2 ^{abxy}	3.3-8.4 ^{abxy}	3.5-7.3 ^{abxy}	3.5-5.3 ^{bxy}	3.0-5.9 ^{bx}	3.5-5.0 ^{bx}	4.1-6.7 ^{abx}
ULT	4.50	4.85	4.60	4.40	3.95	4.10	4.00	4.70
	3.3-6.6 ^{ax}	3.7-8.0 ^{aby}	3.2-5.5 ^{aby}	3.5-4.9 ^{aby}	3.3-4.6 ^{by}	3.5-4.9 ^{bx}	3.4-4.8 ^{bx}	3.6-5.8 ^{abx}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal;

^{a,b,} within row means not having a common superscript differ significantly ($P < 0.05$).

^{x,y,} within column means not having a common superscript differ significantly ($P < 0.05$).

Table 15. Treatment effects on metabolic variables pre and post-transport. Values are expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	NEFA (µmol/l)							
Control	0.63	0.40	0.38	0.37	0.56	0.46	0.56	0.39
	0.28-1.13 ^{ax}	0.07-0.71 ^{bx}	0.11-0.88 ^{bx}	0.06-1.01 ^{bx}	0.06-1.21 ^{abx}	0.19-1.20 ^{bx}	0.15-1.08 ^{abx}	0.06-0.83 ^{bx}
RT	0.58	0.68	0.68	0.64	0.70	0.53	0.29	0.07
	0.24-1.5 ^{ax}	0.12-1.5 ^{aby}	0.1-1.56 ^{aby}	0.28-1.9 ^{aby}	0.07-1.44 ^{abx}	0.09-1.25 ^{abx}	0.02-1.29 ^{by}	0.03-0.11 ^{by}
ULT	0.60	0.64	0.61	0.70	0.66	0.62	0.31	0.07
	0.18-1.37 ^{ax}	0.07-0.98 ^{aby}	0.33-1.21 ^{aby}	0.35-1.5 ^{aby}	0.24-1.63 ^{abx}	0.06-1.37 ^{abx}	0.03-1.35 ^{by}	0.03-0.11 ^{by}
	βHB (mmol/l)							
Control	0.29	0.24	0.26	0.31	0.31	0.30	0.22	0.24
	±0.09 ^{ax}	±0.06 ^{bx}	±0.08 ^{abx}	±0.10 ^{abx}	±0.12 ^{abx}	±0.10 ^{abx}	±0.08 ^{bx}	±0.07 ^{bx}
RT	0.29	0.28	0.31	0.35	0.32	0.26	0.18	0.19
	±0.10 ^{ax}	±0.11 ^{abx}	±0.11 ^{abx}	±0.12 ^{abx}	±0.11 ^{abx}	±0.09 ^{abx}	±0.07^{bx}	±0.48 ^{by}
ULT	0.33	0.29	0.32	0.32	0.35	0.28	0.23	0.22
	±0.09 ^{ax}	±0.14 ^{abx}	±0.11 ^{abx}	±0.11 ^{abx}	±0.14 ^{abx}	±0.15 ^{abx}	±0.16 ^{bx}	±0.05 ^{bxy}
	Urea (mmol/l)							
Control	5.15	3.20	3.65	4.15	3.70	3.00	3.00	2.90
	2.7-11.4 ^{ax}	1.9-13.0 ^{bx}	2.4-14.5 ^{abx}	2.2-16.6 ^{abx}	2.2-14.7 ^{bx}	1.5-6.3 ^{bx}	2.4-8.5 ^{bx}	1.7-4.5 ^{bx}
RT	4.60	5.40	5.55	5.00	4.70	3.70	3.70	5.35
	2.1-7.1 ^{ax}	4.3-17.7 ^{by}	3.9-21.3 ^{by}	3.5-9.9^{by}	3.2-11.7 ^{aby}	2.2-13.4 ^{aby}	2.3-11.6 ^{aby}	1.9-7.0^{by}
ULT	4.50	5.00	4.65	4.35	4.10	4.15	4.55	4.90
	2.6-6.3 ^{ax}	3.8-7.5 ^{by}	3.3-7.4 ^{aby}	3.3-7.2 ^{abxy}	2.1-12.2 ^{abxy}	2.5-6.8 ^{abxy}	2.3-8.0 ^{abxy}	3.4-7.1 ^{abxy}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal;

^{a,b,} within row means not having a common superscript differ significantly (*P < 0.05).*

^{x,y,} within column means not having a common superscript differ significantly (*P < 0.05).*

Table 16. Treatment effects on haptoglobin, fibrinogen and creatine kinase concentrations pre and post-transport. Values are expressed as Median with minimum and maximum values.

Treatment	d 0 pre-transport	d 3 Arrival in France	d5 post transport	d 6 arrival in Spain	d 8 post-transport	d 10 post transport	d 12 post transport	d 36 post transport
	Haptoglobin ((Hb binding capacity/l)							
Control	0.95	0.46	0.40	0.94	0.80	0.62	0.52	0.17
	0.2-4.72 ^{abx}	0.09-3.53 ^{abx}	0.1-2.66 ^{abx}	0.09-2.11 ^{abx}	0.12-2.36 ^{abx}	0.16-2.19 ^{abx}	0.13-1.57 ^{bx}	0.1-0.82 ^{bx}
RT	0.30	0.37	0.50	0.65	0.63	0.85	0.89	0.23
	0.21-4.01 ^{abx}	0.08-2.82 ^{abx}	0.06-2.51 ^{abx}	0.08-3.26 ^{abx}	0.1-3.45 ^{abx}	0.21-2.48 ^{abx}	0.15-1.59 ^{aby}	0.14-1.01 ^{by}
ULT	0.70	1.18	0.92	0.94	0.83	0.46	0.41	0.24
	0.19-2.65 ^{abx}	0.09-2.9 ^{abx}	0.1-2.71 ^{abx}	0.13-3.0 ^{bx}	0.12-2.98 ^{abx}	0.12-2.84 ^{abx}	0.11-2.03 ^{abxy}	0.16-2.01 ^{by}
	Fibrinogen (mg/dl)							
Control	757.50	903.50	763.00	883.00	865.00	774.00	815.00	533.00
	401-1953 ^{abx}	513-1777 ^{abx}	452-1545 ^{abx}	583-1546 ^{abx}	65-1593 ^{abx}	516-1282 ^{abx}	469-1304 ^{abx}	434-888 ^{bx}
RT	711.00	719.00	677.00	735.00	747.00	674.00	474.00	528.00
	208-1484 ^{abx}	454-1983 ^{aby}	399-1825 ^{abx}	454-1859 ^{abx}	429-1847 ^{abx}	475-1309 ^{abx}	321-913 ^{by}	279-1185 ^{bx}
ULT	690.00	847.50	703.50	915.00	1050.00	660.00	445.00	543.00
	166-1933 ^{abx}	455-1649 ^{abxy}	377-1418 ^{abx}	157-1533 ^{abx}	417-1495 ^{abx}	340-1136 ^{abx}	245-986 ^{by}	247-1115 ^{bx}
	Creatine kinase (CK) (U/L)							
Control	993.00	376.50	465.00	289.00	402.50	192.00	225.00	201.50
	350-4203 ^{abx}	127-2749 ^{bx}	129-1922 ^{bx}	117-766 ^{bx}	175-1017 ^{bx}	114-380 ^{bx}	108-595 ^{bx}	102-494 ^{bx}
RT	458.50	309.00	397.50	269.00	957.00	256.00	136.00	232.50
	160-2102 ^{aby}	172-33000 ^{abx}	186-2048 ^{abx}	156-15885 ^{abx}	444-4545 ^{aby}	158-2200 ^{by}	104-592 ^{bx}	126-322 ^{bx}
ULT	829.50	478.00	378.50	335.50	860.00	278.00	179.00	208.00
	194-10710 ^{abxy}	122-2101 ^{bx}	139-2462 ^{bx}	140-1417 ^{bx}	339-3639 ^{aby}	129-1315 ^{bxy}	73-1761 ^{bx}	139-295 ^{bx}

Control = not transported; RT (transported animals that remained on the transporter at the French lairage for 12h) and ULT animals transported (transported animals that were unloaded at the French lairage for a 12 h rest period) at a stocking density of 0.93m^2 per animal;

^{a,b,} within row means not having a common superscript differ significantly ($P < 0.05$).

^{x,y,} within column means not having a common superscript differ significantly ($P < 0.05$).

4. Effects of transportation stress in young bulls on altered expression of neutrophil genes

4.1 Introduction

Stress caused by transportation affects many aspects of health, production, and welfare of beef cattle. Incidences of bovine respiratory disease (BRD) or "shipping fever" have been documented and studied since the 1950s (Graham, 1953; Ryff and Glenn, 1957). However, respiratory diseases continue to be the leading cause of death in young cattle (Lekeux, 1995) and are thought to be associated with an alteration in immune function induced by transportation (Blecha et al, 1984; Murata et al, 1987; Yagi et al, 2004; Gupta et al, 2006) and increased exposure to pathogens as animals are commingled and transported (Galyean et al., 1999).

Neutrophils are phagocytic innate immune cells that are crucial for acute defense in lungs and other tissues. However, a "neutrophil paradox" exists whereby these normally beneficial leukocytes can also contribute to the pathogenesis of infectious diseases if their proinflammatory activities are not properly regulated (Weiss, 1989; Lekeux, 1995; Cox, 1998; Eruslanov et al., 2005). Because of the cells' ability to rapidly degranulate and spill harmful proteolytic enzymes and reactive oxygen species in the normal course of fighting infections, neutrophils can cause excessive damage to otherwise healthy tissue at the infection focus, exacerbating an already dangerous disease state.

Others have investigated neutrophil phenotypic changes during transportation stress (Murata et al., 1987; Yagi et al., 2004) but not gene expression changes. However, neutrophils are well known targets of exogenously administered and endogenous stress hormones called glucocorticoids (Roth et al., 1981; Weber et al., 2006), responding to these steroids with altered expression of genes key to the regulation of apoptosis (Chang et al., 2004; Madsen-Bouterse et al., 2006), adhesion (Weber et al., 2001; 2004), inflammation (Burton et al., 2005), and many other basic cellular and immune functions (Madsen et al., 2002; 2004, Weber et al., 2006). Because transportation elevates the endogenous glucocorticoid, cortisol, (Murata et al., 1987; Gupta et al., 2006) it was logical to hypothesize in this study that neutrophils of truck transported cattle will also respond to stress with altered gene expression.

The main objective of this study was to examine the expression of candidate neutrophil genes of young cattle subjected to transportation stress. The genes selected for profiling were sensitive to glucocorticoids in other stress models (Chang et al., 2004; Burton et al., 2005, Weber et al., 2006) and included Fas, A1, matrix metalloproteinase-9 (MMP-9), L-selectin, bactericidal/permeability-increasing protein (BPI), transforming growth factor-β receptor type III (subsequently referred to as betaglycan), and glucocorticoid receptor α (GRα). β-actin was also examined as a housekeeping gene (Weber et al., 2001; 2004).

4.2 Materials and Methods

Animals and Transportation

Animals used in this study were 6 Belgian Blue x Friesian bulls, 233 ± 3.0 kg in weight and 282 ± 4 days of age at time of transportation. Bulls were weaned at 10 days of age in the spring of 2004 and purchased by Teagasc Grange Beef Research Centre (County Meath, Ireland). They were housed, fed and cared for according to accepted management practices at the Research Centre. Bulls had *ad libitum* access to water and grass silage (in vitro DM digestibility = 872 g/kg) which was supplemented with 1.5 kg barley/soybean concentrate (CP = 104.6 g/kg DM) per animal per day. The transportation study lasted for 6 weeks; groups of 6 bulls were transported at a time with only one bull per group being intensively bled for neutrophil isolation. Bulls to be transported each week were penned together and not commingled with other bulls. They were transported at a stocking density of 0.85 m^2 for 9hours on a variety of road conditions, speeds, and traffic. In accordance with European Union regulation, a 45 minute rest stop was observed after 4.5 h during which the animals remained on the truck. Animals were unloaded and returned to their original group pen at the end of the 9h journey.

Blood Collection

Blood samples for the isolation of neutrophils were collected at the following time points relative to commencement of transportation at 0h: -24, 0, 4.5, 9.75, 14.25, 24, and 48 h. Bleeding at -24, 0, 14.25, 24, and 48 h occurred in a handling chute in a barn, while bleeding at 4.5 and 9.75 h occurred in a handling chute on the truck. Blood (300 mL/sample) was collected at each time point by jugular venipuncture using 18-gauge 2.5 cm multiple draw needles into a series of 30 mL syringes pre-coated with the anticoagulant ACD (acid citrate dextrose). Each syringe was expelled into a 50-mL conical tube containing 4.0 mL of ice cold ACD. These tubes were immediately placed on ice and taken to the laboratory for further processing. Additional blood (10 mL) was also collected at these time points into vacutainer tubes (BD Biosciences, San Jose, CA) containing heparin for subsequent harvesting of plasma for cortisol determination.

Neutrophil Isolation

Neutrophils were isolated according to an adapted version of a previously described method (Weber et al., 2001). Briefly, ACD anti-coagulated whole blood was centrifuged at 4°C for 20 minutes at 1000 x *g*. Plasma, buffy coats, and approximately 2/3 of the red cell pack were aspirated and discarded. The remaining red cell pack was brought to a volume of 25 mL with sterile ice-cold PBS (phosphate-buffered saline) and aliquoted into 4 conical tubes. Each tube was underlaid with 12 mL of 1.084 g/mL Percoll solution (Amersham Biosciences, Piscataway, NJ) and centrifuged at 22°C for 40 minutes at 400 x *g* to separate mononuclear leukocytes from the neutrophils. The PBS, mononuclear cells, and Percoll were aspirated and discarded, and erythrocytes in the remaining

pellet were lysed exactly as in Weber et al. (2001). Remaining neutrophils were washed in cold PBS and resuspended in a volume of 24 mL to 48 mL of PBS to make a single cell suspension. Isolation time was consistently ≤ 3 h. All materials, solutions, and reagents were sterile; solutions were treated with DEPC (diethylpyrocarbonate) prior to sterilization.

Neutrophil counting and purity

ACD anti-coagulated blood (1 mL) was set aside for determination of total leukocyte numbers (Beckman Coulter Z1 Particle Counter and ZapOglobin lytic reagent, Beckman Coulter, Fullerton, CA). Thin blood smears were prepared on glass slides, air-dried, and stained using the haematology three-step stain for morphological differentiation of cell types (Accralab, Fisher Scientific Company, L.L.C., Middleton, VA). One hundred leukocytes in three microscopic fields were counted to determine the percentage of each cell type. Neutrophil counts were determined by multiplying the mean percentage of neutrophils in each sample by the total leukocyte count (number of cells/mL of whole blood).

Neutrophil purity was verified to be ≥ 95% based on microscopic examination using the Accralab 3-step staining method. Contaminating cells were consistently eosinophils.

RNA Isolation

Isolated neutrophils were immediately lysed in TriReagent (Molecular Research Center, Cincinnati, OH) at 1 x 10^7 cells/mL, allowed to incubate at room temperature for 10 minutes, and frozen at -80° C. Total RNA was extracted according to manufacturer's instructions and treated with Promega RQ1 RNase-free DNase (Madison, WI). Quantity and quality were assessed by using an Agilent Technologies 2100 Bioanalyzer (Palo Alto, CA). All RNA samples were determined to be of good quality as electrophotograms of neutrophil RNA were identical to those presented in Zhang et al. (2005) and Weber et al. (2006).

Standard Curve Generation for Quantitative Real-Time RT-PCR Analysis

Briefly, a 290-840 bp length of each gene's cDNA was amplified by PCR with Taq polymerase and accompanying reagents (Invitrogen, Carlsbad, CA), gel purified, and cloned. PCR products were quantitated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and serially diluted to generate a 6-point standard curve (0.01 – 1000 fg/µl) of DNA for each gene. These dilutions were included on the same plate as test samples for each real time RT-PCR assay (see below). Cycles to threshold (Ct) were graphed and fit with a line of regression. Using the equation of the line of regression, the actual amount of mRNA in femtograms (fg) per nanogram (ng) of starting cDNA was calculated for test samples.

Quantitative Real Time RT-PCR to Determine Changes in Gene Expression

cDNA synthesis from sample RNA was performed with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using 2 μg of total RNA according to manufacturer instructions. cDNAs from -24, 4.5, 9.75, 14.25, and 24h for each of the 6 animals were added to quantitative real-time RT-PCR reactions assays in amounts ranging from 2.5 ng starting cDNA to 40 ng cDNA, so that test sample Ct values fell toward the center of each standard curve. Assays were run in an Applied Biosystems 7000 or 7500 Fast Real-Time PCR system using SYBR Green master mix (Applied Biosystems, Foster City, CA) for 50 cycles of PCR. All samples were run in duplicate. Negative controls excluding any template DNA were included on each plate along with standard curves for the relevant test genes. The five time points for test samples were chosen to determine gene expression changes closest to times of changes in plasma cortisol and neutrophil counts relative to -24h (pre-transportation stress); these values had returned to nadir by 48 h, and this time point was omitted. The 0h time point was omitted and the -24h samples were used to represent true normal gene expression before the animals experienced any handling stress. Real-time PCR primers for each gene were designed using Primer Express software (Applied Biosystems) to fall within the amplicons used for the standard curves and were synthesized by Operon (Huntsville, AL); see Table 18 for primer sequences.

Plasma Cortisol Determination

Plasma was harvested from heparin anti-coagulated blood following its centrifugation at 1600 x *g* at 4°C for 15 minutes and stored at -80°C until subsequent cortisol analysis. Cortisol concentration was measured using a commercially available RIA kit (Corti-cote, ICN Pharmaceuticals, Orangeburg, NY) as described in Fisher et al. (1997).

Statistical Analyses

All data were analyzed using the Proc MIXED procedure of SAS (version 9.1; SAS Institute, Inc.) with time relative to transportation as a fixed effect, animal and group as random effects, and weight, age, and baseline (-24h) values for each variable included as covariates. Any data that failed to meet parametric assumptions were log transformed prior to statistical analyses. Data are presented as raw means ± SEM; p-values presented were derived from analysis of the model described above. Differences between time points were determined using a Tukey-Kramer adjustment for multiple comparisons and considered significant when $P \leq 0.05$; these are expressed as different letters. The overall effect of time relative to transportation was also considered significant when $P \leq 0.05$.

Pearson's correlation coefficients were also calculated to determine any significant relationship between gene expression values and both plasma cortisol and circulating neutrophil

counts using PROC CORR in SAS. R-values and p-values are presented; correlations were considered significant when $P \leq 0.05$.

4.3 Results

As expected, 9h of transportation elicited a classic stress response in the bulls of this study, as evidenced by an acute and pronounced increase in plasma cortisol (Figure 1a) and in circulating neutrophil counts (Figure 1b; $P < 0.001$ for both variables). The peak cortisol response (50.64 ± 4.46 ng/mL) was detected at 4.5 h. While neutrophilia was clearly present at 4.5 h, blood neutrophil counts peaked ($7.2 \times 10^6 \pm 9.3 \times 10^5$ cells/mL) at 9.75 h. A weak correlation existed between these two variables ($r^2 = 0.25$; $P = 0.11$). Both parameters returned to pre-transportation values by 24h. Standard curves were created for each candidate gene according to the procedure of Madsen-Bouterse et al. (2006) to assess gene expression changes using absolute quantification displayed in Figure 2 and using the PCR primers described in Table 17.

Neutrophil expression of several candidate genes changed with the onset of transportation stress (Figure 3). The pro-apoptotic gene, Fas, was down-regulated at 4.5 h by over 3 fold ($P = 0.02$; Figure 3a). In contrast, MMP-9 expression was profoundly increased during peak neutrophilia ($P = 0.003$; Figure 3b). L-selectin increased at 9.75 h ($P = 0.0008$; Figure 3c). BPI was also greatly increased at the same times, peaking at 4.5 h with 457-fold higher expression in neutrophils over pre-stress expression ($P < 0.001$; Figure 3d). Changes in A1, betaglycan and GRα could not be detected ($P = 0.28$, 0.36 and 0.27, respectively; data not shown). The housekeeping gene, β-actin, did not change its expression ($P = 0.30$).

All correlations are displayed in Table 19. Significant correlations included those between Fas and neutrophil counts ($R = -0.41$; $P = 0.02$), MMP-9 and neutrophil counts ($R = 0.42$; $P = 0.009$), and BPI and both cortisol and neutrophil counts ($P = 0.004$ and 0.02, respectively).

4.4 Discussion

Transportation stress can affect feed intake, growth rate, carcass quality, behavior, metabolism, immune function, and the overall health and well-being of cattle (Crookshank et al., 1979; Tarrant et al., 1992; Arthington et al., 2003; Chirase et al., 2004; Yagi et al., 2004; Gupta et al., 2006). Of those researchers who have investigated various aspects of immune function, none have explored specific gene expression changes in the innate immune system's first cellular responder, the neutrophil. This transportation study had three key findings. Firstly, a classical stress response occurred in transported bulls as shown by elevated plasma cortisol and neutrophil counts. Secondly, this transportation stress altered expression of genes important for neutrophil function, some of which correlate with cortisol and neutrophil counts, and these expression changes are not identical to other stress models. Finally, gene expression changes indicate that transportation stress may not

be solely immunosuppressive, and an altered regulation of neutrophil functions may make these cells more detrimental to host defense.

Apoptosis in neutrophils is necessary to regulate the life and death of these inflammatory cells, and Fas and A1 proteins play a large part in inducing or preventing apoptosis, respectively. In this study, Fas was down-regulated with transportation stress, suggesting an increased survival in cells that are normally short-lived. Yagi et al. (2004) observed a decrease in rate of neutrophil apoptosis following transportation stress, and along with others (Gupta et al., 2006; Murata et al., 1987) reported blood neutrophilia as these longer-lived neutrophils adding to the blood pool of cells. Our results suggest that depressed Fas expression may contribute to this scenario. In contrast, A1 is an anti-apoptotic protein in neutrophils whose mRNA and protein abundance in neutrophils are up-regulated by in vitro treatment of the synthetic glucocorticoid, dexamethasone (Madsen-Bouterse et al., 2006). A1 was unchanged in the transportation stress model, indicating that other mechanisms, such as the down-regulation of pro-apoptotic Fas, may be responsible for delayed apoptosis that lead to the pronounced neutrophilia observed in this study.

Matrix metalloproteinase-9 (MMP-9) is a gelatinase found in the granules of neutrophils and is used to degrade basal membranes and extracellular matrix proteins the cells migrate from blood into infected tissue. This protease is also known to cleave the potent neutrophil chemoattractant interleukin-8 into a more active form in a positive feedback loop (Opdenakker et al., 2001); upregulated MMP-9 could lead to an increased recruitment of neutrophils to a site of infection. Consequently, human patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) have highly elevated MMP-9 in bronchoalveolar lavage fluid (Fligiel et al., 2006), and MMPs have been implicated in the pathogenesis of lung injury (reviewed by Moraes et al., 2006). Elevated neutrophil expression of MMP-9 in the current study may imply an increased potential for excessive inflammation during respiratory disease by neutrophils that are also reprogrammed for longer lifespan and in extremely high numbers in the circulation.

L-selectin is an adhesion molecule found on the surface of neutrophils that is responsible for neutrophil tethering on endothelial cells in the rapidly flowing bloodstream and the “rolling” mechanism that occurs while neutrophils survey for signs of infections (reviewed by Kansas, 1996). This study’s results increase of L-selectin gene expression do not agree with those found in the literature. Yagi et al. (2004) reported similar changes in plasma cortisol and neutrophil numbers as this study, suggesting delayed apoptosis of neutrophils and a decrease in cell surface L-selectin as causes behind their observed neutrophilia. A decrease in L-selectin in response to glucocorticoids has also been reported elsewhere (Weber et al, 2001; 2004) in accordance with neutrophilia and a possible increased susceptibility to mastitis in periparturient dairy cows. A difference in the results may be due to the fact that the animals used in this study were intact bulls, while subjects in other related studies were either cows or castrated steers; sex hormones are associated with differences in immune responses (Gaillard et al., 1998; Verthelyi, 2001) and

neutrophil function in particular (Deitch et al., 2006). It may also be that an influx of neutrophils from the bone marrow in response to the stimulus of stress contributed to the observed neutrophilia and increased L-selectin expression as these newly released and more immature neutrophils highly express the gene (van Eeden et al., 1995, 1997). Neutrophils expressing more L-selectin preferentially sequester in the lung (van Eeden et al., 1997). With the marked increase of both neutrophil numbers and L-selectin expression during transportation, a risk of increased tissue damage in the lung exists that may contribute to respiratory disease.

BPI is another granule protein in neutrophils utilized to opsonize Gram-negative bacteria for phagocytosis, activate complement, neutralize LPS, and permeabilize bacterial cell walls (Calafat et al., 2000; Nishimura, 2001). It has also been found to be dramatically increased in bovine neutrophils treated with the synthetic glucocorticoid, dexamethasone, *in vitro* (Weber et al., 2006). Findings in this study show a similar response and an up-regulation of bactericidal activity in transportation-stressed neutrophils, challenging the idea of "immunosuppression" thought to occur following transportation (Blecha et al., 1984; Dixit et al., 2001). These results suggest that the infection-fighting capability of innate immune cells is actually enhanced.

TGF-β is an anti-inflammatory cytokine that is associated with wound healing and increased fibrogenesis in the lung and has been associated with several diseases of the lung (Bartram and Speer, 2004). Because of this, its receptor type III, also known as betaglycan, is used as an antagonist in respiratory diseases such as tuberculosis to reduce additional fibrosis (Hernandez-Pando et al., 2006). Betaglycan was found to be down-regulated in neutrophils affected by dexamethasone treatment *in vitro* (Weber et al., 2006); however, this receptor was not changed in the transportation stress model, indicating a need to investigate further into this pathway in neutrophils.

The glucocorticoid receptor (GR) is usually found in the cytosol bound in a complex with several inhibitory heat shock proteins (hsp). When glucocorticoids are released, they freely enter the cell, bind to their receptor, and induce a conformational change in the GR as the hsp complex dissociates. GRs dimerize, translocate to the nucleus, and activate or repress transcription of responsive genes. Glucocorticoids down-regulate their own receptor as a negative feedback mechanism (Bamberger et al., 1996). Expression of the GR has been shown to decrease in association with elevated cortisol (Preisler et al., 2000) in periparturient cows. The authors are not sure why no change in this receptor's expression was observed in this transportation study as cortisol spiked even higher than that reported by Preisler et al. (2000). Again, this could be an effect of using bulls as study subjects rather than cows.

Altogether, these results confirm an alteration in bovine neutrophil gene expression during transportation stress that is associated with increases in plasma cortisol and circulating neutrophil numbers. Identifying changes in the expression of these inflammatory neutrophil genes involved in regulation of apoptosis, tissue remodeling, margination, and bactericidal function could begin to

reveal a possible signature of imbalanced immunocompetence in transportation-stressed cattle. A wider investigation of other genes affected could improve this profile, as well as validation of protein changes for future biomarkers. Findings in the current study imply that neutrophils may be programmed for longer life, increased proteolytic and bactericidal capabilities, and favored migration into the lung and respiratory system in a potentially hyperactive inflammatory response during transportation stress, enabling increased tissue damage and severity of respiratory disease.

Table 17. Standard curve PCR primer sequences.

Gene	Forward sequence (5'–3')	Reverse sequence (5'-3')	Length (bp)
Fas	ATTCAGGGAAAACCTGCACA	ATTTCTGGAGAAGGCAATGG	838
A1[a]	CCAGGCAGAAGATGACAG	GGTTACAATCCTGCCCCAGTT	284
MMP-9	CAGACCTTTGAGGGCGAACT	TCGTCGAAGTGGGCATCTC	296
L-selectin[b]	CCCAACAACAGGAAGAGTAAG	TGCCAGCCAAATGATAAA	711
BPI	CCCTCCAGCCTTACCAG	ATGCAATTCTTGGAGGAAAT	324
Betaglycan	TTGTTGGGTGACTCGT	AAGGATTTAAAACTGTGGTT	309
GRα	ATCACCAATCAGATACCAAAAT	ATCCTCTCTCTGCAGCACATTTCC	397
β-actin	AAGGCCAACCGTGAGAAGATG	TGCGGTGGACGATGGAG	781

[a] As described in Madsen-Bouterse et al. (2006)

[b] As described in Weber et al. (2006)

Table 18. Quantitative real-time RT-PCR primer sequences, length and melting temperatures (Tm) of the gene product, and starting cDNA concentrations for each gene's PCR reaction.

Gene	Forward sequence (5'–3')	Reverse sequence (5'-3')	Length (bp)	Product Tm (°C)	Starting cDNA (ng)
Fas[a]	TGTAAAGTCAGCTTATACA CAGCAGAAGT	GTGGGCTGCCGCCTATG	104	81	2.5
A1[c]	ACTGCCAGAACAATATTCA ACCAA	GGTTACAATCCTGCCCCA GTT	78	76	20
MMP-9	CGCACGACATCTTTCAGTAC CA	GGAACTCACGCGCCAGT AG	74	78	40
L-selectin[b]	ACGGGAAAAAAGGATTACT ATGGA	GCCTATAGTTGCATATGT ATCAAATTTTCA	144	74	2.5
BPI[b]	TTCAGAAATGATCCAAACA TGAAAC	GCCCTTGGAAGAAACAA TTCC	81	75	10
Betaglycan[b]	TGAGGGTAAAAAGAGTACC CTGAAA	CACGTTGAACAGAGAAA AAGAGTACAA	80	78	10
GRα[a]	TGTGGTTTAAAGAGGGCCA AGA	TTCTACGTTCCCATCACT GAAAAG	74	78	2.5
β-actin[a]	AAGGCCAACCGTGAGAAGA TG	TGCGGTGGACGATGGAG	74	82	2.5

[a] As described in Chang et al. (2004)

[b] As described in Weber et al. (2006)

[c] As described in Madsen-Bouterse et al. (2004)

Table 19. Pearson correlation coefficients (R^2) and p-values for each gene respective to plasma cortisol and neutrophil counts. Genes that were significantly affected by transportation stress are bolded. * represents significant correlations ($P \leq 0.05$); † represents a tendency toward significance ($P \leq 0.10$).

Gene	Plasma Cortisol		Neutrophil Counts	
	r^2	P	r^2	P
Fas	-0.006	0.98	-0.43	0.02*
A1	0.25	0.17	0.02	0.91
MMP-9	0.13	0.48	0.47	0.009*
L-selectin	0.10	0.58	0.03	0.86
BPI	0.51	0.004*	0.42	0.02*
Betaglycan	0.14	0.46	0.21	0.27
GRα	0.10	0.59	0.31	0.10†
β-actin	0.25	0.17	0.20	0.28

Figure 1. Plasma **cortisol** (a) and circulating **neutrophil numbers** (b) are acutely and profoundly increased by transportation stress. The covariates weight, age, and baseline values were included in the model for cortisol analysis and all found to be significant ($P < 0.05$); only baseline neutrophil values were included in the model for neutrophil analysis (weight and age were not significant ($P = 0.29$ and 0.16, respectively). Differences between time points relative to transportation ($P < 0.05$) are represented by different letters. The overall effect of time is also represented on each graph.

Figure 1a)

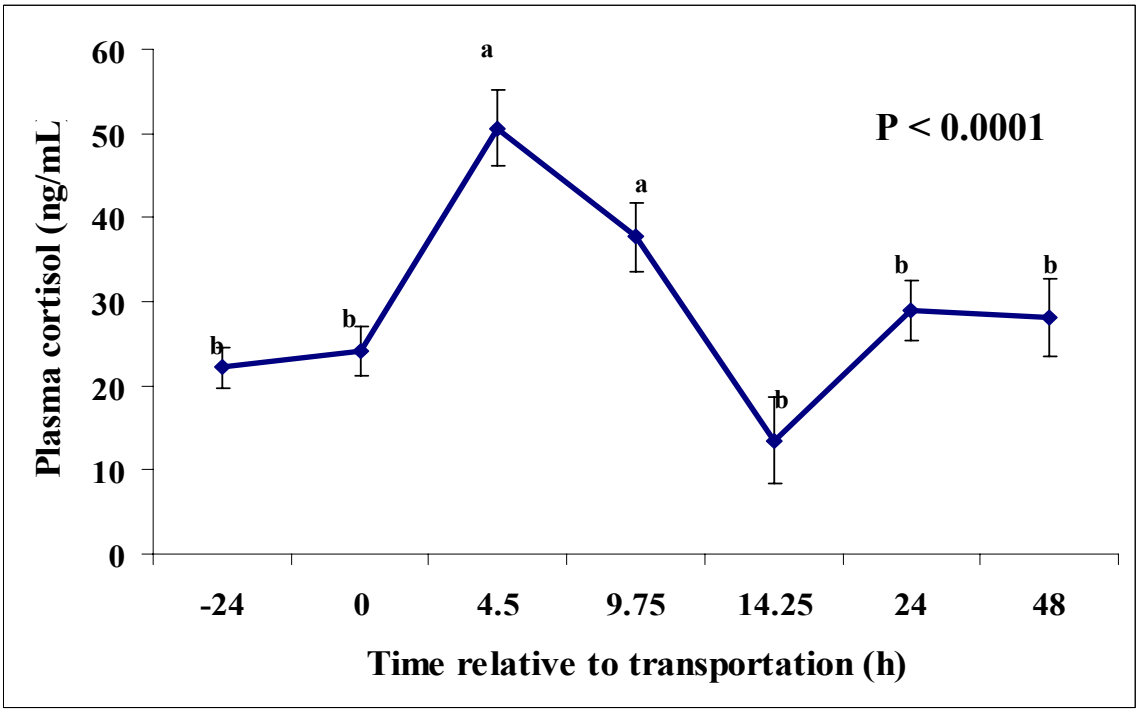


Figure 1 b)

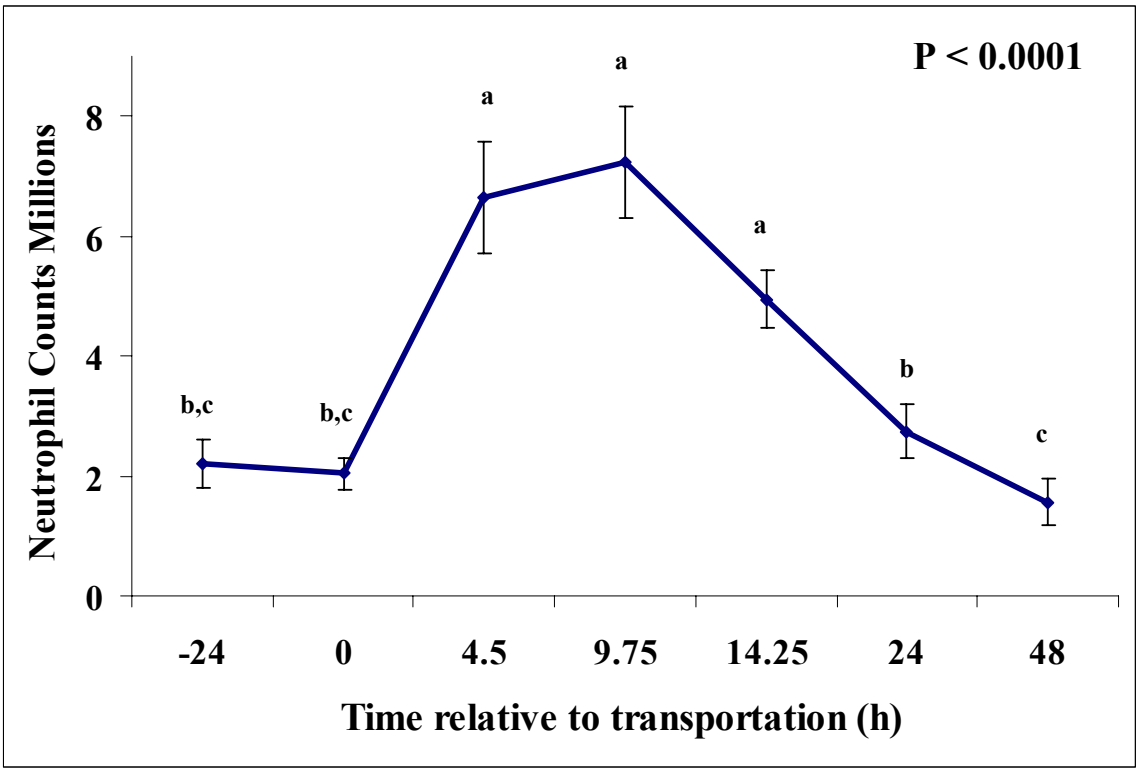


Figure 2. Standard curves for candidate genes. R^2 values > 0.96 for all curves, indicating an excellent linear relationship. Regression equations are shown for each gene and are used to determined absolute quantity of each corresponding gene with transportation-stressed neutrophil cDNA samples. Primer sequences used to generate each standard curve are found in Table 17.

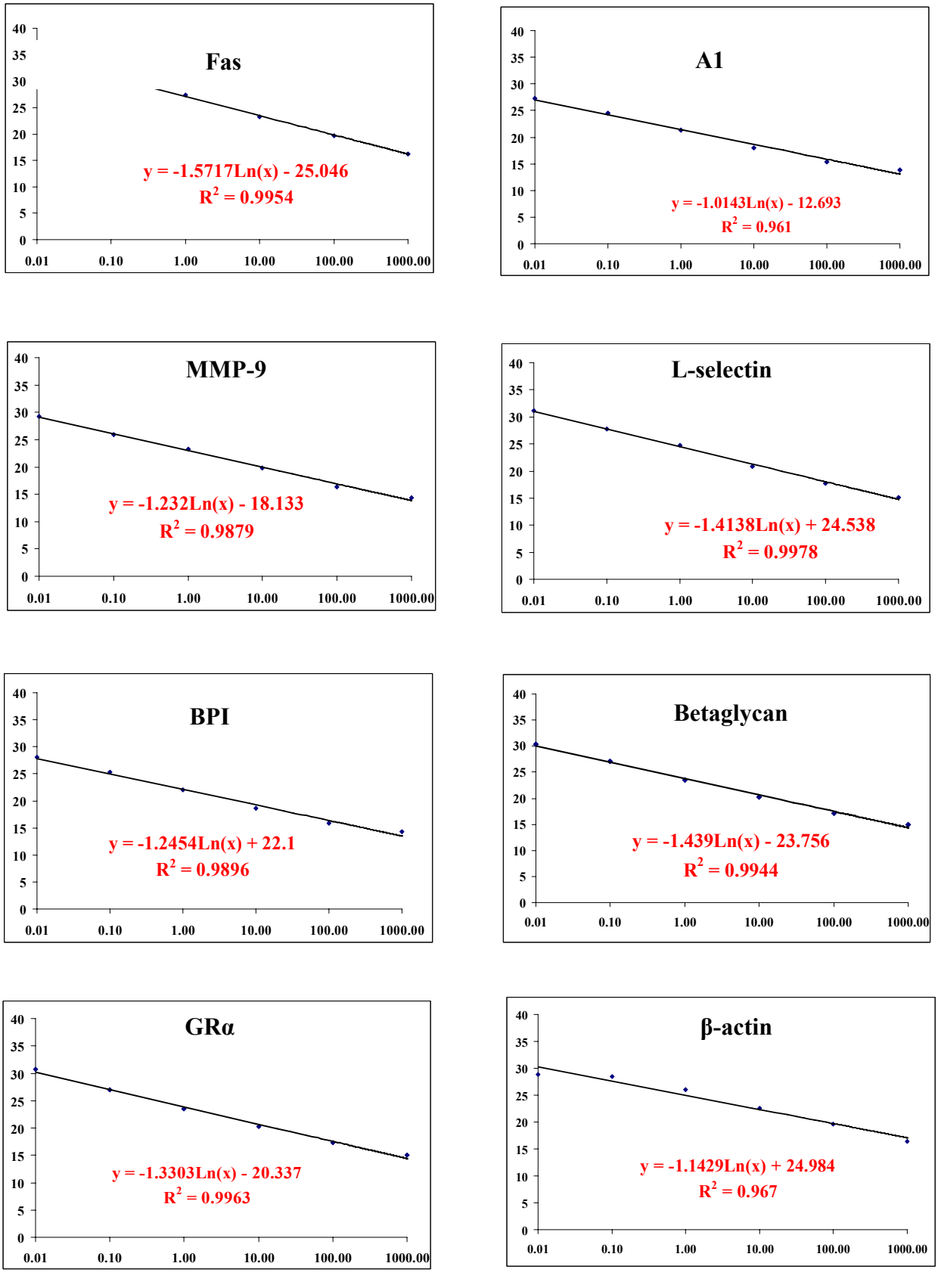
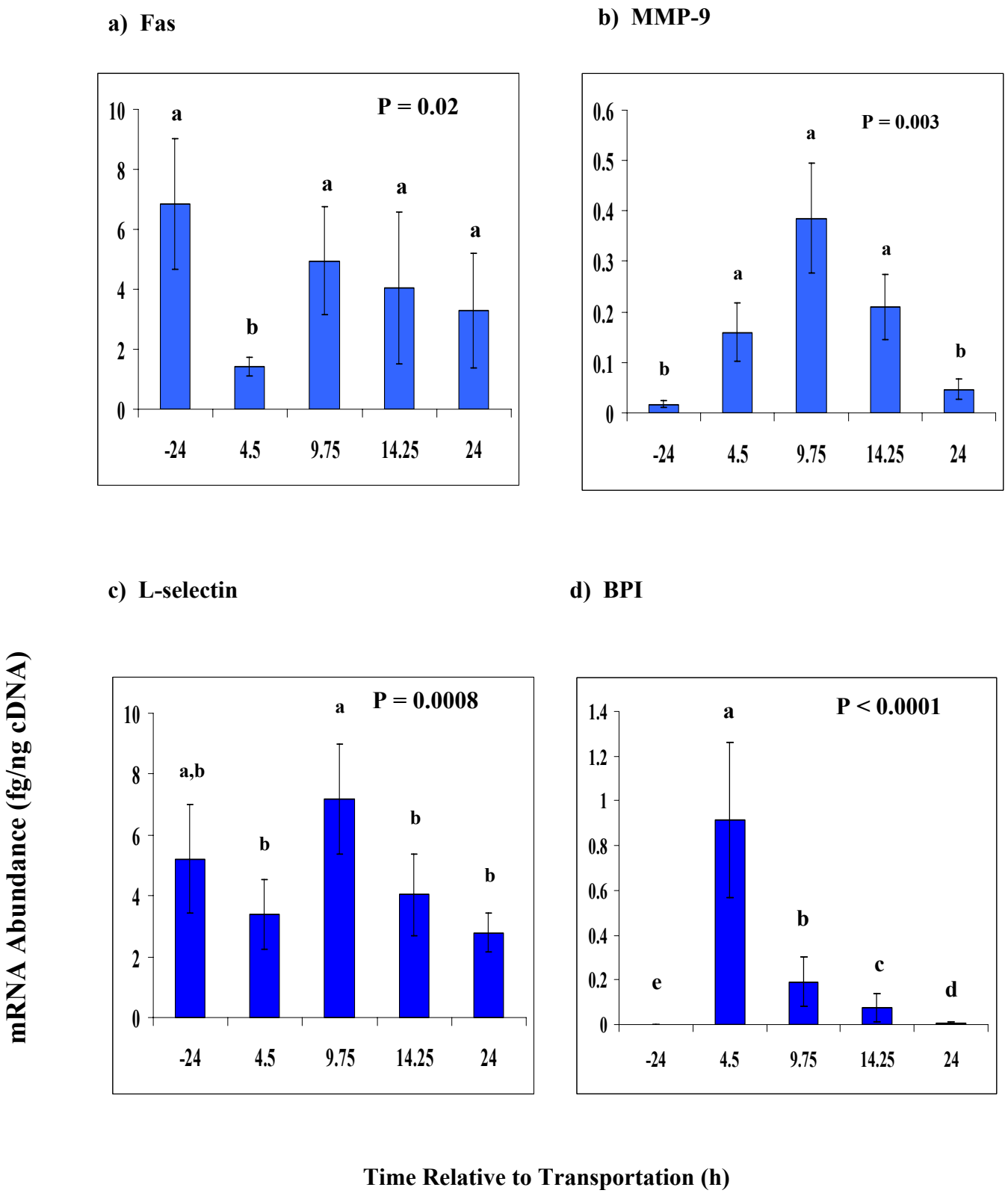


Figure 3. Neutrophil genes associated with functions of apoptosis (**Fas**), tissue remodeling (**MMP-9**), surveillance (**L-selectin**), and bacterial killing (**BPI**) are altered by transportation stress. All values are absolute quantities in femtograms (fg) and obtained by comparing Cycles to Threshold (Ct) to known standard curves for each gene (Figure 2). Bars represented by different letters are different from each other. P values for overall effect of time are also shown.



5. Acknowledgements

The authors gratefully acknowledge the excellent technical help and assistance of M. Donlon, J.A. Farrell, J. Larkin, A. Marley, M. Murray, M. Munnelly, J. Munroe, D. Prendiville, J Price and the farm staff (J. Horan, P. McGovern, H. Mulligan, E. Mulligan, P.Reilly and G. Costelloe) at Teagasc, Grange Beef Research Centre, for care and management of the animals; in the conduct of this study.

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