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An initial investigation into the effects of isolation and enrichment on the welfare of laboratory pigs housed in the PigTurn® system, assessed using tear staining, behaviour, physiology and haematology

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Abstract

*In some parts of the world, the laboratory pig (*Sus scrofa*) is often housed in individual, sterile housing which may impose stress. Our objectives were to determine the effects of isolation and enrichment on pigs housed within the PigTurn® — a novel penning system with automated blood sampling — and to investigate tear staining as a novel welfare indicator. Twenty Yorkshire × Landrace weaner pigs were randomly assigned to one of four treatments in a 2 × 2 factorial combination of enrichment (non-enriched [NE] or enriched [E]) and isolation (visually isolated [I] or able to see another pig [NI]). Pigs were catheterised and placed into the PigTurns® 48 h post recovery. Blood was collected automatically twice daily to determine white blood cell (WBC) differential counts and assayed for cortisol. Photographs of the eyes were taken daily and tear staining was quantified using a 0–5 scoring scale and Image-J software to measure stain area and perimeter. Behaviour was video recorded and scan sampled to determine time budgets. Data were analysed as an REML using the MIXED procedure of SAS. Enrichment tended to increase proportion of time standing and lying laterally and decrease plasma cortisol, tear-stain area and perimeter. There was a significant isolation by enrichment interaction. Enrichment given to pigs housed in isolation had no effect on plasma cortisol, but greatly reduced it in non-isolated pigs. Tear-staining area and perimeter were highest in the NE-I treatment compared to the other three treatments. Eosinophil count was highest in the E-NI treatment and lowest in the NE-I treatment. The results suggest that in the absence of enrichment, being able to see another animal but not interact may be frustrating. The combination of no enrichment and isolation maximally impacted tear staining and eosinophil numbers. However, appropriate enrichment coupled with proximity of another pig would appear to improve welfare.*

Keywords: *animal welfare, cortisol, enrichment, isolation, pigs, tear staining*

Introduction

Refinement is the attempt to enhance animal welfare by reducing the amount of stress inflicted on those animals housed in our care (Russell & Burch 1959) and control peripheral variables which have the potential to reduce research data validity (Reinhardt & Reinhardt 2002). To refine the husbandry and use of laboratory animals, less invasive and validated methods for acquiring data and measuring stress must be developed and a perceived sense of control given to the animal through the use of environmental enrichment.

Laboratory animals are routinely subjected to procedures that have been demonstrated to induce stress (Balcombe *et al* 2004). Blood collection is one of the most common procedures conducted on laboratory animals. Stress during blood collection is induced by pain of the procedure and by handling and restraint of the animal during the procedure. Effects are seen in significant changes in corticosterone and cortisol (Armario *et al* 1986; DeBoer *et al* 1990) and immunoglobulin concentrations (Moynihan *et al* 1989, 1990), lymphocyte counts (Moynihan *et al* 1990), heart rate (Line *et al* 1989; Sharp *et al* 2003), prolactin secretion (Seggie & Brown 1975), blood pressure (Sharp *et al* 2001, 2002) and active behaviours (Sharp *et al* 2003). These findings have implications for not only the welfare of the experimental animals, but also the validity of the research performed on them. Therefore, to obtain accurate physiological measures on research animals it is imperative to reduce restraint, handling, and human interaction as much as possible. Automated blood-sampling machines have been designed to do precisely this in a number of different species, including rodents, primates, dogs (*Canis familiaris*), and pigs (*Sus scrofa*). Automated blood-sampling machines avoid the repeated stressors that occur during manual blood sampling by allowing continuous automated access to the circulatory system. This process involves surgically implanting a catheter into a major blood vessel through which direct blood collection can occur via a series of pumps. Although the surgical implantation is invasive, proper surgical techniques as well as pre- and

post-surgical recovery helps to ensure the future samples taken for the experiment are done so with minimum stress (Marchant-Forde *et al* 2012).

Animals housed in impoverished and barren environments, which in some parts of the world is often still the case for animals used in a laboratory research setting, are restricted from carrying out species-specific behaviours and lack control over their surrounding environment. These poor housing conditions often lead to the development of stereotypical or maladaptive behaviours and may have extensive effects on physiological performance, especially of the neurological and endocrine systems (Fox 1986; van de Weerd *et al* 1997; Würbel *et al* 1998). For example, pigs, when housed in isolation, show behavioural and physiological signs of stress, such as increased plasma cortisol concentrations (Stolba & Wood-Gush 1989; Ruis *et al* 2001), decreased body temperature (Ruis *et al* 2001), decreased TNF-α (Tuchscherer *et al* 2004), and increased frequency of behaviour associated with anxiety and stress (Herskin & Jensen 2000; Tuchscherer *et al* 2006). An accepted method to reduce stress in housed animals is to implement appropriate environmental enrichment. Environmental enrichment involves the enhancement of an animal's physical or social environment and is increasingly viewed as an essential research component (Guide for the Care and Use of Agriculture Animals in Research and Teaching [GUCAA] 2010).

Practical and inexpensive non-invasive methods for measuring stress are also needed to monitor animal welfare. In rats (*Rattus norvegicus*), chromodacryorrhoea, also called 'bloody tears', is a non-invasive, and qualitative method for assessing stress or disease (Mason *et al* 2004). Chromodacryorrhoea is the overproduction of porphyrin, a red secretion produced by the Harderian gland, which is found in most vertebrates (Chieffi *et al* 1996). Casual observations from our previous study (DeBoer *et al* 2013), in which pigs housed in non-enriched environments appeared to show more dark-red/brown tear staining, led us to investigate a similar phenomenon in pigs as a possible novel indicator of stress.

Therefore, the objectives of this initial study were: i) to determine the physiological and behavioural effects of isolation and environmental enrichment in pigs housed within a novel system which allowed for automated blood sampling; and ii) to determine whether tear staining in pigs could be a non-invasive indicator of stress. Our hypotheses were that: i) the combination of enrichment and no visual isolation would be least challenging, and the combination of visual isolation and no enrichment would be most challenging, for the pig in terms of welfare measures; and ii) that tear staining would be greatest in those pigs housed in visual isolation with no enrichment.

Materials and methods

All surgical and experimental procedures in this experiment were approved by the Purdue University Animal Care and Use Committee prior to conducting the experiment (PACUC approval 09-055). The animals used in this study were returned to the Purdue University Animal Sciences Research and Education Center (ASREC) pig herd at the end of the experiment. This experiment was conducted with one replicate per month between September 2010 and January 2011.

Study animals and housing

Thirty crossbred (Yorkshire × Landrace) commercial pigs (17.0 [± 1.5] kg) from ASREC were used as test subjects. Twenty pigs were used as experimental animals during data collection and the remaining ten were used as companions. We used data from previous studies in our laboratory on pigs (Mack *et al* 2014; Marchant-Forde *et al* 2014) to carry out power analyses to determine appropriate sample size, using G*Power (Version 3.1.9.2, University of Dusseldorf, Dusseldorf, Germany) as detailed in Faul *et al* (2009). The power analyses of eight blood parameters and one behavioural measure, showed that five of the parameters would have appropriate power (0.8 and above) with a total sample size of 20 or fewer animals. This, combined with cost, influenced our sample size decision. No data were available to carry out *a priori* power analysis for tear-staining measures. The pigs were selected from the farm population based on similar weights (target weight: 16 kg) and prior to the experiment were housed in groups in flat-deck weaner pens (1.4 × 1.4 m; length × width), with perforated metal floors, and *ad libitum* access to water and Purdue's feed mill standard swine nursery phase 4 diet, containing 17.0% crude protein and 3.35 kcal g^{-1} digestible energy, fed from a feed hopper. Within each replicate, the pigs were selected from different litters and pens. Six animals were used per replicate — four pigs as test subjects with an additional two pigs serving as 'companion' animals — over five replicates but four test subject animals were excluded from the study due to catheter failure.

For each replicate, the six pigs were transported 17 km from ASREC to Purdue University's Veterinary Animal Holding Facility. Upon arrival, pigs were immediately placed in pairs into three raised, perforated, metal-floored, holding pens (2.4 × 1.2 m; length × width) for acclimatisation. Partitions between pens were made of stainless steel mesh so that pigs had visual, olfactory, auditory and limited tactile communication with pigs in neighbouring pens. Pigs had *ad libitum* access to the same nursery phase 4 diet and had water provided from a nipple drinker *ad libitum*.

After five days of acclimatisation, four of the pigs underwent surgical catheterisation and were randomly assigned to one of four treatments within the PigTurn® (BASi, West Lafayette, IN, USA) housing system. The treatments were a 2 × 2 factorial combination of enrichment (non-enriched [NE] or enriched [E]) and isolation (visually isolated [I] or able to see one of the two companion pigs [NI]; see Figure 1), giving four treatments: E-I (n = 5); E-NI (n = 4); NE-I (n = 3); and NE-NI (n = 4). Enrichment included the provision of both a rubber mat (cut into a trapezoid with approximately 0.5 m^2 area) and a mirror (durable, cut to fit completely into one of the eight sides of the PigTurn®). Within the PigTurn®, the mat was positioned directly beneath the mirror. Both of these enrichment items were chosen on the basis of previous work (DeBoer *et al* 2013), which showed that both of these elements were beneficial enrichments as defined by Würbel and Garner (2007) in that they provided, respectively, a

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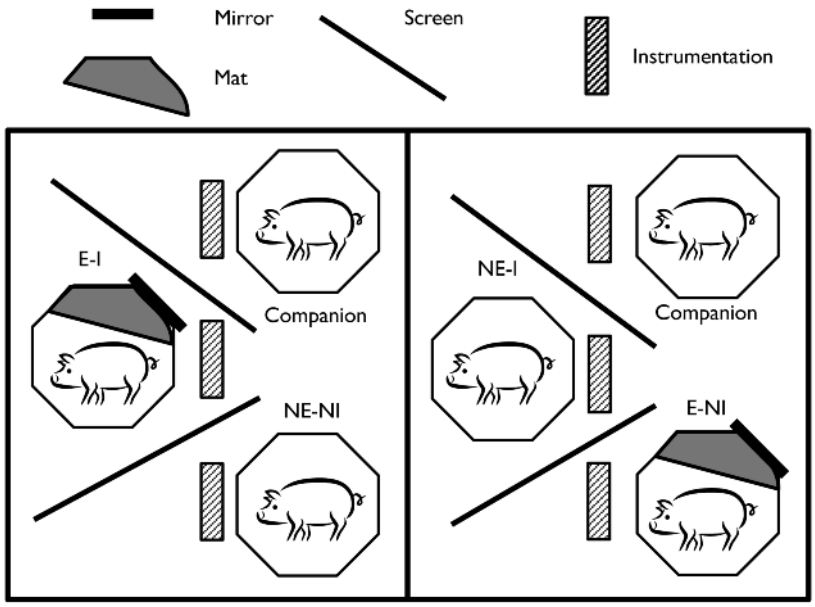
Figure 1



Room plan used for enrichment study showing the four treatments: E-I, E-NI, NE-I and NE-NI which combined non-enriched (NE) or enriched (E) and visually isolated (I) or able to see one of the two companion pigs (NI). Treatment location was rotated between and within rooms between replicates.

preferred comfortable lying area and a social buffer in times of perceived threat (DeBoer *et al* 2013). The mat was bolted onto the rubber-coated perforated metal floor and the mirror was also attached firmly to the pen wall to prevent damage. Both were cleaned daily to maintain effectiveness.

For a full description of the PigTurn® housing system, see Marchant-Forde *et al* (2012) but, briefly, the PigTurn® consists of a single-animal, octagonal-shaped pen with 1.12 m^2 floor area, which allows a catheterised pig to be attached to an automatic blood-sampling system, yet move freely and avoid catheter twisting by counter-rotating against the direction of the pig's movement (see Figure 2). After 48 h post-surgery recovery in the holding pens, each pig was moved into a PigTurn® and tested for seven days. While within the PigTurn®, pigs were fed 0.6 kg of standard swine nursery phase 4 diet per day and provided water *ad libitum*, which was checked on a daily basis. Artificial light was kept on between 0600 and 1800h. The same human entered the room every morning around 0800h for pen cleaning, testing drinkers for proper functioning, and feeding, lasting approximately 30 min. The treatments were rotated with every replication so that the treatments were completely balanced across the two rooms to the end of the study. The pigs used for this study were naïve to the enrichment objects.

Surgical procedures

Pigs used as companions did not undergo surgery, whereas experimental pigs placed into the PigTurn® housing system underwent catheterisation surgery, five days after arrival. All four pigs underwent surgery on the same morning. For catheterisation, feed was withdrawn 12 h before surgery and anaesthesia was induced by an intramuscular (IM) injection of 2.2 mg kg^{-1} of bodyweight

Figure 2

The PigTurn® housing system showing a catheterised pig wearing the harness housed within the octagonal pen, the tether attached to the optical sensor arm and the cabinet unit.

Table 1 DeBoer-Marchant-Forde descriptive scale used for evaluation of tear-stain scores.

Score	Description
0	No signs of any staining
1	Staining is barely detectable and area stained does not extend below the eyelid
2	Staining is obvious and area stained is approximately < 50% of total eye area
3	Staining is obvious and area stained is approximately 50–100% of total eye area
4	Staining is severe, area stained is approximately ≥ 100% of total eye area, and area stained does not extend below the mouth line
5	Staining is severe, area stained is > 100% of total eye area, and area stained extends below the mouth line

(BW) each of tiletamine, zolazepam (combined as Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA), ketamine (Ketaset, Fort Dodge Animal Health) and xylazine (Sedazine, Fort Dodge Animal Health). Anaesthesia was maintained by cone delivery of 1 to 4% of isoflurane with oxygen. Effective anaesthesia was tested by ensuring that palpebral reflexes were diminished, interdigital pinch yielded no response, and there was absence of movement to physical stimuli. Instruments were packed and autoclaved. Surgery was carried out using sterile technique.

For the surgery, pigs were placed in dorsal recumbency with forelimbs pulled caudally and an incision was made over the jugular fossa. The external jugular vein was isolated and two loose ligatures of 2-0 non-absorbable suture (Ethilon, Ethicon Inc, Somerville, NJ, USA) were placed around the vein, 2 cm apart. The incision was packed with sponges soaked in sterile isotonic saline and the pig was rolled to lateral recumbency. The dorsal cervical exit site was re-scrubbed and draped. An approximate 3-cm incision was made and bluntly dissected 3 to 4 cm subcutaneously towards the ventral cervical incision. A trocar was passed from the dorsal site to the ventral cervical incision as an assistant protected the vessels. The stylet of the trocar was withdrawn and the catheter was fed through. The trocar was withdrawn as the surgeon set the 7-french, double-lumen, central venous catheter (Arrow International, Reading, PA, USA) into the dorsal incision. A mattress suture of 2-0 absorbable material (Monocryl, Ethicon Inc, Somerville, NJ, USA) in the subcutaneous tissue secured the catheter and the same suture was used to close the dorsal incision in a continuous pattern. The pig was then brought back to dorsal recumbency and jugular catheter installed by making a small nick in the vessel and utilising a vessel pick to feed the catheter towards the heart. Placement was verified by the ease of pulling blood into the catheter using a sterile syringe pre-filled with isotonic saline. When the surgeon was satisfied with placement, the ligatures on either side of entry of the catheter into the jugular were tied and the incisions closed. An H-harness was fitted to the pig and the catheter end was attached to the harness using a plastic cable tie. The catheter was locked with 500 µl of heparinised (300 units ml^{-1}) glycerol and the pig was given an IM analgesic injection of flunixin meglumine (Banamine, Merck Animal Health, Summit, NJ, USA) at 2.2 mg kg^{-1} of BW and an IM antibiotic injection of Cefazolin (Excede, Zoetis, Florham Park, NJ, USA) at 50 mg kg^{-1} of BW. Each pig was then transported to an individual holding pen for recovery after voluntary movement was evident. Towels were used under and over the animal to maintain body temperature and continuation of recovery was in a darkened room with monitoring every 15 to 30 min until the animal was standing steadily. After standing, lighting was increased. All surgeries took approximately 45–60 min and were carried out between 0800 and 1200h.

Approximately 48 h post surgery (0700h on Day 1), the experimental pigs were walked 20 m down a corridor to the experimental rooms and placed individually into a PigTurn®, where each pig's harness was hooked up to the tether and the pen rotation system was activated. The companion pigs were placed into the PigTurn® system the day of surgery. The catheter was attached to a 2-m catheter extension fixed to the automatic sampling system (Culex-L, BASi, West Lafayette, IN, USA), which was programmed to tend the catheter at 6 min intervals, ie to push 1 ml of heparinised (10 units ml^{-1}) saline into the pig to maintain catheter patency.

Experimental procedures

Once in the PigTurn®, blood was sampled two times a day over seven days. Automated blood collection was controlled by an automated sampling system (Culex, BioAnalytical Systems Inc, West Lafayette, IN, USA). The sampling system (Culex) was programmed to collect one 1-ml blood sample 2 h after the programme was started, timed to begin immediately after the person responsible for daily health checks and husbandry left that morning (at approximately 0900h). These samples, used to analyse cortisol, were then collected without human presence by the Culex system. During sampling, the blood was drawn up through the catheter and the extension line into a plastic reservoir. The blood sample was then pushed from the bottom of the reservoir into sealed EDTA vials contained within a chilled carousel. A sensor within the Culex measures the haematocrit level to ensure the sample contained whole undiluted blood. Once the sample was collected, the remaining blood in the reservoir and the extension line and 1 ml of heparinised saline was pushed back into the pig. Catheter patency was maintained continuously by periodic automated flushing of small amounts of heparinised saline. The total volume of the implanted catheter and catheter extension was 6.6 ml.

At 1300h, a human entered the room and drew a 3-ml sample into an EDTA tube through the Culex for the haematology analysis. The 1-ml samples were removed from the carousel and, along with the 3-ml samples, placed on ice. The 3-ml samples were run immediately for WBC differential counts. The 1-ml samples for cortisol analysis were centrifuged 13,000 × g for 15 min at 4°C for separation of plasma. Plasma was stored at –80ºC until analysed.

Tear staining

On the day of entry to the PigTurn®, the pigs' faces were wiped clean using wet wipes. Data for the tear staining were then collected each day at 1400h. The experimenter re-entered the room and took digital photographs of the left and right sides of each pig's face, using a digital camera (Nikon D-60, Nikon Corp, Tokyo, Japan). Photographs were analysed using a descriptive scoring scale (DeBoer-Marchant-Forde [DMF] Scale; Table 1, Figure 3) and pigs were assigned a score

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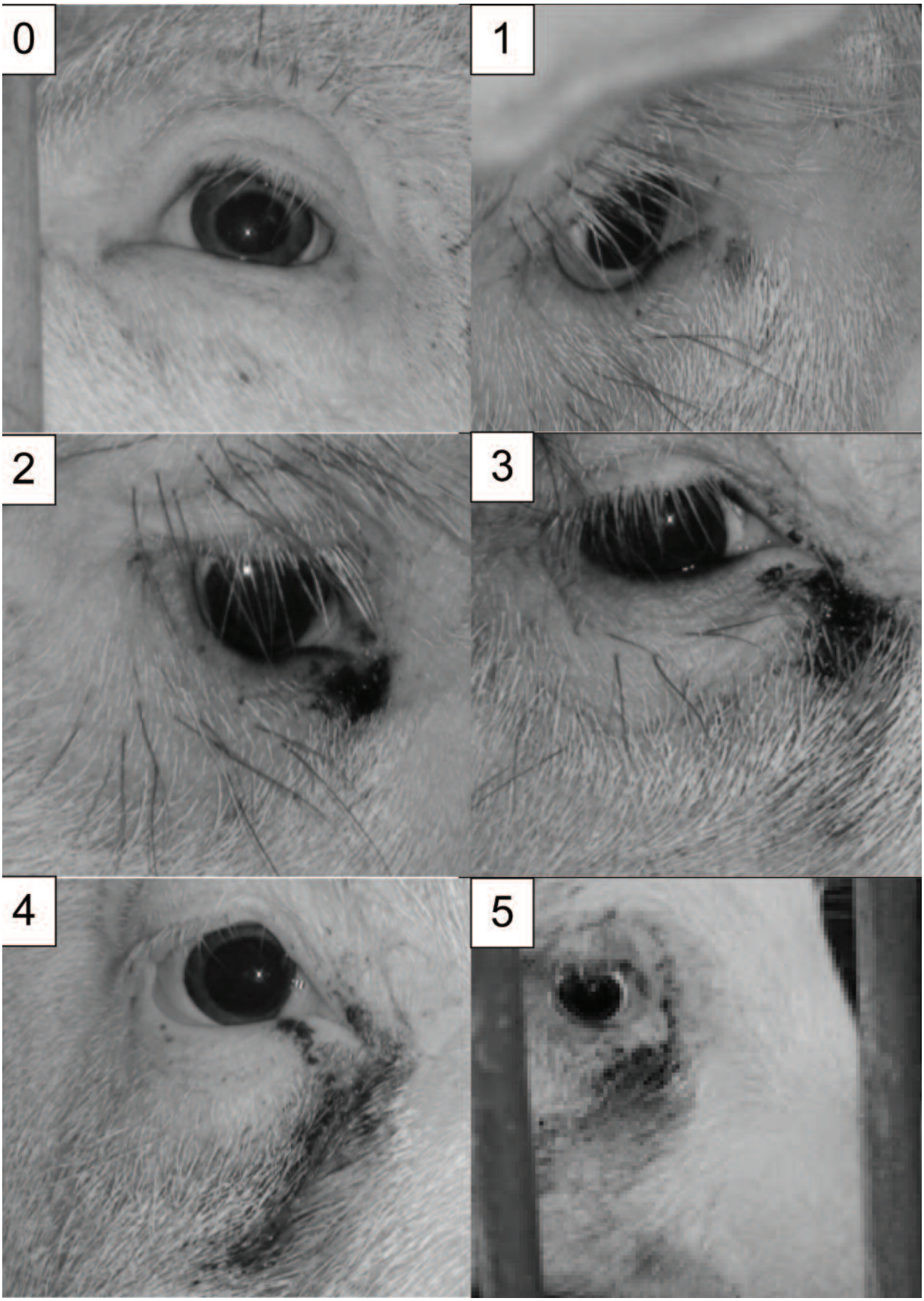
Figure 3



Example photographs showing the relative amounts of tear staining as scored using the DeBoer-Marchant-Forde 0–5 descriptive scale.

Table 2 Ethogram used for evaluation of time-budget behaviours and postures and their categories.

Category	Behaviours	Description
Maintenance	Eat	Head in feeder
	Drink	Mouth in contact with nipple drinker
	Eliminate	Excretion of either urine or faeces
Walk	Walk	Feet moving in a way that advanced the animal
Investigate	Root floor	Snout in contact with the woven wire floor
	Pen interaction	Snout in contact with material comprising the pen
	Alert	Head upright and ears erect
	Nose mat	Snout in contact with mat
	Nose mirror	Snout in contact with mirror
Inactive	Inactive	No behaviours being performed
	Postures	**Description**
	Standing	Pig is supporting its bodyweight equally on all four legs
	Lying sternally	Pig is lying upright with chest touching the ground
	Lying laterally	Pig is lying on its side with shoulder touching the ground
	Sitting	Front half of pig is upright, while hindquarters are touching the floor

between 0 (no staining) and 5 (severe staining). Two experienced observers, who were blind to the treatments, scored the same photographs and the score was averaged for each side of the face separately. The photographs were also analysed using the freeware Image-J software (NIH, Rockville MD, USA), which enabled area and perimeter of stain to be calculated using the width of the harness as a reference distance. Left and right eyes were analysed separately as preliminary analyses of this and other studies has shown side differences in staining.

Behaviour recording

Pigs were video recorded from 0600 to 1800h each day. Video was recorded using a Digital Video Recorder (DVR) (Inter-pacific, CV-S4DVRLX, USA) and cameras (Panasonic, WV-CL 350, Japan) with detachable lens (Computar lens, TG4Z2813FCS-31, USA). Each camera was mounted to the ceiling above each tested PigTurn® pen and connected to the DVR. The video was analysed by one highly experienced observer for time budget information by scan sampling every 10 min. For each observation four behaviours and four postures were recorded for the tested individual using the elements described in Table 2. Using the PigTurn®'s own recording software, the number of bouts (total number of times the PigTurn® rotated to the left or right) per day and the total duration (total time the PigTurn® rotated to the left or right) per day were recorded automatically.

Cortisol

Quantification of cortisol concentration was performed though a commercially available radioimmunoassay (I^{125}) kit purchased through DiaSorin (Stillwater, Minnesota, USA) and carried out according to manufacturer's instructions. The kit has been previously validated for pigs (Haussmann *et al* 2000). Samples were run in duplicates and re-run if CV > 12%. Intra-assay CV was < 6% and inter-assay CV was 10.6%. Sensitivity of the assay is 0.21 µg dl^{-1}.

Differential WBC count

Blood samples were run approximately 15–30 min after collection for WBC differential using a Hemavet 950 Hematology Analyzer (Drew Scientific, Dallas, TX, USA). Until then, the samples were placed on ice until a few minutes before being run, allowing them to come to room temperature for the HemaVet analysis. Blood cell measures consisted of haematocrit percentage; counts of total white blood cells and counts and percentages of basophils, eosinophils, neutrophils, monocytes, and lymphocytes.

Statistical analysis

All data were tested for normality and transformed appropriately where necessary. Data were analysed as a repeated measures mixed model (REML) using the MIXED procedure, or a correlation (PROC CORR) where appropriate, in SAS for Windows (2005, SAS Institute, Cary, NC, USA). The data for sex were not different in preliminary analysis and not included in the statistical model. Room and replicate were not included in the model because of the balanced treatments. The model included the fixed effects of isolation, enrichment and day and their interactions. Pig was treated as a random effect in the analysis and was nested within the treatments. Significant results ($P < 0.05$) were examined with LS Means and LS Mean Slices to further describe the relationship between the tested interactions. Numerical data are presented as back-transformed means (± SEM).

Results

Tear staining

Tear staining developed quickly from Day 1 and continued to increase over the course of the study for most measures (Figure 4). When looking at the left eye, isolation significantly affected all measures of tear staining. Pigs that were isolated had greater stain area (0.93 [± 0.13] vs 0.49 [± 0.12] cm^2; $P < 0.01$) and perimeter (4.86 [± 0.43] vs 3.06 [± 0.33] cm; $P < 0.01$) than those that were non-isolated and a higher stain DMF score (1.82 [± 0.12] vs 1.24 [± 0.17]; $P < 0.01$). Also, with the left eye, enrichment affected stain area and perimeter. Pigs housed in non-enriched PigTurns® had greater stain area (0.92 [± 0.20] vs 0.50 [± 0.05] cm^2; $P < 0.01$) and perimeter (4.62 [± 0.50] vs 3.30 [± 0.25] cm; $P < 0.01$) than those that were in enriched PigTurns®. In terms of treatment, NE-I pigs had greater left eye stain perimeters and tended to have greater left eye stain areas than all other treatments (Figure 4, Table 3).

With the right eye, isolation affected area of tear staining and tended to affect DMF score. Pigs that were isolated had

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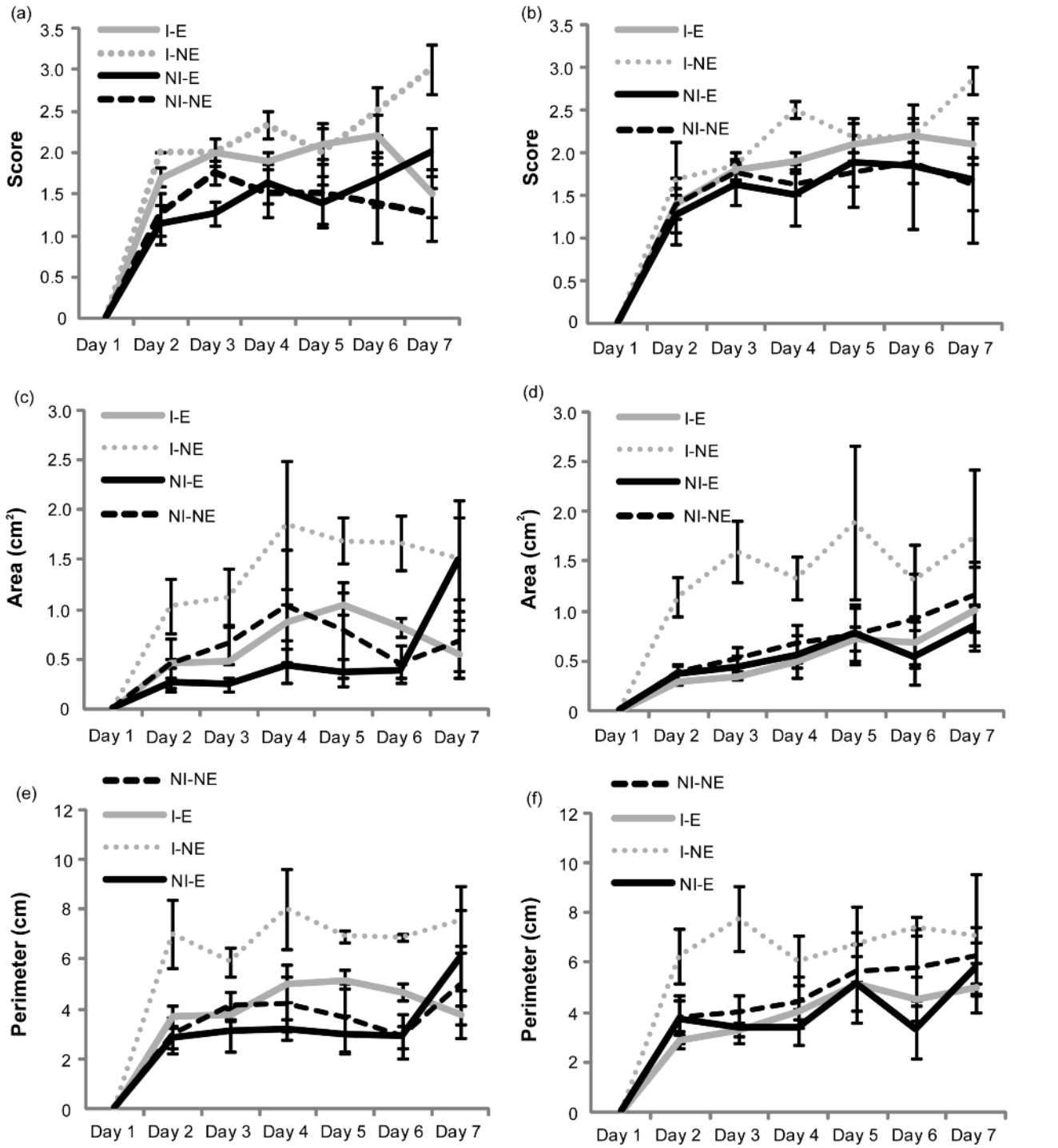
Figure 4



LS Means (± SEM) of (a) left eye tear-stain score, (b) right eye tear-stain score, (c) left eye tear-stain area, (d) right eye tear-stain area, (e) left eye tear-stain perimeter and (f) right eye tear-stain perimeter of pigs assigned to each treatment over the course of seven-day experiment.

greater stain area (0.90 [± 0.12] vs 0.55 [± 0.14] cm^2; $P < 0.05$) and tended to have a higher stain DMF score (1.71 [± 0.08] vs 1.41 [± 0.16]; $P = 0.06$) than those that were non-isolated. Also, with the right eye, enrichment affected stain area and perimeter. Pigs housed in non-enriched PigTurns® had greater stain area (0.95 [± 0.15] vs 0.50 [± 0.12] cm^2; $P < 0.01$) and perimeter (5.07 [± 0.49] vs 3.54 [± 0.50] cm; $P < 0.05$) than those that were in enriched PigTurns®. In terms of treatment, NE-I pigs had greater right eye stain perimeters than all other treatments (Table 3, Figure 4).

Table 3 Mean (± SEM) of various physiological and immunological measures in pigs subjected to isolation and enrichment treatment.

	Treatment				Main effects		Interaction effect
	Enr (E)		Not Enr (NE)		Enrichment	Isolation	Enr × Iso
Measures	**Iso (I)**	**Not Iso (NI)**	**Iso (I)**	**Not Iso (NI)**	***P*-value**	***P*-value**	***P*-value**
Physiological							
Cortisol (μg dl^{-1})*	1.48 (± 0.18)	1.10 (± 0.20)	1.49 (± 0.24)	2.15 (± 0.20)	< 0.05	ns	< 0.05
Immunological							
Total WBC count (K μl^{-1})	13.0 (± 0.9)	14.1 (± 1.1)	12.6 (± 1.2)	12.5 (± 1.1)	ns	ns	ns
Basophils (K μl^{-1})	0.014 (± 0.005)	0.021 (± 0.006)	0.018 (± 0.006)	0.018 (± 0.005)	ns	ns	ns
Eosinophils (K μl^{-1})*	0.22 (± 0.06)	0.55 (± 0.07)	0.15 (± 0.08)	0.31 (± 0.06)	< 0.05	< 0.05	ns
Lymphocytes (K μl^{-1})	6.75 (± 0.69)	6.39 (± 0.77)	6.24 (± 0.83)	6.76 (± 0.77)	ns	ns	ns
Monocytes (K μl^{-1})*	0.62 (± 0.12)	0.73 (± 0.14)	0.92 (± 0.15)	0.61 (± 0.13)	ns	ns	ns
Neutrophils (K μl^{-1})	5.35 (± 0.75)	5.51 (± 1.03)	5.36 (± 0.92)	5.61 (± 0.95)	ns	ns	ns
Basophils (%)	0.10 (± 0.04)	0.14 (± 0.04)	0.15 (± 0.05)	0.12 (± 0.04)	ns	ns	ns
Eosinophils (%)*	1.92 (± 0.47)	4.06 (± 0.55)	1.39 (± 0.6)	2.37 (± 0.52)	0.07	< 0.05	ns
Lymphocytes (%)	52.7 (± 4.0)	49.0 (± 4.7)	48.5 (± 4.7)	53.5 (± 4.4)	ns	ns	ns
Monocytes (%)*	5.22 (± 0.70)	5.59 (± 0.78)	7.58 (± 0.84)	4.37 (± 0.79)	ns	ns	< 0.05
Neutrophils (%)	39.1 (± 4.7)	41.1 (± 6.4)	43.0 (± 5.6)	40.0 (± 5.8)	ns	ns	ns
HCT (%)	25.8 (± 1.1)	24.9 (± 1.3)	26.4 (± 1.3)	24.5 (± 1.2)	ns	ns	ns
Neutrophil:Lymphocyte	0.79 (± 0.13)	1.13 (± 0.18)	0.82 (± 0.25)	0.82 (± 0.33)	ns	ns	ns
Tear staining: left eye							
Area (cm^2)	0.60 (± 0.05)	0.41 (± 0.05)	1.26 (± 0.21)	0.57 (± 0.19)	< 0.01	< 0.01	0.08
Perimeter (cm)	3.70 (± 0.25)	2.90 (± 0.25)	6.01 (± 0.61)	3.22 (± 0.41)	< 0.01	< 0.01	< 0.05
Score	1.64 (± 0.12)	1.24 (± 0.10)	1.99 (± 0.13)	1.23 (± 0.24)	ns	< 0.01	ns
Tear staining: right eye							
Area (cm^2)	0.51 (± 0.12)	0.49 (± 0.12)	1.28 (± 0.13)	0.62 (± 0.17)	< 0.01	< 0.05	< 0.05
Perimeter (cm)	3.52 (± 0.34)	3.55 (± 0.63)	5.88 (± 0.41)	4.25 (± 0.67)	< 0.05	ns	ns
Score	1.64 (± 0.09)	1.39 (± 0.30)	1.88 (± 0.06)	1.43 (± 0.03)	ns	0.06	ns

* Denotes significant day effects.
iso: isolation treatment; enr: enrichment treatment.

Behaviour and posture

Behaviour was not influenced by isolation (REML: $F_{3,32} = 0.16$; $P > 0.05$) or enrichment (REML: $F_{3,32} = 1.61$; $P > 0.05$) treatments. Although not statistically significant, both isolation (REML: $F_{3,32} = 2.90$; $P = 0.0502$) and enrichment (REML: $F_{3,32} = 2.78$; $P = 0.057$) showed a strong tendency to influence posture. Enriched animals tended to spend a greater proportion of time standing (0.34 [± 0.03]) and lying laterally (0.34 [± 0.03]) and less time lying sternally (0.35 [± 0.03]) compared to the non-enriched animals (stand: 0.31 [± 0.02], lying sternally: 0.40 [± 0.02], lying laterally: 0.27 [± 0.02]). Additionally, isolated pigs tended to spend a greater proportion of time lying laterally (0.35 [± 0.02]) than those not isolated (0.27 [± 0.03]). Animals that were not isolated tended to spend a greater proportion of time lying sternally (0.40 [± 0.03]) than those that were isolated (0.35 [± 0.02]). There were no differences between treatments in total number of bouts of rotation of the PigTurn® (REML: $F_{1,14} = 0.47$; $P > 0.05$) nor total rotation duration (REML: $F_{1,14} = 0.66$; $P > 0.05$).

Cortisol concentration

The presence of enrichment influenced cortisol concentration (REML: $F_{6,67} = 6.47$; $P < 0.05$; Figure 5). Pigs without enrichment had higher cortisol concentrations (1.82 [± 0.16] μg dl^{-1}) than those that were enriched

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Figure 5

Mean (± SEM) cortisol concentration (μg dl^{-1}) of treatment interactions. Different superscripts indicate differences in pair-wise comparisons (Tukey: $P < 0.05$).

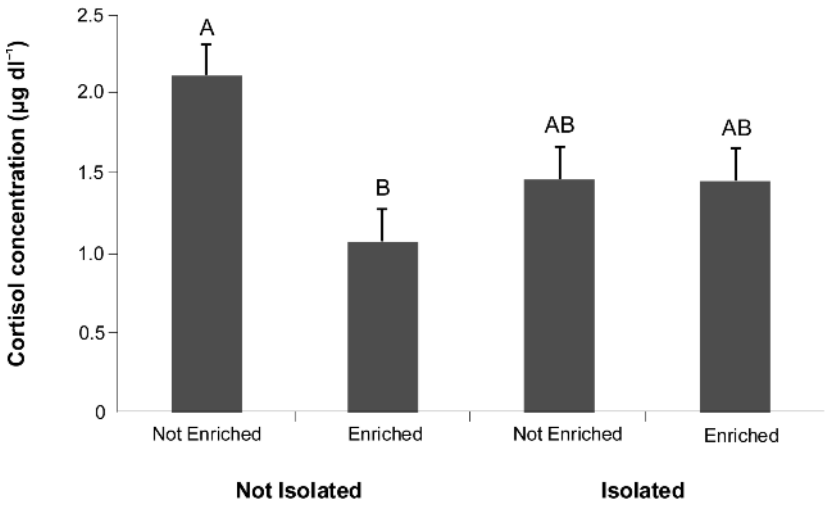
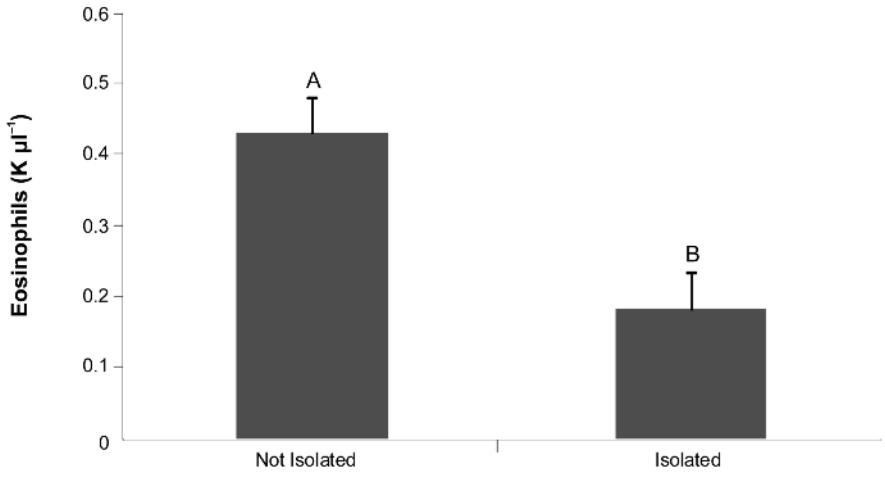


Figure 6

Mean (± SEM) of eosinophil count (K μl^{-1}) of isolation treatment. Different superscripts indicate differences in pair-wise comparisons (Tukey: $P < 0.05$).



(1.29 [± 0.14] μg dl^{-1}). Isolation had no effect on cortisol concentration ($P > 0.05$). However, an isolation by enrichment interaction was seen (REML: $F_{6,67} = 6.42$; $P < 0.05$). Pigs that were not isolated and not given enrichment had higher concentrations of cortisol (2.15 [± 0.20] μg dl^{-1}) than those that were not isolated and given enrichment (1.10 [± 0.20] μg dl^{-1}).

White blood cell counts and percentages

Only eosinophil count and percentage were influenced by both isolation (REML: $F_{1,64} = 8.74$; $P < 0.05$; see Table 3) and enrichment treatments (REML: $F_{1,64} = 3.71$; $P < 0.05$; see Table 3, Figure 6). Animals which were not isolated had higher eosinophil counts than those that were isolated (0.43 [± 0.06] vs 0.18 [± 0.05] K μl^{-1}; $P < 0.05$). Enriched animals were also found to have higher eosinophil counts than those housed without enrichment (0.39 [± 0.05] vs 0.23 [± 0.05] K μl^{-1}; $P < 0.05$). Eosinophil percentages were higher in those not isolated (3.2 [± 0.4]%) than those that were (1.7 [± 0.4]%; $P < 0.05$). Additionally, an isolation by enrichment interaction effect was found to influence monocyte percentage (see Table 3). Animals which were both isolated and without enrichment had significantly higher monocyte percentages than those not isolated and not enriched. For basophil, lymphocyte, and neutrophil counts and percentages, as well as haematocrit and total WBC percentages, treatment did not have an effect ($P > 0.05$).

Discussion

The objectives of this study were to determine if providing enrichment objects, which could be used within the potential limitations of a laboratory setting, would have beneficial effects in terms of welfare measures on pigs housed individually, and to investigate whether tear staining could be a potential non-invasive indicator of welfare. The enrichment items (rubber mat and mirrors) were chosen as a result of previous work (Elmore *et al* 2010; DeBoer *et al* 2013) and were deemed to be beneficial enrichments, in that they are

biologically relevant and would benefit the animal's welfare without negative welfare consequences (Würbel & Garner 2007). We also examined a factor of social isolation, defined in our study as whether the pigs could or could not see another pig within the same room. Overall, although the study was limited by small sample size, the presence of enrichment would appear to have positive effects, whereas the effects of isolation/non-isolation were equivocal, perhaps indicating that our perceived beneficial effect of having visual contact with another pig is not actually perceived by the pig as beneficial. Our *a priori* power analyses using behavioural, physiological and haematological data from our laboratory showed that with 20 pigs, there would be enough power in the sample size to give reliable results (power greater than 0.80) in many of the parameters measured. However, catheter failures reduced our overall sample size to 16 pigs thereby reducing our power. *Post hoc* analyses using the tear-staining data generated in this study indicate that even with only 16 animals, we achieved power ranging from 0.99 to 0.86 across the six measures reported in Table 3. Further studies with larger numbers of animals would be beneficial for more explicit conclusions to be drawn on the haematological data in particular, but the interpretation and conclusions regarding the tear-staining results are valid with this sample size.

Tear staining in pigs is a well-known phenomenon, but is only described in the literature as a symptom indicative of atrophic rhinitis (Jackson & Cockroft 2007; Register 2012) or environments with high levels of dust and/or ammonia (Straw *et al* 2006). To our knowledge, this current study is the first report of the use of tear staining in pigs as a potential welfare indicator. Pigs in this study were all clinically healthy and as the study was carried out in laboratory housing, the air quality was excellent and uniform across all treatments. Therefore, the differences seen across treatments are real treatment effects, and are not due to incidental environmental or health differences. Tear staining has been described in rodents in response to stress (Mason *et al* 2004; Burn *et al* 2006), and the red-brown chromodacryorrhoea is attributed to secretion of porphyrins from the Harderian gland (Harkness & Ridgway 1980) under autonomic and endocrine control (Payne 1994). The pig also has well-developed Harderian glands capable of secreting porphyrins (McCafferty & Pinkstaff 1970) and it is therefore possible that the increased staining seen in pigs housed in the non-enriched/isolated treatment is a result of Harderian gland activation. More research is required to validate this finding, but preliminary results of further studies would appear to show that staining is correlated with social rank when pigs are group housed (Marchant-Forde & Marchant-Forde 2014) and also with measures of HPA and SAM axis activation (DeBoer & Marchant-Forde 2013).

There was no effect of enrichment or isolation on the behavioural elements that we recorded. Ordinarily, it might be expected that pigs with enrichment would show enrichment-object-directed activity as seen in a laboratory setting (Smith *et al* 2009) and on-farm (Guy *et al* 2013). However, the enrichment objects used in this study were designed to improve the pig's comfort rather than be interactive. It is known that deformable and suspended point-source objects in particular stimulate the pig's exploratory behaviour (Averós *et al* 2010), but also that enrichment *per se*, does not impact overall activity that is not object-directed (Guy *et al* 2013). Therefore, the lack of an enrichment effect on behaviour is not unexpected.

The lack of an isolation effect however, is perhaps more surprising. It might be expected that pigs housed in visual isolation, but with the other sensory inputs of pigs nearby, might show increased disturbance or restlessness, with more time spent alert, walking and interacting with the pen. However, the fact that this did not occur is perhaps indicative that our non-isolated treatment was in fact more disturbing than envisaged. A study by Herskin and Jensen (2000) examined different degrees of isolation on pigs in a laboratory setting and found that their equivalent of our non-isolated treatment — that is pigs which were housed individually, but could see other pigs — increased stress-related behaviours compared with group housing and housing individually but with close proximity and limited tactile communication through wire mesh pen dividers. Thus, our isolated and non-isolated treatments perhaps did not actually differ very much in the pig's perception.

There were strong tendencies for differences in posture between treatments, with enriched and isolated treatments spending more time lying laterally and decreased time lying sternally compared to non-enriched and non-isolated treatments. Rubber mats have been shown previously to increase time spent lying laterally in pigs (Tuyttens *et al* 2008; Elmore *et al* 2010) and this posture is associated with comfort and restfulness (Street & Gonyou 2008). Again, this result might be expected in our enriched treatments, but it is more surprising that our visually isolated pigs spent more time in the most restful posture compared to our non-isolated pigs.

Differences in cortisol concentration between treatments demonstrated that the provision of enrichment has an effect on the hypothalamo-pituitary-adrenal (HPA) axis and possibly immune function. Animals not given enrichment had higher cortisol levels compared to those given enrichment. Higher cortisol concentrations are associated with stress (Wiepkema & Koolhaas 1993; De Jonge *et al* 1996; Moberg & Mench 2000). The provision of enrichment has previously been shown to reduce cortisol concentrations in pigs in production environments (Warnier & Zayan 1985; Klont *et al* 2001). Additionally, the impact of enrichment was greatest in those animals that were not isolated. Non-isolated animals housed without enrichment had significantly higher levels of cortisol than non-isolated animals housed with enrichment; while no difference was found between isolated animals housed with or without enrichment. Therefore, the increase in cortisol in animals not isolated but not enriched may be due to frustration caused by an inability of the pig to bring itself in closer proximity to the companion seen across the way. Frustration due to various reasons has been

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previously implicated in higher levels of cortisol and increased activity (Wood-Gush *et al* 1975; Lewis 1999). Individual housing with the ability to see a conspecific has also been found to induce frustration measured by posture changes, frequency of escape attempts and frequency of pawing behaviour (Herskin & Jensen 2000). In our study, we found no significant behaviour changes, but non-isolated animals tended to spend more time lying sternally than ventrally. This reduced restfulness has also been observed in animals known to be frustrated (Lewis 1999).

Isolation has been found in previous studies to significantly impact the welfare of the animals, seen with an increase of cortisol production in pigs (Stolba & Wood-Gush 1989; Ruis *et al* 2001). One possible reason for the lack of difference in cortisol concentration between our isolated-enriched and our isolated-non-enriched treatments is that our animals were not completely isolated; they had no visual ability to see each other, but they were housed in the same room with other pigs on study. This may have provided the animal with enough olfactory and auditory stimulation from the visually obstructed pigs to reduce the stress of isolation.

Isolation and enrichment both affected eosinophil counts and percentage, with pigs housed in isolation and pigs housed without enrichment having lower numbers than non-isolated and enriched pigs. Eosinophils are multifunctional cells involved in initiation and propagation of diverse inflammatory responses and also modulators of innate and adaptive immunity (Rothenberg & Hogan 2006). Decreases in eosinophils have been documented in numerous cases of stress in mammals (Dreyfuss & Feldman 1952; Louch *et al* 1953; Dreyfuss & Czaczkes 1959; Forssberg *et al* 1961; Jain 1993) and specifically in transport stress in pigs (Averós *et al* 2009) although the role of eosinophils in the stress response has yet to be understood. Monocytes are cells with phagocytic function, which both circulate in the blood and also migrate into tissue becoming macrophages in response to inflammation. In general, monocytes increase in response to stress and a previous study in pigs has demonstrated an increase in monocyte count in pigs exposed to high ammonia levels (von Borell *et al* 2007). The results of the current study are in accordance with our hypothesis that the combination of no enrichment and visual isolation would be most stressful and the NE-I treatment had highest percentage monocytes and numerically highest monocyte count.

Conclusion

Our results indicate that overall, tear staining may be a useful, non-invasive measure of welfare. The presence of enrichment had positive effects. We found that non-isolated pigs that were not given enrichment had higher cortisol levels than non-isolated pigs given enrichment. Additionally, isolated pigs housed with no enrichment had the highest tear staining and monocyte levels and lowest eosinophil levels. However, being able to see another pig, but not interact, may increase some indicators of stress. Larger studies are needed to fully confirm these initial findings.

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