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HEALTH AND WELFARE

Transportation stress in young bulls: alters expression of neutrophil genes associated with the regulation of apoptosis, tissue remodeling, margination, and bactericidal function

Stress caused by transportation affects many aspects of health, production, and welfare of beef cattle. Incidences of bovine respiratory disease (BRD) or “shipping fever” have been documented and studied since the 1950s, and continue to be the leading cause of death in young cattle and are thought to be associated with an alteration in immune function induced by transportation and increased exposure to pathogens as animals are comingled. Neutrophils are phagocytic innate immune cells that are crucial for acute defense in lungs and other tissues. However, a “neutrophil paradox” exists, whereby, these normally beneficial leukocytes can also contribute to the pathogenesis of infectious diseases if their pro-inflammatory activities are not properly regulated. Because in the normal course of fighting infections the cell can rapidly degranulate and spill harmful proteolytic enzymes and reactive oxygen species, neutrophils can cause excessive damage to otherwise healthy tissue at the infection site thus, exacerbating an already dangerous disease state.

Others have investigated neutrophil phenotypic changes during transportation stress but not gene expression changes. Because transportation elevates the endogenous glucocorticoid, cortisol, it was logical to hypothesize that neutrophils in truck transported cattle would also respond to stress with altered gene expression. The main objective of this study was to examine the expression of candidate neutrophil genes of young cattle subjected to transportation stress. The genes selected for profiling were sensitive to glucocorticoids in other stress models and included Fas, A1, matrix metalloproteinase-9 (MMP-9), L-selectin, bactericidal/permeability-increasing protein (BPI), transforming growth factor- β receptor type III (subsequently referred to as betaglycan), and glucocorticoid receptor α (GR α). β -actin was also examined as a housekeeping gene.

Animals used in this study were 6 Belgian Blue x Friesian bulls, 233 ± 3.0 kg in weight and 282 ± 4 days of age at time of transportation. They were housed, fed and cared for according to accepted management practices at the Research Centre. Bulls had *ad libitum* access to water and grass silage (*in vitro* DM digestibility = 872 g/kg) which was supplemented with 1.5 kg barley/soybean concentrate (CP = 104.6g/kg DM) per animal per day. The transportation study lasted for 6 weeks; groups of 6 bulls were transported at a time with only one bull per group being intensively bled for neutrophil isolation. Bulls to be transported each week were penned together and not mixed with other bulls. They were transported at a stocking density of 0.85 m² for 9 hours on a variety of road conditions, speeds, and traffic. In accordance with European Union regulation, a 45 minute rest stop was observed after 4.5 h during which the animals remained on the truck. Animals were unloaded and returned to their original group pen at the end of the 9 h journey.

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

Briefly, ACD anti-coagulated whole blood was centrifuged at 4°C for 20 minutes at 1000 x g. Plasma, buffy coats, and approximately 2/3 of the red cell pack were aspirated and discarded. The remaining red cell pack was brought to a volume of 25 ml with sterile ice-cold PBS (phosphate-buffered saline) and aliquoted into 4 conical tubes. Each tube was underlaid with 12 ml of 1.084 g/ml Percoll solution (Amersham Biosciences, Piscataway, NJ) and centrifuged at 22°C for 40 minutes at 400 x g to separate mononuclear leukocytes from the neutrophils. The PBS, mononuclear cells, and Percoll were aspirated and discarded, and erythrocytes in the remaining pellet were lysed. Remaining neutrophils were washed in cold PBS and resuspended in a volume of 24 ml to 48 ml of PBS to make a single cell suspension. Isolation time was consistently ≤ 3 h. All materials, solutions, and reagents were sterile; solutions were treated with DEPC (diethylpyrocarbonate) prior to sterilisation.

ACD anti-coagulated blood (1ml) was also set aside for determination of total leukocyte numbers (Beckman Coulter Z1 Particle Counter and ZapOglobin lytic reagent, Beckman Coulter, Fullerton, CA). Thin blood smears were prepared on glass slides, air-dried, and stained using the haematology three-step stain for morphological differentiation of cell types (Accralab, Fisher Scientific Company, L.L.C., Middleton, VA). One hundred leukocytes in three microscopic fields were counted to determine the percentage of each cell type. Neutrophil counts were determined by multiplying the mean percentage of neutrophils in each sample by the total leukocyte count (number of cells/ml of whole blood). Neutrophil purity was verified to be $\geq 95\%$ based on microscopic examination using the Accralab 3-step staining method. Contaminating cells were consistently eosinophils.

Isolated neutrophils were immediately lysed in TriReagent (Molecular Research Center, Cincinnati, OH) at 1×10^7 cells/ml, allowed to incubate at room temperature for 10 minutes, and frozen at -80° C. Total RNA was extracted according to manufacturer's instructions and treated with Promega RQ1 RNase-free DNase (Madison, WI). Quantity and quality were assessed by using an Agilent Technologies 2100 Bioanalyser (Palo Alto, CA).

Standard curves were created for each candidate gene to assess gene expression changes using absolute quantification and using the PCR primers described in Table 57. Briefly, a 290-840 bp length of each gene's cDNA was amplified by PCR with Taq polymerase and accompanying reagents (Invitrogen, Carlsbad, CA), gel purified, and cloned. PCR products were quantitated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and serially diluted to generate a 6-point standard curve (0.01 – 1000 fg/ μ l) of DNA for each gene. These dilutions were included on the same plate as test samples for each real time RT-PCR assay. Cycles to threshold (Ct) were graphed and fit with a line of regression. Using the equation of the line of regression, the actual amount of mRNA in femtograms (fg) per nanogram (ng) of starting cDNA was calculated for test samples.

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

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amplicons used for the standard curves and were synthesised by Operon (Huntsville, AL); see Table 58 for primer sequences.

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

As expected, transportation elicited a classic stress response in the bulls of this study, as evidenced by an acute and pronounced increase in plasma cortisol (Figure 13) and in circulating neutrophil counts (Figure 14) ($P < 0.001$ for both variables). The peak cortisol response (50.64 ± 4.46 ng/ml) was detected at 4.5 h. While neutrophilia was clearly present at 4.5 h, blood neutrophil counts peaked ($7.2 \times 10^6 \pm 9.3 \times 10^5$ cells/ml) at 9.75 h. A weak correlation existed between these two variables ($r^2 = 0.25$; $P = 0.11$).

Plasma cortisol concentrations were elevated at 4.5 and 9.75 h, peaking at 50.64 ng/ml ± 4.46 ng/ml ($P < 0.0001$), confirming that the animals were stressed by transportation. Blood neutrophil counts were elevated between 4.5 and 14.25 h ($P < 0.0001$), reaching a peak over 3-fold higher than -24 h. Fas expression was profoundly down-regulated ($P = 0.02$) by transportation stress, while MMP-9, BPI, and L-selectin were up-regulated ($P = 0.003$, < 0.001 , and $.002$, respectively). All correlations are displayed in Table 59. Significant correlations included those between Fas and neutrophil counts ($R = -0.41$; $P = 0.02$), MMP-9 and neutrophil counts ($R = 0.42$; $P = 0.009$), and BPI and both cortisol and neutrophil counts ($P = 0.004$ and 0.02 , respectively) (Table 49). Changes in betaglycan, GR α , and A1 could not be detected, and no change in the housekeeping gene β -actin was observed. Taken together, these gene expression changes and massively increased neutrophil numbers indicate that the transportation stress scenario may enable these proinflammatory cells to create excessive tissue damage with their longer life, increased proteolytic and bactericidal potential, and increased ability to marginate and migrate to sites of infection, causing a greater risk for severe respiratory disease.

These results confirm an alteration in bovine neutrophil gene expression during transportation stress that is associated with increases in plasma cortisol and circulating neutrophil numbers. Identifying changes in the expression of these inflammatory neutrophil genes involved in regulation of apoptosis, tissue remodeling, margination, and bactericidal function could begin to reveal a possible signature of imbalanced immunocompetence in transportation-stressed cattle. A wider investigation of other genes affected could improve this profile, as well as validation of protein changes for future biomarkers. Findings in the current study imply that neutrophils may be programmed for longer life, increased proteolytic and bactericidal capabilities, and favored migration into the lung and respiratory system in a potentially hyperactive inflammatory response during transportation stress, enabling increased tissue damage and severity of respiratory disease.

Table 57: Standard curve PCR primer sequences

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Length (bp)
Fas	ATTCAGGGAAAACCTGCACA	ATTTCTGGAGAAGGCAATGG	838
A1	CCAGGCAGAAGATGACAG	GGTTACAATCCTGCCCCAGTT	284
MMP-9	CAGACCTTTGAGGGCGAACT	TCGTCTGAAGTGGGCATCTC	296
L-selectin	CCCAACAACAGGAAGAGTAAG	TGCCAGCCAAATGATAAA	711
BPI	CCCTCCAGCCTTACCAG	ATGCAATTCTTGGAGGAAAT	324
Betaglycan	TTGTTGGGTGACTCGT	AAGGATTTAAAACGTGTGGTT	309
GR α	ATCACCAATCAGATACCAAAAT	ATCCTCTCTCTGCAGCACATTTCC	397
β -actin	AAGGCCAACCGTGAGAAGATG	TGCGGTGGACGATGGAG	781

Table 58: Quantitative real-time RT-PCR primer sequences, length and melting temperatures (T_m) of the gene product, and starting cDNA concentrations for each gene's PCR reaction

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Length (bp)	Product T _m (°C)	Starting cDNA (ng)
Fas	TGTAAAGTCAGCTTATACACAGCAGAAGT	GTGGGCTGCCGCCTATG	104	81	2.5
A1	ACTGCCAGAACAATATTCAACCAA	GGTTACAATCCTGCCCCAGTT	78	76	20
MMP-9	CGCACGACATCTTTCAGTACCA	GGAACCTCACGCGCCAGTAG	74	78	40
L-selectin	ACGGGAAAAAAGGATTACTATGGA	GCCTATAGTTGCATATGTATCAAATTTTCA	144	74	2.5
BPI	TTCAGAAATGATCCAAACATGAAAC	GCCCTTGGAAGAAACAATTCC	81	75	10
Betaglycan	TGAGGGTAAAAAGAGTACCCTGAAA	CACGTTGAACAGAGAAAAAGAGTACAA	80	78	10
GR α	TGTGGTTTAAAGAGGGCCAAGA	TTCTACGTTCCCATCACTGAAAAG	74	78	2.5
β -actin	AAGGCCAACCGTGAGAAGATG	TGCGGTGGACGATGGAG	74	82	2.5

Table 59: Pearson correlation coefficients (R^2) and P-values for each gene, respective, to plasma cortisol and neutrophil counts

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

* represents significant correlations ($P \leq 0.05$)

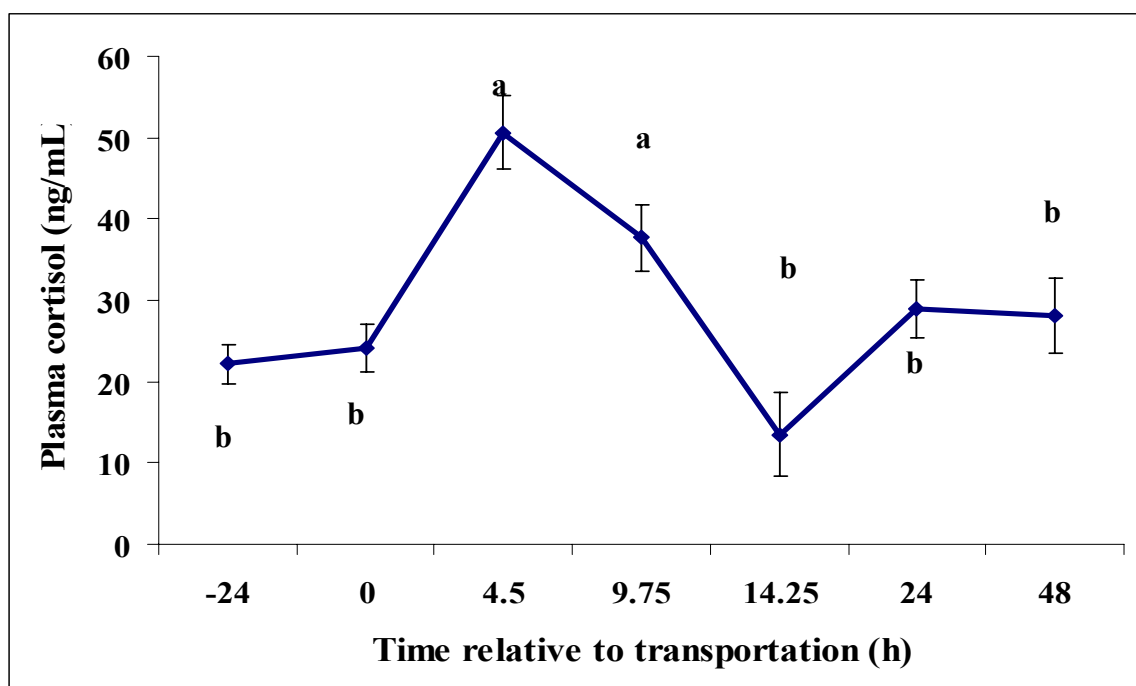


Figure 13. Effect of transportation time on plasma cortisol concentrations. Differences between time points are represented by different letters.

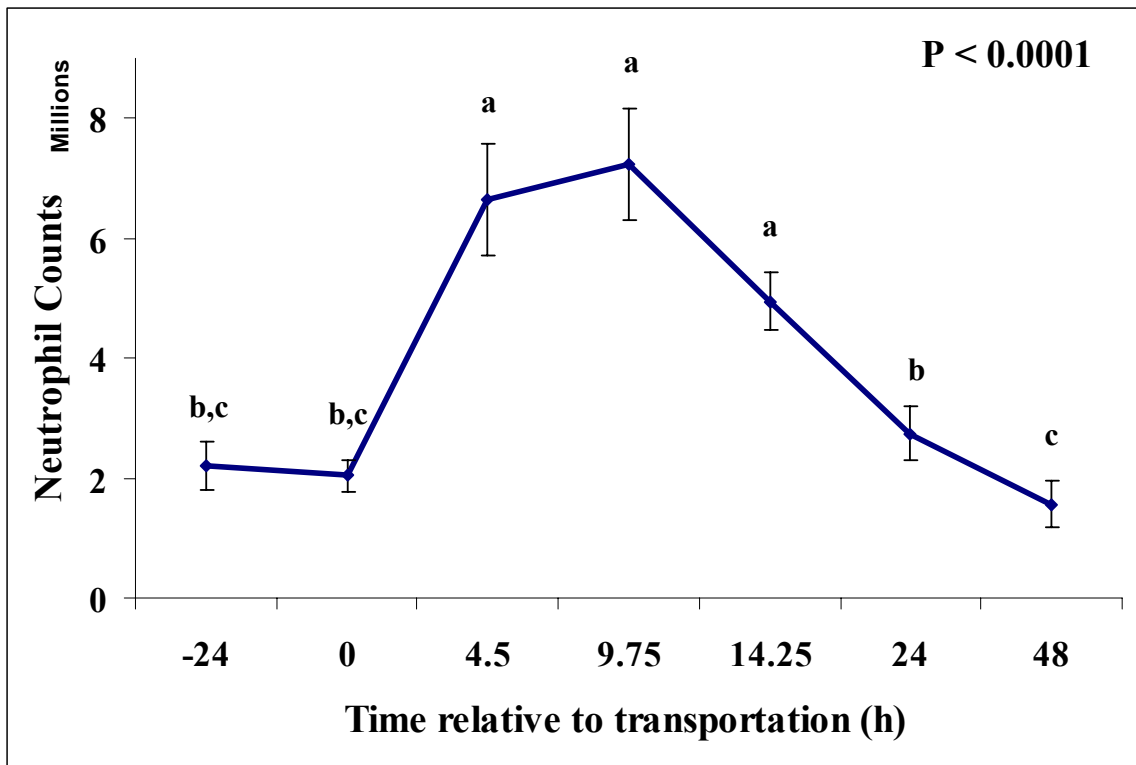


Figure 14. Effect of transportation on time on neutrophil counts. Differences between time points relative to transportation ($P < 0.05$) are represented by different letters.

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Gene expression profile of bovine neutrophils during transportation stress

Transportation acts as a stressor for cattle in that it activates the hypothalamic-pituitary-adrenal (HPA) axis and results in dramatically increased plasma glucocorticoid (cortisol) concentrations. Neutrophils are also known targets of glucocorticoids, which alter the cells' gene and protein expression and phenotype. Transportation stress has also been associated with changes in the expression of genes important in neutrophil-mediated immunity, in particular, key genes that regulate their apoptosis, adhesion, tissue remodeling, and bactericidal activity. Given the prominence of neutrophil's as a target of stress and in innate immune responses during bacterial infections, it is conceivable that measurements of altered neutrophil gene expression during stress may be effective indicators of altered immune status and thus susceptibility to infectious diseases. Thus, a broader investigation of the neutrophil transcriptome during transportation stress was performed in the current study in order to elucidate possible functional themes and a signature of changes that may illuminate future biomarkers of disease susceptibility. To follow through with this two-pronged approach, a two-color cDNA microarray experiment was designed using 42 BOvine Total Leukocyte (BOTL-5) arrays that were developed at Michigan State University Center for Animal Functional Genomics (CAFG).

The current study investigated for the first time the transcriptome response of blood neutrophils from beef bull calves before, during, and after truck transportation stress. The investigation had two objectives: 1), to identify themes in neutrophil gene expression changes and 2), to discover candidate biomarkers potentially related to the risk of severe respiratory diseases following stress. Genes involved in immune response, apoptosis, and wound healing were confirmed as differentially expressed in this study, and mRNA abundance changes occurred at the same times that elevated plasma cortisol and circulating neutrophil counts were observed. Expression changes in several genes was observed with as yet unknown function; these too may prove to be useful in determining innate immune status and disease susceptibility following transportation stress.

Animals used in this study were 6 Belgian Blue x Friesian bulls, 233 ± 3.0 kg in weight and 282 ± 4 days of age at time of transportation. Blood samples were collected from 6 bulls at the following time points relative to commencement of transportation at 0 h: -24, 0, 4.5, 9.75, 14.25, 24, and 48 h. Bleeding at -24, 0, 14.25, 24, and 48 h occurred in a handling chute in the cattle holding yard, while bleeding at 4.5 and 9.75 h occurred in a handling chute on the truck. Blood (300 ml/sample) was collected by jugular venipuncture using 18-gauge 2.5 cm multiple draw needles into a series of 30 ml syringes pre-coated with the anticoagulant ACD (acid citrate dextrose). Each syringe was expelled into a 50-ml conical tube containing 4.0 ml of ice cold ACD. These tubes were immediately placed on ice and taken to the laboratory for further processing.

Isolated neutrophils suspended in PBS were pelleted by centrifugation ($300 \times g$ for 10 min at $4^\circ C$), and PBS was aspirated. Cell pellets were immediately lysed in TriReagent (Molecular Research Center, Cincinnati, OH) at 1×10^7 cells/ml, incubated at room temperature for 10 min, and flash frozen at $-80^\circ C$. These frozen samples were then shipped to Michigan State University for extraction of RNA and to perform the microarray experiment. Briefly, total RNA was extracted according to TriReagent manufacturer's instructions and treated with Promega RQ1 RNase-free DNase (Madison, WI) to remove any contaminating DNA. Quantity and quality of the RNA samples were assessed using an Agilent Technologies 2100 Bioanalyzer (Palo Alto, CA). All RNA samples were deemed to be of good quality as evidenced by electrophotograms that were identical to those presented for human neutrophils and for bovine neutrophils.

Bovine Total Leukocyte (BOTL) arrays developed at the Michigan State University Center for Animal Functional Genomics (CAFG) were used. The 5th generation (BOTL-5) of these arrays was used in the current experiment. This microarray contains approximately 1,056

unique genes spotted in duplicate. Arrays were hybridised in a connected loop design with each loop representing one bull, as shown in Figure 15. Samples from each time point were represented twice within each loop, labeled once with a green fluorescent dye (Alexa 555, Invitrogen, Carlsbad, CA) and once with a red fluorescent dye (Alexa 647, Invitrogen). The -24 h samples were connected directly to 4.5 and 9.75 h samples on every loop to reflect times of peak plasma cortisol responses, blood neutrophil counts, and changed expression of candidate genes. The remaining samples were randomly placed in the loop. The direction of the dye was also reversed in 3 of the 6 loops.

cDNA synthesis was performed (46° C for 180 min) using the Superscript Plus Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA) and dye coupled with Alexa Fluor dyes 555 and 647 according to the manufacturer's instructions. Reactions were spiked with 1.25 ng of lambda Q synthetic mRNA, which served as an internal control. Hybridisation was performed as in Madsen et al. (2004). Briefly, cDNA was incubated in a 70° C water bath, while arrays were incubated at 75° C prior to sample loading. Arrays were hybridised for 18 h in a GeneTAC Hybridisation Station (Genomic Solutions, Ann Arbor, MI) under the following conditions: 65° C for 3 h, 55° C for 3 h, and then 50° C for 12 h. Hybridisation was followed by two medium-stringency washes at 50° C, two high-stringency washes at 42° C, and two washes with a postwash buffer at 42° C (all buffers from Genomic Solutions). Arrays were immediately rinsed in 2X SSC and then in ddH₂O and centrifuged in a 50 ml conical tube at 500 x g for 5 minutes to dry. Arrays were then immediately scanned with a GeneTAC LS IV scanner (Genomic Solutions) and accompanying software (version 3.1). Spot aligning was performed with the MolecularWare DigitalGENOME software (Cambridge, MA), and total spot intensities were exported as Microsoft Excel spreadsheets.

Total spot intensities were subjected to LOESS (locally-weighted regression and smoothing scatter plot) normalisation to minimise potential dye biases. First, M/A scatter plots were constructed whereby log₂ intensity ratios, $M = \log_2(\text{Alexa } 647/555)$, were plotted against mean log₂ intensities, $A = (\log_2 \text{Alexa } 555 + \log_2 \text{Alexa } 647)/2$, for each array using a procedure described by Yang et al. (2002). M/A plots were examined before and after the LOESS procedure was performed by SAS. Normalised data were back-transformed before being statistically analysed for significance. The LOESS-transformed log intensities were analysed statistically using a two-step mixed model. In this approach, the first modeling step normalises data across all genes and arrays, while the second step analyses gene-specific data to test the effect of interest, time relative to transportation. The model employed for the first step was as such:

$$\log(y_{ijklmno}) = \mu + T_i + D_j + A_l + B_m + R_n + A(B)_{kl} + \epsilon_{ijklmno}$$

in which $y_{ijklmno}$ represents LOESS-normalised fluorescent intensities, μ is an overall mean intensity, T_i is the fixed effect of time i relative to initiation of transportation, D_j is the fixed effect of dye j , A_l is the random effect of array l , B_m is the random effect of bull (or loop) m , R_n is the random effect of array print batch n , $A(B)_{lm}$ is the random effect of array l within bull m , and $\epsilon_{ijklmno}$ is a stochastic error (assumed to be normally distributed with mean 0 and variance σ^2).

The model for the second step was as such:

$$\hat{\epsilon}_{ijklmopqr} = \mu + T_{ki} + D_{kj} + A_{kl} + B_{km} + R_{kn} + A(B)_{klm} + P(A)_{klp} + S(P)_{kpq} + e_{ijklmopqr}$$

in which all effects have the same definition as in the first step of the model, but now they are specific for each gene and carry an additional index k . Also, $P(A)_{klp}$ is the random effect of patch p with array l , and $S(P)_{kpq}$ is the random effect of spot q with patch p . The error term $e_{ijklmopqr}$ were assumed to have independent normal distributions using gene-specific variances σ_k^2 .

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The analyses were performed using the MIXED procedure of SAS. Pair-wise comparisons between -24 h and 4.5, 9.75, and/or 14.25 h relative to initiation of transportation were considered as these times yielded significant changes in plasma cortisol concentration, blood neutrophil count, and expression of several candidate genes. Gene expressions were declared significantly differentially expressed across sample times when $P \leq 0.05$.

Genes whose expression was significantly altered by transportation stress were identified by their “BOTL” number as spotted on the BOTL-5 arrays by MSU’s Center for Animal Functional Genomics (CAFG). The CAFG’s interactive website (www.cafg.msu.edu) was then used to determine TIGR cluster numbers within the Bos Taurus Gene Index (www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=cattle) and putative gene names and functions. Each gene was assigned an ontological grouping after an extensive search of the PubMed (www.ncbi.nlm.nih.gov), UniProt (www.uniprot.org), and GO (www.godatabase.org) databases. Information gleaned from these databases searches was used to group the differentially expressed genes into ontological clusters as shown in Figure 16.

The expression of 88 genes was significantly ($P \leq 0.05$) changed by transportation stress, as highlighted by differences in mRNA abundance between -24 h and 4.5, 9.75, and/or 14.25 h (Table 60 and Figure 16). Seventeen of these genes were differentially expressed at more than one time point. While realising that many genes are multifunctional and could justifiably be categorised into ontological clusters in a number of ways, an exhaustive database and literature search was preferred and grouped the 88 differentially expressed genes into ontological clusters that most reflected neutrophils as defenders and also damagers of infected tissue. The aim was to further identify themes or pathways of neutrophil genes affected by transportation stress in cattle. The ontological clusters and number of genes included in each are shown in Figure 16 and were as follows: signal transduction (16), immune response (15), protein trafficking (7), apoptosis (6), transcriptional regulation (6), ribosomal (6), wound healing (5), mitochondrial (3), metabolic enzymes (2), translational regulation (2), ubiquitin pathways (2), protein activation (1), RNA processing (1), steroid (1), and cell structure (1). The identification and/or function of 14 affected genes was not available in the databases searched and were thus grouped in a cluster called “unknown.”

Because of the key roles of neutrophils in defense and damage to lung tissue during respiratory disease outbreaks following transportation stress, genes from the immune response, apoptosis, and wound healing clusters were chosen to validate by qRT-PCR. Four genes from the unknown cluster, were also selected, because these genes were highly differentially expressed at more than one time point relative to the initiation of transportation. A total of 31 genes were subjected to qRT-PCR validation, and the gene expression changes of 14 of these genes were confirmed or considered at the $P < 0.10$ level. Immune response genes included interleukin-8 (IL-8) precursor (increased; $P = 0.001$), erythropoietin precursor (increased, $P = 0.005$), interferon- γ receptor (IFN γ R) (decreased, $P = 0.02$), eotaxin-2 like protein (increased, $P = 0.04$), protein-tyrosine phosphatase non-receptor type substrate1 precursor (SHPS-1) (increased, $P = 0.04$), semaphorin 4A (increased, $P = 0.06$), and intercellular adhesion molecule-3 (ICAM-3) (increased, $P = 0.06$). Validated genes in the apoptosis cluster included cyclin-dependent kinase inhibitor 1 (p21) (increased, $P = 0.009$) and caspase 13 (decreased, $P = 0.05$). Platelet-derived growth factor α (PDGF α) (decreased; $P = 0.004$) and peroxisome proliferators activated receptor γ (PPAR- γ) (increased; $P = 0.09$) were the confirmed wound healing genes. Expression changes of 3 of the 4 unknown genes were increased: Brain protein I3 ($P = 0.02$), *Bos taurus* hypothetical proteins LOC515425 ($P = 0.04$), and *Bos taurus* hypothetical protein LOC615942 ($P = 0.05$). Expression profiles of these validated genes are shown in Figures 17-21.

A gene expression function associated with immune response functions was also studied. Particularly notable were the pronounced up regulations of genes encoding master regulatory proteins involved in neutrophil adhesion, chemotaxis, and activation, including IL-8, eotaxin-2-like protein, ICAM-3, semaphorin 4A, and erythropoietin.

Interleukin-8 is a potent neutrophil chemoattractant that also activates the cells for degranulation, adhesion, and bactericidal activities. In addition, IL-8 is understood to be linked to the expression and function of key inflammation regulatory proteins in neutrophils, such as pro-apoptotic and the gelatinase MMP-9.

The transportation stress study also studied the effect on neutrophil apoptosis regulating genes. In particular, transportation stress down regulated the expression of caspase-13 while up regulating p21 expression by over 800-fold. Caspase-13 is an inflammatory mediator that is activated by caspase-8, which is pivotal in apoptotic pathways initiated by members of the tumor necrosis factor (TNF) family, including Fas. Its down-regulation, concurrent with Fas down regulation during transportation stress and neutrophilia suggests that there would be a significant delay in neutrophil apoptosis around the time of shipping.

A third theme pursued in this study was wound healing as represented by changes in neutrophil gene expression of platelet-derived growth factor α (PDGF α) and peroxisome proliferator activated receptor γ (PPAR- γ). In addition to their bactericidal functions at the site of infection, neutrophils perform key tissue remodeling and wound healing roles.

In this study, we also observed expression changes in several neutrophil genes with as yet unknown functions. Three of these genes showed profound up regulation during the peak stress response. Because one of the goals of this study was to identify a signature of gene expression profile associated with transportation stress, these unknown genes along with the reported immune response, apoptosis, and wound healing genes should be considered as potential biomarkers of stress, immune status, and disease susceptibility in future studies.

In conclusion, we have investigated for the first time transcriptome changes in bovine neutrophils following transportation stress. We have confirmed expression changes in genes involved in immune response, apoptosis, and wound healing, as well as a signature of changes in genes whose functions are as yet unknown. With these new discoveries, a greater understanding of neutrophil function following transportation stress as well as identification of biomarkers of respiratory disease susceptibility may allow the development of preventative therapies to combat the disease in transported cattle.

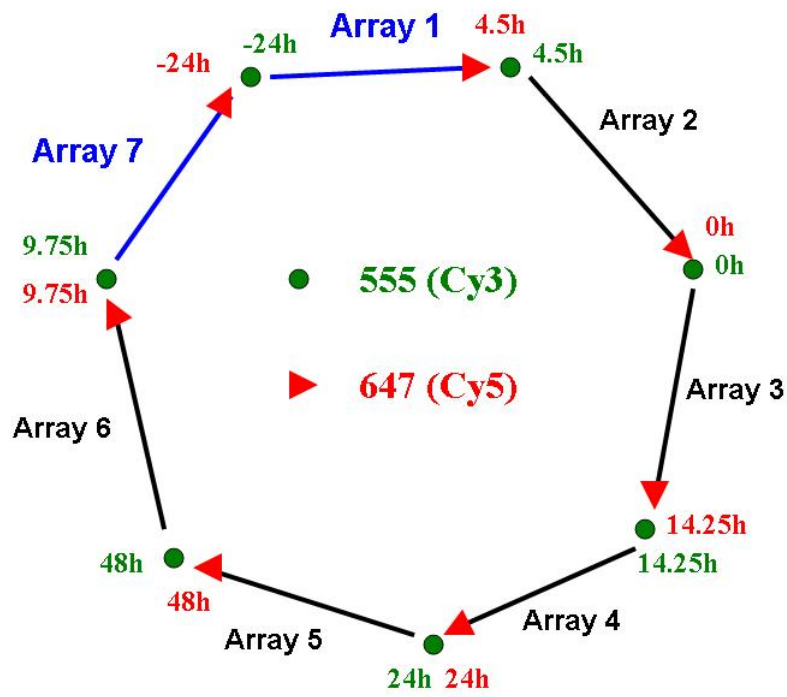


Figure 15. Microarray loop design.

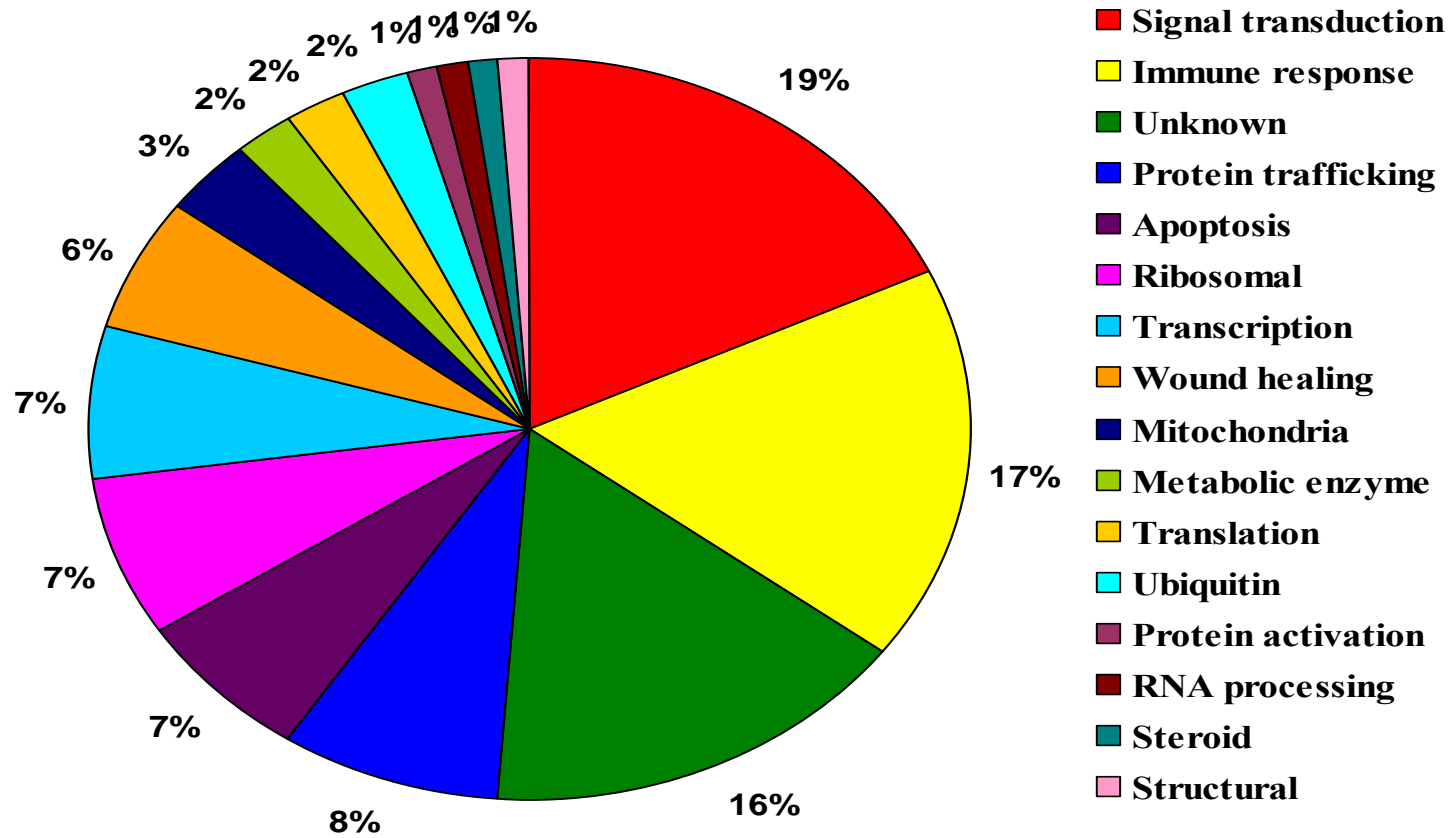


Figure 16. Transportation stress alters the expression of 88 neutrophil genes, clustered into 16 ontological groups.

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Table 60: Gene names, identification numbers, functions, and P-values for the 88 genes differentially expressed between -24 h and 4.5, 9.75, and/or 14.25 h relative to initiation of transportation. * indicates genes chosen for validation by Q-RT-PCR

Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Cbp/p300-interacting transactivator 2; MSG-related protein 1	Regulation of transcription from Pol II promoter	BOTL0100008_A06	TC225633	Transcriptional regulation	0.00001
Human ribosomal DNA complete repeating unit †	Unknown	BOTL0400015_PCR	TC258301	Unknown	0.0002
Non-receptor tyrosine kinase JAK1	Protein-tyrosine kinase activity, Janus kinase activity	BOTL0100004XF08R	TC245135	Signal transduction	0.0004
Tyrosine-protein kinase receptor EPH #	Kinase activity	BOTL0400007_PCR BOTL0400212_PCR	TC231293	Signal transduction	0.00063 0.043
Bos taurus mitochondrial DNA complete sequence †	Mitochondrial	BOTL0100002XF06R	TC236722	Mitochondrial	0.0006
Inhibitor of apoptosis protein-1*†	Negative regulation of apoptosis	BOTL0100009_H08	TC228230	Apoptosis	0.0007
Serine/threonine protein phosphatase 6; PP6 †	Phosphatase activity	BOTL0100001XE01R	TC227460	Signal transduction	0.0007
Bos taurus hypothetical LOC515425*†	Unknown	BOTL0100003XA05R	TC251467	Unknown	0.0008
Homo sapiens abhydrolase domain containing 2	Unknown	BOTL0100013_A04	TC225778	Unknown	0.001
Sad1/unc-84-like protein 2 †	Positioning of nucleus, nucleus-centrosome connection, spindle pole	BOTL0100013_B08	TC229885	Structural	0.003
RPL6 protein †	Structural constituent of ribosome	BOTL0100012_G11	TC262065	Ribosomal	0.003

Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Wee1 tyrosine kinase	Protein-tyrosine kinase activity	BOTL0400084_PCR	TC259191	Signal transduction	0.003
Elongation factor 1 A-1; Elongation factor Tu; EF-Tu †	Translation elongation factor activity	BOTL0100002XF08R	TC223210	Translational regulation	0.004
Transforming growth factor- β receptor type I; TGF- β RI*	TGF- β signaling, anti-fibrinogenesis	BOTL0400063_PCR	TC232881	Wound healing	0.005
C-X-C chemokine receptor type 4; CXCR4*	Regulation of cell migration	BOTL0400164_PCR	TC247479	Immune response	0.006
Tyrosine-protein kinase LYN †	Protein-tyrosine kinase activity, intracellular signaling cascade	BOTL0100006XH06R	TC225577	Signal transduction	0.00604
C alpha catalytic subunit of the bovine cAMP-dependent protein kinase	Kinase activity	BOTL0100008_F07	TC278964	Signal transduction	0.007
Human 18S ribosomal RNA †	Ribosomal	BOTL0400511_PCR	TC275263	Ribosomal	0.007
Protein tyrosine phosphatase, non-receptor type 2, isoform 2	Protein tyrosine phosphatase activity	BOTL0100004XC08R	TC248647	Signal transduction	0.008
Proprotein convertase subtilisin/kexin type 6 precursor	Proteolysis and peptidolysis, serine-type endopeptidase activity	BOTL0400610_PCR	TC251477	Protein activation	0.008
TAR DNA-binding protein-43; TDP-43	Splicing inhibition, nuclear ribonucleoprotein	BOTL0100007_C10	TC245192	RNA processing	0.009
Cyclin-dependent kinase 5 activator 1 precursor †	Protein kinase activity	BOTL0100002XE10R	TC264541	Signal transduction	0.009
Tat interactive protein	Regulation of transcription	BOTL0400425_PCR	TC245256	Transcriptional regulation	0.009

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Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Brain protein I3; Bri3 *#	Unknown	BOTL0100009_F05 BOTL0100008_H06	TC245047	Unknown	0.012 0.034
40S ribosomal protein S2 †	Protein biosynthesis, structural constituent of ribosome	BOTL0100002XF03 R	TC242665	Ribosomal	0.012
Strawberry notch homolog, Sno	Notch signaling pathway	BOTL0100003XE03 R	TC225827	Signal transduction	0.013
Homo sapiens ubiquitin-conjugating enzyme E2G 1	Ubiquitin ligase	BOTL0100011_D10	TC253248	Ubiquitin pathway	0.013
Beta-2-microglobulin precursor; Lactollin*	Constituent of MHC Class I molecule	BOTL0100001XE12 R	TC243013	Immune response	0.015
Interleukin-8; IL-8; CXCL8*	Neutrophil chemotaxis, inflammatory and immune response	BOTL0400317_PCR	TC227230	Immune response	0.015
Human DNA sequence from clone RP3-331H24 on chromosome 6	Unknown	BOTL0100003XD06 R	TC240574	Unknown	0.015
Bos taurus hypothetical protein LOC615942 *†	Unknown	BOTL0100002XH11 R	TC247791	Unknown	0.016
Stromelysin 1 PDGF-responsive element-binding protein; AR1; TCF20*	Transcription factor activity	BOTL0400424_PCR	TC252936	Transcriptional regulation	0.017
Growth factor receptor-bound protein 2; SH2/SH3 adapter GRB2	G-protein coupled receptor protein signaling pathway, cell-cell signaling	BOTL0100004XF04 R	TC263427	Signal transduction	0.019
Mitochondrial import receptor subunit TOM20	Protein targeting to mitochondrion, protein translocase activity	BOTL0100010_A03	TC385929	Mitochondrial	0.019

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Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Cyclin-dependent kinase inhibitor 1; p21*	Negative regulation of apoptosis, cell cycle arrest	BOTL0400469_PCR	TC241141	Apoptosis	0.019
Methylosome protein 50; MEP50	binds histone H2A, mediates transcriptional repression	BOTL0100008_E04	TC226498	Transcriptional regulation	0.02
RAF proto-oncogene serine/ threonine-protein kinase; Raf-1	Intracellular signaling cascade, protein amino acid phosphorylation	BOTL0400030_PCR	TC243392	Signal transduction	0.02
CDC10 protein	Vesicle targeting	BOTL0100001XE05R	TC244574	Protein trafficking	0.02
Erythropoietin precursor; EPO*	Erythropoiesis, cell-cell signaling, stress response	BOTL0400219_PCR	TC256299	Immune response	0.022
Peroxisome proliferator activated receptor γ ; PPAR- γ *	Anti-inflammatory, transcription factor activity	BOTL0100002XF07R	TC291290	Wound healing	0.022
Secretory granule proteoglycan core protein precursor; Serglycin*	Sorting of proteins in neutrophil granules	BOTL0100003XE07R	TC276510	Immune response	0.023
Adhesion regulating molecule 1 precursor; ADRM-1*	Cell adhesion, protein binding	BOTL0100012_H07	TC224168	Immune response	0.023
Interleukin-16; IL-16*	Associated with a neutrophilic infiltration, chemotactic activity	BOTL0400314_PCR	TC240761	Immune response	0.025
Amyloid-like protein 1 precursor; APLP-1*†	Aggregation of proteins, apoptosis	BOTL0400429_PCR	TC290958	Apoptosis	0.025
Intercellular adhesion molecule-3 precursor; ICAM-3*	Cell-cell adhesion, integrin binding	BOTL0400304_PCR	TC290460	Immune response	0.025
Xpo1 protein	Protein transporter activity, protein-nucleus import	BOTL0400595_PCR	TC256597	Protein trafficking	0.025
Mitochondrial uncoupling protein 2; UCP 2	Proton transport, energy pathways, mitochondrial transport	BOTL0100001XG10R	TC243478	Mitochondria 1	0.025

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Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Translocating chain-associating membrane (TRAM) protein	Signal recognition particle receptor	BOTL0100008_G03	TC251726	Protein trafficking	0.025
Semaphorin 4A precursor; Sema4A *	Stimulates T-cells, plexin and neuropilin binding	BOTL0100003XE04R	TC223804	Immune response	0.026
Bos taurus similar to CG33556-PA; LOC538493	Unknown	BOTL0100006XD03R	TC260389	Unknown	0.026
Fibrinogen-like protein 2; Flg2 *	Pathogenesis of fibrin deposition, prothrombinase	BOTL0100001XC01R	TC247187	Wound healing	0.026
Eotaxin-2-like protein; CCL24*	Cell-cell signaling, chemotaxis, inflammatory and immune response	BOTL0400598_PCR	TC229379	Immune response	0.026
Syntaxin 5	Intracellular protein transport, vesicle-mediated transport	BOTL0100013_F09	TC225459	Protein trafficking	0.026
Steroidogenic factor 1; STF-1	Steroid hormone receptor activity, transcriptional activator activity	BOTL0400471_PCR	TC230899	Steroid	0.026
GRAF protein	GTPase activating protein	BOTL0400488_PCR	TC253924	Signal transduction	0.027
Nuclear envelope membrane protein	Localized to nuclear membrane	BOTL0100007_C06	TC245085	Unknown	0.028
Thyroid receptor interacting protein 8; TRIP-8; Jumonji domain containing protein 1C	Hormone-dependent transcriptional activation, histone demethylase	BOTL0100013_C01	TC228702	Transcriptional regulation	0.028
Platelet-derived growth factor α ; PDGF α *	Pathogenesis of fibrosis, growth factor activity, angiogenesis	BOTL0400542_PCR	TC227506	Wound healing	0.028
Homo sapiens Wiskott-Aldrich syndrome protein interacting protein; WASPIP	Signal transduction	BOTL0100004XH07R	TC232833	Signal transduction	0.029

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Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Homo sapiens hypothetical protein DKFZP434I0714	Unknown	BOTL0100009_H04	TC238899	Unknown	0.029
Phosphoglycerate kinase; PGK-1 protein	Gluconeogenesis, glycolysis	BOTL0100012_F07	TC262774	Metabolic enzyme	0.029
TRK-fused gene/anaplastic lymphoma kinase (Ki-1) fusion protein long form; TFG protein	Signal transduction	BOTL0100004XG10R	TC242510	Signal transduction	0.03
TNF receptor associated factor 6; TRAF6*	T-helper 1 type immune response, signal transduction, apoptosis	BOTL0400653_PCR	TC246905	Apoptosis	0.03
Bos taurus BAC CH240-36112	Unknown	BOTL0100002XF12R	TC250087	Unknown	0.03
Bos taurus similar to CG6597-PA; LOC508759*†	Unknown	BOTL0100003XG01R	TC230701	Unknown	0.032
Bos taurus similar to FGFR1 oncogene partner 2	Centrosomal protein, involved in microtubule dynamics and organization	BOTL0100002XH09R	TC259865	Protein trafficking	0.032
Zinc-finger protein DZIPl1; Homo sapiens DAZ interacting protein 1	Unknown	BOTL0100011_C08	TC252937	Unknown	0.032
Ribosomal protein †	Ribosomal	BOTL0100002XG01R	TC262522	Ribosomal	0.033
40S ribosomal protein p40 (C10 protein)	Ribosomal	BOTL0100002XD08R	TC262451	Ribosomal	0.034
Ring finger protein 149	Ubiquitin-protein ligase activity, proteolysis and peptidolysis	BOTL0100006XG02R	TC245450	Ubiquitin pathway	0.034
Manganese superoxide dismutase*	Superoxide dismutase activity, metal ion binding	BOTL0100002XG05R	TC262121	Immune response	0.036

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Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Bcl-2-related protein A1*	Negative regulation of apoptosis	BOTL0400057_PCR	TC226055	Apoptosis	0.037
Methionyl-tRNA synthetase; MetRS	Methionine-tRNA ligase activity, protein biosynthesis	BOTL0400596_PCR	TC276399	Translation	0.037
CCAAT/enhancer binding protein β ; C/EBP β *	Transcription factor activity, inflammatory and immune response	BOTL0100013_E09	TC245257	Immune response	0.039
Transmembrane emp24 domain-containing protein 7 precursor	Intracellular protein transport	BOTL0100013_D05	TC247560	Protein trafficking	0.04
Protein-tyrosine phosphatase non-receptor type substrate 1 precursor; SHPS-1*	Negative regulation of immune cells	BOTL0400418_PCR	TC246452	Immune response	0.04
Interferon- γ receptor alpha chain precursor; IFN- γ R*	Response to virus and pathogenic bacteria, signal transduction	BOTL0400305_PCR	TC288112	Immune response	0.04
Caspase-13 precursor; Evolutionarily related interleukin-1beta converting enzyme; ERICE*	Induction of apoptosis	BOTL0400409_PCR	TC301774	Apoptosis	0.042
60S ribosomal protein L12	Protein biosynthesis, ribosome biogenesis, ribosomal large subunit assembly and maintenance	BOTL0100002XE08R	TC243106	Ribosomal	0.043
Acyl-CoA synthetase	Lipid biosynthesis, fatty acid degradation	BOTL0100008_F04	TC221480	Metabolic enzyme	0.044
Guanine nucleotide-binding protein beta subunit-like protein; Receptor of activated protein kinase C 1; RACK1	G-protein coupled receptor protein signaling pathway	BOTL0100007_E07	TC262565	Signal transduction	0.044
Bone morphogenetic protein 4 precursor; BMP-4*	Homeostasis and repair of various tissues, cell growth and maintenance	BOTL0400108_PCR	TC249563	Wound healing	0.047

Gene Name	Function	BOTL number	TIGR number	Ontological Cluster	P-value
Smad interacting protein 1; SMADIP1	Transcriptional repressor activity	BOTL0400028_PCR	TC234944	Transcriptional regulation	0.047
Integrin $\alpha 4$ *	Chemotaxis, cell adhesion, cell migration	BOTL0100004XG08R	TC267276	Immune response	0.048
Unknown	Unknown	BOTL0400573_PCR	Not available	Unknown	0.048
Signal recognition particle 19 kDa protein; SRP19	Signal recognition particle, protein targeting, cotranslational membrane targeting	BOTL0100009_F07	TC246030	Protein trafficking	0.048

* Genes that were chosen for validation by qRT-PCR.

† Genes that were differentially expressed at more than one time point relative to initiation of transportation.

Genes that were identified at more than one location on the BOTL-5 array and yielded 2 separate BOTL numbers. These numbers both corresponded to one TIGR number.

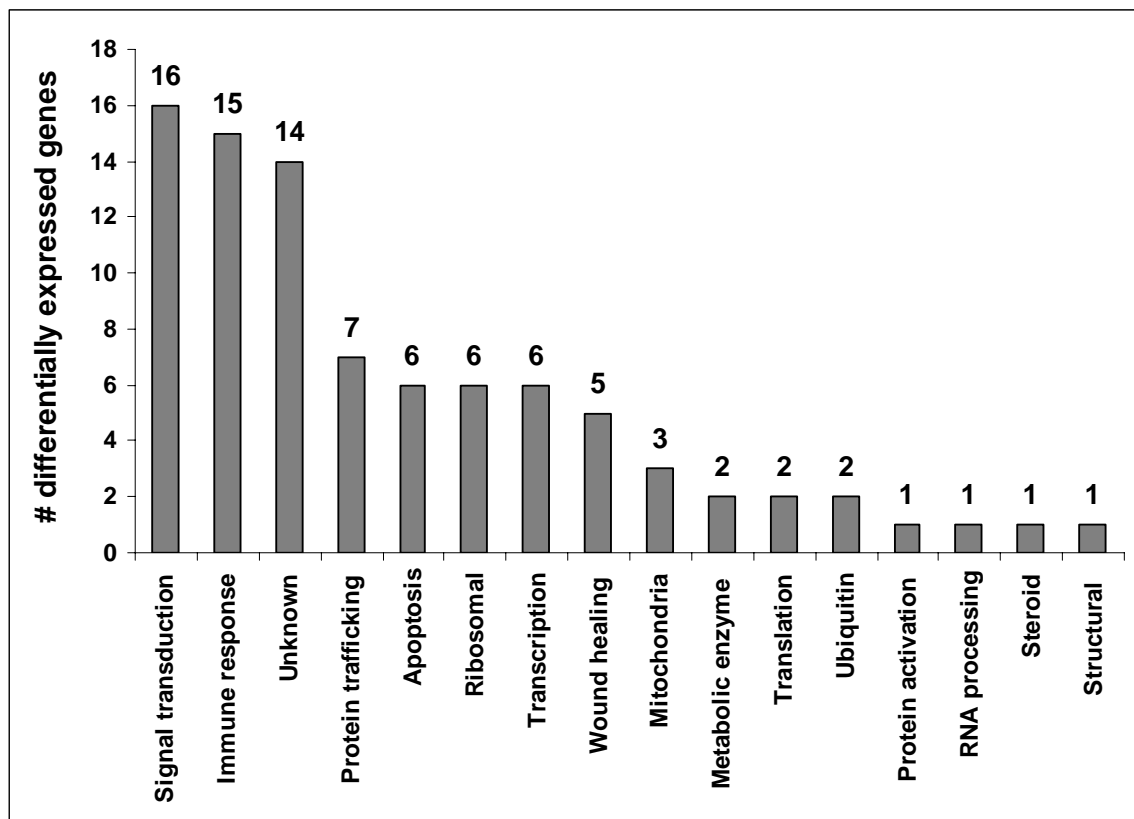


Figure 17. Ontological clusters of neutrophil genes significantly affected by transportation stress between -24 h and 4.5, 9.75, and/or 14.25 h.

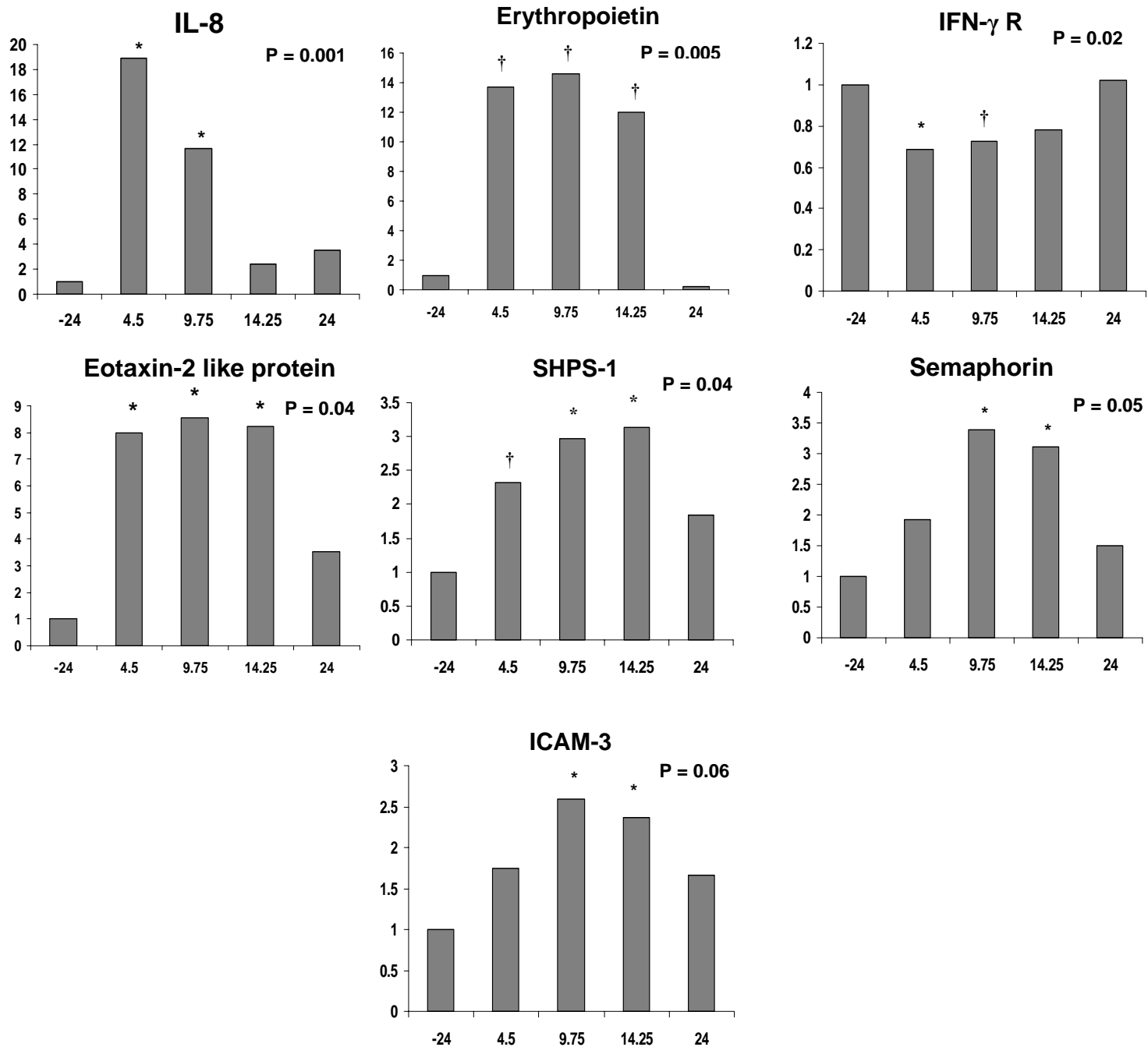


Figure 18. Expression profiles of genes involved in neutrophil immune response and affected by transportation stress. * denotes $P < 0.05$; † denotes $0.05 < P < 0.10$ as different from -24 h pre-transportation.

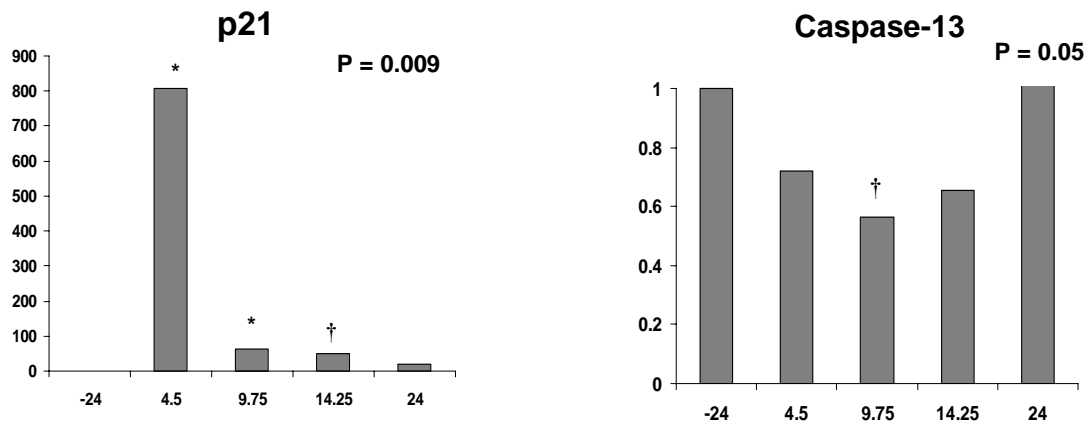


Figure 19. Expression profiles of genes involved in apoptosis and affected by transportation stress. * denotes $P < 0.05$; † denotes $0.05 < P < 0.10$ as different from – 24 h pre-transportation.

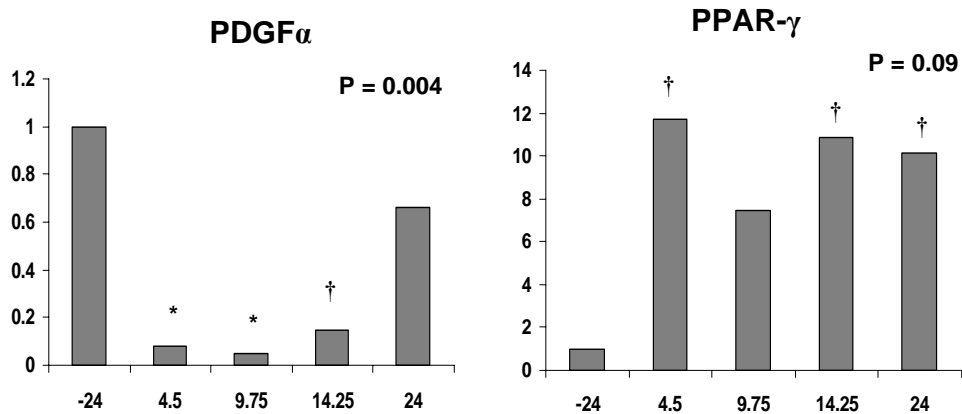


Figure 20. Expression profiles of genes involved in wound healing and affected by transportation stress. * denotes $P < 0.05$; † denotes $0.05 < P < 0.10$ as different from – 24 h pre-transportation.

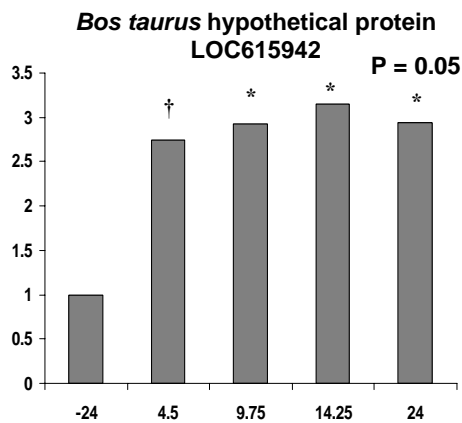
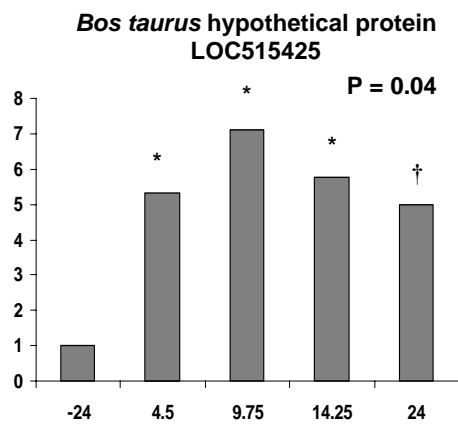
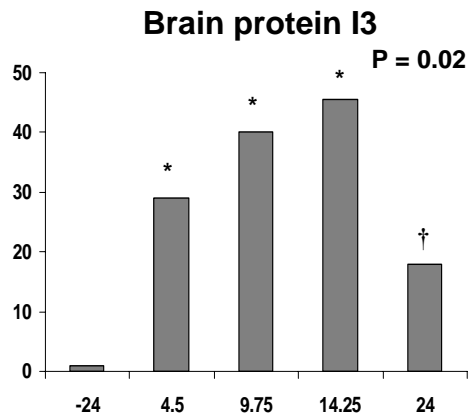


Figure 21. Expression profiles of genes whose function is as yet unknown. * denotes $P < 0.05$; † denotes $0.05 < P < 0.10$ as different from -24 h pre-transportation.

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Effect of floor type on the welfare and performance of finishing beef steers

There is limited scientific evidence regarding the effects of different floor types on animal well-being. Previous studies have measured the performance and welfare of finishing cattle in response to a range of space allowances with concrete slatted floors. This study investigated the effects of placing mats or wood-chips on concrete slatted floors on production, behavioural, physiological and immunological responses of finishing beef steers.

Continental crossbred (n=124) and Holstein-Friesian (n=20) beef steers (total = 144; mean body weight = 503 ± (s.e. 4.29) were blocked by breed and BW and were randomly assigned to one of four treatments: 1) Concrete slats alone, 2) Mat 1 3) Mat 2) and 4) Wood-chips. Animals were accommodated in a roofed building with concrete slatted floors. There were four pens per treatment, with nine steers per pen at a mean space allowance of 2.73m²/head. The mats and wood chips overlaid the concrete slats. Blood samples were collected by jugular venipuncture on days 0, 23, 45, 65, 107, 128 and 148. Dirt scoring and live weights were measured on days 0, 23, 45, 65, 86, 107, 128 and 148. A detailed pathological examination of the four hooves of each animal was recorded prior to and at the end of the study. Behavioural observations were recorded over 5 time periods throughout the experiment. Animals had *ad libitum* access to a total mixed ration (TMR) of grass silage and rolled barley on 50:50 dry matter (DM) basis. Animals were slaughtered after 149 to 150 days. Carcass weights, kill-out rates, kidney and channel fat weights were determined. The pen of cattle was the experimental unit. Performance data were subjected to ANOVA using a model that accounted for treatment and replicate blocks. Physiological and haematological measurements were tested in a one-way ANOVA (repeated measures) by PROC MIXED, using a means statement with a Tukey option to detect treatment differences. Dirt scores, hoof scores and behavioural data were not normally distributed and were analysed by PROC GLM, using ranked data, in a Kruskal-Wallis test and a Wilcoxon signed rank test was used to detect treatment differences.

No laminitis was observed. The number of lesions on the hooves of animals on Mat 1 and Mat 2 and wood chip treatments were greater (P<0.05) than the animals on concrete slats (Table 61). Dirt scores did not differ (P>0.05) between mat or slat treatments but were lower (P<0.05) than for the wood chip treatment with an overall significant treatment x time interaction (P<0.001). Live weight gain and carcass characteristics did not differ (P>0.05) between treatments (Table 61). The lying and standing times did not differ (P> 0.05) for cattle on concrete slats or wood-chips. The proportion of animals lying was greater for those on Mat 1 and Mat 2 compared to concrete slats and wood chips. Animals on the wood-chip treatment showed increased eating times compared to all other treatments.

Table 61: Performance and welfare characteristics of finishing beef steers. Values are expressed as Mean \pm s.e.m.

	Slats	Mat 1	Mat 2	Wood-chips
Total DM intake (kg DM/day)	9.8 \pm 0.30	9.9 \pm 0.06	9.8 \pm 0.17	10.1 \pm 0.09
Live weight gain (g/day)	1160 \pm 50	1140 \pm 50	1110 \pm 40	1180 \pm 50
Carcass weight (kg)	350.9 \pm 4.40	353.9 \pm 4.43	348.5 \pm 5.43	346.3 \pm 5.98
Kill-out (%)	52 \pm 0.43	53 \pm 0.69	52 \pm 0.39	51 \pm 0.34
Kidney channel fat (kg)	11.4 \pm 0.37	12.3 \pm 0.54	12.4 \pm 0.39	11.0 \pm 0.60
FCE (DMI/LWG)	8.6 \pm 0.38	8.8 \pm 0.38	8.8 \pm 0.15	8.6 \pm 0.26
Lying %	49.6 ^a \pm 0.38	51.2 ^b \pm 0.39	51.0 ^b \pm 0.38	49.5 ^a \pm 0.39
Standing %	50.1 ^a \pm 0.38	48.3 ^b \pm 0.39	48.8 ^b \pm 0.39	50.2 ^a \pm 0.39
Hoof scores ¹ (lesions)	40.9 ^a \pm 2.91	65.8 ^b \pm 2.92	71.9 ^b \pm 2.92	55.6 ^c \pm 2.92
White line lesions ¹	22.5 ^a \pm 2.29	35.3 ^{bc} \pm 2.19	41.0 ^b \pm 2.22	33.8 ^c \pm 2.22
Dirt scores ²	31.9 ^a \pm 1.20	32.0 ^a \pm 1.22	31.8 ^a \pm 1.20	49.4 ^b \pm 1.20

^{abc} Within row, means with the same superscripts are not significantly different ($P \geq 0.05$).

¹The total number of lesions/animal at the end of the study; ²Dirt scores; sum of 16 body parts each on a cleanliness scale of 1 (clean) to 5 (dirty).

In conclusion, under the conditions of the present study, there was no evidence to suggest that housing animals on slats alone was detrimental to their performance or welfare, based on the performance measurements and welfare indices (immune and metabolic indices) that were evaluated. Placing mats or wood-chips over the concrete slats did not alter either animal production or welfare.

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Effect of sea transport from Ireland to the Lebanon on performance, behaviour and physiology of bulls

There are concerns about the long distance transport of animals by sea and its effects on animal welfare. The objective was to examine the stress response in bulls induced by the stages of transport during a sea journey of approximately 12 days from Ireland to the Lebanon and to determine the environmental conditions on board the ship.

Fifteen hundred Holstein-Friesian bulls were assigned to 5 decks of a shipping vessel and transported from Ireland to the Lebanon in October 2005. One hundred and eleven Holstein-Friesian bulls, mean BW = 429 (\pm s.d. 59.2)kg were randomly assigned to one of three treatments. Controls were housed at Grange Beef Research Centre either at a stocking density of 1.7m²/head (n = 27) or 3.4m²/head (n = 27), while those transported (n = 57) were penned at 1.7m²/head. The animals on the ship were allocated to one of 5 decks (Deck 1 (lower), (n=11); Deck 2, (n=10); Deck 3 (n=10); Deck 4 (n=8) and Deck 5 (upper), n=2 pens; ((n=9 and n=9)). Animals had *ad libitum* access to hay and water and were fed 2 kg of concentrates/head/day. Live weights, rectal temperature and blood samples (jugular venipuncture) were collected from transported and control animals before the journey (day-1) and on days 3, 6, 9 and 11. Blood samples were collected into heparinised tubes, centrifuged

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and the plasma separated for subsequent analysis of: β -hydroxy butyrate (β HB), total protein, albumin, the acute phase protein (haptoglobin), the stimulated lymphocyte production of interferon (IFN)- γ determined from harvested supernatant following a whole-blood culture. The haematological variables including red blood cell (RBC) numbers, haemoglobin (Hb), haematocrit (packed cell volume (PCV)), mean corpuscular volume (MCV) and white blood cell (WBC) numbers were also determined. Data were analysed using SAS/STAT. Physiological and haematological measurements were tested in a one-way ANOVA by PROC MIX, using a means statement with a Tukey option to detect treatment differences.

A matched-pair t-test was used to detect differences pre- and post-transport for each treatment. Physiological, haematological and live-weight data and rectal temperature measurements were analysed using the repeated measures procedure in PROC GLM to detect differences in treatments while controlling for time effects. Measurements for albumin, haptoglobin and β HB, were not normally distributed and were analysed by PROC GLM, using ranked data in a Kruskal-Wallis test with a Tukey option to detect treatment differences. A Wilcoxon signed rank test was used to detect differences pre- and post-transport for each treatment.

Bulls travelling by sea for 12 days had greater ($P < 0.05$) live weight gain (4.4%) compared with control animals housed at 1.7m²/bull or 3.4m²/bull (2.0% loss and 0.13% gain, respectively). Behavioural observations showed that lying time percentage was greater ($P < 0.05$) for control bulls. Control bulls housed at 3.4m² space allowance showed greatest ($P < 0.05$) lying time overall. Eating activity was similar across all treatments. There was no ($P > 0.05$) difference in rectal temperature, pre- and post-transport. Haematocrit values were lower ($P < 0.05$) by day 3 and returned to baseline by day 6. IFN- γ concentrations were not different ($P > 0.05$) between treatments. Lymphocyte percentage increased ($P < 0.05$) by day 3 but returned to baseline by day 9 in transported bulls. In control bulls, lymphocyte percentage increased on days 3, 6, and 9 ($P < 0.05$) and returned to baseline by day 11. Neutrophil percentage was lower in transported bulls on day 3 ($P < 0.05$) and returned to baseline by day 11. Control bulls had lower neutrophil percentage on days 3, 6 and 9 ($P < 0.05$) and returned to baseline by day 11. Transported bulls had greater cortisol concentrations ($P < 0.05$) pre-transport than control bulls. On day 3, control bulls (3.4m²) had greater ($P < 0.05$) cortisol concentrations than transported bulls on Deck 2 (Figure 22). On day 6, control bulls (3.4m²) had lower ($P < 0.05$) cortisol concentrations than transported bulls on Decks 1, 3 and 5. On day 9, control bulls (3.4m²) had lower cortisol concentrations than transported bulls on Decks 1 and 3. On days 6 and 9, control bulls (1.7m²) had lower ($P < 0.05$) cortisol concentrations than transported bulls on Deck 3 and greater ($P < 0.05$) cortisol concentrations than transported bulls on Deck 2. On day 11, all control bulls had greater ($P < 0.05$) cortisol concentrations than transported bulls on Deck 2. The mean ambient temperature during the sea journey ranged from 14°C to 23°C, while the temperature on the decks of the ship ranged from 18.1°C to 25.2°C.

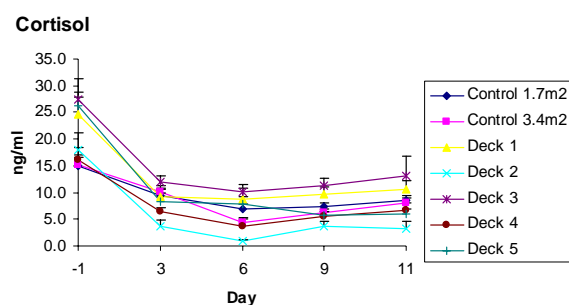


Figure 22. Cortisol concentrations, pre-transport (day -1) and on days 3, 6, 9, and 11 of the sea journey.

Physiological and haematological responses of transported bulls returned to pre-transport levels by day 3 while the physiological variables (neutrophil and lymphocyte numbers, non-esterified fatty acid concentrations) remained altered ($P < 0.05$) compared with baseline. Transport of bulls for 12 days by sea did not impact negatively on animal welfare.

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Temporal patterns of inflammatory gene expression in local tissues after banding or burdizzo castration in cattle

The exposure of a cell to external stimuli triggers complex intracellular signalling cascades that result in finely tuned alterations in gene expression, enabling the cell to react in an appropriate manner via regulation of cell growth and division, differentiation, metabolism and many other functions. There is now compelling evidence that stress responses can cause clinically relevant immunosuppression as well as other types of immune system dysfunction. Techniques used to castrate male cattle include the application of rubber rings or tightened latex bands (referred to as banding), surgical removal of the testicles, and use of a burdizzo instrument to crush the testicular cords. Castration of male cattle has been shown to elicit physiological stress, inflammatory reactions, pain-associated behaviour, suppression of immune function, and a reduction in performance.

Banding or burdizzo castration causes ischemia, which in turn leads to necrosis. Acute inflammation is initiated and sustained by the participation of tumour necrosis factor α (TNF- α), interleukin-1 (IL-1), interferon-gamma (IFN- γ) and IL-6, 8 and 10, etc. The sequential production of these cytokines by macrophages and other cells is essential for inflammation. Real-Time Quantitative RT-PCR has been successfully applied to quantify cytokine gene expression. The hypothesis was that bovine local tissues around the castration site respond to the changed blood flow (ischemia) associated with banding or burdizzo castration by altering the expression of cytokine genes involved in inflammatory functions. The objective was to monitor local tissue inflammatory cytokine gene expression profiles (IL-1, 6, 8, 10, IFN- γ , TNF- α) by quantitative real-time RT-PCR.

Sixty continental \times beef bulls (12 ± 0.2 month old; 341 ± 3.0 kg) were blocked by weight, and randomly assigned to one of three treatments (20 animals / treatment): 1) untreated control (Con); 2) banding castration at 0 min (Band); 3) Burdizzo castration at 0 min (Burd). Bulls were housed in individual tie-stalls from d -14 (day of treatment = d 0) to acclimatise them to handling, restraint and their housing environment. Animals had *ad libitum* access to water and grass silage. Band animals were castrated (time = 0 min) with latex rubber bands applied and tensioned to the neck of the scrotum using the Callicrate Smart Bander (St. Francis, KS, USA) following the instrument guidelines. Burdizzo castration (time = 0 min) was performed on the Burd animals (10 sec crushing applied to each spermatic cord). As part of the castration procedure, gentle manual restraint of the bulls was used to facilitate the operator. Animals in the control group were sham handled for a period equivalent to the time required to perform the castration procedure in the remaining treatment groups. At the time of tissue sample collection, a latex rubber band was applied above the Band or Burd castration site or equivalent site in control animals, so as to avoid ascending infection due to the open wound. At each time of 12 h, 24 h, 7 d, and 14 d post-treatment, samples of the testis, epididymis and scrotal skin (skin around the procedure site (ST), and the middle scrotum (SM)) were collected surgically from 5 animals from each group (Figure 23). Tissue samples were dissected and frozen in liquid nitrogen immediately and then stored at -80°C until analysed. All procedures were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876, and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations, 1994.

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Tissue samples were broken into small pieces after removal from the liquid nitrogen. After weighing, tissue samples (~50 mg) were placed in a liquid nitrogen pre-cooled 14 ml round-bottom culture tube, followed by the addition of 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA). Tissue was homogenised using a Polytron homogeniser (KINEMATICA AG, Switzerland). Total RNA was isolated from the tissue suspension following TRIzol Reagent manufacturer's instructions. The isolated RNA was DNase (Promega, WI, USA) treated, re-extracted using phenol/chloroform (Sigma-Aldrich, MO, USA), followed by precipitation using RNase-free 3M sodium acetate and pure ethanol (Sigma-Aldrich, Dublin, Ireland). Following isolation and purification, RNA concentration and purity were determined using an UV-1601 PC spectrophotometer (SHIMADZU, Japan) set at 260 and 280 nm readings. RNA quality was also checked by 28S and 18S rRNA band visualisation following gel electrophoresis. RNA was converted into first-strand cDNA using SuperScript™ III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions.

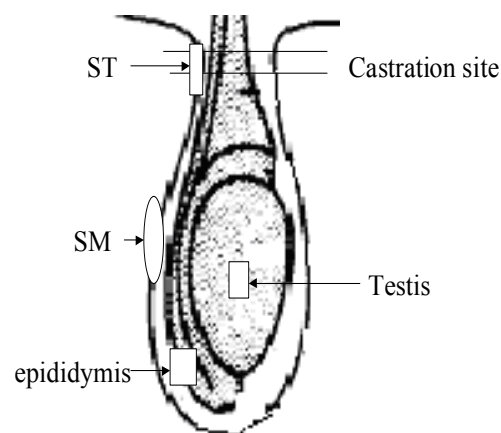


Figure 23. Diagrammatic representation of scrotal tissue sampling sites.

Quantitative real-time PCR was performed using the SYBR Green PCR master Mix (Applied Biosystems, UK). Seven gene-specific primer pairs were designed using Primer Express Software (Applied Biosystems) and synthesised at MWG (UK). β -actin was included as an endogenous reference in all real-time PCR analyses. The real-time PCR was performed using an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). 10 μ l of sample containing 0.5 μ l cDNA, 300 nM of forward and reverse primers, and SYBR Green PCR master Mix, was placed in each well of a 384-well plate. The samples were run in triplicate. Real-time PCR conditions were as follows: stage 1 of 50 °C for 2 min; stage 2 of 95 °C for 10 min and followed by stage 3 of 40 repeats of 95 °C for 15 sec and 60 °C for 1 min. Results were recorded as relative gene expression changes after normalising for β -actin gene expression, and calculated using the $2^{-\Delta\Delta C_t}$ method. The average C_T values of control animals at each time point were the calibrator used to determine relative gene expression changes.

Electrophoresis data showed that at 7 d post-treatment, RNA isolated from all the testicle samples of castrates, and epididymis and middle scrotum samples from Band castrates were degraded with the disappearance of both 28 S and 18 S rRNA bands (Figure 24). At 14 d post-castration, all the RNA samples from castrated animals were degraded and were not included in further real-time PCR assays. Other RNA samples had spectrophotometer absorbance ratios (260:280) in range of 1.8 to 2.3.

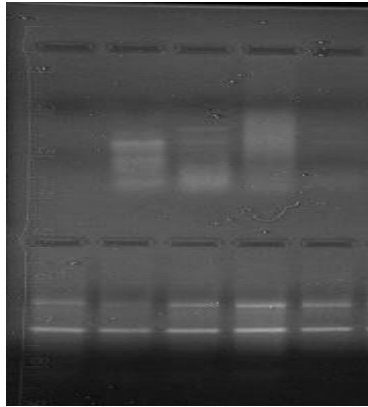


Figure 24. Electrophoresis of middle scrotum samples (7 d post castration). Upper line: 5 samples of Band castrates. Lower line: 5 samples of Burd castrates.

12 h post-castration showed that Burd had greater IFN- γ mRNA gene expression in the testis than Band ($P = 0.048$) and Con ($P = 0.02$) (Figure 25). Band animals had greater ($P = 0.04$) IL-6 mRNA levels than Burd; Burd had lower ($P = 0.02$) TNF- α mRNA levels than Con. At 24 h after castration, Burd had greater IFN- γ mRNA level than Band ($P = 0.02$) and Con ($P = 0.01$); Burd animals had greater IL-8 mRNA level than Band ($P = 0.03$) and Con ($P = 0.03$); Burd had lower ($P = 0.04$) TNF- α mRNA levels than Con.

12 h post-castration, Burd animals had greater IL-6 mRNA gene expression in the epididymis levels than Band ($P = 0.0008$) and Con ($P = 0.0005$) animals. (Figure 26). Burd had greater IL-8 mRNA level than Band ($P = 0.002$) and Con ($P = 0.001$); Burd had greater ($P = 0.049$) IL-10 mRNA level than Band. At 24 h post-castration, Burd had greater IL-6 mRNA level than Band ($P = 0.01$) and Con ($P = 0.01$); At d 7, Burd had greater ($P = 0.02$) IL-6 mRNA level than Con. No differences were found among treatments in ST and SM (data not shown). No IFN- γ amplifications were detected in samples of epididymis, ST and SM from all animals.

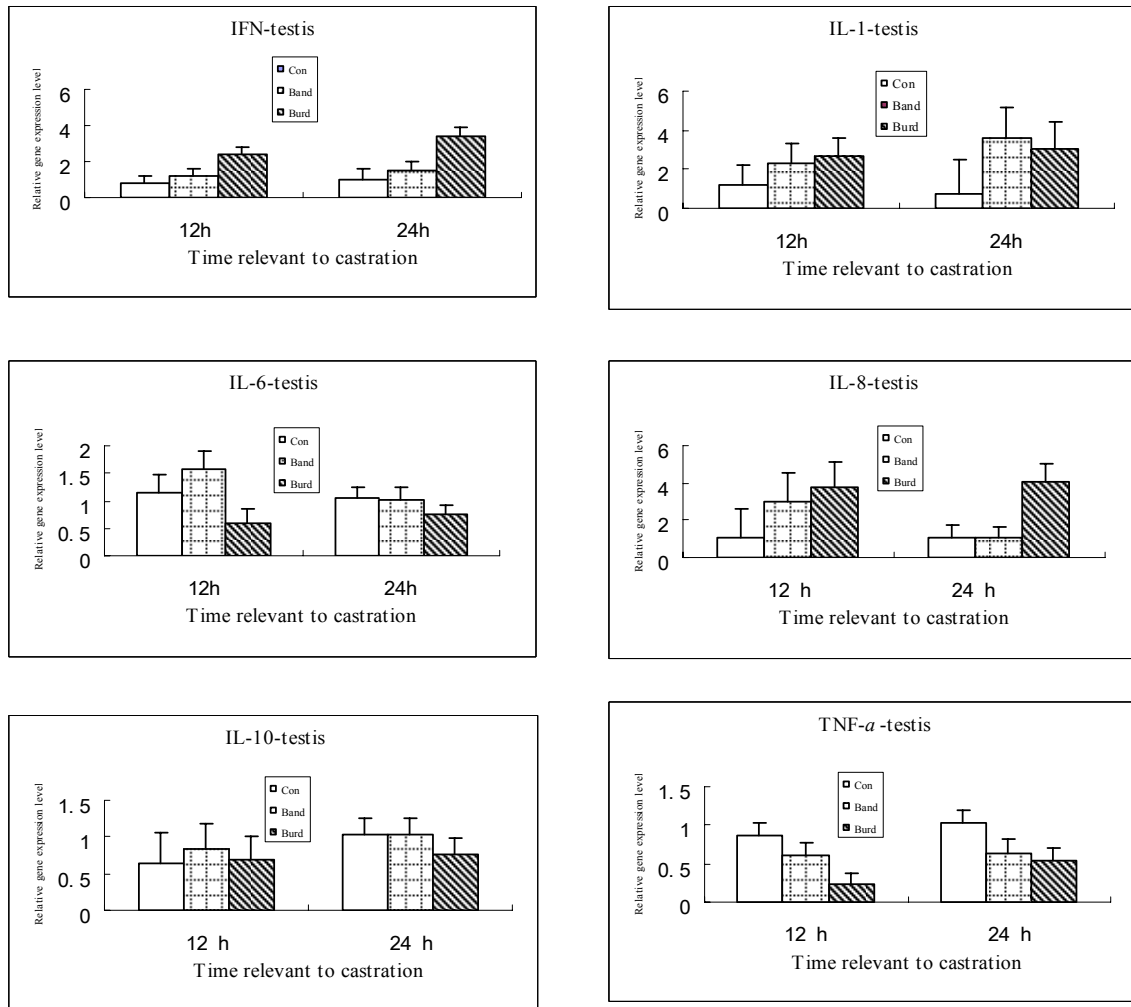


Figure 25. Mean relative expression level (\pm SE) of cytokine genes (12 and 24 h relative to castration) in the testis of bulls left untreated (Con), or castrated by banding (Band) or Burdizzo (Burd) methods.

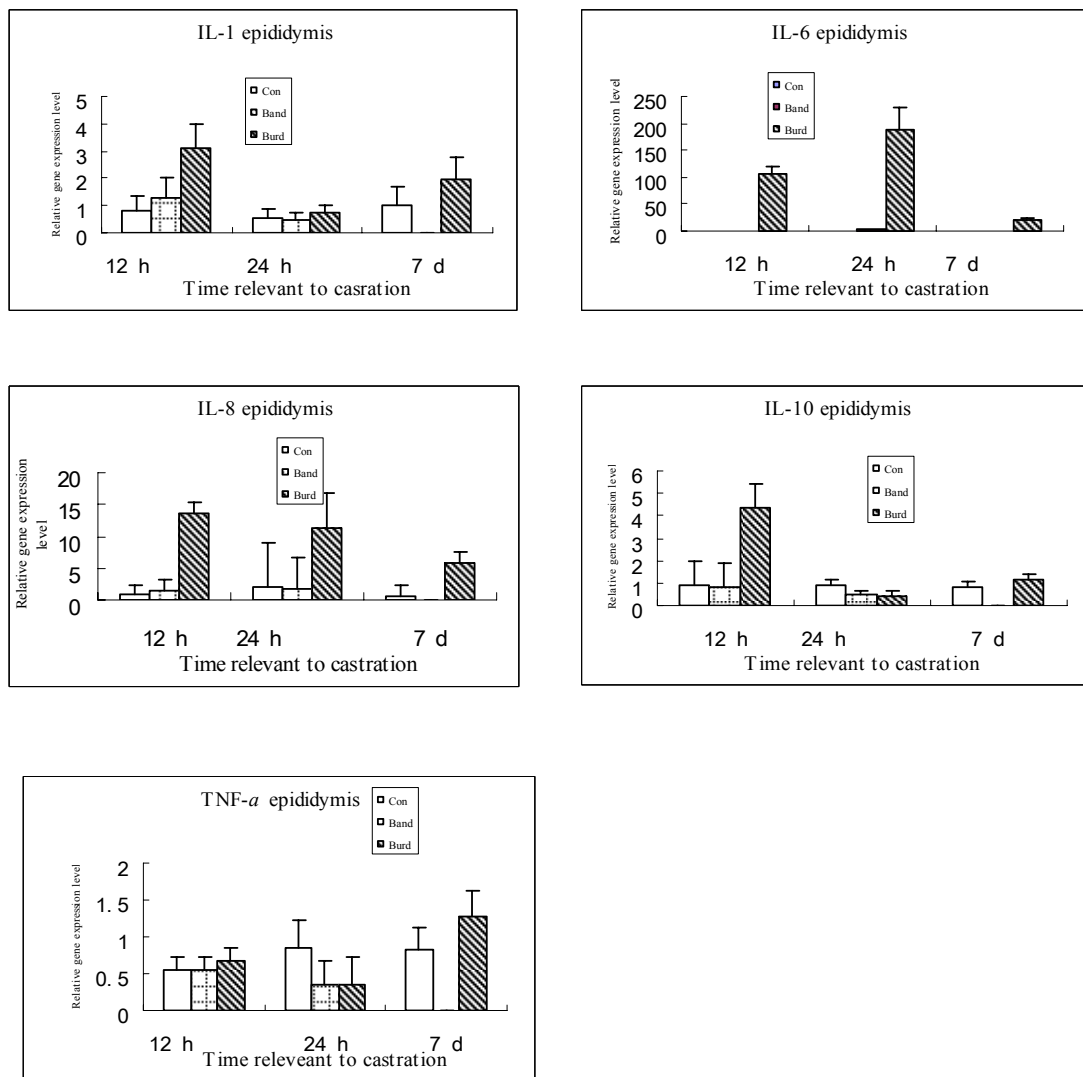


Figure 26. Mean relative expression level (\pm SE) of cytokine genes in the epididymis (12, 24h and 7days relative to castration) of bulls left untreated (Con), or castrated by banding (Band) or Burdizzo (Burd) methods.

In the present study, RNA isolated from the testis of all castrates at 7 d post-castration was degraded. The epididymis and middle scrotum samples from Band castrates were degraded by 7 d post-castration, while the tissue RNA isolated from Burd castrates were not. This may suggest that banding causes more rapid necrosis or atrophy damage to the epididymis and scrotum than burdizzo. This is in accordance with the different principles of these two castration techniques. Banding causes ischemia to testicles, epididymis and also scrotum, leading to ischemic necrosis of the testicles, eventually testicular atrophy and sloughing of the scrotum. Burdizzo castration is based on the principle that crushing destroys the spermatic cord carrying blood to the testicles but that the skin of the scrotum remains intact as clamping of the burdizzo on each spermatic cord is not overlapped.

Burd castrates had greater IFN- γ mRNA levels than control and Band animals in the testis. IFN- γ has been found in the testis, particularly in the Sertoli and Leydig cells. IFN- γ up-regulates a variety of pro-inflammatory parameters such as IL-12, inducible nitric oxide synthase (iNOS), caspase-1. Moreover, data suggest that IFN- γ may also be able to enhance

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activation of the pro-inflammatory transcription factor nuclear factor- κ B (NF- κ B) under certain conditions. Hence, the finding in this study may suggest that Burd caused more severe acute inflammation than Band. However, IFN- γ also has the potential to direct inflammatory responses by inhibiting production of pro-inflammatory IL-1 not affected and IL-8, by up-regulating the production of cytokine antagonists such as IL-1Ra and IL18BP, by inducing expression of members of the SOCS family of regulatory proteins, and by induction of apoptosis in leukocytes and local resident cells.

Band castrates had greater IL-6 transcripts than Burd castrates in the testis, this may suggest that banding castration caused more severe inflammatory responses in the testis than burdizzo. However, Burd castrates had greater IL-6 mRNA level than Band and Con animals in the epididymis; this may suggest Burd caused more significant acute inflammatory responses in the epididymis than Band.

Burd had greater IL-8 mRNA levels than Band in the testis and epididymis, this may suggest burdizzo castration lead to a greater acute inflammatory response than Band. IL-8 enhances inflammation by enabling immune cells to migrate into tissues and is a powerful inducer of chemotaxis for neutrophils.

It's not clear why Burd animals had lower levels of TNF- α mRNA than control animals. TNF- α is produced by activated monocytes and macrophages, and expression of TNF- α has been found in pachytene spermatocytes and round spermatids, and in activated macrophages isolated from the testis. Similar to IL-1, TNF- α inhibits Leydig cell steroidogenesis, and its localisation to the post-meiotic germ cells also indicates possible involvement in the process of spermatogenesis. For example, TNF- α might play a role in controlling the efficiency of the spermatogenic process, inhibiting germ cell apoptosis by regulating the level of Fas ligand (FasL).

It was also not clear why there were no successful IFN- γ amplifications detectable in samples of the epididymis, although the reaction conditions for these tissue samples were the same as those for testis samples. In conclusion, banding causes more damage to the epididymis and scrotum than burdizzo. Burd causes more severe acute inflammatory responses than Band in the testis and epididymis, in terms of pro-inflammatory cytokine gene expression. Further research is required to relate the severity of castration induced inflammation with inflammatory cytokine gene expression.

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Effects of banding or burdizzo castration of bulls or cortisol infusion on neutrophil phagocytosis and respiratory burst, CD62-L expression, and serum Interleukin-8 concentration

The application of rubber rings or tightened latex bands (referred to as banding) and the use of burdizzo instrument to crush the testicular cords are two methods used to castrate cattle. Castration by these techniques has been shown to elicit physiological stress indicated by acutely increased plasma cortisol concentrations.

Observational studies have described relationships between increasing cortisol concentrations and altered trafficking, phagocytic ability and CD62L expression of blood neutrophils around parturition. The hypothesis was that banding or burdizzo castration, which increases cortisol concentrations, would affect phagocytosis and respiratory burst, CD62L expression in neutrophils and serum interleukin-8 concentrations.

Thirty-two Holstein - Friesian bulls (14 months old, 505 ± 7.8 kg) were blocked by weight into 4 groups and assigned to one of four treatments (n = 8 animals / treatment): 1) sham handled control (Con); 2) banding castration alone (Band); 3) burdizzo castration alone (Burd); 4) cortisol infusion (Cort). On day -14, eight animals (2 animals / treatment) were tied into tie stalls per week (day of treatment = d 0). The experiment was completed in 4 weeks. Animals had *ad libitum* access to water and grass silage.

Each week, 8 animals were catheterised on d -1. Band animals were castrated (time = 0 min) with latex bands applied and tensioned to the neck of the scrotum using the Callicrate Smart Bander (St. Francis, KS 67756, USA) following instrument guidelines. Burdizzo castration (time = 0 min) was performed in the Burd bulls following the procedure of Fisher *et al.* (1996). As part of the castration procedure, gentle manual restraint of the bulls was used to facilitate the operator. Animals that received cortisol (corticosterone) were administered with *i.v.* doses of aqueous 0.2% (w/v) hydrocortisone sodium succinate (Solu-Cortef; Pharmacia and Upjohn Ltd., Knowhill, Milton Keynes, UK) solution prepared in sterile 0.9% (w/v) saline. Each Cort bull received 16 mg hydrocortisone at 0 h (time of castration), 8 mg at 0.25 and 0.5 h, and 4 mg at 0.75 and 1 h, followed by 2 mg at 1.5, 2, 3, 3.5, 5, 5.5, 6, 7 and 8 h relative to the time of castration in Band or Burd animals. Following each hydrocortisone injection, 5 ml sterile saline was administered to flush the catheters. Animals in Band, Burd and Con groups were given an equivalent volume of sterile 0.9% saline via the catheter at the same time Cort animals receiving hydrocortisone. Animals in the control group were sham handled for a period equivalent to the time required to perform the castration procedure in the castration groups.

At -2h, 2h, 6h, 12h, 24h, 48h, d3 and d6, relative to treatment time, heparin anti-coagulated blood samples were collected for neutrophil phagocytosis and respiratory burst assays, and for cortisol assay; ACD blood samples were collected for G1 and CD62L flow cytometric assay; serum samples were collected for IL-8 assay. Two more samples were collected at 0.5 h and 1 h for cortisol measurement. Plasma and serum samples were collected after centrifugation at $1,600 \times g$ at 8 °C for 15 min, and stored at -20 °C until analysed. The other samples were processed immediately after collection. All procedures were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876, and the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994.

Plasma cortisol concentrations were determined using a commercially available radio-immunoassay kit (Corti-cote, ICN Pharmaceuticals, Orangeburg, NY, USA) adapted and validated for bovine plasma. The intraassay CV (n = 4) for samples containing 1.5 and 16.4 ng of cortisol/ml were 24.1 and 8.3%, respectively, and the interassay CV (n = 3) for the same samples were 21.5, and 7.6%. Immunostaining and fluorescence activated flow cytometry were used to analyse neutrophil surface CD62-L and circulating neutrophil percentage. Immunostaining was performed in 96-well microtiter plates (VWR Scientific, Chicago, IL, USA) using a whole blood assay. Briefly, 1ml aliquant of whole blood was aliquot into a 5ml test tube (SARSTEDT, Germany). Tubes were centrifuged at 1200 rpm ($250 \times g$) for 5 min at 4 °C. After aspiration of supernatants, 3 ml of BD FACS lysing solution (BD Biosciences, Oxford, UK) was added into each tube to lyse red blood cells for 10 min at room temperature. The remaining leukocytes were suspended in 1.5ml of sheath fluid (Coulter® Isoton® II Diluent) (Beckman Coulter UK Ltd., UK) and counted using a Z1 Coulter® Particle Coulter (Beckman Coulter™, LABPLAN Ltd., Ireland) (the counts were recorded as WBC). 100µl aliquants of cell suspension (1×10^6 cells) were added to a series of three wells (per sample), one that received 100µl of PBS (pH 7.2) containing 0.01% bovine serum albumin (PBS-BSA), one that received 100µl of anti-G1 monoclonal antibody (clone MM20A, IgG1) (VMRD, WA, USA), and the third that received 100µl of anti-CD62L monoclonal antibody (clone BAQ92A, IgG1) (VMRD). Both monoclonal antibodies were diluted to 14 µl/ml in

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PBS-BSA before addition to the plate wells. Plates were sealed and incubated for 30 min at 4 °C. After the incubation, 100µl of cold PBS-BSA was added to each well and plates centrifuged at 1200 rpm for 5 min. Using the same centrifugation step, all wells were washed twice using 200µl of cold PBS-BSA per well, with gentle aspiration of supernatants between washes. After washes, 100µl of PE-conjugated goat-anti-mouse IgG1 antibody (1:400 diluted in PBS-BSA; Caltag Laboratories, CA, USA) was added and plates sealed and incubated for 15 min at 4 °C. After addition of 100µl of cold PBS-BSA to each well, plates were centrifuged as before and supernatants aspirated. After two washes and centrifugations, the cell pellet was resuspended in 200 µl of sheath fluid and then further diluted in 800 µl of sheath fluid for immediate flow cytometric acquisition of neutrophil G1 and CD62L expression data on a Partec CyFlow Flow Cytometer (Partec GmbH, Munster, Germany) (Figure 27).

The percentage of neutrophils was determined by multiplying the percentage of the G1-stained cells (G1 is a granulocyte marker) in the gate set for neutrophils (G1 gating) and the percentage of gated cells (the neutrophil gate) in the whole leukocyte population. Surface CD62L expression was recorded as percentage of CD62L staining positive cells and the mean fluorescence intensity in the gated neutrophils in which at least 90% of the cells are G1 positive.

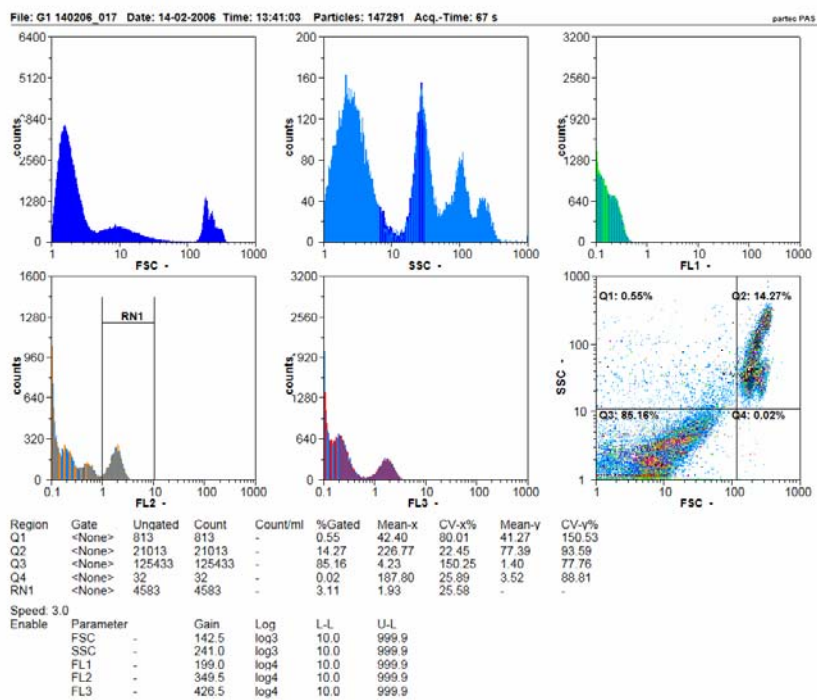


Figure 27. G1 gating using Flow Cytometry.

The phagocytosis and respiratory burst were analysed using the Phagotest® kit and Bursttest (Phagoburst®) kit (Orpegen Pharma, Heidelberg, Germany) with whole, heparinized blood following the manufacturer's instructions with the modifications by Kampen *et al.* (2004), on a Partec CyFlow Flow Cytometer. Samples were analysed in duplicate. Data were collected from 30,000 cells per sample, and analysis was done using FloMax software (Attachment 6.2; 6.3). The results of the phagocytosis assay were presented as percentage of phagocytosing cells and the mean fluorescence intensity in the gate set for neutrophils. The results of the Bursttest assay were presented as percentage of phagobursting cells and the mean fluorescence intensity in the neutrophil gate.

Serum IL-8 concentration was measured using a human IL-8 DuoSet (R&D Systems, UK) following the manufacturer's instructions. Each plate had standards and samples were run in duplicate. The optical density (OD) values were obtained using a TECAN SUNRISE Absorbance Reader (TECAN Austria GmbH, Austria). The standard curves (four parameter logistic [4-PL] curve-fit) were generated and IL-8 concentrations automatically calculated by using WIACALC software (Pharmacia-LKB, Uppsala, Sweden). In order to validate the assay for bovine serum samples, a spike and recovery test was carried out according to an experimental protocol (R&D Systems Europe, UK). The recovery of spiked IL-8 standard was 92.1% (should be in the range of 80-120%), and the assay had linearity for bovine serum samples. Two bovine serum samples were included in each ELISA assay as a quality control. The intra-assay CV ($n = 2$) for samples containing 30.3 and 111.5 pg/ml of IL-8 was 6.3 and 9.8%, respectively, and the inter-assay CV ($n = 7$) for the same samples was 8.4, and 7.5%, respectively.

The results showed that there was a treatment ($P < 0.0001$) and time effect ($P < 0.0001$), but no group effect ($P = 0.57$). There was no difference ($P = 0.87$) among the four treatments in pre-treatment plasma cortisol concentration (Table 62). Band and Burd castrates had greater ($P = 0.008$; 0.0004 ; respectively) cortisol concentration than Con animals at 0.5 h post-treatment, while Cort bulls had greater cortisol concentration compared with Band ($P < 0.0001$) and Burd ($P < 0.0001$) castrates at 1 h post-treatment, animals in Band ($P < 0.0001$), Burd ($P = 0.0006$), and Cort ($P < 0.0001$) had greater cortisol concentration than Con bulls, while no difference ($P = 0.40$) between Band and Cort animals, but Cort animals had greater ($P = 0.04$) cortisol concentration than Burd castrates at 12 h post-treatment, Cort animals had greater ($P = 0.04$) cortisol concentration than Con bulls. 24 h post-treatment, Burd animals had greater cortisol concentration than Con bulls ($P = 0.03$) and Band castrates ($P = 0.02$) at 48 h post-treatment, Burd animals had greater ($P = 0.04$) cortisol concentration than Con bulls.

There was no interaction between treatment and group ($P = 0.31$), and treatment and time ($P = 0.13$) for white blood cell (WBC) counts. WBC was not different ($P = 0.41$) among the four treatments prior to treatment at 12 h post-castration, Band castrates had lower ($P = 0.04$) WBC number than Burd castrates, while WBC numbers of castrates from both Band and Burd were not different from Con ($P = 0.24$; 0.33 , respectively) and Cort ($P = 0.25$; 0.33 ; respectively) animals. No other difference was found.

Table 62: Mean plasma cortisol concentration (LSM ± SEM; ng/ml) of males castrated by banding (Band) burdizzo (Burd), left untreated (Con), or injected with cortisol (Cort)

Time relative to castration	Treatments			
	Con	Band	Burd	Cort
- 2 h	6.9 ± 3.86	8.0 ± 3.86	11.1 ± 3.86	7.5 ± 3.86
0.5 h	6.7 ± 4.44 ^b	24.7 ± 4.44 ^a	32.2 ± 4.44 ^a	61.2 ± 4.44 ^c
1 h	5.0 ± 2.99 ^c	27.2 ± 2.99 ^{ab}	21.5 ± 2.99 ^a	31.0 ± 3.20 ^b
2 h	11.2 ± 5.10	22.7 ± 5.10	10.0 ± 5.10	20.1 ± 5.10
6 h	5.1 ± 3.61	2.6 ± 3.61	12.1 ± 3.61	6.4 ± 3.61
12 h	5.4 ± 3.37 ^a	8.0 ± 3.37 ^{ab}	8.5 ± 3.60 ^{ab}	16.0 ± 3.37 ^b
24 h	4.1 ± 2.88 ^a	3.5 ± 2.88 ^a	13.5 ± 3.07 ^b	8.0 ± 2.88 ^{ab}
48 h	7.0 ± 2.57 ^b	11.1 ± 2.57 ^{ab}	14.7 ± 2.57 ^a	8.6 ± 2.57 ^{ab}
72 h	8.4 ± 2.58	8.7 ± 2.58	10.1 ± 2.58	8.4 ± 2.58
6 d	7.7 ± 2.37	12.0 ± 2.37	7.8 ± 2.37	11.2 ± 2.37

^{a, b, c}Means within a row that do not have common superscripts differ (P < 0.05).

In the data on percentage of phagocytosing neutrophils (phago %), there was no interaction between treatment and group (P = 0.51), and treatment and time (P = 0.97) (Table 63). At 2 h before treatment, there were no differences (P = 0.91) in phago % among the four treatments. No treatment effects were found in phago % at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 6 d post-treatment. There were no differences among the four treatments in the mean fluorescence intensity of phagocytosing neutrophils at those time points.

The data on percentage of neutrophils that performed respiratory burst (burst %) showed there was no interaction between treatment and group (P = 0.92), and treatment and time (P = 0.32). At 2 h prior to treatment, there were no differences (P = 0.63) in burst% among the four treatments. At 72 h post-treatment, Burd castrates had greater burst% compared with Band castrates (P = 0.048) and Con (P = 0.01). There were no differences among the four treatments in burst% at 2 h (P = 0.83), 6 h (P = 0.65), 12 h (P = 0.88), 24 h (P = 0.64), 48 h (P = 0.20), and 6 d (P = 0.74) post-treatment. There were no differences among the four treatments in the mean fluorescence intensity of neutrophils that performed respiratory burst at those time points.

Table 63: Percentage of phagocytosing neutrophils(LSM ± SEM) of males castrated by banding (Band), burdizzo (Burd), left untreated (Con), or injected with cortisol (Cort)

Time relative to treatment	Treatments				Treatment effect
	Con	Band	Burd	Cort	
- 2 h	53.3 ± 4.96	50.3 ± 4.96	53.1 ± 4.96	55.4 ± 4.96	P = 0.91
2 h	52.3 ± 5.10	55.3 ± 5.10	55.2 ± 5.10	58.0 ± 5.10	P = 0.89
6 h	49.3 ± 5.58	46.7 ± 5.58	52.6 ± 5.58	53.6 ± 5.58	P = 0.81
12 h	52.9 ± 3.36	55.3 ± 3.36	52.6 ± 3.59	55.2 ± 3.36	P = 0.92
24 h	52.4 ± 3.92	50.5 ± 3.66	56.5 ± 3.66	60.1 ± 3.66	P = 0.27
48 h	56.5 ± 3.49	54.4 ± 3.49	59.2 ± 3.49	59.3 ± 3.49	P = 0.71
72 h	48.8 ± 3.49	51.0 ± 3.49	51.8 ± 3.49	52.4 ± 3.49	P = 0.89
6 d	53.6 ± 4.59	55.0 ± 4.59	57.5 ± 4.59	52.5 ± 4.59	P = 0.88

The percentage of G1 positive cells (G1%) in the leukocytes, showed there was no interaction between treatment and group ($P = 0.50$), and treatment and time ($P = 0.10$). At 2 h before treatment, there were no differences ($P = 0.65$) in G1% among the four treatments (Table 64). At 2 h post-treatment, Burd castrates had greater G1% compared with Con ($P = 0.001$) and Cort ($P = 0.002$) bulls at 24 h post-treatment, Burd castrates had greater G1% compared with Band castrates ($P = 0.02$), Con ($P = 0.02$), and Cort ($P = 0.009$) bulls. 48 h post-treatment, Burd castrates had greater G1% compared with Band castrates ($P = 0.03$), and Cort ($P = 0.03$) bulls at 72 h post-treatment, Burd castrates had greater G1% compared with Band castrates ($P = 0.03$), Con ($P = 0.04$), and Cort ($P = 0.005$) bulls. 6 d post-treatment, Burd and Band castrates had greater G1% compared with Cort ($P = 0.04$; 0.02 ; respectively) bulls.

The mean fluorescence intensity (G1Mean) of G1 positive neutrophils, showed there were no treatment and group interactions ($P = 0.48$), and no interaction between treatment and time ($P = 0.17$). At 2 h before treatment, there were no differences ($P = 0.31$) in G1Mean among the four treatments at 2 h post-treatment, Band castrates had lower G1 Mean compared with Burd castrates ($P = 0.009$), and Con bulls ($P = 0.02$). 48 h post-treatment, Band castrates had lower G1Mean compared with Burd castrates ($P = 0.02$), Con bulls ($P = 0.004$), and Cort bulls ($P = 0.004$) at 6 d post-treatment, Burd had lower G1Mean compared with Con ($P = 0.04$), and Cort ($P = 0.02$) bulls.

Data on the percentage of CD62L positive neutrophils (CD62L%) showed there were no interactions between treatment and group ($P = 0.75$), and between treatment and time ($P = 0.35$). At 2 h prior to treatment, there were no differences ($P = 0.74$) in CD62L% among the four treatments. There were no differences among the four treatments in CD62L% at 2 h ($P = 0.35$), 6 h ($P = 0.44$), 12 h ($P = 0.65$), 24 h ($P = 0.47$), 48 h ($P = 0.48$), 72 h ($P = 0.65$), and 6 d ($P = 0.79$) post-treatment.

Data on the mean fluorescence intensity of CD62L positive neutrophils (CD62LMean) revealed there were no interactions between treatment and group ($P = 0.49$), and between treatment and time ($P = 0.18$). At 2 h prior to treatment, there were no differences ($P = 0.66$) in CD62LMean among the four treatments. There were no differences among the four treatments in CD62LMean at 2 h ($P = 0.98$), 6 h ($P = 0.97$), 12 h ($P = 0.89$), 24 h ($P = 0.62$), 48 h ($P = 0.69$), 72 h ($P = 0.77$), and 6 d ($P = 0.29$) post-treatment.

Table 64: The percentage of G1 positive cells in the leukocytes (Lsmeans \pm SEM) of males castrated by banding (Band), burdizzo (Burd) or left untreated (Con), or injected with cortisol (Cort)

Time relative to treatments	Treatment				Treatment effect
	Con	Band	Burd	Cort	
- 2 h	28.4 \pm 4.72	26.8 \pm 4.72	33.0 \pm 4.72	24.8 \pm 4.72	$P = 0.65$
2 h	25.3 \pm 4.51 ^b	36.9 \pm 4.51 ^{ab}	48.3 \pm 4.51 ^a	26.9 \pm 4.51 ^b	$P = 0.004$
6 h	30.5 \pm 5.27	36.9 \pm 5.27	35.6 \pm 5.27	37.7 \pm 5.27	$P = 0.77$
12 h	26.9 \pm 5.06	30.5 \pm 5.06	36.5 \pm 5.41	25.4 \pm 5.06	$P = 0.46$
24 h	26.0 \pm 5.89 ^a	26.3 \pm 5.51 ^a	45.5 \pm 5.51 ^b	23.5 \pm 5.51 ^a	$P = 0.03$
48 h	32.7 \pm 5.34 ^{ab}	28.9 \pm 5.34 ^a	47.4 \pm 5.72 ^b	29.3 \pm 5.35 ^a	$P = 0.08$
72 h	27.1 \pm 5.23 ^a	26.7 \pm 5.23 ^a	43.3 \pm 5.23 ^b	20.8 \pm 5.23 ^a	$P = 0.03$
6 d	24.0 \pm 5.33 ^{ab}	35.3 \pm 4.98 ^a	38.6 \pm 4.98 ^a	20.4 \pm 4.98 ^b	$P = 0.04$

^{a, b}Means within a row that do not have common superscripts differ ($P < 0.05$).

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There were no treatment and group interactions ($P = 0.75$), and no treatment and time interaction ($P = 0.06$) for serum IL-8 concentrations. There were no differences ($P = 0.13$) in serum IL-8 concentrations among the four treatment groups before treatment (Table 65) at 2 h post treatment, Burd castrates had lower serum IL-8 concentrations compared with Con ($P = 0.026$) and Cort ($P = 0.035$) bulls. Band castrates had similar IL-8 concentrations as Burd castrates ($P = 0.62$), Con ($P = 0.07$) and Cort ($P = 0.10$) bulls at 48 h post treatment, Cort bulls had greater ($P = 0.01$) IL-8 concentration compared with Burd castrates, while they were not different from band castration ($p=0.19$) or control bulls ($p=0.06$).

This research group have previously reported that hydrocortisone infusion successfully mimicked the cortisol response profile of burdizzo-castrated calves and there was no effect of castration or cortisol mimic on leukocyte numbers (from d 1 to 35 post-infusion). However infusion of pharmacological levels of cortisol resulted in a transient suppression of IFN- γ production, increased plasma glucose, insulin and GH concentrations. In the present study, greater dosage of hydrocortisone was injected trying to induce a greater plasma cortisol concentration so to investigate the effects of cortisol on neutrophil functioning. A greater peak concentration (61.2 ± 4.44 ng/ml) was achieved by hydrocortisone injection than that induced by either banding (24.7 ± 4.44) or burdizzo (32.2 ± 4.44) castration.

Table 65: The mean serum IL-8 concentration (LSM \pm SEM; pg/ml) of males castrated by banding (Band), burdizzo (Burd), left untreated (Con), or injected with cortisol (Cort)

Time relative to treatment	Treatments			
	Con	Band	Burd	Cort
- 2 h	78.4 \pm 12.11	55.0 \pm 12.11	44.6 \pm 12.11	78.9 \pm 12.11
2 h	79.9 \pm 11.53 ^b	49.7 \pm 11.53 ^{ab}	41.4 \pm 11.53 ^a	77.6 \pm 11.53 ^b
6 h	70.5 \pm 12.45	52.3 \pm 11.64	48.9 \pm 11.64	70.0 \pm 11.64
12 h	63.1 \pm 11.93	42.5 \pm 11.93	52.0 \pm 12.75	54.0 \pm 11.93
24 h	55.3 \pm 15.23	60.1 \pm 14.24	44.0 \pm 14.24	62.1 \pm 14.24
48 h	53.5 \pm 12.44 ^{ab}	65.0 \pm 12.44 ^{ab}	43.0 \pm 12.44 ^a	88.7 \pm 12.44 ^b
72 h	70.8 \pm 11.89	61.7 \pm 11.89	61.9 \pm 11.89	72.7 \pm 11.89
6 d	96.2 \pm 16.55	67.4 \pm 16.55	86.9 \pm 16.55	80.9 \pm 16.55

^{a, b}Means within a row that do not have common superscripts differ ($P < 0.05$).

There was no difference in WBC between castrates or Cort bulls and control animals. This is in agreement with our previous findings. Burd induced a greater granulocyte percentage compared to control bulls. Similarly, others have reported a greater neutrophil percentage on d 2 after burdizzo castration. It is not clear why no neutrophilia was found in Band castrates, although there was no difference in the cortisol response profile between banding and burdizzo castrated animals. Hydrocortisone infusion had no effect on granulocyte percentage compared to controls. These findings indicate that the increased cortisol concentration is not the only reason for neutrophilia after castration. In contrast, severe glucocorticoid challenge by dexamethasone administration can cause neutrophilia. There was also a decrease in mean G1 expression per cell indicated by the mean G1 fluorescence intensity in Band and Burd castrates. The reason is not clear, and this may possibly warrant the need to further explore the functions of G1 expression in neutrophils.

There was no difference in both the percentage of cells expressing CD62L and the mean CD62L expression level per cell (indicated by the mean CD62L fluorescence intensity), among the four treatments. Apparently this is the first time CD62L has been studied in

castration. The results presented indicate banding or burdizzo castration would not affect the capacity of neutrophils to adhere to blood vessel endothelia, and hydrocortisone infusion on its own would not effect neutrophil trafficking. Generally, banding or burdizzo castration and hydrocortisone injection did not affect serum IL-8 concentration. This finding may suggest that a mild degree of localised injury or stress after banding or burdizzo castration and hydrocortisone infusion had no effect on serum IL-8 concentration. In conclusion, banding or burdizzo castration and hydrocortisone infusion did not induce leukocytosis, while burdizzo castration did induce a neutrophilia; neutrophil functioning in terms of phagocytosis and respiratory burst, and serum IL-8 concentration were not compromised by banding or burdizzo castration and hydrocortisone infusion. These findings suggest non-surgical castration is unlikely to induce a severe systemic inflammatory response.

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