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END OF PROJECT REPORT
Physiological and behavioural aspects of housing stress in cattle.

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1.0 Overall summary

The effect of various space allowances on pituitary, adrenal, immune responses and performance was investigated in 72 Holstein x Friesian bulls. Bulls (403 ± 3.5 kg) were blocked by weight and randomly assigned into two groups (familiar, F and unfamiliar, UF) x three (1.2, 2.7 and 4.2 m^2 per bull; n = 24 bulls per space allowance) treatments and housed for 83 days in 18 pens (n = 4 per pen). Blood samples were collected on day –1, 0, 3, 14, 36 and 77 with respect to mixing and housing on day 0. The bulls were administered with adrenocorticotrophic hormone (ACTH) on day 3 and corticotrophin-releasing hormone (CRH) on days 14, 36 and 77. The basal cortisol concentrations were not affected (P>0.05) by mixing of familiar and unfamiliar bulls. On day 3, basal cortisol was greater (P<0.05) in the bulls housed at 1.2 than those at 2.7 and 4.2 m^2 space allowances while no effect was observed in ACTH-induced plasma cortisol concentration among treatments. Following CRH administration there was no effect (P>0.05) of treatment and treatment x time on ACTH. On day 14, interferon-γ production was lower (P<0.05) in the bulls housed at 4.2 vs 2.7 m^2 and was intermediate but not (P>0.05) different for those housed at 1.2 m^2. Bulls housed at either space allowances had (P<0.05) neutrophilia, lymphopenia, eosinopenia and decreased haemoglobin on day 3 compared with day 0. The liveweight gain from days 0 to 83 was lower (P< 0.05) in bulls housed at 1.2 compared with those at 2.7 and 4.2 m^2. Housing bulls at 1.2 m^2 space allowance had a detrimental effect on their growth and was associated with an acute rise in plasma cortisol concentration (on day 3) compared with space allowances of 2.7 and 4.2 m^2/bull.

The effect of transporting bulls for 12-h by road previously housed for 96 days at three space allowances (1.2, 2.7, 4.2 m^2 per bull) on adrenal, haematological, immune responses, body temperature and performance was investigated. Holstein Friesian bulls (n = 72; BW = 403 ± 3.5 kg) were allocated to one of two treatments, T (transport for 12-h; n = 16 per space allowance) and C (control; n = 8 per space allowance). Basal cortisol concentrations and interferon (IFN) -γ production from cultured lymphocytes were not different (P>0.05) following the housing period. Removing bulls from their home pens and walking them to the pre-loading crush facility, loading on to the transporter, and unloading the bulls following the 12-h road journey, increased (P<0.001) cortisol concentration. Bulls housed at 4.2 m^2 had greater (P<0.05) cortisol concentrations than bulls housed at 1.2 m^2 at loading, unloading, on return to the crush holding facility, and bulls housed at 1.2 m^2 had greater (P<0.05) cortisol concentrations than bulls housed at 2.7 and 4.2 m^2 in their home pens after transport. There was an increased (P<0.05) cortisol response in T than in C bulls to ACTH administration. Transport reduced (P<0.05) IFN-γ production, increased (P<0.05) neutrophils, eosinophils, packed cell volume, red blood cell numbers, haemoglobin and decreased lymphocyte % and body weight. In conclusion, while transport increased cortisol and reduced immune response in the short-term, the changes were within normal physiological ranges suggesting that 12-h road transport had no adverse effect on welfare status over a long-term period.

The effect of repeated regrouping and relocation (R&R) on behaviour of steers was investigated. Seventy-two Holstein-Friesian (14 month old; 441 ± 3.2 kg) steers were assigned to either control (n = 30; C) or regrouped (n = 42; R) treatments and housed 6 per pen in 12 pens. The R steers were exposed to six R&R over 84 days. New pen cohorts were allowed to stabilise for 14 days and none of the R steers were allowed to share the same pen or pen-mates, where or with whom, they were previously housed. Control steers were housed in the same pen with the same pen-mates. Each steer was marked on its back with an individual identification code. Twelve cameras were used to observe and record behaviour for each pen allowing observation of all individual steers continuously for a week following each R&R. The following behaviours

were recorded for each steer: lying, standing, eating, drinking, head- to -head contact with another animal while not eating, head contact with the body of another animal and bodily contact with none, one, two or three steers. Behaviour was observed by instantaneous scan sampling after each R&R, at 2 min intervals for 2 h on day 1; at further 20 min intervals on days 1 and 2; and at 120 min intervals from day 3 to 7. Where appropriate, the % of time spent in each behaviour was calculated from the data on total counts in each behavioural category. The total count data were analysed by χ^2 statistics for all behavioural categories. Steers were weighed before each R&R. Average daily gain from day 0 to 84 was analysed by ANOVA. During the first 2 h observation period following mixing, R steers displayed a greater ($P < 0.05$) % of time standing (following the first to sixth R&R), eating (first to fourth and sixth R&R) and drinking (first, third and fourth R&R) than control steers. In the 20 min observational period, a greater % ($P < 0.05$) of time was spent standing, eating and drinking in R than in C steers following each R&R. In the 120 min observation period, R steers spent a greater ($P < 0.05$) %of time lying with less body contact behaviours than C steers, and these findings increased in the fourth, fifth and sixth R&R. These data suggest that there was partial adaptation to repeated R&R at the first two R&R followed by complete adaptation at the third and subsequent R&R, with no detrimental effect on animal performance.

The effect of repeated regrouping and relocation (R&R) of cattle on hypothalamic-pituitary-adrenal (HPA) axis, immune function, blood biochemical, hematological variables and ADG, was investigated. Seventy-two Holstein-Friesian (14-mo-old; 441 ± 7.2 kg) steers were assigned to either control (n = 30; C) or regrouped (n = 42; R) treatments and housed 6 per pen in 12 pens. The R steers were exposed to 6 R&R over 84 d. New pen cohorts were allowed to stabilize for 14 d and none of the R steers were allowed to share the same pen or pen-mates where or with whom they were previously housed. Control steers were housed in the same pen with the same pen-mates. Steers were blood sampled 2 h before and 2 h after the first, third and sixth R&R. Steers were weighed the day before each R&R. Median area under the plasma cortisol curve (AUC) was higher ($P < 0.05$) in R than C steers after the first R&R. Following the first, third and sixth R&R the median ACTH AUC was similar ($P > 0.005$) between the treatments. Cortisol AUC in R steers decreased ($P < 0.001$) following the third and sixth compared with the first R&R. However, cortisol AUC in response to exogenous ACTH (following administration of dexamethasone at -12 h) after the third R&R were higher in C than R steers. Corticotrophin-releasing hormone induced cortisol and ACTH AUC were not different ($P \geq 0.10$) in C vs R after the sixth R&R. There were no differences ($P \geq 0.10$) among treatments in haptoglobin, fibrinogen and Con A-induced interferon-γ after the first, third and sixth R&R. Albumin, urea and NEFA were higher ($P \leq 0.05$) in R than C steers after the first R&R. Beta hydroxy-butyrate and glucose levels were higher ($P \leq 0.05$) in R than C, while no ($P \geq 0.05$) changes in the protein and globulin levels were found in C vs R after the sixth R&R. White blood cell, differential and total count, red blood cell and platelets numbers were not different ($P \geq 0.05$) in C vs R after the first and third R&R. Lymphocyte numbers and mean corpuscular volume were higher ($P \leq 0.05$) in R than C steers after the sixth R&R. There was no ($P \geq 0.05$) difference in the overall ADG in C vs R. In conclusion, steers exposed to R&R responded with increased plasma cortisol, albumin, urea and NEFA. Repeated R&R did not have a detrimental effect on immune and production parameters.

2. Pituitary, adrenal, immune and performance responses of Holstein-Friesian bulls housed on slatted floors at various space allowances.

2.1 Introduction

Housing protects animals from adverse weather conditions and provides structured management (feeding, drinking, health check etc.) under controlled conditions. However, insufficient space allowance induces a repeated state of stress that alters activity of the pituitary-adrenal axis, immune function, behaviour and growth rate (Ingvartsen and Andersen, 1993; Fisher et al., 1997a, b, c). Previous studies with cattle housed at different space allowances reported either no changes in the basal cortisol concentrations (Fisher et al., 1997a), increased (Friend et al., 1977) or decreased (Benek et al., 1984; Fisher et al., 1997b) cortisol responses to exogenously administered adrenocorticotropic hormone (ACTH). Hickey et al. (2003) reported attenuation of lymphocyte proliferation *ex vivo* immune functions tests for cattle at 2 m^2 compared with 1.5 m^2 space allowance. In contrast, Fisher et al. (1997a) found no differences in *ex vivo* cellular immune function between space allowances of 1.5, 2.0, 2.5 and 3 m^2 per heifer. In addition, changes in cattle blood immune cells following exposure to stressful stimuli have been examined previously (Kent and Ewbank, 1983; Swanson and Morrow-Tesch, 2001).

Stressful stimuli may induce changes in white blood cell (WBC) numbers and packed cell volume (Kent and Ewbank, 1983). Reduced cattle productivity with a decreasing space allowance from 4.7 to 1.5 m^2 per animal (Ingvartsen and Andersen, 1993) in slatted floor units was reported. Previous studies have measured the growth of finishing cattle in response to a range of three or more space allowances and reported that increasing population density can reduce growth rate (Smith et al., 1981; Morrison and Prokop, 1983).

The objectives of the present study were to examine the effect of mixing mature beef bulls (with and without previous contacts) and housing at 1.2, 2.7 and 4.2 m^2 space allowances. The hypotheses were that (i) familiar animals (F; with previous contact) would be less stressed compared with unfamiliar (UF; with no previous contact) when exposed to chronic stress (inadequate space allowances); and (ii) chronic stress alters the functioning of the hypothalamic-pituitary-adrenal (HPA) axis and associated stress indicators. Indices used to measure the stress responses were plasma cortisol and ACTH responses following exogenous ACTH and corticotrophin-releasing hormone (CRH) administration, respectively; concanavalin A (Con A) and phytohaemagglutin (PHA) induced interferon (IFN)-γ production, haematological variables, feed intake and performance.

2.2 Materials and Methods

Treatments

Seventy-two 14-month-old Holstein-Friesian bulls (mean ± S.E. BW = 403 ± 3.5 kg) were blocked by weight and assigned, using a completely randomised design, to two groups (familiar, F and unfamiliar, UF) and within group, randomly allocated to one of three space allowance treatments. The treatments were 1.2, 2.7, and 4.2 m^2 individual space allowance.

Animal housing and management

Before assignment to the individual space allowances, bulls were weighed and housed in 6 pens (n = 12 bulls per pen; with an individual space allowance of 4 m^2 per bull) from day –30 (day of treatment = day 0) to acclimatize them to handling and restraint. On day 0, bulls were

assigned to one of 18 (n = 4 bulls per pen) slatted floor pens, in two locations (location 1 = 12 pens; location 2 = 6 pens) 50 m apart with six pens per row. The bulls were allocated alternatively into pens to one of the three space allowances and two groups (F and UF; n = 12 bulls per space allowance per group) using a 3 x 2 factorial design. The bulls for the F group were allowed to share the same pen mates in the new location with whom they were previously housed during the acclimatisation period. The bulls in the UF groups were individually taken out, mixed and not allowed to share the same pen mates in the new pen location with whom they were previously housed during the acclimatisation period.

The dimensions of the new pens were 4.5 x 1.1 m, 4.5 x 2.4 m, and 4.5 x 3.8 m for the 1.2, 2.7 and 4.2 m^2 per bull space allowance, respectively. A steel mesh (3.8 x 1.6 m) was used to separate the sides of each pen. The feed face was 4.5 m along the front of each pen for all space allowances. The bulls were housed for 83 days in the respective space allowances. Table 1 provides a calendar of events throughout the experimental period. Animals were given access to grass silage (DM content (mean values) = 190.2 g/kg) supplemented with barley plus soybean mix (mean values on DM basis, crude protein = 146 g/kg, crude fibre = 41.9 g/kg, acid hydrolysable oil = 39 g/kg, ash = 58.6 g/kg) per bull daily. Bulls had free access to water in their individual pens.

Catheterisation

To facilitate frequent blood collection, bulls were fitted aseptically with indwelling jugular catheters on days 2, 13, 35 and 76 post housing. The procedure was performed as previously described (Ting et al., 2003), using 12-gauge Anes spinal needles (Popper and Sons) and polyvinyl tubing (approximately 1.47 mm internal diameter; Ico Rally Corp., catalogue No. SVL 105-18 CLR) attached to a blunt 18-gauge needle at the blood collection end. All catheters were exteriorised on the neck of the animals, filled with sterile 3.5% sodium citrate solution, and plugged with a stopper. Catheters were secured in place in re-sealable patches with the aid of adhesive cement (Big Bull Hip Tag Cement; Bigual Supply, Co., Elysian, MN, USA), Velcro, and zinc oxide wrapping bandages. After catheterisation, animals were returned to their home pens and catheters were maintained patent for 32 hours by flushing with 1 mL of 3.5% sodium citrate after each blood collection.

ACTH challenge

The induced release of plasma cortisol following stimulation of the adrenal cortex gland to a standardised dose of ACTH (1.98 IU/kg metabolic BW; Friend et al., 1977 and Fisher et al., 1997a) was tested on day 3 of treatment, in two bulls from each pen (n = 12 per treatment), which were randomly chosen at the start of the experiment. Dexamethasone (20 μg/kg BW; Faulding Pharmaceuticals) was administered (n = 12 per treatment) intramuscularly (IM) at 2000 to 2030 GMT to all the bulls undergoing ACTH challenge (Synacthen Ampoules, Novartis Pharmaceuticals Ltd.) on the next morning at 0800-0830 h GMT. Both dexamethasone and ACTH administrations to bulls were staggered and carried out within 30 min intervals. Two further bulls from each pen (n = 12 per treatment) also received 5 mL saline (0.89% [weight/volume, wt/vol] sterile) intravenously (IV) (via catheter) at the time of dexamethasone and ACTH administration to serve as controls.

Immediately following the administration of dexamethasone, ACTH and normal saline catheters were flushed with 2 mL of 3.5% sterile sodium citrate solution. Blood samples were collected into tubes containing lithium heparin as anticoagulant from bulls through jugular catheters at -48, -24, 0, 23, 45, 67, 89, 119, 149, 179, 209, 239 and 269 minutes relative to the time of ACTH administration at 0 minutes for bulls in each treatment for plasma cortisol assay. Heparinised plasma was separated after centrifugation at 1600 g and stored at –20^{o}C until

assayed within six weeks. Plasma cortisol concentrations were determined using commercially available RIA kit (Corti-cote, ICN Pharmaceuticals), adapted and validated for bovine plasma by Fisher et al. (1997a). The intra-assay CV (n = 6) for samples containing 5.3, 17.9 and 56.7 ng of cortisol/mL were 16.3, 9.5 and 9.7%, respectively, and the interassay CV (n = 15) for the same samples were 15.8, 11.9 and 13.8%.

CRH challenge

The induced release of ACTH following stimulation of the pituitary gland with exogenous CRH, was determined on days 14, 36 and 77, in two bulls from each pen (n = 12 per treatment). One bull administered with bovine (b) CRH on day 14 had been subjected to the ACTH challenge and the second was administered with normal saline (0.89% [wt/vol] sterile) previously on day 3 from all treatments. bCRH was administered IV at a concentration of 0.3 µg/kg body weight as previously validated and described (Gupta et al., 2004). Two further bulls, from each pen served as controls and received 2 mL 0.89% [wt/vol] sterile saline (one received ACTH and the second saline on day 3) on days 14, 36 and 77.

Immediately following the administration of bCRH and normal saline, catheters were flushed with 2 mL of 3.5% sterile sodium citrate solution. The same animals were administered with bCRH on day 36 and 77 from all the treatments. Blood sample collection commenced on all days at 0900 h GMT, three samples at 24 min intervals each were collected before the IV administration (through jugular catheter) of either bCRH or saline. Subsequent blood samples were collected through the jugular catheters at 23, 45, 67, 89, 119, 149, 179, 209, 239 and 269 min relative to the time of bCRH and normal saline administration.

All blood samples were collected into iced tubes containing EDTA anticoagulants and were centrifuged at 2000 g, at 4 ºC for 15 min within 30 min of collection and plasma was frozen and stored at -80ºC until assayed for ACTH. Commercially available RIA kits were used to determine the plasma levels of ACTH (Diagnostic Products Corporation) within six weeks of collection. The intra-assay CV (n = 6) for samples containing 45.3 and 112.8 pg of ACTH/mL were 18.9 and 16.4%, respectively, and the interassay CV (n = 12) for the same samples were 11.8 and 15.1% respectively.

Stimulated lymphocyte production of interferon-γ

The in vitro production of IFN-γ following stimulation of lymphocytes by the novel mitogen Con A and PHA in whole blood cultures (heparinised) were determined for all bulls (n = 12 per treatment) on days –1, 0, 3, 14, 36, and 77 following the procedure described by Ting et al. (2003), except that the cultures were stimulated with either phosphate buffer saline (PBS) alone or Con A (20 µg in 1.5 mL blood) or PHA (20 µg in 1.5mL blood) for 24 h at 37 ºC and in an atmosphere of 5% CO_2. In brief, duplicate 1.48 mL aliquots of blood were cultured in 24-well culture plates (Costar Corporation) with 20 µL of phosphate buffer saline containing either 1.0 mg/mL of Con A, 1.0 mg/mL of PHA or no additive incubated for 16 h at 37 ºC and an atmosphere of 5% CO_2 in air. The culture plates were then centrifuged at 1,600 g, at 4 ºC for 20 min; the supernatant harvested and frozen at –20 ºC until it was assayed for IFN-γ production, using an ELISA procedure (Rothel et. al., 1990; CSL Biosciences). The in vitro Con A and PHA stimulated IFN-γ production was calculated by subtracting the absorbance at 450 nm of wells that received PBS alone from the absorbance of wells that received either Con A or PHA, respectively.

Haematology

Unclotted (EDTA) whole blood samples collected from catheterised bulls from all treatments on days –1, 0, 3, 14, 36, and 77 were analyzed for red blood cell (RBC) number, haemoglobin (Hb) concentration and packed cell volume (PCV) using an automated electronic particle analyser (Celltac, MEK-6108K) within 1 h of blood sampling. For each sample a thin blood smear was prepared on a grease-free glass slide (Gold star micro slides, Chance Proper Ltd) for differential white blood cell (WBC) counting. The smears were air-dried and stained using the haematology three-step stain for differentiation of morphological cell types (Accralab, Fisher Scientific Company). One hundred cells, including neutrophils, band cells, basophils, eosinophils, monocytes and lymphocytes were counted under the microscope at 40 x.

Environmental condition, health and production

The study was conducted from February to May. The mean daily air temperature and relative humidity in the housing facility were recorded using four Tiny Talk data loggers (Radionics) in each location. During the experiment the general health status of the bulls was recorded on a daily basis and animals were assessed for injury and body lesions. The complete record of any clinical signs and their medical treatment were maintained. The feed offered and refusals were recorded for each pen twice weekly throughout the study. The body weights were measured at two-week intervals throughout the experimental period.

Statistical analyses

Statistical analyses were performed using the general linear model (GLM) procedure of Statistical Analysis System version 8.2 (SAS Inst. Inc.). The data was analysed initially for the effect of F and UF groups and effect of location. When no ($P>0.05$) main effect and interaction were found, the data for the F and UF groups were pooled and were further analysed for the main effect of space allowances. The effect of location on ambient temperature and relative humidity was also examined. Although shed height was greater in location 2, no differences ($P>0.05$) were observed in both ambient temperature and relative humidity; therefore the location effect was excluded from the analyses. Data that failed the test of normality and/or homogeneity of variance (Sphario-Wilk test, $P<0.05$); using procedure UNIVARIATE) were subjected to suitable transformation and analysed with an ANOVA or analysed non-parametrically using Wilcoxon and Kruskall-Wallis procedures (Zar, 1999). The percentages (%) were transformed angularly $\{(x = 180/\pi) \times \arcsin [\sqrt{(p/100)}]$, where p is a percentage $[0<P<100; \pi = 3.1416]\}$.

The average feed intakes were determined using the means of each of the subsequent four feed intakes for two weeks, and calculated by subtracting feed refusal from that offered. The daily weight gain was determined using the means of each bodyweight measurement, recorded at two-week intervals until the end of the experiment. The data for average daily feed intake (ADFI) and average daily gain (ADG) were analysed using a randomised complete block design for the main effect of treatments using repeated measure ANOVA. The data for the neutrophils %, band cells %, basophils %, eosinophils %, monocytes %, lymphocytes %, RBC, PCV, Hb, both Con A- and PHA- induced IFN-γ production were analysed by repeated measure ANOVA. The model included the effect of space allowance, time and their interaction. Where significant the pre-housing values (values at day –1) were included as a covariate. Following a significant F test for space allowances, time and their interaction, means were compared using the probability of difference (PDIFF) procedure of SAS on the least square means and was applied to determine statistical differences.

The cortisol and ACTH data following ACTH and/or CRH challenge were rationalised into a single variable by calculating the area under the curve vs time (integrated response) from the time of the ACTH/CRH/saline administration until the final sample of the day using a linear trapezoidal rule (Veissier et al., 2001). Mean pre-treatment basal plasma cortisol and/or ACTH concentration was included as covariates. In addition to AUC, for each bull, the peak cortisol/ACTH concentrations and time to reach peak cortisol/ACTH concentrations post ACTH and/or CRH challenge were determined. To observe over time effects for ACTH responses following CRH challenge, data were analysed using a repeated measure ANOVA, the statistical differences were determined by Tukey's studentised range test (Zar, 1999).

2.3 Results

Plasma cortisol and ACTH

Mean basal plasma cortisol concentrations from 0 to 48 min in bulls (n = 12 bull per treatment administered with saline) housed at 1.2, 2.7 and 4.2 m^2 space allowances were 4.03, 2.85 and 3.25 ng/mL, respectively (Fig. 1 lower panel). The mean basal plasma ACTH concentrations from 0 to 48 min in the bulls (n = 24 bull per treatment) housed at 1.2, 2.7 and 4.2 m^2 space allowances were 61.0, 60.4 and 57.9 pg/mL, respectively. The administration of dexamethasone (n = 12 bulls per treatment) at -12 h (before administration of exogenous ACTH) reduced the mean (0 to 48 min) plasma cortisol concentration to 0.6 ng/mL consistently among all treatments (Fig. 1 upper panel) with no difference (P>0.05) between the treatments. On day 3, before administration of ACTH / saline the integrated basal plasma cortisol concentration was greater (P<0.05) in the bulls housed at 1.2 than 2.7 and 4.2 m^2 individual space allowances (Table 2).

ACTH challenge

Integrated (AUC) and peak plasma cortisol concentrations following exogenous administration of ACTH on day 3 post housing was not different (P>0.05) among treatments (Table 2). Following exogenous ACTH administration, the time to reach peak plasma cortisol concentrations tended (P=0.094) to be earlier in the bulls housed at 1.2 than 2.7 m^2 space allowance and it was intermediate for the bulls housed at 4.2 m^2 space allowance treatment. Following saline administration on day 3, the integrated plasma cortisol concentration was greater (P<0.05) in the bulls housed at 1.2 than those at 2.7 and 4.2 m^2 individual space allowances (Table 2).

There was a treatment x group effect (P=0.045) for the integrated plasma cortisol concentration following exogenous ACTH administration. In the group housed at 2.7 m^2 space allowance, this was indicated by the greater (P=0.011) AUC for plasma cortisol among the F (5690.4 ± 552.04 ng. mL^{-1}.min) than in the UF (4551.6 ± 408.97 ng. mL^{-1}.min) group. Integrated plasma cortisol response was greater (P=0.005) in the F bulls housed at 2.7 (5690.4 ± 552.04 ng. mL^{-1}.min) than 1.2 (4291.9 ± 476.72 ng. mL^{-1}.min) m^2 space allowance. The AUC for plasma cortisol was also greater (P=0.017) among the F bulls housed at 2.7 vs 4.2 m^2 (5690.4 ± 552.04 vs 4663.9 ± 457.55 ng. mL^{-1}.min) space allowance.

CRH challenge

The data for the plasma ACTH concentrations following bCRH and saline administration on day 14, 36 and 77 are presented in Table 3. The administration of exogenous bCRH vs saline increased (P=0.001) plasma ACTH area under the curve for bulls within each space allowance.

Pre bCRH and/or saline administration, ACTH concentrations did not differ (P>0.05) between treatments and there was no (P=0.959) treatment x time effect. There was a significant effect of time on the basal plasma ACTH concentration (P=0.001), the concentration on day 77 was greater than on days 14 and 36.

Following bCRH administration there was no effect (P>0.05) of treatment and treatment x time on the integrated plasma ACTH, peak ACTH and time to reach peak ACTH concentrations. However there was a significant time effect, bCRH administration increased the integrated ACTH response (P = 0.008) on day 14 compared with 36 and was intermediate but not different (P=0.099) on day 77. The time to reach peak plasma ACTH concentration was greater (P=0.002) on day 14 than on days 36 and 77 following exogenous bCRH administrations. Following saline administration the treatment effect was indicated by the increased (P=0.012) integrated plasma ACTH concentrations among the bulls housed at 2.7 m^2 than those at 4.2 m^2 and it was intermediate but not different (P=0.104) among the bulls housed at 1.2 m^2 space allowances while for the time effect, the integrated ACTH concentration was intermediate and different (P=0.001) on day 36 than on days 14 and 77 and it was greater (P=0.007) on day 14 than on day 77.

Stimulated lymphocyte production of interferon γ

The pre-treatment IFN-γ production from stimulated lymphocytes via novel mitogens Con A and PHA was not different (P>0.05) among bulls housed at 1.2, 2.7 and 4.2 m^2 space allowance. The effect of mixing (F or UF) was negligible on days 14, 36 and 77. On days 0 (P=0.081), 3 (P=0.330), 36 (P=0. 492) and 77 (P=0.091) IFN-γ production in response to Con A was not different among treatments (Fig. 2; upper panel). However, on day 14, Con A induced IFN-γ was less (P=0.051) in the bulls housed at 4.2 vs. 2.7 m^2 and was intermediate but not different (P=0.09) for those housed at 1.2 m^2 (Fig. 2; upper panel). In response to PHA, IFN-γ production was not different (P>0.05) among treatments on days 0, 3, 14, and 77 (Fig. 2; lower panel), however, on day 36 there was a tendency (P=0.071) for greater IFN-γ production among the bulls housed at 4.2 than those at 1.2 m^2 (Fig. 2; lower panel).

White blood cell differential count

The data for white blood cell differential counts are presented in Table 4. The pre-treatment % of neutrophils, lymphocytes, monocytes, eosinophils, basophils, and band cells were not (P>0.05) different among treatments. There was no (P>0.05) treatment and treatment x time effect in neutrophil, lymphocyte, band cell, eosinophil and monocyte %. There was no time (P>0.05) effect in band cell, basophil and monocyte %.

Over time, neutrophil % increased (P=0.002) and lymphocyte % decreased (P=0.001) on day 3 and 14 compared with day 0, while on day 36 and 77 no differences (P>0.05) were observed. Reduced (P< 0.05) eosinophil % was observed on days 3 compared with days 0, 14, 36 and 77.

Blood cell counts

The data for blood cell counts are presented in Table 5. The pre-treatment values of the RBC, PCV and Hb were not different (P>0.05) among treatments. There was no (P>0.05) effect of treatment on RBC, PCV, Hb and total white blood cells post treatment. However a treatment x time interaction (P<0.05) on RBC and Hb was observed. On day 36 RBC and Hb were lower (P<0.001) in the bulls housed at 4.2 than those at 1.2 and 2.7 m^2. On day 77 the bulls housed at 1.2 m^2 had greater (P<0.001) RBC and Hb compared with bulls housed at 4.2 m^2 but was intermediate and not different (P>0.05) than those at 2.7 m^2 space allowance. On day 3 after housing, the RBC and Hb in all treatments were lower than on day 0 (P<0.05). These were still

lower ($P< 0.05$) on day 14 except in the bulls housed at 1.2 m^2. The Hb increased ($P< 0.05$) from day 14 to 77 in bulls housed at 1.2 m^2 space allowance, while, no difference ($P>0.05$) was observed in RBC from day 3 to 77. There was no difference ($P>0.05$) in the RBC on days 36 and 77 in the bulls housed at 2.7 and 4.2 m^2 space allowance while Hb increased ($P<0.05$) from day 36 to 77. The time effect on the PCV is indicated by the decreased ($P=0.001$) values from day 0 to 3, 14, 36 and 77.

Environmental condition, health and production

The mean daily air temperature (location 1: 9.3°C (range 0.7 to 27.0°C); location 2: 9.0°C (range 0.3 to 22.3°C) and relative humidity (location 1: 82.1% [range 41.2 to 100.0%]; location 2: 83.7% (range 41.2-100.0%) in the two housing sheds were not different ($P>0.05$). There were no ($P>0.20$) effects of space allowances (1.2, 2.7, and 4.2 m^2space allowances) on ADFI (33.1 ± 0.70, 36.5 ± 0.82, and 35.7 ± 1.90 kg per pen per day, respectively) throughout the study. The overall ADG from days 0 to 83 was lower ($P=0.001$) in bulls housed at 1.2 (0.59 ± 0.050 kg per day) compared with 2.7 (1.32 ± 0.087 kg per day) and 4.2 (1.26 ± 0.222 kg per day) m^2 individual space allowance with no difference ($P>0.05$) in ADG between bulls housed at 2.7 (1.32 ± 0.087 kg per day) and 4.2 (1.26 ± 0.222 kg per day) m^2 individual space allowances. No obvious sign of injury or morbidity associated with reduced space allowance were detected during the experiment.

2.4 Discussion

In this study, basal plasma cortisol and ACTH concentrations were within normal biological ranges and were similar to a previous study reported by Gupta et al. (2004). The basal cortisol concentrations were not affected by mixing of familiar and unfamiliar bulls. However, exposure of the bulls to a restricted (1.2 m^2 per bull) compared with generous (2.7 and 4.2 m^2 per bull) space allowances increased basal plasma cortisol concentrations on day 3 of treatment indicating that bulls housed in the restricted space allowance were stressed. This finding is in accordance with the suggestion of Ingvartsen and Anderson (1993), that insufficient space allowance induces a threat to the well being of cattle and leads to deleterious effects on performance and health. Housing bulls at a restricted space allowance activated their adrenal glands with increased plasma cortisol concentrations within 3 days. However sensitivity to exogenous Dexamethasone and ACTH was not altered which was somewhat unexpected. Perhaps the increased cortisol following the acute stress of housing at the restricted space allowance decreased the adrenal responsiveness by the saturation of ACTH receptors in the adrenal gland. There was a tendency for a reduction in the time to reach peak cortisol concentrations in the 1.2 m^2 space allowance compared with a space allowance of 2.7 m^2. Therefore short-term housing at restricted space allowance increased cortisol and tended to reduce the response time for cortisol secretion following exogenous ACTH administration.

Unlike basal plasma cortisol concentrations, cortisol response induced by exogenous ACTH administration may provide an independent index of adrenocortical sensitivity (Moberg and Mench, 2000). There are differences in the results for increased or decreased cortisol response following ACTH administration under stressful situations among farm animals. Studies examining the effects of housing cattle at restricted space allowance (Benek et al., 1984; Fisher et al., 1997b) and restricted movements in bulls (Ladewig and Smidt, 1989) have reported a reduced cortisol response, while, studies related to the social stress of regrouping and relocation in calves (Veissier et al., 2001) and ewes (Sevi et al., 2001) reported increased cortisol responses following exogenous ACTH administration. The decreased sensitivity of the adrenal gland to ACTH challenge in the UF group of bulls housed at 2.7 and 4.2 m^2 space allowances might

indicate down regulation of the pituitary-adrenal axis or increased sensitivity of the pituitary to cortisol negative feedback. Initial exposure to the mixing stressor coupled with the housing stressor may have resulted in the desensitisation of the pituitary-adrenal axis in the UF group. These findings are in agreement with Gupta et al. (2005), which reported a decreased cortisol response (i.e. decreased sensitivity of the adrenal gland) to ACTH challenge following regrouping and relocation of steers compared with non-regrouped and relocated steers. There was increased adrenal sensitisation in the F bulls housed at 2.7 m^2 compared with the UF. In contrast, housing F bulls for three days in a restricted space allowance altered the sensitivity of the adrenal glands, indicating chronic housing stress, which is not supportive of our hypothesis regarding familiar animals being less stressed.

The present study failed to demonstrate that the CRH induced ACTH response was affected by space allowance. This could be due to the failure of the lowest space allowance to induce sufficient stress and bring about changes in the activity of the pituitary-adrenal axis. CRH challenge has provided an independent method to test the sensitivity of the pituitary and adrenal gland during chronic stress (Gupta et al., 2004). The results of the present study suggests that using CRH induced ACTH release as an independent index at focal time points may not provide conclusive evidence on the effects of a range of space allowances as potential husbandry chronic stressors. The increased ACTH concentrations and time to reach peak ACTH concentrations observed on day 14 of housing indicates a degree of up-regulation of the pituitary gland that was extended up to day 36. However lack of changes on day 77 on the functioning of the pituitary gland support the hypothesis that pituitary desensitisation occurs in bulls in response to ongoing stress while the longer duration of the stressor modified the functioning of the pituitary gland and led to either adaptation or regulatory changes in the pituitary and/or adrenal gland to avoid the health of animals at risk. This hypothesis was further supported by the greater ACTH response on day 14 that tended to be lower on day 77 following saline administration.

Earlier studies that used mitogen and/or antigen-stimulation *in vitro* assays for the production of cytokines (e.g. IFN-γ), derived from lymphocyte proliferation, suggested that this was a useful indicator for studying stress in bovines (Ting et al., 2003; Earley and Crowe, 2002). Both Con A (Earley and Crowe 2002) and PHA (Gupta et al., 2005) (T-cell antigen and mitogen), respectively, have been used as a measure of immune responsiveness in previous studies. In this study, the Con A induced IFN-γ was lower on day 14 in the bulls housed at 4.2 vs. 2.7 m^2 and was intermediate for those housed at 1.2 m^2. These findings are not consistent with that of Fisher et al. (1997a,b) who reported no effect on immune response of housing beef heifers from 1.5 to 3.0 m^2 average space allowance but are in close agreement with Hickey et al. (2003) who reported an attenuation of the immune response at space allowances <2.0 m^2 for steers.

The relationship between stress induced activation of the HPA axis and alteration of immune functions is well established (Ladewig, 2000), however, in some circumstances e. g. exogenous cortisol administration within physiological ranges does not suppress immune function (Fisher et al., 1997c; Ting et al., 2004). Studies in animal husbandry (castration, Ting et al., 2003; regrouping and relocation of steers, Gupta et al., 2005) stress models have shown the suppression of IFN-γ production following peak cortisol response after treatment. In the present study there was not a major effect relevant to the hypothesis tested on the lymphocyte stimulated IFN-γ production. Overall little or no changes in immune response occurred over time in bulls housed at a range of space allowances suggesting adaptation of the immune system to chronic low-grade housing stress. Use of interferon-γ in cattle for detecting effects of chronic housing stress on the immune system may warrant further investigation.

Cattle blood immune cells (e.g. WBC, RBC, PCV etc.) are sensitive indicators of the physiological or patho-physiological responses of an animal to stress (Radostits et al., 1994). Neutrophilia (including band cell %), lymphopenia and eosinopenia that were observed in this study on day 3 were not in agreement with Fisher et al. (1997b) and Hickey et al. (2003), who reported no effect on white cell population from day 0 to day 96 in heifers housed at 1.5 or 3.0 m^2 average individual space allowance and steers housed at 1.5, 2.0, 3.0 and 4.0 m^2 per steer. Following housing, relative neutrophilia (including band cell %), lymphopenia and eosinopenia persisted until day 36. It has been previously reported that increased neutrophil, decreased lymphocyte and eosinophil % occurred following a variety of animal husbandry stressors of cattle including transportation (Murata et al., 1987), handling and diseases of bacterial origin (Radostitis et al., 1994). Eosinopenia observed in this study on day 3 post housing in the bulls housed at a range of space allowances are indicative of altered homeostasis. Neutrophilia and lymphopenia are common findings in stressed animals and are associated with changes in the WBC trafficking and release from the bone marrow by elevated concentrations of glucocorticoids (Dunn, 1989). In the present study, elevated concentrations of plasma cortisol may be related with altered WBC differential population. Therefore these data suggests that bulls housed at 1.2 m^2 space allowance have reacted to the restricted space allowance with altered body homeostasis.

A change in blood cell composition indicates a response to restore animal homeostasis when exposed to abrupt physical conditions (Radostits et al., 1994). Decreased RBC, PCV, and Hb values in this study after housing bulls in the range of space allowances on day 3 indicate housing stress and this effect persisted until day 36 indicating chronic housing stress (i.e. a time effect with no main treatment effect). These findings are in contrast with Hickey et al. (2003) and Fisher et al. (1997b) in steers and heifers, respectively. Throughout the study bulls housed at a range of space allowances did not recover to their pre treatment RBC, PCV and Hb values. The overall picture of the blood cell count indicate that bulls housed at restricted (1.2 m^2) and generous (4.2 m^2) space allowances seem to be affected more than bulls housed at 2.7 m^2 space allowance. However, the observed changes were within the physiological ranges of cattle suggesting that health of the bulls was not compromised.

Although there were no changes in the overall average daily feed intake among all the treatments, the average daily gain was lower among the bulls housed at 1.2 m^2 than those at 2.7 and 4.2 m^2/bull. This indicates that insufficient space allowance may have decreased the efficiency of feed conversion (Ingvartsen and Anderson, 1993). These findings are consistent with other findings, Fisher et al., (1997a,b) recorded that heifers of up to 468 kg bodyweight over a period of 140-141 days gained 0.65, 0.70 and 0.69 kg at space allowances of 2.0, 2.5 and 3.5 m^2, respectively, but only 0.52 kg at a space allowance of 1.5 m^2. Similarly, Smith et al., 1981, reported a significantly lower daily live-weight gain at 1.3 m^2 per steer and only a subtle decline in daily live-weight gain between space allowances of 2.32 and 1.58 m^2 per steer. The decreased growth rate of cattle at low space allowances may be attributed to the space available at the feed manger or insufficient space induced stress changes (Ingvartsen and Anderson, 1993). In the current study, the feed mangers were of constant length, however, activation of the HPA-axis may suppress feed conversion efficiency among bulls housed at restricted space allowances (Ingvartsen and Anderson, 1993).

In conclusion, housing bulls at 1.2 m^2 space allowance (restricted space allowance) increased acute plasma cortisol concentrations (day 3) compared with either 2.7 or 4.2 m^2 space allowances. On day 3 post housing, exogenous ACTH administration failed to detect altered adrenal reactivity in bulls housed at the range of space allowances. However, ACTH

administration revealed that the previous exposure of mixing had effects on altering the sensitivity of the adrenal gland for further exposure to stress. Time effects of CRH challenge revealed that pituitary responsiveness decreased over time irrespective of space allowance. Housing finishing bulls at a reduced space allowance (1.2 vs 2.7 and 4.2 m^2) did not alter ADFI, but reduced performance (ADG) without causing substantial effects on blood immune cells. Housing bulls at reduced space allowance (1.2 m^2) had a detrimental effect on their growth and was associated with raised plasma cortisol concentrations and reduced daily gain compared with space allowances of 2.7 and 4.2 m^2 per bull.

Table 1. Calendar of events including allocation to groups, space allowances and procedures performed on the bulls

Day	Event	[a] Procedure performed
-30	Bulls housed for acclimatization (n = 90)	Body weight (BW)
-1		BW, haematology and IFN-γ production measurements
0	[b] Mixing and housing of bulls (n =72)	[b] Haematology and IFN-γ production measurements
2		Catheterisation and [c] dexamethasone (n = 6 per group per space allowance) challenge
3		Haematology and IFN-γ production measurements, ACTH (n = 6 per group per space allowance) and saline (n = 6 per group per space allowance) challenges
13		BW, catheterisation
14		Haematology, IFN-γ production measurements, CRH (n = 6 per group per space allowance) and saline (n = 6 per group per space allowance) challenges
27		BW
35		Catheterisation
36		Haematology, IFN-γ production measurements, CRH (n = 6 per group per space allowance) and saline (n = 6 per group per space allowance) challenges
41		BW
55		BW
69		BW
76		Catheterisation
77		Haematology, IFN-γ production measurements, CRH (n = 6 per group per space allowance) and saline (n = 6 per group per space allowance) challenges
83		BW

[a] For details on blood sample collection see the text.
[b] On day 0 bulls were housed in 18 (n = 4 bulls per pen) slatted floor pens. The bulls were allocated alternatively into pens to one of the three space allowances and two groups (Familiar, F and Unfamiliar, UF).
[c] Dexamethasone was administered to all the bulls undergoing ACTH challenge on the next morning.

Table 2: Effect of housing bulls at 1.2, 2.7 and 4.2 m^2 space allowance on plasma cortisol concentrations following ACTH (1.98 i.u. ACTH/kg metabolic body weight) and saline (5 mL, 0.89% [wt/vol] administration on day 3 post housing.

Variables [a]	Space allowances, m^2 per bull			*P*
	1.2	2.7	4.2	Values[b]
Post-ACTH cortisol AUC, ng. mL^{-1}.min	4825 ± 332.9	5118 ± 364.9	5057 ± 313.2	NS
Post-saline cortisol AUC, ng. mL^{-1}.min	1031^{x} ± 86.5	821^{y} ± 116.9	758^{y} ± 94.6	0.05
Post-ACTH peak [c] cortisol, ng/mL	28.5 ± 2.0	29.8 ± 1.7	29.8 ± 1.6	NS
Post-ACTH interval [d] to peak cortisol, min	88.0 ± 9.7	111.0 ± 8.8	93.0 ± 8.8	0.094
Basal cortisol AUC [e], ng. mL^{-1}.min	138.2^{x} ± 29.8	83.8^{y} ± 20.3	91.2^{y} ± 19.2	0.049

[a] The mean ± SEM are presented for the post ACTH and saline administration area under the cortisol vs. time curve (AUC), post ACTH peak, interval to peak cortisol and AUC for basal cortisol.
[b] Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[c] Peak cortisol is presented for the bulls administered with ACTH.
[d] Peak interval was time taken to reach peak values following ACTH administration.
[e] Cortisol concentrations at -48, -23, -22 minutes (before administration of ACTH and saline) were averaged and included as a significant ($P<0.05$) covariate.
[x,y] Means within a row without common superscript are different ($P<0.05$)

Table 3: Effect of housing bulls at 1.2, 2.7 and 4.2 m^2 space allowance on plasma ACTH concentrations following bCRH (0.3 /kg body weight) and saline (5 mL, 0.89% [wt/vol] administration.

Variables [a]	Space allowance									Pooled SEM	*P* values [b]		
	1.2 m^2/bull			2.7 m^2/bull			4.2 m^2/bull						
Days	14	36	77	14	36	77	14	36	77		Treat [c]	Time[d]	Treat x time
[e] Basal ACTH AUC, pg. mL^{-1}.min	2765	2811	3240	2703	2741	3121	2649	2544	3049	242.8	NS	0.001	NS
Post-CRH ACTH AUC, pg. mL^{-1}.min	24970	20548	22657	25594	23123	22833	26719	21706	24294	1746.8	NS	0.029	NS
Post-saline ACTH AUC, pg. mL^{-1}.min	15164	12267	13732	16425	12809	15345	14795	11115	13318	528.4	0.042	0.001	NS
Post-CRH peak[f] ACTH, pg/mL	185	226	197	196	229	218	210	203	207	20.9	NS	NS	NS
Post-CRH interval [f] ACTH, min	33	23	24	43	28	23	39	34	27	6.2	NS	0.002	NS

[a] The means on day 14, 36 and 77 are presented for the post CRH and saline administration area under the cortisol vs. time curve (AUC), post ACTH peak, interval to peak ACTH and AUC for pre bCRH and saline administration basal ACTH.
[b] Non-significance, defined as $P > 0.05$, is denoted as NS.
[c] Treat = 1.2, 2.7 and 4.2 m^2 space allowances.
[d] Time = day 14, 36 and 77.
[e] ACTH concentrations at -48, -23, -22 minutes (before administration of bCRH and saline) were averaged and included as a significant ($P < 0.05$) covariate.
[f] Peak ACTH is presented for the bulls administered with bCRH.
[f] Peak interval was time taken to reach peak values following CRH administration.

Table 4: The effect [a] of housing bulls at 1.2, 2.7 and 4.2 m^2 space allowance on mean (± SEM) differential white blood cell counts

Variables, %	Space allowance, m^2/bull	Days [b]						*P*-value [c]		
		-1	0	3	14	36	77	Treat [d]	Time	Treat x time
Neutrophil	1.2	36.0 ± 1.88	36.2 ± 1.34	54.7 ± 4.04	45.6 ± 2.07	41.3 ± 2.29	38.2 ± 2.32	NS	0.001	NS
	2.7	36.5 ± 1.61	35.8 ± 2.11	57.0 ± 4.50	39.1 ± 2.18	35.5 ± 1.78	38.8 ± 2.17			
	4.2	35.8 ± 1.79	35.5 ± 1.50	55.7 ± 4.12	39.0 ± 3.23	34.8 ± 1.83	40.1 ± 1.55			
Lymphocyte	1.2	55.9 ± 1.62	52.1 ± 1.39	33.9 ± 3.71	44.2 ± 1.76	45.7 ± 2.14	49.9 ± 1.66	NS	0.001	NS
	2.7	53.2 ± 1.61	51.5 ± 1.94	29.9 ± 3.38	49.2 ± 1.71	52.2 ± 1.42	49.3 ± 1.97			
	4.2	55.0 ± 1.83	50.9 ± 1.19	32.1 ± 3.56	48.9 ± 2.93	51.2 ± 1.43	49.9 ± 1.50			
Band cell	1.2	0.5 ± 0.13	0.3 ± 0.09	0.4 ± 0.15	0.08 ± 0.06	0.08 ± 0.05	0.08 ± 0.06	NS	NS	NS
	2.7	0.7 ± 0.18	0.3 ± 0.15	0.2 ± 0.13	0.08 ± 0.06	0.08 ± 0.06	0.12 ± 0.07			
	4.2	0.7 ± 0.15	0.1 ± 0.07	0.4 ± 0.22	0.1 ± 0.00	0.21 ± 0.12	0.08 ± 0.06			
Eosinophil	1.2	2.4 ± 0.43	2.3 ± 0.38	1.2 ± 0.33	1.8 ± 0.33	3.0 ± 0.48	3.3 ± 0.63	0.092	0.001	NS
	2.7	4.8 ± 1.03	3.9 ± 0.81	1.8 ± 0.97	2.9 ± 0.45	3.5 ± 0.87	2.8 ± 0.53			
	4.2	3.3 ± 0.49	3.1 ± 0.70	1.1 ± 0.35	2.6 ± 0.50	2.5 ± 0.47	1.8 ± 0.39			
Basophil	1.2	0.10 ± 0.90	0.1 ± 0.07	0.17 ± 0.07	0.13 ± 0.09	0.12 ± 0.07	0.08 ± 0.00	0.034	NS	NS
	2.7	0.30 ± 0.10	0.1 ± 0.04	0	0.04 ± 0.00	0	0			
	4.2	0.30 ± 0.11	0.1 ± 0.04	0.08 ± 0.0	0.13 ± 0.00	0.13 ± 0.09	0.40 ± 0.24			
Monocyte	1.2	5.1 ± 0.48	9.0 ± 0.68	9.6 ± 0.74	8.3 ± 0.97	9.6 ± 0.86	8.5 ± 0.96	NS	0.067	NS
	2.7	4.6 ± 0.41	8.4 ± 0.75	11.0 ± 1.13	8.7 ± 1.00	8.8 ± 1.00	9.0 ± 0.88			
	4.2	4.9 ± 0.53	10.4 ± 0.71	10.5 ± 1.27	9.4 ± 0.65	11.0 ± 1.26	7.7 ± 0.67			

[a] Data presented are angular transformed for individual percentages.
[b] Pre housing value on day-1 included as covariate.
[c] Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[d] Treat = 1.2, 2.7 and 4.2 m^2 space allowances.

Table 5: Effect of housing bulls at 1.2, 2.7 and 4.2 m^2 space allowance on haematological variables. The means on day -1, 0, 3, 14, 36 and 77 are presented for red blood cells, packed cell volume, haemoglobin and total white blood cells

Variables	Space allowance per bull	Days [a]						Pooled SEM	*P* value [b]		
		-1	0	3	14	36	77		Treat [c]	Time	Treat x time
Red blood cell concentration, x 10^{12}/L	1.2 m^2	8.70	8.94	7.78	7.51	7.81	7.56	0.14	NS	0.001	0.029
	2.7 m^2	8.90	9.05	7.98	7.63	7.51	7.24	0.12			
	4.2 m^2	8.50	8.86	7.78	7.38	7.03	6.77	0.14			
Packed cell volume, %	1.2 m^2	28.90	29.45	25.96	25.27	26.60	26.17	0.43	NS	0.001	NS
	2.7 m^2	29.10	29.47	26.40	25.72	25.99	25.56	0.48			
	4.2 m^2	28.60	29.47	26.39	25.56	24.78	24.60	0.39			
Haemoglobin, concentration, x g/dL	1.2 m^2	10.30	10.36	9.27	8.98	9.42	9.92	0.13	NS	0.001	0.017
	2.7 m^2	10.50	10.46	9.56	9.07	9.15	9.68	0.12			
	4.2 m^2	10.30	10.44	9.45	8.96	8.79	9.40	0.12			
Total white blood cell, x 10^9/L	1.2 m^2	8.76	10.90	13.05	8.47	8.48	8.25	0.10	NS	0.001	NS
	2.7 m^2	8.43	10.49	12.70	7.89	7.76	8.78	0.11			
	4.2 m^2	8.49	10.20	14.26	7.93	7.66	8.69	0.11			

[a] Pre housing value on day-1 included as covariate.
[b] Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[c] Treat = 1.2, 2.7 and 4.2 m^2 space allowances.

Figure 1: Effect of intravenous administration of ACTH (1.98 i.u. ACTH/kg metabolic body weight; upper panel) and saline (5 mL, 0.89% [wt/vol]; lower panel) on mean ± SEM plasma cortisol in the bulls housed at 1.2 (—*—), 2.7 (—□—) or 4.2 (—Δ—) m^2 individual space allowance. The ACTH (n = 12 per space allowance) and saline (n = 12 per space allowance) were administered at time 0. Pre-challenge baseline samples were collected for 48 minutes before the challenge. Dexamethasone was administered at -12 hours before the ACTH administration. Bulls receiving saline also received saline at the time of dexamethasone administration.

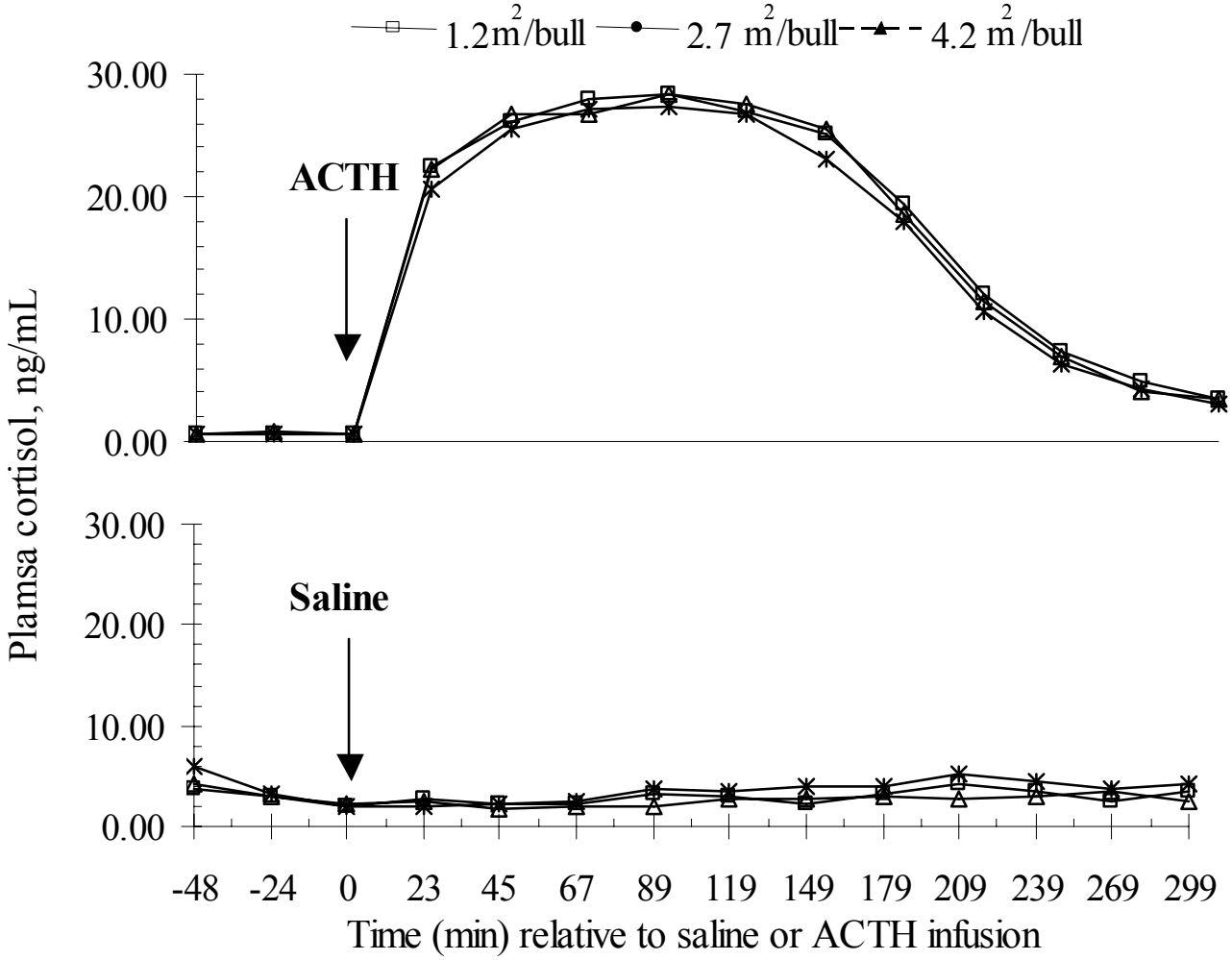
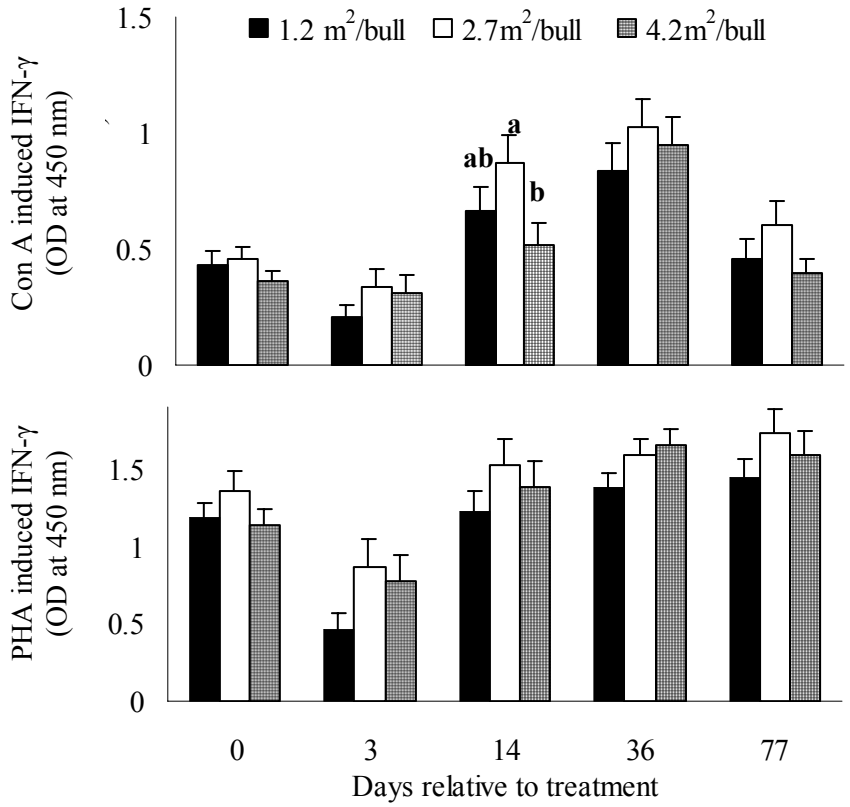


Figure 2: Effect of housing bulls at 1.2, 2.7 and 4.2 m^2 individual space allowance on concanavalin (Con; upper panel) A and phytohaemagglutin (PHA; lower panel) induced in vitro interferon (IFN)-γ production from cultured whole blood of bulls at day 0 and days 3, 14, 36 and 77 of housing. Data are presented as the mean ± SEM. [a,b] Within day means without common superscripts are different ($P< 0.05$).



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3. Effects of 12-hour transportation by road on adrenal, haematological and immune responses, body weight and temperature of bulls previously housed on slatted floors at 1.2, 2.7 and 4.2 m^2 space allowance

3.1 Introduction

Farm animals housed at different space allowances are routinely transported as a common management practice in the beef industry. Animals housed at reduced space allowance have been reported to show a degree of chronic stress and associated physiological changes (Ingvartsen and Andersen, 1993; Fisher et al., 1997a, b, c; Hickey et al., 2003a). From an animal welfare and an economic point of view, the transport of animals is regarded as an acute physical stressor stimulating an associated psychological response (Van de Water et al., 2003). Stress disrupts the internal homeostasis of animals by inducing changes in the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Haddad et al., 2002). Studies for groups of beef cattle housed at restricted space allowances have reported reduced cortisol response to exogenous adrenocorticotrophic hormone (ACTH) (Beneke et al., 1984; Fisher et al., 1997a). In contrast, Friend et al. (1977) measured an increased cortisol response to ACTH in dairy cows subjected to a lower space allowance compared with controls. Several studies in cattle have shown altered cortisol response to transportation stress (Murata et al., 1987; Knowles, 1999; Van de Water et al., 2003). The magnitude of the cortisol response in cattle following transport has been related to the pre-transport rearing conditions (e.g. farm facility and market), loading, unloading and post transport rearing conditions. However, it was suggested that basal or induced adrenal response to ACTH in cattle might alter in response to the magnitude of the stressor (e. g. regrouping and relocation of steers, Gupta et al., 2005) and time of sampling after the commencement of the stressor (Fisher et. al., 1997a). Therefore the regulation in the HPA axis secretion (e.g. cortisol) to stress is stimulus specific (Armario et al., 1988) and may not be associated with the return to the normal functioning of the HPA axis (Sakellaris and Vernikos-Danellis, 1975).

Previously *in vitro* stimulated lymphocyte interferon (IFN)-γ production has been used as a reliable measure of immune activation to common husbandry stressors such as castration (Earley and Crowe, 2002; Ting et al., 2003) and weaning (Hickey et al., 2003b) in cattle. Hickey et al. (2003a) reported the attenuation of lymphocyte stimulated IFN-γ production for cattle at 2.0 m2 compared with 1.5 m2 space allowance. In contrast, Fisher et al. (1997b) found no difference in lymphocyte stimulated IFN-γ production between space allowances of 1.5, 2.0, 2.5 and 3.0 m2 per heifer. In addition, changes in cattle blood immune cells (e.g. white blood cells, packed cell volume) following exposure to stressful stimuli (e.g. housing, transport), have been examined previously (Fisher et al., 1997a; Kent and Ewbank, 1986; Swanson and Morrow-Tesch, 2001). Blecha et al. (1984) reported increased neutrophil numbers and reduced lymphocyte blastogenic responses in steers transported for 10 h.

Earlier studies have measured the growth of finishing cattle in response to a range of three or more space allowances and reported that increasing population density can reduce growth rate (Smith et al., 1981; Morrison and Prokop, 1983). Transport disrupts the homeostasis of animals (Warriss, 2004), therefore, there is an increase in metabolic activity and demand for energy. Dixit et al. (2001) have reported increased body temperature in cows after 14 h of transport. Transported animals have been reported to lose body weight ranging

from 2 to 11 percent after 5 to 15 h of transportation (Knowles, 1999; Swanson and Morrow-Tesch, 2001).

The integrated approach of the animals' physiological and immunological systems to the repeated exposure of more than one stress stimuli (Moberg and Mench, 2000) in a production system is often complex. Therefore, the objective of this study was to investigate the effect of 12-h transport by road of mature bulls previously housed at different (1.2, 2.7, 4.2 m^2 per bull) space allowances. Indices used to measure the stress responses were plasma cortisol, concanavalin A (Con A) and phytohaemagglutin (PHA) induced IFN-γ production, haematological variables, body temperature and performance.

3.2 Materials and Methods

Treatment

Seventy-two 14-month-old Holstein x Friesian bulls (mean ± s. e. body weight = 403 ± 3.5 kg) were blocked by body weight and randomly allocated to the one of three (1.2, 2.7, 4.2 m^2 per bull) space allowances (S. Gupta et al., unpublished). The bulls were housed for 96 days in the respective space allowances. On day 97 bulls were assigned either a transport (T) or a control (C) treatment. On day 97, 98 and 99 a total of forty-eight bulls (n = 16 per day) were transported (T treatment) and 24 bulls (n = 8 per space allowance) were not transported and served as controls (C treatment).

Animal housing and management

Before assignment to the individual space allowances, bulls were weighed and housed in 6 pens (n = 12 bulls per pen; with an individual space allowance of 4 m^2 per bull) from day –30 (day of treatment day = 0) to acclimatise them to handling and restraint. On day 0, bulls were assigned to one of 18 (n = 4 bulls per pen) slatted floor pens, representing two locations (location 1 and 2) 50 meters apart with 6 pens per row. The bulls were allocated alternatively to one of the three space allowance. The dimensions of the new location pens were 4.5 x 1.1 m, 4.5 x 2.4 m, and 4.5 x 3.8 m for the 1.2, 2.7 and 4.2 m^2 per bull space allowance, respectively. A steel mesh (3.8 x 1.6 m) was used to separate the sides of each pen (S. Gupta et al., unpublished). The feed face was 4.5 m long for all space allowances. Animals were given access to grass silage (dry matter content (mean values) = 190.2 g per kg supplemented with barley plus soybean mix (mean values on dry matter basis, crude protein = 146 g per kg, crude fibre = 41.9 g per kg, acid hydrolysable oil = 39 g per kg, ash = 58.6 g per kg) per bull daily. Bulls had free access to water in their individual pens.

Experimental procedures

Forty-eight bulls were transported over a 3 day period from the following treatments; T treatment from location 1 were transported on day 97 [bulls from 1.2 (n = 8), 2.7 (n = 4), 4.2 (n = 4) m^2 space allowance], 98 [bulls from 1.2 (n = 4), 2.7 (n = 8), 4.2 (n = 4) m^2 space allowance] and 99 [bulls from 1.2 (n = 4), 2.7 (n = 4), 4.2 (n = 8) m^2 space allowance]. Each day, bulls were brought from their home pens to the crush holding facility, (20 m apart), weighed, loaded in the transporter and assigned to four pens (n = 4 per pen) in the transporter. Bulls were loaded in the transporter at 2100 GMT each day and transported by road for 12-h with 45 min where the transporter was stopped to facilitate driver's rest, in compliance with

National driving laws. After completion of the journey at 0900 GMT the next day, bulls were blood sampled in the transporter, unloaded, taken to the crush holding facility, blood sampled, weighed and brought to their home pens and blood sampled. Bulls had free access to water and feed in their home pens. On day 100 the C animals in location 2 [1.2 (n = 8), 2.7 (n = 8), 4.2 (n = 8) m^2 space allowances] were brought to the cattle holding facility, blood sampled, weighed, kept for 1 h and brought back to their home pens and blood sampled.

Transport vehicle, environmental conditions, health and production

The study was conducted in May. On the evening of the journey, the animals were loaded into 4 fan-ventilated pens on a transporter (total floor area of 30.96m^2) at a stocking density of 1.9 m^2 per animal, and transported by road on an air suspension articulated transporter. The transport route encompassed all types of road but the majority of the time was spent on national highways with average traffic mainly between Grange Research Centre, County Meath and County Cork. During the transport, the transporter was driven at an average speed of 70 (range 60 to 80) km per h. The transporter was fitted with sensors for measuring ambient temperature (^oC), relative humidity (RH; %), carbon dioxide (CO_2; ppm), air velocity (m per s) and vapour density (g per m^3) continuously during transport. The sensors were switched on and off at the start and end of the journey, respectively. The data was recorded every 10 min interval using Tiny Talk data loggers (Radionics, Dublin, Ireland) for temperature and RH and using Qrae, logging systems CO_2, air velocity and vapour density. The mean ambient temperature, RH, CO_2, air velocity and vapour density during the journey in the truck was 13.23^{o}C (range: 9.8 to 18.5^{o}C), 90.31% (range: 68.2 to 99.9%), 0.05 ppm (range: 0.03 to 0.11 ppm), 0.85 m per s (range: 0 to 3.96 m per s) and 11.75 g per m^3 (range: 5.9 to 18.0 g per m^3), respectively. There were water troughs in the truck. The mean daily air temperature and relative humidity in the housing facility of two locations were recorded. The mean daily air temperature [location 1: 11.5°C (range: 7.9 to 14.9°C); location 2: 11.7°C (range: 8.3 to 14.3°C)] and relative humidity [location 1: 80.0% (range: 57.9 to 90.8%); location 2: 77. 9% (range 57.4 to 86.0)] in the two housing locations were not different (P>0.05). The general health status of the bulls was recorded on a daily basis and animals were assessed for injury and body lesions.

Catheterisation

To facilitate intensive blood collection, bulls were fitted aseptically with indwelling jugular catheters on day 97, 98 and 99 (i.e. every morning before transport). The procedure was performed according to the method of Ting et al. (2003), using 12 gauge Anes spinal needles (Popper and Sons, Inc., New Hyde Park, NY, USA) and polyvinyl tubing (approximately 1.47 mm internal diameter; Ico Rally Corp., Palo Alto, CA; USA, catalogue No. SVL 105-18 CLR) attached to a blunt 18-gauge needle at the blood collection end. On day 97, sixteen bulls from 1.2 (n = 8), 2.7 (n = 4) and 4.2 (n = 4) m^2 space allowance were allocated to T treatment and brought to the crush holding facility from their home pens, catheterised from 0600 to 0700 GMT. Similar methods were repeated on day 98 ([1.2 (n = 4), 2.7 (n = 8), 4.2 (n = 4) m^2 space allowances] and 99 [1.2 (n = 4), 2.7 (n = 4), 4.2 (n = 8) m^2 space allowances] bulls assigned to T treatment. On day 99 C treatment bulls [1.2 (n = 8), 2.7 (n = 8), 4.2 (n = 8) m^2 space allowances] were catheterised from 0600 to 0730 GMT. All catheters were exteriorised on the neck of the animals, filled with sterile 3.5% sodium citrate solution, and plugged with a stopper. Catheters were secured in place in resealable patches with the aid of an adhesive cement (Big Bull Hip Tag Cement; Biguel Supply Co., Elysian, MN, USA), Velcro, and zinc oxide wrapping bandages. After catheterisation, animals were returned to their home pens and catheters were maintained patent for 32 h by flushing with 1

mL of 3.5% sodium citrate after each blood collection and with 2 mL of 3.5% sodium citrate before transportation.

Physiological measurements

Blood samples were collected from T and C treatments. Bulls in the T treatment were blood sampled before transport at 1900 and at 1915 in their home pens (two samples at 15 min interval each in the home pen; home pen1), 1945 (one sample in the crush holding facility; crush1), 2030 (after loading in the transporter; load), after 12-h of transport at 0830 (before unloading in the transporter; unload), 0900 (after unloading in the crush holding facility; crush2), 1000, 1024, 1048 GMT in the home pens (home pen2) for subsequent cortisol (lithium heparin anticoagulant) assay. Heparinised blood samples for stimulated lymphocyte production of IFN-γ, and blood samples containing EDTA as anticoagulant for routine haematology and white blood cell (WBC) differential populations, were collected at 1900 in the home pen1, at 0900 in the crush2, after 24 and 168 h of transport at 0900 GMT in the home pen2 from the T treatment. In parallel, blood samples were collected from C bulls on day 99 of the study, at 1900, 1915, 1945 and 2030 in the home pen, 0800, 0900 (in the crush holding facility), and at 1000, 1024, 1048 GMT in the home pen for cortisol in the heparin containing anticoagulants tubes. Blood samples for stimulated lymphocyte production of IFN-γ, routine haematology and white blood cell (WBC) differential populations were collected from C bulls on day 99 at 1900 GMT, after 14 h, 24 h and 168 h relative to the time of first blood sampling in the home pens. The induced release of cortisol following stimulation of the adrenal glands to a standardised dose of ACTH (1.98 IU per kg metabolic body weight; Friend et al., 1977 and Fisher et al., 1997a) was tested in two bulls from each pen following each day of transport at 1048 GMT in T (n = 8 per space allowance) and on day 100 in C (n = 4 per space allowance), which were randomly chosen at the start of the transport treatment experiment. Normal saline (5 mL, 0.89% sterile) was administered at 1048 GMT to two bulls from both T (n = 8 per space allowance) and C (n = 4 per space allowance) treatment pens. Immediately following the administration of ACTH (Synacthen ampoules, Novartis Pharmaceutical Ltd., UK) and normal saline catheters were flushed with 2 mL of 3.5% sterile sodium citrate solution. Blood samples were collected in tubes containing lithium heparin from bulls at 1111, 1133, 1155, 1217, 1247, 1317, 1347, 1417, 1447, 1517 and 1547 GMT (in the home pens) for subsequently cortisol assay. All blood samples were separated by centrifugation at 1,600 x g at 4°C for 15 min and plasma was stored at -20°C for cortisol assay. Heparinised whole blood samples collected and cultured for *in vitro* mitogen stimulated lymphocyte production of IFN-γ were separated by centrifugation at 300 x *g* at 8^{o}C for 15 min, and subsequently stored at –20^{o}C until assayed. The rectal and surface body (neck, rump and tail area) temperatures were monitored before and after transport from the T and C bulls using a digital electronic thermometer (Jorgen Kruse A/S; Model VT-801BWC Lot No 0701) and LASER device (Raynger MX4, Infrared laser thermometer, Raytek, Radir Ltd, Douglas House, Simson Road, Bletchley, Milton Keynes, UK), respectively.

Assay procedures

Commercially available RIA kits were used to determine the plasma levels of cortisol (Corti-cote, ICN Pharmaceuticals, Orangeburg, NY, USA) as described by Fisher et al. (1997a) within 4 week after the collection. The intraassay CV (n = 6) for samples containing 5.3, 17.87 and 56.71 ng of cortisol per mL were 16.34, 9.54 and 9.69%, respectively, and the interassay CV (n = 15) for the same samples were 15.83, 11.90 and 13.79%. *In vitro* lymphocyte IFN-γ production was determined in whole blood cultures following the procedures described in Ting et al. (2003), except that the cultures were stimulated with either

phosphate buffer saline alone, Con A (20 μg in 1.5 mL blood) or PHA (20 μg in 1.5 mL blood) for 24 h at 37^{o}C and in an atmosphere of 5% CO_2. In brief, duplicate 1.48 mL aliquots of blood were cultured in 24-well culture plates (Costar Corporation, Cambridge, MA) with 20 μL of phosphate buffer saline containing either 1.0 mg per mL of Con A, 1.0 mg per mL of PHA or no additive incubated for 16 h at 37ºC and an atmosphere of 5% CO_2 in air. The culture plates were then centrifuged at 1,600 x g at -4ºC for 20 min; the supernatant was harvested and frozen at –20ºC until it was assayed for IFN-γ production, using an ELISA procedure (Rothel et. al., 1990; CSL Biosciences, Parkville, Victoria, Australia). The *in vitro* Con A and PHA stimulated IFN-γ production was calculated by subtracting the absorbance at 450 nm of wells that received PBS alone from the absorbance of wells that received Con A and PHA, respectively. Red blood cell (RBC), Packed cell volume (PCV), haemoglobin (Hb) concentrations and platelet counts were determined for unclotted (EDTA) whole blood samples using an automated electronic particle analyser (Celltac, MEK-6108K, Nihon-Kohdon, Tokyo, Japan) within 1 h of blood sampling. Thin blood smears on grease free glass slides (Gold star micro slides, Chance Propper Ltd, UK) were prepared for WBC differential population counts. The smears were air-dried and stained using the haematology three step stains for differentiation of morphological cell types (Accralab, Fisher Scientific Company, L.L.C., 8365 Valley Pike, Middleton, VA 22645-0307, USA). One hundred cells, including neutrophils, band neutrophils, basophils, eosinophils, monocytes and lymphocytes were counted under the microscope at 40X.

Statistical analyses

Statistical analyses were performed using the general linear model (GLM) procedure of the Statistical Analysis System version 8.2 (SAS Inst. Inc. Cary, NC, USA). The data were analysed initially for the possible effects of transporting bulls across three days and their interactions with space allowances (model included effect of days, space allowances and their interactions), and effect of locations using PROC GLM. There was no effect (P>0.05) of days and days x space allowances among the T treatment bulls. The difference in the ambient temperature and relative humidity were tested for the location effect. Although the shed height was greater in location 2, no differences (P>0.05) were observed in both ambient temperature and relative humidity, therefore the location effect was excluded from the data. Data that failed the tests for normality and/or homogeneity of variance (Shapiro-Wilk test, P <0.005; using procedure UNIVARIATE) were either subjected to suitable transformation or analysed non-parametrically with either Wilcoxon or Kruskal-Wallis procedures based on the rank transformation where appropriate. The percentages (%) were transformed angularly {(x =180/π) x arcsin [√(p/100)], where p is a percentage [0 <p<100; π = 3.1416]}. For cortisol analysis the values before (1900 and 1915 GMT; basal) and after (1000, 1024, 1048 GMT; home pen2) transport treatment were averaged, and the medians were compared following ranks. The data for the neutrophil %, lymphocyte %, band %, platelets, RBC, PCV, haemoglobin, IFN-γ production for both Con A and PHA induced, rectal, shoulder, belly and rump temperatures were analysed by two-way ANOVA. The model included the effect of treatment, space allowance and their interactions in the home pen1, before transport treatment, crush1, load, unload, crush2 and in home pen2, post transport treatment depending upon the samples collected for the required variables. For the body weight data pre-transport treatment values at the home pen1 were subtracted from the post-transport and after 1 week of transport treatment and similarly analysed. Where appropriate the pre transport treatment values were included as a covariate. Following significant F test for the treatment, space allowance and their interactions, means were compared using the probability of differences (PDIFF) procedure of SAS on the least square means and was applied to determine statistical differences. The data relating to the ACTH/saline challenge for the cortisol analyses, for each

bull, area under the cortisol versus time curve (integrated response) was calculated from the time of the ACTH and/or saline administration until the final sample of the day using a linear trapezoidal rule (Veissier et al., 2001). Three cortisol concentrations in the home pen2 at the interval of 22 min each pre ACTH challenge were recorded and included as significant covariates. In addition to AUC, for each bull, the peak cortisol concentrations and time to reach peak cortisol concentrations post ACTH/saline challenge were identified, data were analysed by ANOVA using GLM, the statistical differences were determined using PDIFF procedure of SAS on the least square means. Data from four (n = 2, 1.2 m^2; n = 1, 2.7 m^2; n = 1, 4.2 m^2 space allowance) animals were excluded from the post ACTH challenge analyses due to loss of catheters during intensive blood sampling.

3.3 Results

Plasma cortisol

The basal median (plasma cortisol concentration at 1900 and 1915 GMT in the home pen1) plasma cortisol concentrations in the C and T bulls were less than 3.72 ng/mL. The data for the cortisol concentrations are presented in the Table 6. There was no difference (P>0.05) in treatment, space allowance and treatment x space allowance for the basal median plasma cortisol concentrations. At Crush1, bulls assigned to the T treatment had greater (P=0.0001) median plasma cortisol concentrations than C bulls. There was a treatment x space allowance effect (P=0.0019) at loading in the median plasma cortisol concentrations. The median plasma cortisol concentrations were greater (P=0.005) in the bulls housed at 4.2 than those at 1.2 m^2 space allowance of the T treatment at loading in the transporter. At loading, the median plasma cortisol concentrations were greater (P=0.015) in the bulls housed at 1.2 than those at 4.2 m^2 space allowance of the C treatment. The loading of the T bulls into the transporter previously housed at 1.2, 2.7 and 4.2 m^2 space allowance increased (for 1.2 m^2, P=0.007; 2.7 m^2, P=0.001 and for 4.2 m^2, P=0.001, respectively) median plasma cortisol concentrations compared with C bulls housed in the corresponding space allowances. Following 12-h of transport, at unloading there was a treatment (P<0.05) and space allowance (P<0.05) effect. This was indicated by the greater (P=0.0006) median plasma cortisol concentrations in the T than the C bulls at unloading. The bulls housed at 4.2 m^2 had greater (P=0.0026) median plasma cortisol concentrations than the bulls housed at 1.2 and 2.7 m^2 space allowance. Following unloading after the 12-h road journey, there was a tendency (P=0.076) for greater median plasma cortisol concentrations in the bulls housed at 4.2 m^2 than those at 1.2 and 2.7 m^2 space allowance. Following the 12-h road journey, the bulls in the home pens housed at 1.2 m^2 space allowance had greater (P=0.053) median plasma cortisol concentrations (mean of plasma cortisol concentrations at 1000, 1024 and 1048 GMT) than the bulls housed at 2.7 and 4.2 m^2 space allowances.

ACTH challenge

The adrenal glands of the T bulls responded acutely and showed an increased integrated cortisol response to the exogenous administration of ACTH than the C bulls housed at 1.2 (P=0.002), 2.7 (P=0.044) and 4.2 (P=0.026) m^2 space allowance, respectively (Table 7). Following saline administration, no differences (P>0.05) were observed in the T versus C bulls housed at 1.2, 2.7 and 4.2 m^2 space allowance, respectively. Bulls in the C treatment, housed at 2.7 m^2 space allowance and administered exogenous ACTH had greater (P=0.045) integrated cortisol response than the bulls housed at 1.2 m^2 space allowance. Integrated cortisol response in the T bulls administered with saline and housed at 1.2 m^2 was greater

(P=0.046) than those at 4.2 m^2 space allowance. The integrated cortisol response was not different (T, P=0 974; C, P=0.797) in the bulls housed at 1.2, 2.7 and 4.2 m^2 space allowance for the T and C treatment, administered with ACTH and saline, respectively. The peak cortisol concentrations following the administration of ACTH in the bulls housed at 1.2 (P=0.007) and 4.2 (P=0.047) m^2 space allowance were greater in the T than the C bulls. The time to reach peak cortisol concentrations following administration of ACTH was greater (P<0.05) in the T than the C bulls housed at 1.2 m^2 space allowance. There was no difference (P>0.05) in the time to reach peak cortisol concentrations in the T compared with the C treatment bulls housed at 2.7 and 4.2 m^2 space allowance. There was a clear response (T, P=0.001; C, P=0.001) to exogenously administered ACTH when compared with the saline for the integrated mean plasma cortisol concentrations within the T and C treatments.

Stimulated lymphocyte production of interferon-γ

There was no (P>0.05) effect of treatment, space allowance and treatment x space allowance before transport in the home pens, at 24 h and 168 h following transport on *in vitro* IFN-γ production (Table 8). Following transport, there was a treatment and space allowance main effect, indicated by the suppression (P=0.001) of the mean Con A induced IFN-γ production in the T compared with C bulls and suppression (P=0.049) of the mean Con A induced IFN-γ production in the bulls housed at 4.2 compared with those at 1.2 and 2.7 m^2 space allowance. Mean PHA induced IFN-γ production tended to be lower (P=0.067) in the bulls housed at 4.2 than those at 1.2 and 2.7 m^2 space allowance following transport when animals were blood sampled in the crush holding facility.

White blood cell differential counts

There was a greater (P=0.001) neutrophil % in the C compared with the T bulls in the home pens pre-transport. Following transport, the neutrophil % was greater (P<0.05) in the T compared with the C treatment in the crush holding facility and at 24 h after transport (Table 9). Bulls in T treatment housed at 2.7 and 4.2 m^2 space allowance tended (P=0.089) to have greater neutrophil % than the C treatment in the crush holding facility following transport. There was a main treatment effect on the lymphocyte %, described by the greater (P=0.018) lymphocyte % before transport treatment in the T than in the C bulls. The lymphocyte % was not affected (P>0.05) by transport, however it tended (P=0.078) to be greater in the bulls previously housed at 2.7 than those at 4.2 m^2 space allowance when sampled in the crush holding facility (crush2). Twenty-four h following transport, the lymphocyte % was greater (P=0.002) in the C compared with the T bulls. There was no (P>0.05) treatment, space allowance and treatment x space allowance effect at 168 h. The eosinophil % was greater (P<0.05) in the T than C bulls in the home pens prior to transport, in the crush holding facility following transport, and at 24 h following transport. No effects (P>0.05) of the treatment, space allowance and treatment x space allowance were observed for the basophile and monocyte %. The band cells %, which are an immature form of neutrophils, were greater (P<0.05) in the T bulls housed at 4.2 m^2 space allowance than C bulls, when sampled in the crush holding facility (crush2).

Blood cell counts

The data for the blood cell counts are presented in the Table 10. The main space allowance effect was indicated by greater (P<0.05) RBC numbers in the bulls housed at 1.2 and 2.7 than those at 4.2 m^2 space allowance before transport in the home pens, crush 2 and at 168 h after transport. However, at 24 h following transport, bulls housed at 1.2 m^2 had a

greater (P=0.012) RBC count than the bulls housed at 2.7 and 4.2 m^2 space allowance. PCV tended to be greater (P=0.067) in the T than in the C bulls in the crush holding facility following transport. The treatment x space allowance effect at 24 (P=0.0261) and 168 (tendency, P=0.0563) h post transport treatment was indicated by the greater PCV in the T than in the C bulls housed at 4.2 m^2 space allowance. The Hb concentration was affected by the main effect of treatment with greater (P=0.0474) Hb concentrations in the T than in the C bulls at crush 2. At 24 (P=0.053) and 168 (P=0.048) h post transport there was a treatment x space allowance effect. At 24 and 168 h, bulls in C housed at 1.2 m^2 space allowance had greater (P<0.05) Hb concentrations than bulls housed at 2.7 and 4.2 m^2 space allowance and was greater (24 h, P=0.021; 168 h, P=0.042) in the T than in the C bulls housed at 4.2 m^2 space allowance.

Body weight and general health

There was a greater (P=0.0001) loss of body weight following transport in the T than in the C bulls (Table 11). Overall, the mean bodyweight loss was 2.5% in the C and 6% in the T bulls, respectively. Following transport, there was a greater tendency (P=0.0852) of weight loss in the bulls housed at 2.7 than those at 1.2 m^2 space allowance. At 168 h post transport, there was a tendency towards increased weight gain (P=0.061) in T compared with the C bulls. One week following transport, the weight loss had recovered in the T bulls and was only 0.5% different from their initial weight, while, C bulls had a 2% difference in the body weight compared with initial body weight. No obvious signs of injury or illness were detected during the experiment therefore the general health status of the animals was good.

Temperature data

No (P>0.05) effect of treatment, space allowance, or treatment x space allowance was observed in the rectal, shoulder, belly and rump temperature data.

3.4 Discussion

In this study, the basal cortisol concentrations were within normal physiological ranges and were not affected by the long-term exposure of bulls to a restricted (1.2 m^2/bull) compared with generous (2.7 and 4.2 m^2/bull) space allowances. Reduced space allowance threatens the well being of cattle and may have deleterious effects on performance and health (Ingvartsen and Anderson, 1993). Armario et al. (1988) and Klemcke (1994) reported that following chronic intermittent stressors, associated changes occur in the functioning of the pituitary-adrenal axis, which are not apparent when considering basal hormonal concentrations. No differences occurred in basal concentrations or following saline administration in C bulls housed in the range of space allowances indicating that there was no effect of space allowance on basal plasma cortisol concentrations. The control bulls kept at 1.2 m^2 space allowance had reduced cortisol concentrations indicating chronic stress, and when exposed to an acute stress challenge (induced cortisol release following exogenous ACTH administration) showed altered homeostasis. Studies examining the effects of housing cattle at restricted space allowance (Beneke et al., 1984; Fisher et al., 1997a, b) and restricted movements in bulls (Ladewig and Smidt, 1989) have reported a reduced cortisol response to ACTH challenge and the findings are in agreement with the results of the present study. The lower levels of cortisol reported in finishing beef heifers (Fisher et al., 1997b) are due to sex

differences (Tennessen et al., 1984). Removing bulls from their home pens to a crush holding facility prior to loading on the transporter, increased cortisol concentrations and this increase was maintained during transport and when the animals were unloaded following transport. These findings are in agreement with Kent and Ewbank (1986) who reported similar findings following 6 to 18 h transport journeys in young (Kent and Ewbank, 1986; Knowles, 1999) and adult cattle (Tennessen et al., 1984; Warriss et al., 1995). However, Blecha et al. (1984) have demonstrated no change in plasma cortisol in controls and shipped steers. This difference could be attributed to the habituation to handling of the bulls in the present study. There is evidence in the literature that suggests chronically stressed rats (Mizoguchi et al., 2001) and depressed humans (Anisman, 2002), have differentially regulated glucocorticoid negative feed back mechanisms in response to acute stress. It the current study it may be postulated that the coping mechanism that actively decreased the elevated cortisol concentrations to near basal cortisol levels in bulls housed at 2.7 and 4.2 m^2 may be due to enhanced negative feedback responses when bulls were returned to their home pens.

Exogenous ACTH increased adrenal responsiveness (i.e. greater cortisol release) in transported compared with control bulls housed at the range of space allowances, suggests a degree of activation of the adrenal gland. Thus transport stress may enhance the enzyme affinity of the cortisol secreting mechanism (Båge et al., 2000). The findings of the ACTH challenge in the transported bulls, suggest that the transportation stress was of sufficient potential to alter desensitisation induced by chronic low-grade housing stress to further sensitisation of the adrenal gland. The desensitisation of the pituitary-adrenal axis to one type of stressor does not extend to a different type of stressor, i.e. desensitisation of the pituitary-adrenal axis is stressor specific. The difference in the concentrations of induced cortisol to exogenous ACTH challenge when compared with saline within the same transport treatment was due to a drug effect and was as expected.

Earlier studies that used mitogen and/or antigen-stimulation *in vitro* assays for the production of cytokines (e.g. IFN-γ), derived from lymphocyte proliferation, suggested that this was a useful indicator for studying stress in bovines (e.g. castration: Ting et al., 2003; Earley and Crowe, 2002). Both Con A (Earley and Crowe 2002) and PHA (Gupta et al., 2005), T-cell antigen and mitogen, respectively, have been used as a measure of immune responsiveness in previous studies. In the current study, housing cattle for 96 days at 1.2, 2.7 and 4.2 m^2 space allowances has not affected the IFN-γ production following stimulation by both Con A and PHA. These findings are consistent with those of Fisher et al. (1997b) with finishing beef heifers but not in agreement with Hickey et al. (2003a) with steers who reported an attenuation of the immune response at a space allowance of $\leq$2.0 m^2 per steer. The relationship between stress-induced activation of the HPA axis and alteration of immune functions is well established (Anisman, 2002), however, in some circumstances e. g. exogenous cortisol administration within physiological ranges does not suppress immune function (Fisher et al., 1997c; Ting et al., 2004). Studies in various stress models in cattle (castration, Ting et al., 2003; regrouping and relocation in steers, Gupta et al., 2005) have shown the suppression of IFN-γ production following peak cortisol response after the stressor. The probable explanation of no change in IFN-γ in this study is due to the lack of apparent changes in the cortisol concentrations in bulls housed in the restricted to generous space allowances for 96 days or chronic low-grade housing stress induced by the restricted space allowance may have further modified the immune response to overcome any effects. Recently, Engler et al. (2004) has shown that rats exposed repeatedly to a stressor causes substantial mobilisation of leukocytes from the bone marrow and subsequently affects redistribution of these cells via circulation into the spleen. The increased cortisol concentration following the acute stress stimuli of transport, suppressed the *ex vivo* immune

functions, and this effect was more pronounced in bulls housed at the more generous than the restricted space allowances. The suppression of lymphocyte proliferation responses following shipping of calves (Blecha et al., 1984; Murata et al., 1987) and steers (Tarrant et al., 1992) for greater than 4 h transport duration have been previously reported.

Blood cell constituents maintain a physiological balance between the environmental conditions and the animal's body by restoring normal homeostasis. The changes in the composition of the blood cells reflect the physiological or patho-physiological response of the animal to stress (Radostits et al., 1994). The increased neutrophil % in the C and increased lymphocyte, eosinophil % in the T bulls following housing bulls in the range of space allowances was not an expected result and are not in agreement with Hickey et al. (2003a). However the neutrophilia and lymphopenia following transportation observed in this study are in agreement with previously reported findings following a variety of stressors, including transportation stress (Blecha et al., 1984; Murata et al., 1987; Tarrant et al., 1992). But the eosinophilia observed was not in agreement with that of Blecha et al. (1984) and Tarrant et al. (1992). Eosinophilia is commonly associated with antigen-antibody reactions and has the function of detoxification by inactivating histamines or histamine-like toxic materials (Radostits et al., 1994). Eosinophilia observed in this study may be the response to the change in the physical environment that triggered histamine release. The neutrophilia (including the increased band cell) and lymphopenia in bulls housed at the generous space allowances following transport is evidence that 2.7 and 4.2 m^2 space allowances were not of sufficient potential to pre-condition the immune system for further exposure to stress but had immune suppression consequences. Neutrophilia, lymphopenia and eosinophilia persisted until 24 h post transport, however, at 168 h post transport the effect was diminished, indicating that short-term responses for changes in the differential populations. Blecha et al. (1984) also reported breed specific apparent lack of neutrophilia and lymphopenia after 1 week of shipping. Neutrophilia and lymphopenia is a common finding in stressed animals and is associated with changes in WBC trafficking and release from the bone marrow by elevated concentrations of glucocorticoids (Dunn, 1989). In the present study, the elevated concentrations of cortisol may be correlated with altered WBC differential populations. Therefore this data concludes that bulls have reacted to transportation stress with short-term immune system changes.

Despite the availability of water in the housing facilities before transport, in the transporter during transport and in the home pens after transport, the increases in the RBC, PCV and Hb at all time points after treatment, suggests the involvement of factors other than dehydration of bulls. Tarrant et al. (1992) reported increased RBC, PCV and Hb following transportation of steers while Lambooy and Hulsegge (1988) reported increased haematocrit in pregnant heifers. By contrast Blecha et al. (1984) reported no changes in the PCV in shipped calves. The bulls housed in the restricted space allowance seem to be affected more than the bulls housed in generous space allowances. The elevated PCV and Hb continued until 168 h post transport in the bulls housed at 4.2 m^2 space allowance seems to be of no consequence and probably not related to the treatment effect.

A review of road transport of cattle concluded that approximately 3 to 11% of body weight loss occurs with greater losses associated with increased duration of the journey (Knowles, 1999). The 6% decrease in the body weight in this study could be due to reduced gut fill. Simply moving and blood sampling control animals produced a 2% weight loss. The reason for this weight loss in C bulls is not known as there was no change in the quality and quantity of the feed offered and there were no recorded incidences of the disease among the

control bulls. The rectal, shoulder, belly and rump temperatures of bulls before and after transportation were within the normal clinical ranges for beef cattle (Radostitis et al., 1994).

In conclusion, housing bulls for 96 days in a range of space allowances did not affect basal cortisol response and immune function. ACTH administration may be a beneficial indicator for assessing the effects of chronic stress during the housing of cattle. ACTH challenge failed to differentiate between the increased adrenal response of cattle undergoing chronic stress and if subsequently exposed to the acute stress of transport. The physiological data indicate that loading bulls on a transporter, transporting for 12-h and subsequently unloading reduced body weight, suppressed Con A and PHA induced IFN-γ production, and produced neutrophilia, eosinophilia and lymphopenia. Transportation also affected the normal body homeostasis by increasing the packed cell volume, red blood cell and haemoglobin levels. While transport increased cortisol and reduced immune response in the short-term, the changes were within normal physiological ranges suggesting that 12-h road transport had no adverse effect on welfare status over a long-term period

Table 6: Effect of transporting bulls previously housed at various space allowances on plasma cortisol. The concentrations (ng per mL) are presented as median, minimum (min) and maximum (max) for plasma cortisol

		Control			Transport			P[a]		
Space allowance per bull		1.2 m^2	2.7 m^2	4.2 m^2	1.2 m^2	2.7 m^2	4.2 m^2	Treat[b]	Space[c]	Treat x space
Home pen1[d]	Median	2.18	3.29	3.60	2.89	2.10	3.72	NS	NS	NS
	min-max	(0.60 - 6.05)	(0.60-8.81)	(0.47-7.86)	(0.60-26.51)	(0.48-11.44)	(0.97-18.96)			
Crush1[e]	Median	1.55	1.18	1.13	5.12	4.67	5.85	0.0001	NS	NS
	min-max	(1.15-2.76)	(0.60-2.99)	(0.60-1.48)	(0.60-17.50)	(0.60-19.04)	(2.57-19.71)			
Load	Median	1.95	0.96	0.60	3.55	5.19	8.64	0.0001	NS	0.0019
	min-max	(0.59-3.29)	(0.60-2.37)	(0.60-1.12)	(1.30-17.99)	(1.49-31.03)	(2.80-17.74)			
Unload	Median	3.21	2.24	8.37	9.03	8.52	11.31	0.0006	0.0026	NS
	min-max	(1.74-12.19)	(0.73-5.58)	(4.01-16.62)	(1.62-82.62)	(1.19-34.27)	(2.21-77.82)			
Crush2[e]	Median	2.71	5.18	11.21	7.01	6.18	8.41	NS	0.0755	NS
	min-max	(1.49-10.12)	(1.23-21.95)	(3.46-20.18)	(0.65-68.77)	(0.52-21.05)	(1.59-68.92)			
Home pen2[f]	Median	2.92	1.50	1.91	1.61	1.40	2.48	NS	0.0532	NS
	min-max	(1.50-5.70)	(0.95-3.21)	(0.49-3.26)	(0.60-10.42)	(0.47-6.19)	(1.08-8.88)			

[a]Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[b]Treat = treatment; C-control; T-transport.
[c]Space = 1.2, 2.7, 4.2 m^2 individual space allowance.
[d]Mean of cortisol concentrations at 1900 and 1915 GMT in the home pens before transport.
[e]Crush holding facility before and after transport. For Crush 1, controls were not in crush, but still in their home pens.
[f]Mean of the cortisol concentrations at 1000, 1024 and 1048 GMT in the home pens after transport.

Table 7: Effect of transporting bulls previously housed at various space allowances on plasma cortisol following administration of ACTH (1.98 i. u. ACTH per kg metabolic body weight)

Variables[a]	Treatment	Space allowance per bull				
		1.2 m^2	2.7 m^2	4.2 m^2	Pooled s.e.	P^d
Post-ACTH cortisol AUC, ng.mL^{-1}.min	Control (n = 12)	5816^{b*}	9352^{c*}	7607^{bc*}	1246.2	0.05
	Transport (n = 20)	12423*	12642*	12301*	788.4	NS
Post-saline cortisol AUC, ng.mL^{-1}.min	Control (n = 12)	920	820	833	109.1	NS
	Transport (n = 24)	989^{b}	816^{bc}	593^{c}	106.6	0.041
Post-ACTH peak[e] cortisol, ng per mL	Control (n = 12)	36.3*	45.1	42.8*	3.24	NS
	Transport (n = 20)	64.5*	59.1	66.7*	4.87	NS
Post-ACTH interval[f] to peak cortisol, min	Control (n = 12)	93*	128	141	9.7	NS
	Transport (n = 20)	130*	120	113	9.1	NS

[a]Cortisol concentrations at 1000,1024,1048 GMT in the home pens were averaged and included as a significant (P<0.05) covariate. ACTH/saline was administered to the bulls in the home pens following 12-h road journey. The least square means are presented for the post ACTH and saline administration area under the cortisol versus time curve (AUC), post-ACTH peak cortisol and interval to peak cortisol

[bc]Means with different superscripts within variable for each treatment differ, $P \leq 0.05$.

[*]Means within variable between treatments differ, $P \leq 0.05$.

[d]Non-significance, defined as $P \geq 0.05$, is denoted as NS.

[e]Peak cortisol is presented for the bulls administered with ACTH only.

[f]Peak interval was time taken to reach peak values following ACTH administration.

Table 8: The effect of transporting bulls previously housed at various space allowances on mean concanavalin A (Con A) and phytohaemagglutin (PHA) induced *in vitro* interferon-γ production (optical density @450nm) from cultured whole blood

	Control			Transport			Pooled	*P*[a]		
Space allowance per bull	1.2 m^2	2.7 m^2	4.2 m^2	1.2 m^2	2.7 m^2	4.2 m^2	s.e.	Treat[b]	Space[c]	Treat x space
Con A										
Pre-transport, home pen	0.87	0.72	0.46	0.65	0.86	0.65	0.140	NS	NS	NS
Crush2[1]	0.48	0.67	0.48	0.32	0.39	0.10	0.092	0.001	0.049	NS
24 h post-transport	0.78	1.23	0.67	0.79	0.67	0.66	0.200	NS	NS	NS
168 h post-transport	0.57	0.54	0.47	0.72	0.77	0.70	0.155	NS	NS	NS
PHA										
Pre-transport, home pen	1.68	1.33	1.18	1.54	1.76	1.49	0.201	NS	NS	NS
Crush2[1]	1.34	1.52	1.19	1.12	1.42	0.81	0.185	NS	0.067	NS
24 h post-transport	1.30	1.62	1.18	1.22	1.32	1.09	0.246	NS	NS	NS
168 h post-transport	0.74	0.68	1.29	1.35	1.46	1.51	0.216	NS	NS	NS

[a]Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[b]Treat = treatment; C - control; T- transport.
[c]Space = 1.2, 2.7, 4.2 m^2 space allowance.
[1]Crush holding facility after transport.

Table 9. The effect[a] of transporting bulls previously housed at various space allowances on differential white blood cell counts

Space allowance per bull		Control			Treatment			Pooled s. e.	P[b]		
		1.2 m^2	2.7 m^2	4.2 m^2	1.2 m^2	2.7 m^2	4.2 m^2		Treat[c]	Space[d]	Treat x space
Neutrophils, %	Pre-transport, home pen	40.3	41.8	40.8	36.0	36.3	34.5	1.04	0.0001	NS	NS
	Crush 2[1]	39.9	38.9	33.4	45.2	46.9	49.3	1.65	0.0001	NS	0.0896
	24 h post-transport	39.6	39.7	39.1	47.9	39.8	44.5	1.26	0.004	NS	NS
	168 h post transport	42.7	40.1	40.5	40.1	40.2	41.0	1.00	NS	NS	NS
Lymphocytes, %	Pre-transport, home pen	43.0	44.1	48.4	47.1	47.1	44.6	1.20	0.0189	NS	NS
	Crush 2[1]	41.6	47.6	36.9	41.4	40.8	39.4	1.69	NS	0.0783	NS
	24 h post-transport	44.4	45.4	39.2	35.3	40.4	45.6	1.40	0.0002	NS	NS
	168 h post transport	39.9	43.8	43.5	44.1	44.2	39.9	1.03	NS	NS	NS
Eosinophils, %	Pre-transport, home pen	8.3	6.8	9.9	10.5	8.8	6.8	0.83	0.0203	NS	NS
	Crush 2[1]	8.0	5.8	3.9	5.3	5.3	7.9	1.03	0.0312	NS	NS
	24 h post-transport	3.4	3.5	8.4	10.1	8.8	7.1	0.88	0.0001	NS	NS
	168 h post transport	6.8	5.6	7.6	8.8	7.8	7.5	0.64	NS	NS	NS
Basophils, %	Pre-transport, home pen	4.4	4.6	8.0	8.0	7.7	3.5	0.70	NS	NS	NS
	Crush 2[1]	6.5	4.0	4.2	6.7	4.8	6.2	0.80	NS	NS	NS
	24 h post-transport	3.2	4.6	4.8	4.5	6.4	4.9	0.75	NS	NS	NS
	168 h post transport	4.5	4.2	5.5	6.2	6.1	4.6	0.45	NS	NS	NS
Monocytes, %	Pre-transport, home pen	10.1	7.8	8.8	9.4	10.1	7.7	0.76	NS	NS	NS
	Crush 2[1]	8.4	7.8	6.7	8.0	4.3	6.6	0.98	NS	NS	NS
	24 h post-transport	8.8	10.6	8.5	9.1	8.0	10.2	0.88	NS	NS	NS
	168 h post transport	9.3	9.0	8.2	9.9	7.7	8.4	0.54	NS	NS	NS
Band, %	Pre-transport, home pen	7.4	6.4	9.8	9.6	9.0	8.7	1.10	NS	NS	NS
	Crush 2[1]	2.1	0.6	2.2	6.9	2.0	20.9	1.96	0.0013	0.0043	0.0135
	24 h post-transport	11.2	12.9	8.0	5.5	5.8	7.0	1.81	NS	NS	NS
	168 h post transport	8.6	7.3	12.9	5.7	4.2	5.8	0.89	NS	NS	NS

[a]Data presented are angular transformed for individual percentages. [b]Non-significance, defined as $P \geq 0.05$, is denoted as NS. [c]Treat = treatment; C - control; T- transport. [d]Space = 1.2, 2.7, 4.2 m^2 space allowance. [1]Crush holding facility after transport.

Table 10. The effect of transporting bulls previously housed at various space allowances on haematological variables

		Control			Treatment			Pooled s.e.	P^a		
Space allowance per bull		1.2 m^2	2.7 m^2	4.2 m^2	1.2 m^2	2.7 m^2	4.2 m^2		Treat[b]	Space[c]	Treat x space
Red blood cell, x 10^{12}/L	Pre-transport, home pen	8.1	7.5	6.9	7.7	7.5	7.2	0.15	NS	0.0019	NS
	Crush 2[1]	8.4	7.9	7.5	8.2	8.1	7.8	0.17	NS	0.0358	NS
	24 h post-transport	8.2	7.2	6.8	7.9	7.6	7.3	0.14	NS	0.0001	NS
	168 h post transport	8.5	8.0	7.4	8.0	8.2	7.8	0.15	NS	0.0125	NS
Packed cell volume, %	Pre-transport, home pen	27.8	26.5	25.3	27.0	27.2	27.2	0.50	NS	NS	NS
	Crush 2[1]	28.7	28.0	27.5	29.1	29.1	29.3	0.49	0.0698	NS	NS
	24 h post-transport	28.6	25.5	24.8	27.2	27.0	26.9	0.49	NS	0.0076	0.0261
	168 h post transport	29.4	29.0	27.2	28.5	29.9	29.8	0.5	NS	NS	0.0563
Haemoglobin, x g/dL	Pre-transport, home pen	10.2	9.9	9.3	10.2	10.2	10.1	0.17	NS	NS	NS
	Crush 2[1]	10.7	10.5	10.4	10.9	11.0	11.0	0.18	0.0474	NS	NS
	24 h post-transport	10.8	9.7	9.4	10.3	10.2	10.2	0.17	NS	0.0076	0.0530
	168 h post transport	10.9	10.7	10.2	10.6	11.2	11.0	0.16	NS	NS	0.0476

[a]Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[b]Trans = treatment; C - control; T- transport.
[c]Space = 1.2, 2.7, 4.2 m^2 space allowance.
[1]Crush holding facility after transport.

Table 11. [a]The effect of transporting bulls previously housed at various space allowances on body weight (BW)

Space allowance per bull	Control			Transport			Pooled s.e.	P[b]		
	1.2 m^2	2.7 m^2	4.2 m^2	1.2 m^2	2.7 m^2	4.2 m^2		Treat[c]	Space[d]	Treat x space
Pre-transport	483	564	558	501	560	555	1.3	NS	0.0001	NS
Post-transport (Crush 2)	-12.63	-16.00	-11.75	-25.00	-29.75	-29.93	1.226	0.0001	0.0852	NS
Post-transport (168 h)	-11.38	-7.13	-7.25	-2.06	-5.19	-6.07	1.768	0.061	NS	NS

[a]Data are least square means and analysed by subtracting the values of post transport at crush holding facility (crush 2) and 168 h from the pre-transport values. [b]Non-significance, defined as $P \geq 0.05$, is denoted as NS.
[c]Trans = treatment; C - control; T- transport.
[d]Space = 1.2, 2.7, 4.2 m^2 space allowance.

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4. Effect of repeated regrouping and relocation on behaviour of steers

4.1 Introduction

Grouping, regrouping and relocation (R&R) of animals are common husbandry practices in the management of farm animals (Veissier et al., 2001; Sevi et al., 2001; Bøe and Færevik, 2003). Introducing one individual animal or an entire group of animals into an established herd or making a new herd affects the social relationship; not only for the introduced cattle or group but also for the whole herd (Keeling and Gonyou, 2001; Veissier et al., 2001; Bøe and Færevik, 2003). Negative effects of grouping on the production performance of farm animals have been reported (Sevi et al., 2001). Therefore, an understanding of social stress is increasingly necessary in farm animal husbandry as most animals are housed in groups rather than in individual stalls or pens.

Animals react to the abrupt breaking of social relationships with altered behaviour as a first biological response (Moberg and Mench, 2000) that may lead to stress. Altered behaviour is the major factor affecting production and performance, with further potential effects on animal health, welfare and farm profitability (Keeling and Gonyou, 2001; Bøe and Færevik, 2003). Increases in aggression, locomotion and social stress occurred following grouping of unfamiliar farm animals (Venediktova et al., 1974; Tennessen et al., 1985; Bøe and Færevik, 2003). It has been shown that the presence of unfamiliar conspecifics in the immediate environment of animals or mixing of unacquainted conspecifics elicits aggression and disruptive social behaviour in poultry (Siegel, 1976) and pigs (Ladewig, 2000). Veissier et al. (2001) has reported a clear modification in the behavioural patterns of calves immediately after regrouping. Hasegawa et al. (1997) showed that regrouping of cattle increased aggression and changed behavioural patterns and duration. Increased agonistic behaviour was reported in cows (Brakel and Leis, 1976) and sheep (Sevi et al., 2001) following regrouping. Regrouping of familiar animals also resulted in negative effects on production traits such as feed intake and growth rate (Krohn and Konggaard, 1980; Hasegawa et al., 1997). Given frequent ownership transfers as stock age and progress through the market and production systems, animals are frequently moved from familiar surroundings or cohorts into new lots or herds. An awareness of the potential social and performance effects of such transfers can potentially help owners alleviate negative aspects and possibly expand profit margins.

Most previous studies on regrouping and relocation focused on young calves and cows. We have peviously reported that steers responded to regrouping and re-penning with increased basal plasma cortisol, and changes in metabolic activity (Gupta et al., 2005). Repeated R&R reduced the sensitivity of the adrenal to ACTH with no effect on the pituitary following CRH challenge compared with controls. The aim of the present study was to investigate the effect of repeated regrouping and relocation on the range of behaviours and production performance of finishing beef steers.

4.2 Materials and Methods

All procedures in this study were conducted under an experimental licence 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) Regulation, 1994.

Treatments

Seventy-two Holstein x Friesian (14 month old; mean body weight (BW) = 441 ± 3.2 kg) steers were blocked by BW and randomly assigned to either control (n = 30; C) or regrouped (n = 42; R) treatments. Steers were housed in 12 pens with 6 steers in each pen at a space allowance of 2.8 m^2 per steer. The pens for both treatments were alternated within the shed.

Animal housing and management

Before assignment to treatment, steers were weighed and housed from day -35 (day of treatment = day 0) to adapt them to handling, and were restrained in the novel housing environment. Steers were housed for 84 days in a twelve-pen slatted-floor facility with two rows of six pens facing each other. The dimension of each pen was 4.5 x 3.8 m with a feed face of 4.5 m long. A steel mesh with dimensions of 3.8 x 1.6 m separated the sides of each pen. The pens were continuously illuminated throughout the experiment. The steers had *ad libitum* access to grass silage and supplemented with 2.5 kg of barley /soybean mix concentrate ration. The grass silage had a dry matter (DM) content (mean values) = 224 g/kg, *in vitro* DM digestibility = 887 g/kg of DM, pH 4.2, and the concentrate ration (mean values) was composed of crude fibre = 41.9 g/kg, crude protein = 155 g/kg, acid hydrolyzable oil = 39 g/kg, ash = 58.6 g/kg per animal daily. Steers had free access to water in their pens.

Regrouping and relocation

Regrouped steers were exposed to six R&R events from day 0 to 84. Following each R&R new pen cohorts (n = 6 steers per pen) were allowed to stabilise for 14 days (Table 12). In each R&R, none of the R steers were allowed to share the same pen or pen-mates, where or with whom, they were previously housed. Control steers were housed in the same pen with the same pen-mates for the duration of the experimental study. On the day of R&R, steers from the R treatment were individually taken out from their pens and were regrouped and taken to their new pens. Each R&R of R steers was staggered and was carried out between 0800 and 0830 GMT. Immediately after R&R, the housing facility was closed and steers with their new pen cohorts were allowed to interact for 2 h without human interference. During this time, steers had access to water only.

Behaviour measurements

Equipment. Twelve black and white cameras were used to record the behaviour of the steers in each pen. Each camera was fixed in front of the individual pen so as to give a clear view of the whole pen. The video cameras were connected to a video tape recorder via a multi-vision system (Robot, monochrome duplex multiplexer), which allowed pictures from all cameras to be viewed on one screen at a time. The pictures from all the cameras were marked with individual pen number and calibrated with time and date settings.

Observation. Each steer was marked on its back with an individual identification code. A neck collar was fitted on some of the animals for identification during behavioural observation to differentiate between steers with the same body colour. For each pen, behavioural observations commenced on days 0, 14, 28, 42, 56, and 70 to coincide with the six regrouping and relocations of steers (Table 1). Behaviour was continuously recorded for one week after the six regrouping and relocations. Steers were observed by instantaneous scan sampling. The interval between scans was 2 min for 2 h on day 1 immediately after regrouping and relocation, followed by every 20 min for the remainder of days 1 and 2. From day 3 to day 7 each steer was observed every 120 minutes. Each steer was observed for behavioural activity and body contact. In the behavioural activity category, steers were observed for the following: lying down: head supported by the neck, head not supported by the neck (chin on the floor, on the body or on another steer); standing: with or without moving; eating (head in the trough); drinking; head to head contact except while eating; head contact with the body of another steer; and mounting. In the contact category the criteria were: no body contact with other steers and contact with one, two or three steers.

Environmental conditions and body weight

The mean daily air temperature and relative humidity in the housing facility were continuously recorded using Tiny Talk data loggers (Radionics, Dublin, Ireland). The mean daily air temperature and relative humidity in the housing facility were 16.9ºC (min-max 5.8 to 25.9ºC) and 82.0% (min-max 38.4 to 100.0%) during the experimental period from April to June. The BW of steers was recorded on seven different occasions, on day -1 and days 13, 27, 41, 55, 69 and 83 to determine average daily gain (ADG; kg/day).

Statistical analyses

All statistical analyses were performed using SAS software Version 8.2 (SAS Institute Inc. Cary, NC, USA). The behavioural data was analysed within either the first 2 h period (i.e. scan sampling at 2 min) or during the period from 24 h post-mixing up to day 7 (scan sampling at 20 min or 120 min). A count of the total number of occurrences of each behaviour or contact was made for each scan time point. The expected number of occurrences of each behaviour or contact was then calculated. A chi-square statistic was used to determine the difference between the count total and the expected frequencies. For presentation purposes and for a more meaningful understanding of the data, percentage time values were calculated from the total count data for behaviours of considerable duration such as lying, standing etc. A probability of $P < 0.05$ was chosen as the level of significance for the statistical tests (Zar, 1999).

4.3 Results

Behaviour observations during each 2 h period after each regrouping and relocation

Behavioural activity

The data for the activity categories are presented in Table 13. During the first 2 h immediately after the first, second, third, fourth, fifth and sixth R&R, R steers were lying for a lower % of observation ($P = 0.001$) and were 'standing without moving' for a greater % of observation ($P<0.001$). Animals were standing for a greater % of time with movement for a lower % of observations ($P<0.001$) in C than in R steers after the first, fourth and sixth R&R, however there was no ($P > 0.05$) difference between R and C steers for standing with movement following the second, third and fifth R&R. Except at the fifth R&R, the % of time spent eating was greater ($P < 0.05$) in R than in C steers following the first, second, third, fourth and sixth R&R. Following the first, third and fourth R&R, drinking behaviour was greater ($P < 0.05$) in R than in C steers with no ($P > 0.05$) difference following the second, fifth and sixth R&R. No differences ($P > 0.05$) in head to head or head to body contact behaviours between R and C steers were observed following each R&R.

Contact behaviour

The occurrence of no body contact was greater ($P = 0.001$) in R than in C steers following the first, fourth, and sixth R&R (Table 13), while it was greater ($P = 0.001$) in C than in R steers following the third R&R and it was not different ($P > 0.05$) between treatments following the second and fifth R&R. There was a greater ($P < 0.05$) occurrence of 'in contact' with one other steer in C compared with R steers following the first, fourth and sixth R&R, however no differences ($P > 0.05$) were observed between R and C steers following the second, third and fifth R&R. Following the third and sixth R&R there was a greater ($P < 0.05$) occurrence of 'in contact' with two other steers in R than in C steers, while no differences ($P > 0.05$) were observed following the first, second, fourth

and fifth R&R. No difference (P > 0.05) was observed in the occurrence of 'in contact' with three steers following each R&R.

Behaviour observations during the period of scan sampling on days 1 to 7 after each R&R

Behavioural activity following the first regrouping and relocation (Table 14)
On day 1 following the first R&R, R steers spent a lower (P < 0.05) % of observations lying with or without the head supported. However the % of observations standing, (with or without moving) eating and drinking on day 1 following the first R&R was greater (P < 0.05) in R than in C steers. Head to head and head to body contacts were not different (P > 0.05) between C and R steers on day 1 following the first R&R. No differences (P > 0.05) between C and R treatments were observed for lying with the head supported and not supported, standing with or without moving, eating, drinking, head- to -head and head- to -body contacts from days 2 to 7 after the first R&R.

Contact behaviour following the first regrouping and relocation (Table 14)

There was a greater (P < 0.05) occurrence of 'no body contact' in R than in C steers, while the occurrence of 'in contact' with one steer was less (P < 0.05) in R than in C steers on day 1 after the first R&R. There were no differences (P > 0.05) for 'no body contact' or 'in contact' with one or three steers from day 2 to 5 and day 7 following the first R&R. On day 6 after the first R&R there was a greater (P < 0.05) occurrence of 'no body contact' in C than in R steers, while the occurrence of 'in contact' with one steer was lower (P < 0.05) in C than in R steers. On day 3, after the first R&R the occurrence of 'in contact' with two steers was greater (P < 0.05) in C than in R steers. No difference (P > 0.05) was observed for 'in contact' behaviour with two or three steers between R and C steers on day 1 after the first R&R.

Behavioural activity following the second regrouping and relocation (Table 15)

Following the second R&R on day 1, the occurrence of lying with the head supported was less (P < 0.05) in R than in C steers. On day 2, following the second R&R the occurrence of lying with the head not supported was greater (P < 0.05) in R than in C steers, however the occurrence of standing without moving was less (P < 0.05) in R than in C steers. On day 1 following the second R&R the occurrence of standing without moving and eating was greater (P < 0.05) in R than in C steers. On day 1, following the second R&R the occurrence of lying with the head not supported, drinking, head to head and head to body contact were not different (P > 0.05) between C and R steers. Following the second R&R on day 2 the occurrence of lying with the head supported, standing with moving without activity, eating, drinking, head to head and head to body contact was not different (P > 0.05) between C and R steers, while the occurrence of standing without moving was lower (P = 0.001) in R than in C steers. On day 4, the occurrence of lying with the head supported was greater (P = 0.001) and standing without moving was lower (P = 0.001) in R than in C steers. There was no (P > 0.05) difference for lying with the head supported and not supported, standing with or without moving, eating, drinking, head to head and head to body contacts on day 3 and from days 5 to 7 following the second R&R.

Contact behaviour following the second regrouping and relocation (Table 15)

Following the second R&R on days 1, 3 and 5 to 7, the occurrence of 'no body contact' or 'in contact' behaviour with one, two or three steers was not different ($P > 0.05$) between C and R steers. However, on day 2 after the second R&R there was a lower ($P = 0.001$) occurrence of 'no body contact' and 'in contact' with two steers and a greater ($P < 0.05$) occurrence of 'in contact' with one steer in the R treatment than in C steers. On day 4, after the second R&R the occurrence of 'no body contact' was lower ($P = 0.001$) and the occurrence of 'in contact' with one steer was greater ($P = 0.001$) in R than in C steers.

Behavioural activity following the third regrouping and relocation (Table 16)

After the third R&R on day 1, the occurrence of lying with the head supported was lower ($P = 0.001$) in R than in C steers. However, the occurrence of standing without moving, and drinking was greater ($P < 0.05$) in R than in C steers on day 1 following the third R&R. No difference ($P > 0.05$) was observed in the occurrence of lying without head support, standing moving, eating, head to head and head to body contact between C and R steers on day 1 following the third R&R. There was no difference ($P > 0.05$) for lying with the head supported and not supported, standing with or without moving, eating, drinking, head to head and head to body contacts from day 2 to day 7 after the third R&R.

Contact behaviour following the third regrouping and relocation (Table 16)

Following the third R&R, the occurrence of 'no body contact' and 'in contact' behaviours with one, two or three steers was not different ($P > 0.05$) from day 1 to 7 between R and C steers.

Behavioural activity following the fourth regrouping and relocation (Table 17)

On day 1, following the fourth R&R the occurrence of lying down with or without the head supported was lower ($P < 0.05$) in R than in C steers. However, on day 1 following the fourth R&R the occurrence of standing with or without moving, eating and drinking was greater ($P < 0.05$) in R than in C steers. No difference ($P < 0.05$) was observed for head to head and head to body contact on day 1 following the fourth R&R. On day 2, following the fourth R&R, the occurrence of lying with the head supported and drinking was greater ($P = 0.001$), and standing without moving was lower ($P = 0.001$) in R than in C steers. On day 5 following the fourth R&R, the occurrence of standing with or without moving was lower ($P < 0.05$) and occurrence of eating was greater ($P = 0.001$) in R than in C steers. There was no difference ($P > 0.05$) in lying with or without the head supported, standing with or without moving, eating, drinking, head to head and head to body contact on days 3, 4, 6 and 7 after the fourth R&R.

Contact behaviour following the fourth regrouping and relocation (Table 17)

Following the fourth R&R, the occurrence of no body contact was greater ($P < 0.05$) in R than in C steers, while the occurrence of 'in contact' behaviour with one steer was lower ($P = 0.001$) in R than in C steers. There was no ($P > 0.05$) difference in the occurrence of 'in contact' with two steers on day 1 following the fourth R&R. Following the fourth R&R, on days 2 to 7 the occurrence of 'no body contact' and 'in contact' behaviour with one, two or three steers were not different ($P > 0.05$) in R than in C steers.

Behavioural activity following the fifth regrouping and relocation (Table 18)

There was no difference ($P > 0.05$) for lying with or without the head supported, standing with or without moving, eating, drinking, head to head and head to body contact from day 1 to 7 following the fifth R&R.

Contact behaviour following the fifth regrouping and relocation (Table 18)

On day 1, 3, 5, 6, and 7, following the fifth R&R the occurrence of 'no body contact' and 'in contact' behaviour with one, two or three steers was not different ($P > 0.05$) in R compared with C steers. On day 2 and 4 following the fifth R&R, the occurrence of 'no body contact' was greater ($P < 0.05$) and the occurrence of 'in contact' behaviour with one and two steers was lower ($P < 0.05$) in R than in C steers.

Behavioural activity following the sixth regrouping and relocation (Table 19)

Following the sixth R&R on day 1, the occurrence of lying down with or without the head supported was lower ($P < 0.05$) in R than in C steers. The occurrence of standing with or without moving and eating was greater ($P < 0.05$) in R than in C steers on day 1 following the sixth R&R. There was no difference ($P > 0.05$) in drinking, head to head and head to body contact on day 1 following the sixth R&R between R and C steers. On day 2, following the sixth R&R the occurrence of lying with the head not supported was greater ($P < 0.05$) in R than in C steers. On day 3, the occurrence of lying with the head supported was greater ($P = 0.001$) in R than in C steers. There was no difference ($P > 0.05$) in lying with or without the head supported, standing with or without moving, eating, drinking, head to head and head to body contacts from day 4 to 7 following the sixth R&R.

Contact behaviour following the sixth regrouping and relocation (Table 19)

The occurrence of 'no body contact' behaviour was greater ($P = 0.001$) and the occurrence of 'in contact' with one or two steers was lower ($P < 0.05$) in R than in C steers on days 1 and 3 following the sixth R&R. On days 2, 4, 5, 6, and 7, following the sixth R&R the occurrence of 'no body contact' and 'in contact' behaviour with one, two or three steers was not different ($P > 0.05$) in R compared with C steers. However, on day 5 following the sixth R&R, the occurrence of 'in contact' behaviour with two steers was lower ($P < 0.05$) in R than in C steers.

Average daily gain

Overall there was no effect ($P > 0.05$) of repeated R&R on the average daily gain between R and C steers (Table 20). However, there was a tendency ($P < 0.1$) for a decreased ADG in R than in C steers following the third R&R (days 28-41).

4.4 Discussion

Previous studies have reported increased levels of behavioural activity in cattle due to the abrupt breaking of social bonds (Brakel and Leis, 1976; McVeigh and Tarrant, 1983; Fraser 1997). This abrupt breaking of the social bond and increased levels of activity was caused either by intensive husbandry practices (Winckler et al., 2003) or presence of adverse environmental conditions (Barnett and Hemsworth, 1990). Following grouping, farm animals are vulnerable to excessive competition (Grant and Albright, 2001). Barnett et al. (1993) suggested that social stress, caused by the establishment of hierarchies in new groups, poses short and long term welfare problems, arising from injuries and exclusion from resources such as food or lying space. In the present study, the acute exposure (within 2 h) of steers to R&R resulted in behaviour changes compared with the control group and this was characterised mainly by reduced lying and increased standing. The increase in time spent standing in steers may be a sign of the conflicts arising from the competition for resting space; animals in the standing posture may be exposed less to threats than when lying down, or they may be exhibiting a greater active response, in preparation for further change. No change in the time spent standing between treatment and control groups occurred following the second, third and fifth R&R within 2 h of R&R indicating partial adaptation to social stress or the steers were becoming familiarised to the effect of R&R.

Cattle are social animals and ranking within a group occurs based on dominant and submissive behaviour (Grant and Albright, 2001). When cattle are moved from one group to another, a new social order for that group must be established (Keeling and Gonyou, 2001). Several studies have been designed to partition the effect of social versus nutritional factors on eating (e.g. dry matter intake, DMI) associated with regrouping. Generally, studies in cows indicated a 2.5 to 5.0% decrease in DMI due to social disturbance compared with control animals that were not regrouped (Albright, 1978). Konggaard and Krohn (1978) conducted a series of studies to evaluate the effect of social change with no ration changes. The authors reported that after transfer to a new group, the eating time decreased and number of social confrontations increased during the first day. These findings are in contrast to the present investigation where an increase in feeding and drinking behaviour was observed and there was little or no social confrontation as measured by no difference in head- to -head, or head- to -body contact. However, the increase in eating behaviour following R&R supports the findings of Keeling and Gonyou (2001), who reported that forming a new group comprised of cows ready to leave the fresh-cow group, resulted in a substantial increase in DMI. The increased eating behaviour in regrouped steers of the present study may also indicate fragmentation of the meals. Alternatively, the changes in eating behaviour may be due to a displaced agonistic response.

In the present study, there was a greater time spent standing, eating, drinking and a shorter time spent lying by the steers during the first day following each R&R. There were also less body contacts and less head- to -head or head- to -body contacts with other steers. However, this trend did not continue into days 2 and 3 following each R&R. After the third R&R there was an increase in the time spent lying and a decrease in the time spent standing, indicating that steers were becoming habituated to R&R. Overall this indicated that steers adapted, in part, to situations of grouping and regrouping, but the level of adaptation was highly variable.

Veissier et al., 2001 reported that early social bonds between cattle last even if the animals are separated for prolonged periods. In a study by Bouissou and Andrieu, (1977), cattle were found to be less aggressive towards their former group members than towards unfamiliar animals when reintroduced into the same group. Although, steers in the present study were never exposed to familiar group members and location, the stabilisation of a group for 14 days might have established enough social bonds for further changes in activity and contact to the repeated R&R to be minimised. We found that the acute behavioural reaction of the steers to the R&R diminished

with repetition of R&R. The behavioural changes following the fourth R&R onwards indicated that steers adapted to R&R and this was associated with less time spent standing and greater contact with other steers. These findings are in agreement with Sowerby and Polan (1978), who reported that the negative influence of regrouping on behaviour was less in cattle regrouped previously than in cattle having no previous grouping/re-grouping experience. A review of the literature reveals a variation in reaching social stabilisation after grouping in cattle (Kondo et al. 1984; Tennessen et al., 1985; Hasegawa et al., 1997). Tennessen et al. (1985) reported decreased overt aggressive acts of bulls and steers by 10 days after regrouping. Kondo et al. (1984) and Hasegawa et al. (1997) reported that changes in social behaviour and locomotor activity in cattle returned to a basic level between 5 and 15 days after regrouping or introduction of an unfamiliar animal into a group. In a later study, Kondo and Hurnik, (1990) observed that cows with previous social experience form a stable social hierarchy between days 0 and 2 after re-grouping whereas cattle without previous social experience need 2 to 4 days to reach social stabilisation. Grant and Albright (2001) reported that 3 to 7 days following grouping are required for social stabilisation in cows. The findings in the present study indicate that there was a tendency to establish social bonds within 14 days after the first R&R and the time interval to establishment of social bonds and to reaching a stable group in the new cohort decreased with increased R&R. Grouping has reported negative effects on production performance (Sevi et al., 2001). However, repeated R&R of steers did not reduce overall ADG in the present study.

4.5 Conclusions

In conclusion, steers responded to regrouping and relocation with a change in their behaviour. Steers displayed more standing behaviour immediately following regrouping and relocation. However, following repeated regrouping and relocation more lying and body contact behaviours occurred. These data suggest that regrouping and relocation had significant effects on the activities of the animals indicating that partial adaptation to these changes occurred and were not detrimental to the animals. No detrimental effect on performance of steers was observed following repeated regrouping and relocation despite effects on behaviour.

Table 12. Calendar of events and procedures performed on the 72 steers subjected to repeated regrouping and relocation over 84 days

Day	Event[a]	Procedure performed[b]
-35	Steers housed for acclimatisation	Body weight
-1		Body weight
0	First regrouping and relocation	Behaviour observation
13		Body weight
14	Second regrouping and relocation	Behaviour observation
27		Body weight
28	Third regrouping and relocation	Behaviour observation
41		Body weight
42	Fourth regrouping and relocation	Behaviour observation
55		Body weight
56	Fifth regrouping and relocation	Behaviour observation
69		Body weight
70	Sixth regrouping and relocation	Behaviour observation
83		Body weight

[a]Each regrouping and relocation cohorts were allowed to stabilise for 14 days.
[b]Steers were observed by instantaneous scan sampling (by video cameras). The interval between scans was 2 min for 2 h on day 1, immediately after the regrouping and relocation followed by every 20 min during days 1 and 2. From day 3 to day 7 each steer was observed every 120 min.

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.
[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for

Table 13. Behaviour of 72 steers over the first 2 hours following each R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration, presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Regrouping and relocation[2]											
	1		**2**		**3**		**4**		**5**		**6**	
Treatment	C	R	C	R	C	R	C	R	C	R	C	R
Activity												
Lying	33.3	**4.0***	13.3	**5.9***	22.1	**3.2***	40.1*	**6.3***	2.9	**0.2***	61.5	**29.1***
Standing	32.8	**51.8***	38.3	**42.9***	42.7	**51.6***	33.8*	**49.1***	30.5	**34.7***	19.3	**35.8***
Eating	30.1	**38.4***	42.9	**46.2***	31.5	**39.6***	22.5	**37.9***	61.8	59.7	16.3	**31.6***
Drinking	1.8	**3.7***	3.8	4.3	2.6	**4.6***	1.8	**4.7***	3.8	4.5	2.5	3.2
Contact												
Head to head aggression	22	27	13	**6***	10	8	26	41	10	12	4	4
Head to body aggression	11	15	17	12	3	9	7	10	7	6	2	4
No body contact	1076	**1680***	1394	1946	1074	1286	**1452***	2212	**1585***	2148	878	**1888***
Body Contact with one	412	**328***	325	404	201	227	346	326	**175***	260	356	**407***
Body Contact with two	40	53	55	67	3	33	**25***	24	34	64	8	51
Body Contact with three	0	3	2	1	-	-	-	-	-	-	-	-

14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.
* Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 14. Behaviour of 72 steers for 7 days following the first R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment[2]	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity														
Lying	48.6	**33.7***	58.9	60.7	58.5	60.1	53.9	56.7	51.4	58.1	56.7	56.9	57.2	62.1
Standing	23.0	**33.8***	18.4	15.9	17.2	17.1	20.3	18.5	24.7	18.7	20.5	20.5	18.9	18.3
Eating	25.6	**28.7***	20.3	21.0	22.6	21.6	23.1	22.0	21.9	20.4	20.6	20.4	21.1	17.7
Drinking	1.5	**2.6***	2.1	2.1	1.7	1.2	2.2	2.8	1.9	2.8	1.7	1.4	1.7	1.6
Contact														
Head to head aggression	24	33	4	6	-	-	2	0	-	-	2	4	4	2
Head to body aggression	12	15	0	2	-	-	-	-	-	-	-	-	-	-
No body contact	2016	**3103***	1311	1828	295	432	302	427	313	420	315	**415***	317	431
Body contact with one	1004	**1015***	549	772	54	71	58	77	44	80	44	**86***	42	70
Body contact with two	92	127	24	33	5	**1***	-	-	3	4	1	3	1	3
Body contact with three	0	3	0	1	-	-	-	-	-	-	-	-	-	-

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.

[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.

***** Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 15. Behaviour of 72 steers for 7 days following the second R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment[2]	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity														
Lying	39.5	**35.1***	59.0	**62.9***	53.1	54.6	52.7	**62.3***	62.3	61.3	57.5	54.7	53.3	52.4
Standing	27.5	**29.9***	20.8	**17.4***	17.5	17.5	28.9	**20.3***	20.0	16.7	22.5	21.1	14.7	13.3
Eating	29.4	**31.6***	19.0	18.3	28.0	25.5	16.1	15.3	14.4	17.7	17.2	20.6	29.2	32.3
Drinking	2.7	2.9	0.9	1.4	1.4	1.8	1.7	2.2	2.5	3.4	2.2	3.2	2.8	2.0
Contact														
Head to head aggression	15	10	6	2	0	2	2	0	2	4	2	2	-	-
Head to body aggression	18	12	-	-	0	1	-	-	1	1	-	-	-	-
No body contact	2665	3747	1493	**2001***	291	406	337	**438***	296	429	309	453	315	426
Body contact with one	949	1267	539	**867***	59	82	23	**66***	60	71	47	51	37	73
Body contact with two	128	145	38	**29***	4	10	-	-	4	4	4	0	6	4
Body contact with three	2	1	0	1	-	-	-	-	-	-	-	-	2	1

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.

[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.

* Difference between Treatments at each R&R for each item ($P < 0.05$)..

Table 16. Behaviour of 72 steers for 7 days following the third R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment[2]	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity														
Lying	46.3	**39.8***	60.7	60.8	53.8	56.8	52.2	52.4	57.3	62.3	59.2	61.7	50.5	54.4
Standing	27.5	**30.9***	19.8	19.8	27.4	21.5	17.5	18.9	22.5	20.7	21.1	19.1	25.3	22.8
Eating	23.9	25.9	17.7	17.7	16.9	18.7	26.9	26.8	18.1	12.9	15.6	16.1	21.4	20.4
Drinking	1.8	**2.9***	1.7	1.7	1.5	2.6	3.3	2.0	2.0	3.0	4.2	3.2	1.7	2.0
Contact														
Head to head aggression	11	13	3	4	1	2	-		0	6	-	-	4	1
Head to body aggression	3	9	-	-	-	-	-		-	-	-	-	0	1
No body contact	2196	2985	1569	2242	303	438	313	439	316	415	318	431	326	435
Body contact with one	633	86	447	596	74	96	43	64	26	56	42	70	34	65
Body contact with two	33	72	24	60	13	6	4	1	1	3	0	3	0	4
Body contact with three	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.
[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.
* Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 17. Behaviour of 72 steers for 7 days following the fourth R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment[2]	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity														
Lying	49.1	**32.5***	58.5	**59.8***	56.8	60.9	49.4	54.9	45.6	49.2	60.5	63.7	57.7	59.7
Standing	27.5	**33.9***	23.4	**20.1***	25.7	22.4	25.4	18.3	34.4	**16.7***	18.3	16.9	23.9	22.4
Eating	21.0	**29.1***	17.0	17.9	15.0	14.1	22.7	22.8	15.6	**29.4***	20.3	18.1	17.2	14.7
Drinking	1.5	**3.3***	0.8	**2.1***	2.5	1.8	1.8	3.6	4.4	4.8	0.8	1.0	0.8	3.2
Contact														
Head to head aggression	28	47	6	2	0-	4	2	2	-		0	2	-	
Head to bodyaggression	7	10	-	-	-	-	-	-	-		-		1	0
No body contact	2996	**4359***	1587	2162	307	417	289	398	82	112	299	415	299	404
Body contact with one	764	**917***	411	550	44	87	39	69	8	14	58	87	61	93
Body contact with two	37	46	6	54	3	0	2	7	-	-	3	2	0	7
Body contact with three	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.

[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.

* Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 18. Behaviour of 72 steers for 7 days following the fifth R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items[1]	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment[2]	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity	33.8	32.1	56.8	58.8	58.4	55.1	48.1	49.4	60.9	52.8	58.0	56.9	60.0	73.9
Lying	27.2	29.3	21.1	21.1	18.4	19.5	19.7	18.7	19.1	20.7	17.0	20.1	23.3	9.5
Standing														
Eating	35.8	34.8	20.2	17.7	23.3	24.2	31.1	30.0	18.6	25.6	22.5	20.4	16.7	16.7
Drinking	2.5	3.2	1.5	2.3	0.0	1.2	0.6	1.6	1.4	1.0	1.9	2.2	-	-
Contact														
Head to head aggression	19	18	2	4	-	-	2	2	-	-	2	2	-	-
Head to body aggression	7	8	2	0	-	-	-	-	-	-	-	-	-	-
No body contact	3174	4416	1794	**2596**[*]	320	450	292	**439**[*]	302	425	309	424	23	32
Body contact with one	518	706	351	**433**[*]	37	51	61	**57**[*]	58	73	50	71	6	10
Body contact with two	46	91	33	**12**[*]	3	3	7	**8**[*]	0	6	1	9	1	0
Body contact with three	0	1	0	1	-	-	-	-	-	-	-	-	-	-

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.

[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.

* Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 19. Behaviour of 72 steers for 7 days following the sixth R&R [control (C) = 30 steers, regrouped (R) = 42 steers]. Behaviour is divided into activities of considerable duration presented as % time, and contact behaviour with other animals which is presented as a count of the numbers of contacts observed

Items	Days													
	1		**2**		**3**		**4**		**5**		**6**		**7**	
Treatment	C	R	C	R	C	R	C	R	C	R	C	R	C	R
Activity	66.1	**44.3***	62.4	**64.9***	29.2	**60.0***	52.4	56.7	58.6	60.9	53.0	62.9	56.5	56.4
Lying	14.6	**26.1***	18.6	16.6	66.6	36.6	21.2	19.3	21.3	15.9	24.5	18.3	19.0	20.1
Standing														
Eating	17.4	**27.2***	19.3	16.9	4.2	3.3	24.2	21.4	19.0	22.0	21.7	17.7	22.8	22.5
Drinking	1.6	2.2	1.4	1.5	-	-	2.1	2.2	1.1	0.6	0.8	0.8	1.3	0.7
Contact														
Head to head aggression	4	4	-	-	-	-	0	2	0	2	0	2	2	0
Head to body aggression	2	4	-	-	-	-	-	-	0	1	-	-	0	1
No body contact	1979	**2828***	1622	2266	18	**30***	295	402	294	428	308	425	322	452
Body contact with one	623	**709***	364	518	4	**0***	35	60	47	76	52	76	67	90
Body contact with two	8	57	24	30	2	0	-	-	7	**0***	0	2	1	4
Body contact with three	-	-	-	-		-	-	-	-	-	0	1	-	-

[1]Data were analysed on the total count of body contact and time spent in each behavioural category by χ^2 statistic.
[2]Regrouped (R) steers were exposed to six regrouping and relocation events during an 84 day period and each new cohort was allowed to stabilise for 14 days. Control (C) steers were housed in the same pen with the same pen mates throughout the experiment.
* Difference between Treatments at each R&R for each item ($P < 0.05$).

Table 20. Effect of repeated regrouping and relocation (R&R) on the average daily gain (kg/day) of 14-month-old control and regrouped steers during an 83 day experimental period[a].

	Treatment		
Days	Control (n = 30)	Regrouped (n = 42)	*P*- value
-1 to 13	1.52 ± 0.168	1.50 ± 0.119	0.947
14 to 27	0.47 ± 0.112	0.40 ± 0.124	0.669
28 to 41	0.91 ± 0.131	0.63 ± 0.100	0.086
42 to 55	1.56 ± 0.151	1.55 ± 0.101	0.959
56 to 69	-0.03 ± 0.121	0.12 ± 0.177	0.525
70 to 83	0.96 ± 0.141	0.86 ± 0.246	0.755
-1 to 83	0.90 ± 0.038	0.84 ± 0.033	0.261

[a]Control, n = 30; Regrouped, n = 42

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5. Effect of repeated regrouping and relocation on the physiological, immunological and hematological variables and performance of steers

5.1 Introduction

Regrouping and relocation (**R&R**) of animals is a common husbandry practice to create homogenous groups organized by age, weight and production system (Bøe and Færevik et al., 2003). Regrouping may occur by mixing animals on one occasion or repeatedly; and relocation as: change in location once or several times. However, studies (Hasegawa et al., 1997; Sevi et al., 2001) on farm animals indicate that regrouping and relocation is a problem and shown to elicit physiological stress, suppression of immune function and a reduction in performance. Veissier et al. (2001) found increased sensitivity of the adrenal cortex of regrouped calves to ACTH, while Hanlon et al. (1995) reported reduced lymphocyte response to keyhole limpet haemocynin (**KLH**) in regrouped deer. A short-term effect on production performance was reported in ewes to regrouping and relocation (Sevi et al., 2001). In contrast several studies (Kondo et al., 1990; Veissier et al., 2001) indicate that the cattle R&R does not have a detrimental effect on health and they adapt easily to grouping.

The aim in the present study was to evaluate the physiological, immunological and performance responses of steers exposed firstly to an acute stress stimuli and then subsequently to repeated stress stimuli on their welfare. The hypothesis was that repeated R&R of beef steers would decreased sensitivity of the hypothalamus-pituitary-adrenal (**HPA**) axis to corticotrophin-releasing hormone (**CRH**) and ACTH, decrease immune response, increase body metabolism, decrease animal performance and reduce animal welfare. Indices used to measure the function of the HPA axis were cortisol and ACTH response, with and without exogenous ACTH and CRH administration; interferon (**IFN**)-γ and acute phase proteins (haptoglobin and fibrinogen) for immune response; blood biochemical and hematological variables for body metabolism.

5.2 Materials and Methods

All procedures in the study 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) Regulation, 1994.

Treatments

Seventy-two Holstein × Friesian (14-mo-old; mean BW = 441 ± 3.2 kg) steers were blocked by body weight and randomly assigned to either control (n = 30; **C**) or regrouped (n = 42; **R**) treatments and housed 6 per pen in 12 pens alternatively at a space allowance of 2.8 m^2/ steer.

Animal Housing and Management

Before assignment to treatment, steers were weighed and housed from d -35 (day of treatment = d 0) to acclimatize them to handling and restrained. Steers were housed for 84 days on slatted floor pen facilities, six pens facing each other. The dimension of each pen was 4.5 x 3.8 m with a feed trough length of 4.5 m long. A steel mesh with dimensions of 3.8 x 1.6 m separated the sides of each pen. The light was on 24 h and the shed was dimly lighted using infra red lights. The steers had access to grass silage with DM content (mean values) = 224 g/kg, in vitro DM digestibility = 887 g/kg of DM, pH = 4.2, and supplemented with 2.5 kg of barley /soybean mix concentrate ration (mean values) of crude fiber = 41.9 g/kg, crude protein = 155 g/kg, acid hydrolyzable oil = 39 g/kg, ash = 58.6 g/kg per animal daily. Steers had free access to water in their individual pens.

Catheterization

To facilitate intensive blood collection, steers were fitted aseptically with indwelling jugular catheters on d -1 (day before the first R&R), d 27 and 69 (days before the third and the sixth R&R). The procedure was performed according to the method of Ting et al. (2003), using 12 gauge Anes spinal needles (Popper and Sons, Inc., New Hyde Park, NY) and polyvinyl tubing (approximately 1.47 mm i. d.; Ico Rally Corp., Palo Alto, CA; catalogue No. SVL 105-18 CLR) attached to an 18-gauge needle at the blood collection end.

On d –1, four steers from each pen in both C and R treatments were catheterized. The remaining two steers from each pen in both C and R treatments were sham catheterized by piercing aseptically the left jugular vein with the 12 gauge spinal needle and no catheters were fitted. On day 27, four steers were subjected to catheterization in each pen. Two steers from the sham catheterized and two others randomly selected from steers that were catheterized on d -1. The remaining two steers were sham operated in each pen. On day 69, all steers were catheterized. All the catheters were exteriorized on the left side of the neck, flushed and filled with sterile 3.5% sodium citrate solution, and plugged with a stopper. Catheters were secured in place in patches with the aid of an adhesive cement (Big Bull Hip Tag Cement; Biguel supply Co., Elysian, MN), Velcro, and zinc oxide wrapping bandages (Sterotape-Z.O., Steroplast Ltd, Bredbury, U.K. Product Code No. 4050). Sham operated steers were also wrapped with zinc oxide bandages. After completion of the catheterization procedure the steers were returned to their home pen and catheters were maintained patent for 36 h by flushing with 2 mL of 3.5% sodium citrate after each blood collection time.

Regrouping and Relocation

Regrouped steers were exposed to six R&R events from d 0 to 84. Following each R&R new pen (n = 6 steers/pen) cohorts were allowed to stabilize for 14 days. In each R&R, none of the R steers were allowed to share the same pen or pen-mates where or with whom they were previously housed. Control steers were housed in the same pen with the same pen-mates from the beginning to the end of the experimental study. On the day of R&R, steers from R treatment were individually taken out from their pens and were regrouped and taken to their new pens. Each R&R of R steers was staggered and was carried out between 0800 and 0830 GMT. Immediately after R&R, the housing facility was closed and steers in the new pen cohorts were allowed to interact for 2 h without human interference. During this time steers had access to water only. All the steers were kept in their pens except at the time of R&R, weighing, and catheterization procedures.

Physiological Measurements

Basal Plasma Cortisol and ACTH levels. The cortisol and ACTH levels in the plasma of catheterized C and R steers were determined at the first (n = 20 for C; n = 28 for R), third (n = 10 for C and n = 14 for R) and sixth (n = 16 for C and n = 22 for R) R&R. The blood samples were collected at -2, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 h (0 h = first sample after 2 h of R&R). The tubes containing heparinised whole blood were centrifuged (1600 x g at 8ºC for 15 min); the plasma was collected and stored at –20ºC until assayed for cortisol. The blood samples for ACTH determination were collected into the iced tubes containing EDTA anticoagulants, tubes were centrifuged at 2000g, at 4ºC for 15 min and plasma was frozen and stored at -80ºC until assayed. Commercial RIA kits were used to determine the plasma levels of cortisol (Corti-cote, ICN Pharmaceuticals, Orangeburg, NY, Validated by Fisher et al., 1997) and ACTH (Diagnostic Products Corporation, Los Angeles, CA) within 6 wk after the collection. The intraassay CV (n = 6) for samples containing 7.1, 16.5 and 55.8 ng of cortisol/mL were 10.7, 8.1 and 7.5%, respectively, and the interassay CV (n = 17) for the same samples were 22.6, 17.2 and 11.1%. The intraassay CV (n = four to six per assay) for

sample containing 85.6 and 261.1 pg of ACTH/mL were 8.6 and 12.6%, respectively, and the interassay CV (n = 20) for the same samples were 9.7 and 11.3%, respectively.

ACTH challenge. The response of the adrenal cortex gland to ACTH (1.98 IU/kg metabolic BW; Friend et al., 1977a and Fisher et al., 1997a) was tested following the third and sixth R&R on two steers from each pen (n = 14 for R and n = 10 for C treatments) that were randomly chosen at the start of the experiment. The same steers were used for the ACTH challenge at the third and sixth R&R occasions. Dexamethasone (20 µg/kg BW; Faulding Pharmaceuticals Plc, UK) was administered (i.m.) at –12 h to all the steers undergoing ACTH challenge (Synacthen Ampoules, Novartis Pharmaceutical Ltd., UK). Two steers from each pen (n = 14 for R and n = 10 for C treatments) received normal saline (5 mL, 0.9% sterile) as a placebo at the time of both dexamethasone and ACTH injections. Immediately following the administration of dexamethasone, ACTH and normal saline catheters were flushed with 2 mL of 3.5% sterile sodium citrate solution. Blood samples were collected into the tubes containing EDTA (in an ice bath) for ACTH or heparin anticoagulants for cortisol through jugular catheters immediately before the administration of dexamethasone and saline and at -2, -0.25, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 h (-0.25 h = first sample after 2 h of R&R) relative to the time of ACTH and saline administration. Plasma storage and assays were as described above. Plasma samples collected for ACTH determination were above the range of the ACTH standard provided with the kits. These plasma samples were diluted by 1:20, 1:50 and 1:100 as required and concentrations were corrected accordingly before analysis.

CRH challenge. Two steers from each pen (n = 10 for C and n = 14 for R) were administered with CRH at 4 h following the sixth R&R. These steers were administered with normal saline previously at the first and third R&R. In addition, steers from each C (n = 6) and R (n = 8) treatments were administered with normal saline (2 mL, 0.9% sterile). Two blood samples were collected at 0.25 h interval each before the i.v. administration (through jugular catheter) of bovine (**b**) CRH (0.3µg/kg BW; American Peptide Company, Inc. Sunnyvale, CA; Gupta et al., 2004) and normal saline. Subsequent blood samples were collected through the jugular catheters at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3 h relative to the time of bCRH and saline administration for plasma cortisol and ACTH concentrations. Immediately following the administration of bCRH and normal saline, catheters were flushed with 2 mL of 3.5% sterile sodium citrate solution. The plasma storage and assay method for both cortisol and ACTH were as described previously.

Hematology and Biochemistry. Unclotted (EDTA) whole blood samples were collected from catheterized C and R steers, 2 h before and after the first (d 0; n = 20 for C and n = 28 for R), the third (d 28; n = 20 for C and n= 28 for R) and the sixth (d 70; n = 26 for C and n = 36 for R) R&R. They were analyzed for total white blood cell numbers (WBC), red blood cell (RBC), monocyte, lymphocyte numbers, mean corpuscular volume (MCV) hemoglobin (Hb), and platelet numbers using an automated electronic particle analyzer (Celltac, MEK-6108K, Nihon-Kohdon, Tokyo, Japan) within 1 h of blood sampling. Further blood samples containing heparin as anticoagulant were collected for determination of beta-hydroxy butyrate (**β**HB), albumin, globulin, total protein and urea, and containing fluoride anticoagulant for NEFA and glucose determination. In all cases plasma was separated by centrifugation (1,600 x g at 8ºC for 15 min) and stored at –20ºC until assayed using commercial biochemical assay kits (Boehringer Mnnheimin, Germany and Randox private Ltd, UK) on an automated biochemical (SPACE, Schiapperelli Biosynthesis Inc., USA) analyzer.

Immunological Measurements

Stimulated lymphocyte production of interferon-γ. Catheterized steers were blood sampled at 2 h before and after the first (n = 20 for C and n = 28 for R steers), third (n = 20 for C and n = 28 for R steers) and sixth (n = 26 for C and n = 36 for R steers) R&R. The stimulated lymphocyte production of IFN-γ following whole blood culture of heparinised plasma was determined using a modification (Fisher et al., 1997a; Ting et al., 2003) of the procedure described by Wood et al. (1990).

Haptoglobin and Fibrinogen. The whole blood samples collected into heparinised tubes were centrifuged at 3,000 x g, 8ºC for 10 min and plasma stored at -20ºC until assayed for haptoglobin. The haptoglobin proteins in plasma were determined as the hemoglobin binding capacity using a biochemical assay kit (Tridelta Development Ltd., Ireland, and Catalog No. TP801) previously validated for bovine plasma by Skinner et al. (1991). Blood samples collected into sodium citrate tubes were centrifuged at 3,000 x g, at 8ºC for 10 min and plasma stored at -20ºC until assayed for fibrinogen. Fibrinogen, the circulating precursor of fibrin in the blood-clotting cascade, was determined by using a commercial kit (Roche Diagnostics Gmbh, Mannheim, Germany; Catalog No. 524484) adopted for bovine plasma (Earley and Crowe, 2002).

Environmental conditions and production

The mean daily air temperature and relative humidity in the housing facility were recorded continuously using Tiny Talk data loggers (Radionics, Dublin, Ireland). The BW of steers was recorded on eight different occasions, on d -7 and d 13, 27, 41, 55, 69, 83 and d 97 to determine the ADG (kg/day).

Statistical Analysis

All statistical analyses were performed using SAS (SAS/STAT® software Version 8 of the SAS System for Windows. Copyright© 1989-1996 SAS Institute Inc. Cary, NC). The probability plot of the residuals (Shapiro-Wilk test, $P < 0.005$) using the UNIVARIATE procedure was used to determine the normality of the data. The data that showed lack of normality and heterogeneity of variance were analyzed by non-parametric analysis using Kruskall-Wallis and Mann Whitney procedures based on the rank transformation (Zar, 1999). For each steer the median values for area under the cortisol and ACTH vs time curves were analyzed after rank transformation by Kruskall-Wallis one-way ANOVA (Zar, 1999). The area (ng L^{-1}.h) under the cortisol and ACTH vs time curve (**AUC** cortisol and AUC ACTH) were calculated from the time of treatment either R&R or ACTH/CRH/saline administration until the final sample of the day following the first, third and sixth R&R using a linear trapezoidal rule:

$$\sum \{[(C_t + C_{t+1}) \times 0.5] \times \Delta I\}$$

where C_t is the concentration of a plasma cortisol sample in ng/ml of an animal at a time t, and for the next samples C_{t+1} with a time interval of ΔI in hours between them, $\sum$ is the sum of the responses from C_t to n-1 total number of concentration time points (Veissier et al., 2001). Hematological variables (WBC, RBC, platelet, monocyte, lymphocyte numbers, MCV and Hb), globulin, NEFA, glucose, IFN-γ, haptoglobin and fibrinogen were analyzed after rank transformation by ANOVA for the effect of treatment with the pre-treatment values (values before R&R at 2 h) included as a significant covariate. The statistical differences between the treatments were determined by Wilcoxon on sign rank test. To see the adaptation over time for cortisol and ACTH responses data were analyzed after rank transformation with Friedman test for repeated measures and the statistical differences were determined by Tukey studentised range test (Zar, 1999). Data relating to mean albumin, **β**HB, protein, Hb, urea, and

ADG were analyzed parametrically by ANOVA for the main effect of treatment and taking pre-treatment values as a covariate. During the ACTH challenge at both the third (C steer) and sixth (C steer) R&R, and CRH challenge at the sixth R&R (R steers) the catheter, from one steer at each time was lost after the fourth, fifth and sixth blood collection time, therefore, the complete data from these animals were excluded from the statistical analyses.

5.3 Results

Physiological Measurements

Plasma Cortisol and ACTH. The median basal plasma cortisol and ACTH concentrations in the C steers ranged from 0.92 to 13.5 ng/mL and 40.73 to 129.56 pg/mL, with no difference ($P \geq 0.05$) in C and R steers before pre-treatment period at -2 h. Following the first R&R, the median area under the plasma cortisol curve (AUC) from 0 to 180 min, was higher ($P < 0.005$) in R than C steers (Figure 3), with no difference observed among treatments following the third ($P = 0.799$) and sixth ($P = 0.649$) R&R. No differences were found following the first ($P = 0.608$), third ($P = 0.655$) and sixth ($P = 0.690$) R&R from 0 to 180 min for median area under the plasma ACTH curve. Overall, the change in median values for cortisol AUC over time in R steers decreased ($P = 0.001$) following the first vs third and sixth R&R (median 1225.5 vs 737.7 and 966.9 ng/mL), however cortisol AUC in R steers following the sixth R&R was higher ($P < 0.001$) than the third R&R. No differences ($P = 0.182$) were observed following the first, third and sixth R&R in the control steers (data not shown). In contrast, the median values for the ACTH AUC over time in C and R steers, following the third compared with first and sixth R&R (17362.2 vs 12479.2 and 11014.1 for C, $P = 0.001$; 17455.8 vs 12247.9 and 11689.1 ng L^{-1}.h for R $P = 0.003$) was higher.

ACTH Challenge. Plasma Cortisol and ACTH responses to exogenous ACTH and saline for steers are shown in Figure 4. Immediately before the administration of dexamethasone at -12 h and at -2h before the third and sixth R&R the median cortisol and ACTH levels were not different ($P > 0.05$, data not shown) among C vs R steers. Following the 14 h of dexamethasone administration at the third and sixth R&R, the cortisol and ACTH levels decreased (0.05 ng/mL and 41.57 pg/mL, respectively). There was a response to exogenous ACTH compared with saline for median plasma levels of AUC for cortisol and ACTH in C and R steers following the third (R, $P = 0.001$; C, $P = 0.001$ for both cortisol and ACTH) and sixth (R, $P = 0.001$; C, $P = 0.001$ for both cortisol and ACTH) R&R occasions. Following the administration of ACTH after the third R&R lower median plasma levels of AUC cortisol ($P = 0.048$) and ACTH ($P = 0.049$) were observed in R than C steers. However no difference ($P > 0.005$) in median plasma levels of AUC for cortisol and ACTH were observed among treatments at the sixth R&R.

CRH Challenge. The administration of exogenous bCRH increased cortisol (C, $P = 0.001$; R, $P = 0.001$) and ACTH (C, $P = 0.001$; R, $P = 0.002$) levels within each C and R steers (Figure 5) compared with saline administration. There was no effect ($P > 0.05$) of exogenous administration of bCRH on the median plasma levels of AUC for cortisol and AUC for ACTH among C vs R steers after the sixth R&R.

Hematology and Biochemistry. The median WBC, RBC and platelets numbers, monocyte and Hb were not different ($P > 0.05$) in C vs R steers after the first, third and sixth R&R (Table 21). Median MCV and lymphocyte numbers were higher ($P \leq 0.05$) in R than C steers after 6 R&R. Mean albumin (35.2 ± 0.38 vs 34.4 ± 0.44 g/L), urea (4.1 ± 0.11 vs 2.7 ± 0.14 mmol/L) and median NEFA (0.15 vs 0.11 mmol/L) were greater ($P \leq 0.05$) in R than in

C steers following the first R&R, while mean **β**HB (0.31 ± 0.013 vs 0.28 ± 0.012 mmol/L) and median glucose (4.2 vs 4.1 mmol/L) were greater ($P \leq 0.05$) in R than in C steers following the sixth R&R. Mean **β**HB, mean protein, median globulin and median glucose were not different ($P > 0.05$) in C and R steers after the first and third R&R (data not shown). No differences ($P > 0.05$) were observed in the mean albumin, mean urea and median NEFA following the third and sixth R&R. Mean protein and median globulin were not different ($P > 0.05$) following the sixth R&R in C vs R steers (data not shown).

Immunological Measurements

Stimulated lymphocyte production of interferon-γ, Haptoglobin and Fibrinogen. No differences ($P \geq 0.100$) were observed between C vs R steers in their responses to Con A-induced in vitro IFN-γ production following the first, third and sixth R&R (Figure 6). There were no differences ($P \geq 0.100$) among treatments in plasma haptoglobin and fibrinogen concentrations following the first, third and sixth R&R (Figure 7).

Environmental conditions, ADG and General Health

The mean daily air temperature and relative humidity in the housing facility were 16.9ºC (range 5.8 to 25.9ºC) and 81.98% (range 38.40 to 100.00%) during the experimental period from April to June. There was no ($P > 0.05$) effect of repeated R&R on the average daily gain between R and C steers at all time periods (Table 22).

5.4 Discussion

Regrouping and relocation is a conventional management practice of farm animals. Animals respond to the abrupt or repeated breakage of social bonds either by adaptation within normal physiological parameters or lead to production pathology. In the present study, the acute exposure of steers to the first R&R resulted in a specific endocrine response characterized by increased plasma cortisol response in R compared with C steers. However, the first R&R did not affect the release of ACTH from the pituitary gland. The increase in cortisol may have occurred due to the mixing of unfamiliar animals at different locations and was in contrast to the work of Veissier et al. (2001) in calves and Sevi et al. (2003) in ewes, where both authors reported no difference in the basal plasma cortisol levels following regrouping and relocation treatments, and not consistent with Hanlon et al. (1995), who reported lower basal cortisol levels in mixed than control deer. No change in plasma ACTH concentrations in R steers indicated increased glucocorticosteroid receptors expression and negative feedback sensitivity at the pituitary gland. This is in agreement with Broom and Johnson (1993), who reported that plasma ACTH levels could decline rapidly after the application of the acute stressor. The lack of change in plasma cortisol and ACTH levels at the third and sixth R&R indicated that the steers were accustomed to being moved and grouped with strangers. The hormonal adaptations indicated by decreased responsiveness of the HPA axis to the R&R stressor on day 28 onwards and until day 84 suggests that habituation occurred with the possible involvement of the central nervous system, which is the first organ to perceive stress stimuli and control the secretory activity of the HPA-axis. Furthermore these findings also suggests that cortisol might be a better indicator than ACTH to study acute stress responses.

There was an increased integrated cortisol response in C compared with R steers following exogenous ACTH administration at the third R&R. This finding is in contrast with previous studies (Hanlon et al., 1995; Veisser et al., 2001) of social regrouping and relocation induced by a weekly exposure of change in animal partners and their locations. The difference with the previous studies could be attributed to the two wk interval between each R&R, species variation, age and weight of the animals in the present investigation that might have lowered the magnitude of the stress required to stimulate an adrenal response in R steers.

Unlike basal cortisol levels, cortisol response induced by exogenous ACTH administration may provide an independent index of adrenocortical sensitivity (Moberg and Mench, 2000). There are inconsistencies in interpretation of increased (Friend et al., 1985; Janssens et al., 1994) or decreased (Ladewig and Smidt, 1989; Fisher et al., 1997a) cortisol response following ACTH administration under stressful situations among animals. The present findings suggest that decreased sensitivity of the adrenal to ACTH following acute stress stimuli (first R&R) coupled with the chronic effect of subsequent repeated R&R (first, second and third) down regulated the pituitary-adrenal axis or increased the sensitivity of the pituitary to cortisol negative feedback. Furthermore, following repeated exposure i.e., after the sixth R&R, there was no change in cortisol response to ACTH challenge, which indicates a reduction in the adrenal responsiveness to ACTH. The effect could be occurring either at ACTH receptor levels in the adrenal gland, or in the synthesis, release or clearance of cortisol. When considering an adaptive response, the negative effect of R&R seems to be restricted to only a short period. Interestingly, the induced plasma levels of ACTH by exogenous ACTH administration in R steers were lower than C steers, indicating rapid clearance of ACTH in the R&R animals and warrants further investigation. The difference in the levels of induced cortisol and ACTH following ACTH challenge when compared with saline injection within the same treatments was drug effect and was as expected.

In humans, CRH challenge is used as a diagnostic tool to monitor the course of chronic stress due to Cushing's syndrome, adrenal insufficiency and major depressive disorders (O'Connor, 2000). In humans and domestic animals, the duration or level of stress induced increase in circulating ACTH and cortisol levels may be controlled by down regulating the sensitivity of the HPA axis. Therefore, the use of CRH administration in stressed farm animals for testing the sensitivity of pituitary to ACTH release above physiological threshold levels is of benefit. In the present study steers subjected to repeated R&R did not differ from controls in CRH induced ACTH and cortisol responses. The lack of change on the functioning of the HPA axis following the administration of CRH between stressed and unstressed animals is in agreement with studies in pigs (Janssens et al., 1994) and in calves (Veissier et al., 2001). In contrast increased (Garcia-belenguer et al., 1993) release of ACTH following CRH challenge has been reported in food-restricted rats. Fisher et al. (2002b) showed a decrease in ACTH and cortisol response to CRH in cows that were restricted from lying. The present findings confirm that the longer duration of stressor modified the functioning of HPA axis and following the presence of repeated R&R stressor, there are either adaptation or regulatory changes in the hypothalamus and/or pituitary to minimize the stress imposed.

Interferon-γ is an important cytokine that induces a variety of physiologically significant responses that contribute to immunity (Shtrichman and Samuel, 2001). Interferon-γ production has been shown as a reliable measure of immune responsiveness to common husbandry stressors such as castration (Fisher et al, 1997a; Earley and Crowe, 2002; Ting et al., 2003) and weaning (Hickey et al., 2003) in cattle. In the present study, repeated R&R did not induce an immunosuppressive response in the production of IFN-γ in response to a novel mitogen Con A. These findings differed from that of Hanlon et al. (1995), who reported reduced lymphocyte response to KLH following repeated introduction of deer to a new group. Given that the increased concentrations of neuropeptides (CRH and glucocorticoids) are associated with decreased immune responses (Anisman, 2002), the present study showed that IFN-γ production did not appear to be associated with increased levels of cortisol following the first R&R. Furthermore, the findings of the present study indicate that neither initial acute stress exposure (first R&R) nor later acute stress exposure (third and sixth R&R) coupled with the chronic effect of repeated R&R has affected the immune function of the steers. Previous studies (Fisher et al., 1997a; Earley and Crowe, 2002; Ting et al., 2003) have shown the

suppression of IFN-γ following day 1 and 3 of castration stress in calves where there was a peak cortisol within 30 min of castration. On the basis of these it is suggested that daily sampling at least up to two to three days of treatment would be beneficial to predict change in IFN-γ against any stressor.

Initial (at first) and repeated (at third and sixth) R&R of R steers did not alter the concentrations of plasma haptoglobin and fibrinogen compared with C steers. Other cattle (castration, Earley and Crowe, 2002; Ting et al, 2003 and transportation, Phillips et al., 1989) stress models have reported increased plasma concentrations of acute phase proteins such as haptoglobin and fibrinogen (Anisman, 2002). This acute phase protein response is the reaction of the animals to disturbance in its homeostasis caused by infection, tissue injury, neoplastic growth or immunological disorders. In the present investigation the stress induced by repeated R&R was insufficiently mild to induce changes in haptoglobin and fibrinogen plasma levels**.** The correlation of increased acute phase proteins with increased activated T cell proliferation and glucocorticoid in animals (Anisman, 2002) does highlight the need for further research on plasma haptoglobin and fibrinogen and their interaction with the neuroendocrine system during non-inflammatory stressful circumstances**.**

Blood cells are sensitive indicators of physiological and patho-physiological responses in animals. A change in blood cell composition indicates a response to restore animal homeostasis when exposed to abrupt physical conditions (Radostits et al., 1994). The present study showed that initial and repeated (up to third) R&R did not affect the WBC, RBC, platelet, lymphocyte, monocyte numbers, MCV and Hb of R steers. However increased lymphocyte numbers and MCV following the sixth R&R in R compared with C steers are indicative of a slight increase in sensitivity of the R steers during the recovery process associated with changes in the physical environment. These results could be attributed to the fact that involvement of extra adrenal factor might be boosting blood cell composition to increase the number of non-granulocyte subpopulations in blood (Dhabhar et al., 1996). Given that glucocorticoid is responsible for the regulation of circulating concentrations of blood cells (Dhabhar et al., 1996), this study did not concur with these findings. The overall picture of hematological variables indicates that health of the steers in this study was not compromised with the repeated R&R stress and suggests that the regrouping and relocation may not necessarily be a sufficiently potent stressor that disrupts the homeostasis of animals.

Changes in blood metabolites are indicative of energy mobilization, a mechanism necessary to maintain the homeostasis (Moberg and Mench, 2000). Stressful events are typically associated with increased energy demands as well as reduced appetite and this leads to depletion of energy stores, in particular liver glycogens and body fat. The increase in the albumin, urea and NEFA following first R&R indicate increased protein metabolism**.** Given that glucocorticoids are glycogenolytic factors during stress and stimulate gluconeogenesis, to the detriment of body protein, and, together with neuropeptides (CRH), they are central switches for reallocation of energy streams from body growth towards functions promoting immediate survival. Increased plasma glucose and **β**HB, a ketone body in blood, in R steers at sixth R&R are indicating more energy demand during recovery from the chronic effects of repeating R&R to restore homeostasis**.** The other possibility for increases in glucose and **β**HB could be consumption of a starchy diet; however there was no change in the diet of the steers through out the study hence time of blood sampling is more likely to be a factor.

Grouping has reported negative effects on production performance (Sevi et al., 2001). However, repeated R&R of steers did not reduce average daily gain in the present study.

In conclusion, steers responded to regrouping and relocation with increased basal plasma cortisol, and changes in metabolic activity. Repeated R&R reduced the sensitivity of the adrenal to ACTH with no effect on the pituitary following CRH challenge. However there was adaptation of the HPA-axis and metabolic system over time among steers repeatedly

exposed to regrouping and relocation without any detrimental effect on the performance, immune status and health of the animals.

Exposure and duration of a stress or plays an important role in modulating the regulation and functioning of the hypothalamic-pituitary-adrenal axis. Failure to restore levels of cortisol and metabolic activity following the first regrouping and relocation, a acute stress stimuli reflects increased activity of the hypothalamic-pituitary-adrenal axis. Duration of the previous exposure coupled with repeated exposures limited the effect of regrouping and relocation for short periods. Steers can be regrouped and relocated with out detrimental effects on immune and production parameters.

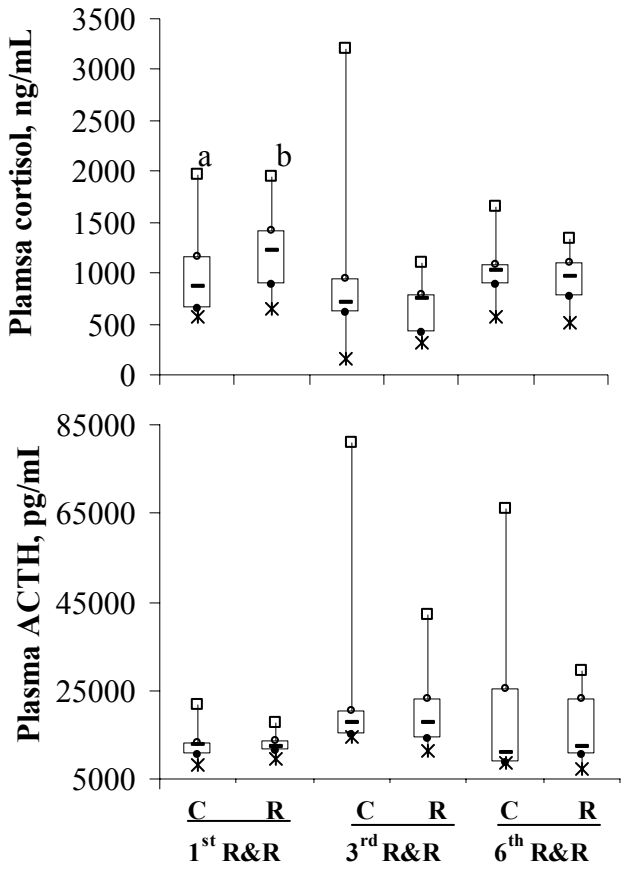

Figure 3. Box-whisker plots of plasma cortisol (upper panel) and ACTH (lower panel) concentrations in control (C) and regrouped (R) steers following first (n = 30 for C, and n = 42 for R), third (n = 10 for C, n = 14 for R) and sixth (n = 10 for C, and n = 14 for R) regrouping and relocation (R&R). The boxes show the median (—), upper (○) and lower (●) quartiles, while the box -whisker plot show the 25th (□) and 75th (x) percentiles of plasma cortisol levels. The integrated plasma cortisol response (area under the curve) was greater ($P < 0.05$; bars with a and b) in R than in C steers following first R&R. There was no difference in the integrated plasma cortisol response following third and sixth regrouping and relocation. No differences were observed for integrated plasma ACTH response following the first, third, and sixth regrouping and relocation.

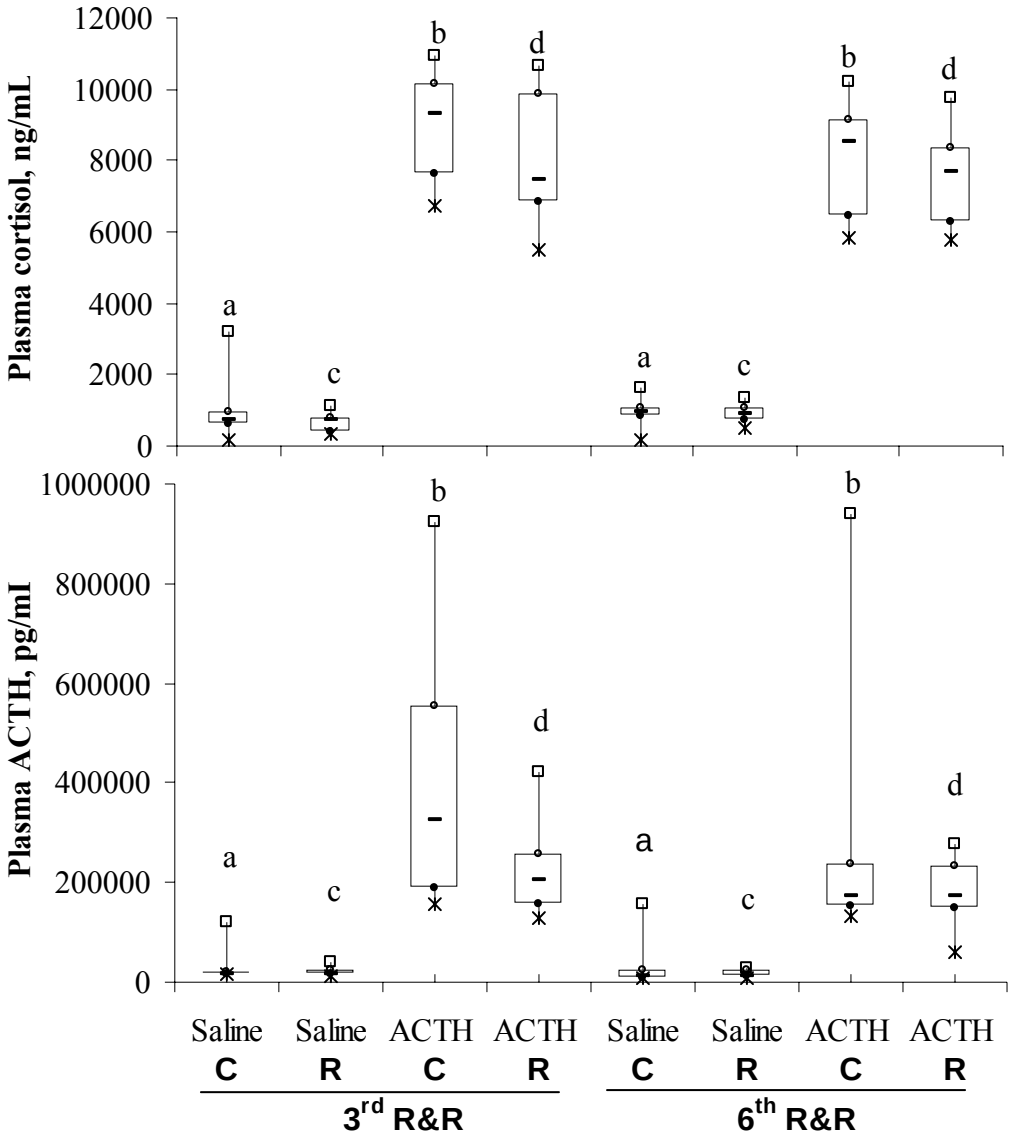

Figure 4. Box-whisker plots of plasma cortisol (upper panel) and ACTH (lower panel) levels, following **exogenous ACTH** (1.98 IU/kg metabolic BW) and saline (5 ml, 0.9 % sterile) administration of control (C) and regrouped (R) steers following the third (n = 10 for C, and n = 14 for R) and sixth (n = 10 for C, and n = 14 for R) regrouping and relocation (R&R). The boxes show the median (—), upper (○) and lower (●) quartiles, while the whiskers show the 25th(□) and 75th (x) percentiles of plasma cortisol and ACTH concentrations. With in each panel, for each regrouping and relocation, bars with a, b for C and c, d for R steers differ ($P < 0.05$).

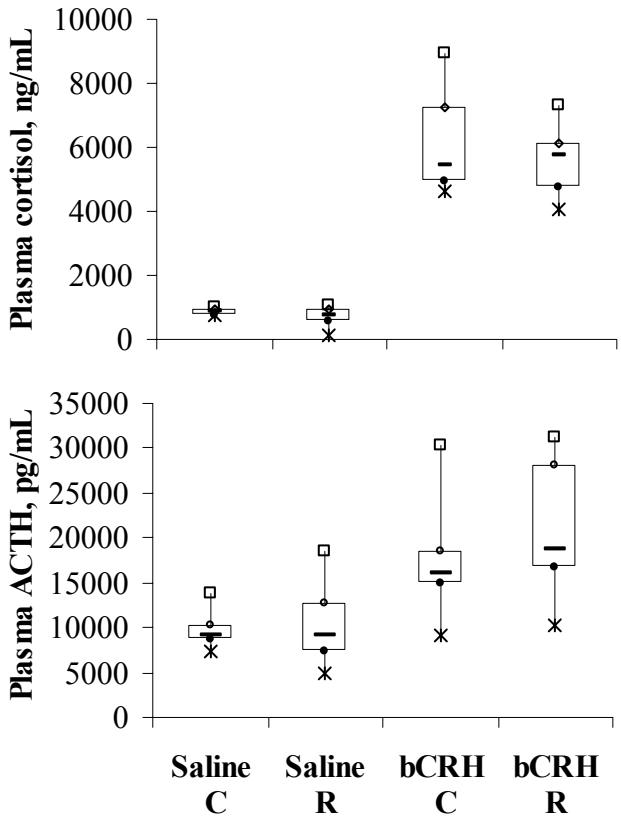

Figure 5. Box-whisker plots of plasma cortisol (upper panel) and ACTH (lower panel) levels, following **exogenous bCRH** (0.3µg/kg BW) and saline (2 mL, 0.9 % sterile) administration of control (C) and regrouped (R) steers following the sixth (n = 10 for C, and n = 14 for R) regrouping and relocation (R&R). The boxes show the median (—), upper (○) and lower (●) quartiles, while the whiskers show the 25^{th}(□) and 75^{th} (x) percentiles of plasma cortisol levels. The integrated plasma cortisol and ACTH levels were greater ($P < 0.005$) in bCRH than saline administered steers within the C (bars with a and b) and R. However no difference in the integrated plasma cortisol and ACTH response following the sixth regrouping and relocation was observed between the C vs R steers.

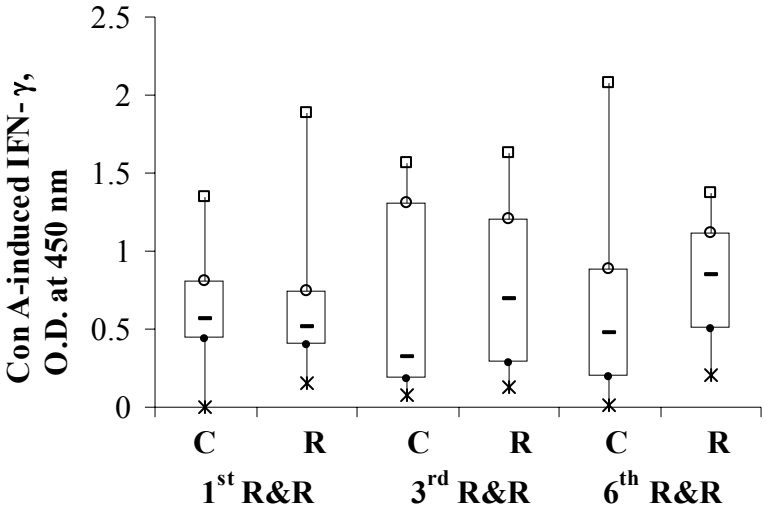

Figure 6. Effect of Concanavalin A (Con A) induced in vitro interferon-γ (IFN-γ) production from cultured whole blood of regrouped (R) and control (C) steers at the first, second and third regrouping and relocation. Data is presented as box-whisker plots, the median (—), upper (○) and lower (●) quartiles, while the whiskers show the 25th(□) and 75th (x) percentiles.

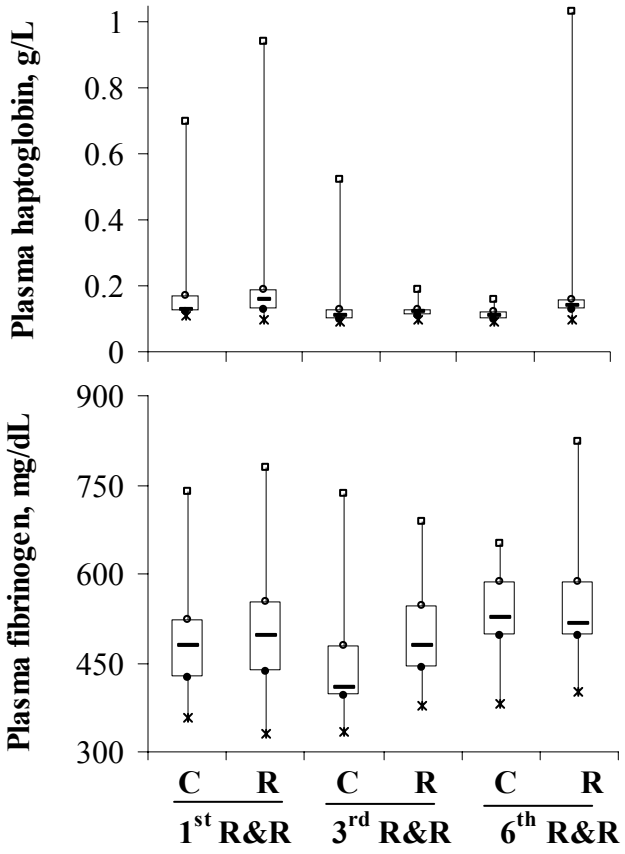

Figure 7. Plasma haptoglobin (upper panel) and fibrinogen (lower panel) of regrouped (R) and control (C) steers at the first, second and third regrouping and relocation. Data is presented as box-whisker plots, the median (—), upper (○) and lower (●) quartiles, while the whiskers show the 25th(□) and 75th (x) percentiles

Table 21. Effect of the first, third and sixth regrouping and relocation (R&R) on the total white blood cell, red blood cell and platelet numbers; mean corpuscular volume, lymphocyte, monocyte and hemoglobin of control and regrouped steers

Items		Control		Regrouped		*P*-value
		Median	Min-max	Median	Min-max	
WBC, x 10^9/L	First R&R	9.2	5.6-15.01	9.2	6.3-16.8	0.333
	Third R&R	9.55	6.7-14.7	10	7.5-13.4	0.515
	Sixth R&R	9.5	6.9-18.2	9.6	5.7-19.1	0.493
RBC, x 10^{12}/L	First R&R	7.15	6.64-7.89	6.82	5.54-8.37	0.307
	Third R&R	6.99	6.15-8.35	7.19	5.88-8.47	0.537
	Sixth R&R	7.05	6.33-7.86	6.85	6.02-8.4	0.769
Platelet, x 10^9/L	First R&R	369	256-598	327	80-537	0.822
	Third R&R	284	202-416	349	270-434	0.387
	Sixth R&R	334	202-539	312	177-480	0.870
MCV, x10^{-15}L	First R&R	35	32-38	35	30-39	0.183
	Third R&R	34.5	31-40	35	30-39	0.663
	Sixth R&R	37[a]	33-39	38[b]	31-40	0.032
Lymphocyte, x 10^9/L	First R&R	8.79	5.54-10.84	8.84	6.13-12.94	0.658
	Third R&R	9.44	6.54-14.34	9.94	7.54-13.24	0.681
	Sixth R&R	8.64[a]	6.14-16.14	9.54[b]	5.74-16.04	0.011
Monocyte, x 10^9/L	First R&R	0.14	0.04-0.64	0.24	0.04-1.53	0.056
	Third R&R	0.19	0.04-0.43	0.14	0.04-1.23	0.189
	Sixth R&R	0.08	0.04-1.33	0.94	0.04-1.24	0.642
Hemoglobin, x g/dL	First R&R	9.25	8.1-10.3	9.1	7.7-10.2	0.145
	Third R&R	9.0	8.7-9.9	9.3	8.3-10.4	0.531
	Sixth R&R	9.4	8.5-10.4	9.4	8.2-10.2	0.232

[a,b] Within rows, medians without a common superscript letter are different ($P < 0.05$).

Table 22. Effect of repeated regrouping and relocation (R&R) on the average daily gain (kg/day) of 14-mo-old control and regrouped steers during an 83 d of experimental period.

	Treatment		
Days	Control (n = 30)	Regrouped (n = 42)	*P*- value
-1 to 13	1.52 ± 0.168	1.50 ± 0.119	0.947
14 to 27	0.47 ± 0.112	0.40 ± 0.124	0.669
28 to 41	0.91 ± 0.131	0.63 ± 0.100	0.086
42 to 55	1.56 ± 0.151	1.55 ± 0.101	0.959
56 to 69	-0.03 ± 0.121	0.12 ± 0.177	0.525
70 to 83	0.96 ± 0.141	0.86 ± 0.246	0.755
-1 to 83	0.90 ± 0.038	0.84 ± 0.033	0.261

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