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Qualitative behavioral assessment of transport-naïve and transport-habituated sheep

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ABSTRACT: Objective and issue-neutral qualitative assessments of livestock behavior could provide a powerful assessment of welfare, augmenting quantitative measures such as autonomic and endocrine changes, which are often difficult to assess under many commercial livestock conditions. We set out to validate the use of qualitative behavioral assessment (QBA) in sheep using controlled experimental conditions (transport as a challenge) and comparing assessments against physiological variables. The behavioral expression of 14 Merino wethers, which had never experienced land transport, were assessed during their first road event (naïve to transport), and then again on their seventh event, 8 d later (habituated to transport). Blood samples were collected immediately before loading and after unloading, and heart rate and core body temperature were measured continuously throughout each event. Continuous video footage recorded during each event was used to provide clips of individual animals that were shown to observers for QBA. There was significant consensus ($P < 0.001$) amongst 63 observers in terms of their assessment of the behavioral expression of the sheep. Transport-naïve sheep were assessed as being more 'alert', 'anxious', and 'aware', whereas transport-habituated sheep were more 'comfortable', 'tired', and 'confident' ($P = 0.015$). Heart rate and heart rate variability, core body temperature and a stress leukogram were greater ($P < 0.05$) in sheep during the first (naïve) event compared with the habituated event, and were significantly correlated with the QBA scores ($P < 0.05$). In conclusion, QBA is a valid, practical and informative measure of behavioral responses to transport.

Key words: behavior, habituated, naïve, qualitative behavioral assessment, stress physiology, sheep, transportation, welfare

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INTRODUCTION

Many animal welfare assessment methods tend to quantify individual behavioral or physiological variables, which are often measured in isolation. By contrast, holistic approaches, which measure multiple aspects of the interaction of an animal with its environment, are likely to capture the most information possible (Wemelsfelder et al., 2000). Qualitative behavioral assessment (**QBA**) quantifies the behavioral expression or "body language" of an animal (Wemelsfelder, 2007) and may therefore be informative regarding how an animal interacts with its environment at that time.

To interpret behavioral expression, it is useful to know how such behavior correlates with measures that reflect physiological state, because most animal welfare measures have largely been derived from physiology (Grandin, 1997; Dwyer and Bornett, 2004). Stockman et al. (2011) found that assessments of the observers of cattle behavioral expression were correlated with physiological variables; heart rate, core body temperature and the stress leukogram were greater for cattle naïve to transport compared with when the same animals were habituated to transport. Such comparison between behavioral assessments and physiological

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variables provides a useful validation of the assessment of behavioral expression in animals.

Exposing animals to a novel environment is known to lead to changes in emotional responses (Boissy, 1995), and repeated exposure results in observably decreased behavioral reactions to a previously novel environment (Jones, 1977). This study uses transport as a novel challenge, because it represents an emotional and physical stressor for the sheep. The purpose of the experiment was to 1) determine whether behavioral assessments could be used to distinguish between sheep that were naïve to transport and sheep that had been transported repeatedly and 2) determine whether behavioral assessments were correlated with physiological measurements.

METHODS

All experimental procedures were reviewed and approved by the animal ethics committee at Murdoch University (Perth, Australia) and Curtin University (Perth, Australia). Responses of sheep on their first exposure to transport ("naïve") were compared with an event undertaken once they had become habituated to the transport conditions (their seventh 90-min event undertaken over 8 d). Continuous video footage was edited to identify individuals within a time window early in each transport event, which were shown to observers for behavioral assessment. These results were compared with physiological variables recorded during each event.

Animal Handling and Transport Events

Fourteen Merino wethers (14-mo old; 46.4 ± 0.4 kg) were sourced from a transport-naïve flock at Muresk Institute, Curtin University, Western Australia. The animals were housed in a group pen throughout the experiment. Animals had ad libitum access to food and water at all times.

Sheep were transported within a single deck trailer 2.0 × 3.6 × 1.6 m (width × length × height) at a stocking rate of 0.45 m^2/head. This space exceeds commercial conditions (c.f., commercial stocking rate of 0.25 m^2/head for 50 kg sheep; Anonymous, 2008) but the additional space was required to facilitate collection of adequate video footage of individuals. The sides and roof of the trailer had similar ventilation to commercial carriers in Western Australia, with 10-cm slats interspersed with 10-cm spaces. The route taken during each transport event included a mixture of principally flat main roads (speed limit 50 to 70 km/h, including stops for traffic lights only) and highways (continuous driving at the speed limit of 70 to 100 km/h). The length of the journey was approximately 65 km and took approximately 90 min. The selection of roads was similar to that experienced by sheep during their transport from adjacent farms to a local shipping port (Fremantle Port) and was largely long straight roads, where possible. The events used to habituate the sheep to transport were similar to the experimental events. The sheep were loaded from a single pen by passing them through a race, directly onto the livestock trailer, and were transported immediately they were loaded. The start time was consistent across each transport event.

Ambient temperature and relative humidity on the trailer were recorded during transport using 2 data loggers (Onset HOBO H8 Pros, #H08-032-IS, OneTemp Pty. Ltd., Adelaide, Australia) to ensure that environmental conditions on each transport event were similar. Each logger was positioned at sheep head height, with one at the front and one at the rear of the trailer. Loggers recorded dry bulb temperature (°C) and relative humidity (%) every 2 s throughout each transport event. Data were averaged over the first 40 min of each transport event for analysis. There was no rain on transport days and animals were transported during similar weather conditions.

Experimental Procedures and Physiological Measurements

Blood Collection and Hematology. Blood (25 mL) was collected via jugular venapuncture (30-mL syringe, 18-gauge needle) immediately before and after each experimental transport event, as the animals were passed through the race towards or away from the livestock trailer. Blood was then aliquoted into 1 lithium heparin tube (Becton Dickinson Pty. Ltd., New South Wales, Australia) and 2 EDTA tubes (Sarstedt Australia, Mawson Lakes, South Australia, Australia) and the tubes were immediately placed on ice after collection. Blood (1 lithium heparin and EDTA tube) was centrifuged at 600 × *g* for 15 min at room temperature and plasma removed and stored at −20°C within 1 h after collection for later analysis.

Lithium heparin plasma was used for assay of glucose, and beta-hydroxybutyrate (**β-OH**). Plasma glucose and β-OH concentrations were determined using a commercial kit (glucose: Olympus kit, Hamburg, Germany, Cat. No. OSR6121; β-OH: Randox kit, Ranbut, Cat. No. RB1007) run on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd.).

Plasma (EDTA) was used for assay of ACTH, cortisol, IGF-1, insulin, and leptin. Plasma ACTH concentration was measured using a solid-phase 2-site chemiluminescent enzyme immunometric assay (Scott-Moncrieff et al., 2003). The limit of detection was 5 pg/mL and a working range of 12 to 1,250 pg/mL. Plasma cortisol concentration was determined using a commercial RIA kit (Clinical Assays, GammaCoat, Cortisol 125I RIA

Kit, DiaSorin, North Ryde NSW, Australia). All samples were assayed in duplicate 25-μL aliquots and run in 1 assay with a limit of detection of 3.5 nmol/L. The intraassay CV for samples containing 46.1 and 85.65 nmol/L was 5.0% and 2.1%, respectively.

Plasma IGF-I concentration was assayed in duplicate by double-antibody RIA with human recombinant IGF-I (ARM4050, Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and antihuman IGF-I antiserum (AFP4892898, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA) after acid-ethanol extraction and cryoprecipitation (Breier et al., 1991). All samples were processed in a single assay with a limit of detection of 0.05 ng/mL. Two control samples containing 0.39 and 2.14 ng/mL were included in the assay and were used to estimate the intraassay CV of 7.3% and 2.3%.

Plasma insulin (Tindal et al., 1978) and leptin (Blache et al., 2000) concentrations were assayed in duplicate by a double-antibody RIA. All samples were processed in a single assay. The limits of detection were insulin: 0.5 μU/mL; leptin: 0.1 ng/mL. Six replicates of 3 control samples containing known hormone concentrations (insulin: 10.62, 3.89 and 2.49 μU/mL; leptin: 0.54, 0.97 and 1.85 ng/mL) were used to estimate the intraassay CV (insulin: 3.9, 1.9 and 5.4%; leptin: 5.1, 4.1 and 3.9%).

The remaining EDTA tube was stored at 4°C for analysis of a complete blood count (**CBC**) within 24 h after collection. Hematological variables analyzed were white blood cell count (**WBC**), red blood cell count (**RBC**), hematocrit (**HCT**), and the numbers of neutrophils, lymphotcytes, eosinophils, basophils, and monocytes (Bayer Advia 120 Hematology System with veterinary software, Pymble, NSW, Australia). The machine was calibrated daily with known controls (high and low) to ensure that all values are within a specified acceptable range and the laboratory also participates in an external hematology quality control program (RCPA Quality Assurance Programs Pty. Ltd., Surry Hills NSW, Australia). The proportion of neutrophils: lymphocytes were calculated for analysis because values for these cell types were autocorrelated and the neutrophil:lymphocyte ratio is a common measure of ruminant stress (Jones and Allison, 2007).

Heart Rate and Heart Rate Variability. Heart rate (**HR**; beats per min, **bpm**) was recorded every 5 s during transport events using external HR monitors (Polar Equine S625X, Polar Electro Öy, Kempele, Finland). Harnesses specifically designed for sheep fitted around the thorax of each individual holding 2 electrodes close to the skin; heart beat signals were transmitted wirelessly and stored on a data logger kept within a pouch on the harness. A patch of wool (5 × 5 cm) was shaved to the skin just behind the left shoulder blade and at the elbow on the right hand side. Water-soluble lubricant was applied to each electrode and the positive electrode positioned at the left shoulder blade and the negative electrode positioned at the girth on the right hand side (30 cm apart, minimum). Heart rate monitors were fitted to sheep as they were passed through the race onto the livestock trailer, immediately after pre-transport blood sampling and were removed after post-transport blood sampling. Heart rate data were downloaded using the Polar IR Interface for USB port. Average HR (bpm) and HR variability (**HRV**, calculated as the SD of beat-to-beat interval, Malik, 2006; von Borell et al., 2007) were calculated for 5-min intervals 10 to 5 min before departure and 5 to 10 min after departure (the same time as collection of video footage for QBA, see below) for comparison

Core Body Temperature. Temperature loggers (iButtons, Maxim Dalla, Sunnyvale, CA) were surgically implanted into the sheep 11 d before the commencement of the study. Loggers recorded temperature every 2 min during the experiments. After retrieval of iButtons at slaughter, data were downloaded using BoxCar Pro software (Onset Computer Corp, Bourne, MA). The loggers were calibrated after retrieval against a high accuracy certified quartz thermometer (Quot 100, Heraeus, Hanau, Germany) in an insulated water bath over the range of 37°C to 41°C. Accuracy after individual calibration was equal to 0.1°C.

Six loggers with staggered start dates were used per sheep to provide continuous logging of core body temperature (**Tcore**) throughout the experiment. The 6 loggers were taped together and dipped in several layers of biologically-inert wax (Elvax, Sasol Wax Pty. Ltd., Durban, South Africa) for waterproofing and protection. When completed, the logger unit had external dimensions of 50 × 20 × 15 mm and a mass of ~20 g. Units were sterilized for 24 h by immersing in 0.5% alcoholic chlorhexidine solution [100ml ChlorhexidineGluconate (50 mg/mL), 150 mL sterile water and 750 ml 70% (vol/vol) ethanol] before implantation.

The units were surgically implanted into the peritoneal cavity in the region of the right paralumbar fossa, as described by Beatty et al. (2008). The sheep were tranquilized with 0.1 mg/kg Xylazine (Ilium/Troy Laboratories, Glendenning, NSW, Australia) administered intramuscularly, and paralumbar nerve blocks provided regional anesthesia (Cakala, 1961).

To take into account circadian patterns of body temperature, comparison of Tcore was carried out for the first 40 min of the 2 experimental (naïve and habituated) transport events, and the same 40-min period averaged over 3 consecutive control (non-transport) days before the naïve event (naïve control) or after the habituated event (habituated control). Average and maximum Tcore were also calculated over 10-min periods for the entire journey.

Statistical Analysis. Two-way repeated-measures ANOVA was used to compare between time points (blood variables: before vs. after transport; HR data: before vs. during transport; Tcore: control days vs. transport days) and between treatments (naïve vs. habituated). Post hoc analysis was carried out using Tukey's Honest Significant Difference (**HSD**) test. Due to some missing data precluding repeated-measures analysis, a factorial ANOVA was used to compare between time points for HR and HRV. Analyses were carried out using Statistica software, version 9 (StatSoft-Inc, Tulsa, OK).

Qualitative Behavioral Assessment

Recording Video Footage for Analysis. Video footage was recorded throughout transport using 2 digital Panasonic SDR-H250 camcorders (Panasonic, Sydney, NSW, Australia) fixed to the front and back of the livestock trailer (above sheep head height, approximately 1.6 m from the trailer floor). Ten of the 14 sheep were clearly visible in the video footage from both the naïve and habituated transport events. One clip (20 to 60 s long) of each individual was chosen from each experimental journey within the first 15 min after departure. Clips were chosen based on the face of the sheep being visible for the majority of the clip and the trailer being in motion at the time. These 20 clips were edited to highlight focal sheep by increasing the opacity of the surrounding animals in the same frame and obscuring identifying numbers when visible in this section of video footage (Adobe Premiere Pro CS3 and Adobe After Effects CS3; San Jose, CA). The 20 clips were shown to observers in randomized order.

Observers and Procedures. Each observer was required to attend 2 sessions. The observers were not told about the experimental treatments or that the sheep were on a livestock trailer. In session 1 (term generation), observers were instructed in the free choice profiling procedures for qualitative assessment (Wemelsfelder et al., 2001; Rousing and Wemelsfelder, 2006), then shown 13 clips of sheep demonstrating a wide range of behaviors (clips included the sheep in their home pen as well as during transport). After watching each clip, observers had 2 min to write down any words that they thought described the behavioral expression of that animal. There was no limit imposed to the number of terms an observer could generate, although terms needed to describe not what the animal was doing but how the animal was doing it (i.e., adjective not adverb). These terms were then alphabetically collated for each observer (thereby effectively randomizing the order of terms) and duplicate terms removed to derive a list of descriptive terms unique to each observer. In session 2 (quantification) observers used their own descriptive terms as quantitative rating scales. Terms were printed in a list, with each term attached to a 100 mm visual analogue scale ranging from min (0) to max (100). Observers watched the 20 clips of individual sheep from the naïve and habituated transport events and quantified each sheep for every descriptive term. For further details of this methodology see Wemelsfelder et al. (2001) and Rousing and Wemelsfelder (2006).

Statistical Analyses. The observer scores were analyed using Generalized Procrustes Analysis (**GPA**) using a specialized software edition written for Françoise Wemelsfelder. Generalized Procrustes Analysis is a multivariate statistical technique that was originally developed in food science, but is still relatively unknown in animal science (for a detailed description of its procedures, see Wemelsfelder et al., 2000). The benefit of this statistical technique is that it enables comparison between observers using different numbers and unique combinations of descriptive terms. Briefly summarized, GPA calculates a consensus or "best fit" profile between observer assessments through complex pattern matching. Because each observer scores the same video footage, the statistic analyses the similarity in scoring patterns between observers. Generalized Procrustes Analysis does not use the terms as fixed reference points but uses the intersample distances used by each observer as the means of comparison. Generalized Procrustes Analysis represents the level of consensus (i.e., the percentage of variation between observers explained) that was achieved. Whether this consensus is a significant feature of the data set, or, alternatively, an artifact of the Procrustean calculation procedures, is determined through a randomization test (Dijksterhuis and Heiser, 1995). This procedure rearranges at random the scores of each observer and produces new permutated data matrices. By applying GPA to these permutated matrices, a "randomized" profile is calculated. This procedure is repeated 100 times, providing a distribution of the Procrustes Statistic indicating how likely it is to find an observer consensus based on chance alone. Subsequently a 1-way *t*-test is used to determine whether the actual observer consensus profile falls significantly outside the distribution of randomized profiles.

Through Principle Components Analysis (**PCA**) the number of dimensions of the consensus profile is reduced to several main dimensions (usually 2 or 3). Each animal receives a quantitative score on each of these GPA dimensions, so that the position of the animal in the consensus profile can be plotted in one or more 2-dimensional plots. Each plot represents each sheep twice (once for each treatment; naïve and habituated) where the positions of the sheep indicates its scores on each

GPA axis. To compare treatments, the GPA scores for each dimension were analyzed using repeated-measures ANOVA with the scores for each sheep for their naïve and habituated transport events compared as the repeated measures.

Interpretation of these consensus dimensions is made possible by correlating the consensus scores with the scores attributed by observers to each descriptive term, producing word charts for individual observers that can be compared for linguistic consistency. From these word charts, a list of terms describing the consensus dimensions can be produced, by selecting terms for each observer that correlated strongly with those dimensions ($r_{19} > 0.7$ on GPA dimension 1, $r_{19} > 0.5$ on GPA dimension 2, $r_{19} > 0.5$ on GPA dimension 3). It should be stressed that all these outcomes are the result of statistical calculation procedures that in no way depend on any interpretation from the experimenter.

Physiological variables were compared with GPA dimension scores for each individual sheep by correlation analyses. A correlation matrix was developed for the proportional changes for each of the blood sample measurements (after transport values divided by before transport values for each of the hormones, metabolites, and hematological variables), HR (values for 5 to 10 min after departure divided by values for 10 to 5 min before departure) and Tcore (transport day values divided by control day values) as well as the scores for each GPA dimension. Critical values were applied according to the number of animals that were included in each analysis (fewer data were included for HR recordings due to difficulties with the recordings).

Statistical analyses were carried out using GenStat 10.2 (VSN International, Hemel Hempstead, Hertfordshire, UK) and Statistica 8.0 (StatSoft-Inc.). Correlation matrices were carried out using Excel (Microsoft Inc., Redmond, WA). Data were tested for normality and homoscedacity and data transformed if required to enable parametric statistics. Data are presented as means ± 1 SD and a statistical level of $\alpha \leq 0.05$ is used throughout.

RESULTS

Sixty-four observers were recruited: 20 male (31%) and 44 female (69%). Eighteen (28%) could be classified as sheep experts (e.g., stock handlers, sheep researchers). Fourteen (22%) were completely inexperienced with sheep; the remaining 32 (50%) observers had limited experience. Observers generated a total of 281 unique terms to describe the sheep they were shown, with an average of 21 ± 7 terms (range 9 to 43) per observer.

The GPA consensus profile explained 53.03% of the variation among the 63 observers, and this differed significantly from the mean of 100 randomized profiles (41.58 ± 0.01%; $t_{99} = 87.5$, $P < 0.001$). Word charts were produced for each of the 63 observers for the first 3 dimensions. The dimensions were largely semantically consistent (i.e., although the observers used different terms, they used them in a similar way so that their meanings were comparable). Terms with the strongest correlation to each GPA dimension are shown in Table 1. The GPA dimension 1 (explaining 42.6% of the variability in the data) was characterized by terms such as

Table 1. Terms with the greatest correlations with dimensions 1, 2 and 3 of the Generalized Procrustes Analysis (GPA) consensus profile. When a term was used by multiple observers, values in parentheses give the number of observers using that term. Terms are sorted in order of numbers of observers using each term and then in decreasing order of correlation with each GPA dimension

Low values	High values
GPA dimension 1 (42.6%)	
Calm (17), relaxed (12), bored (9), happy (9), comfortable (6), content (4), sleepy (3), quiet (2), at ease (2), steady (2), chilled out (2), sure (2), coping, peaceful, balanced, accepting, chilled, mellow, assured, settled, tired, at peace, confident, in control, reassured	Anxious (17), nervous (12), worried (11), agitated (9), frightened (6), distressed (6), alert (6), scared (5), stressed (5), fearful (4), tense (4), concerned (3), apprehensive (3), alarmed (3), panicked (2), jittery (2), aware (2), perturbed (2), jumpy (2), distracted, flighty, attentive, disgruntled, intense, awake, startled, fretting, upset, terrified, wary, afraid, on guard, confused, defensive, looking for a way out, cautious, aroused, trapped, active, responsive.
GPA dimension 2 (9.8%)	
Comfortable (3), tired (2), confident (2), happy (2), scared (2), anxious (2), annoyed (2), content (2), angry, concerned, avoiding, restful, determined, placid, calm, pissed off, defensive, mischievous, fearful, resigned, irritable, grumpy, upset, assured, busy, panic, aloof, withdrawn, absorbed, quiet, weary, occupied.	Alert (15), anxious (5), aware (5), curious (5), interested (4), watchful (3), confused (3), attentive (3), nervous (2), concerned (2), lost (2), observant (2), worried (2), frightened (2), awaiting, defensive, settled, thinking, expectant, inquisitive, defeated, wanting to escape, agitated, tense (2), penned, apprehensive, distressed, scared, bright, questioning, intrigued, afraid, cornered, dominant, definite, confident, fearful, wary, trapped.
GPA dimension 3 (8.2%)	
Curious (7), alert (6), comfortable (5), aware (3), interested (3), relaxed (3), wary (3), inquisitive (3), sure (2), content (2), stressed (2), observant (2), happy (2), calm (2), tense (2), watchful, expectant, stable, conscious, in control, enduring, quizzical, certain, pleasant, dominating, satiated, pleased, looking for escape, confined, mad, active, sleepy, surprised, nervous, concerned, resigned, hesitant, satisfied, worried, confident, purposeful.	Frightened (4), agitated (3), afraid (2), tired (2), scared (2), disturbed (2), nervous (2), worried (2), stoic, sad, distracted, puzzled, depressed, anxious, comfortable, sleepy, terrified, certain, enclosed, steady, calm, lethargic, on edge, annoyed, secure, despair, alert, stressed, resigned, concerned, tense.

'calm', 'relaxed', or 'bored' correlated with the low end of the axis, whereas terms such as 'anxious', 'nervous', or 'worried' were correlated with the high end of the axis. The GPA dimension 2 (9.8%) was characterized by terms such as 'comfortable', 'tired', and 'confident' correlated with the low values of this axis and 'alert', 'anxious', and 'aware' correlated with the high values of this axis. The GPA dimension 3 (8.2%) was associated with terms such as 'curious', 'alert', and 'comfortable' correlated with the low values of this axis, whereas high values were associated with terms such as 'frightened', 'agitated', and 'afraid'.

Positions of individual sheep on the 3 GPA dimensions are shown in Figure 1. There were no significant differences between naïve and habituated sheep on GPA dimension 1 ($F_{1,9} = 3.26$, $P = 0.106$) or GPA dimension 3 ($F_{1,9} = 0.58$, $P = 0.465$). However, naïve sheep received significantly different scores on GPA dimension 2 compared with habituated sheep ($F_{1,9} = 9.01$, $P = 0.015$); transport-naïve sheep were scored as more 'alert', 'anxious', and 'aware', whereas habituated sheep were described as more 'comfortable', 'tired', and 'confident'.

Hormones, Metabolites and Hematological Variables

None of the physiological variables measured (Table 2) had values outside normal ranges (Teare, 2002). Concentration of ACTH during naïve transport was around twice that for the habituated event (treatment: $P < 0.001$) and decreased during both experimental events so that 'after' values were around a third of 'before' values (time: $P < 0.001$). Cortisol concentration was significantly greater during the naïve compared with the habituated event (treatment: $P = 0.003$) and remained at this level for sheep during their naïve event but decreased 46% over the habituated event (treatment × time interaction: $P = 0.043$). Concentration of IGF-I was 25% less during the naïve compared with the habituated event (treatment: $P = 0.001$) and decreased by approximately 10% during transport (time: $P = 0.008$). Leptin concentration was also significantly less during the naïve compared with the habituated event (treatment: $P < 0.001$), but concentrations were maintained during the naïve event and decreased 14% during the habituated event (time: $P < 0.001$; treatment × time interaction: $P < 0.001$). Glucose concentration was significantly greater during the naïve event compared with the habituated event (treatment: $P < 0.001$). There were no measurable differences in insulin or β-OH concentration between treatments or in response to transport.

Hematocrit and RBC numbers were greater (10% and 6% for HCT and RBC numbers, respectively) for the naïve compared with the habituated event (treatment: $P < 0.001$ for each) and both decreased (8% and 7.5% respectively) during transport (time: $P < 0.001$ for each).

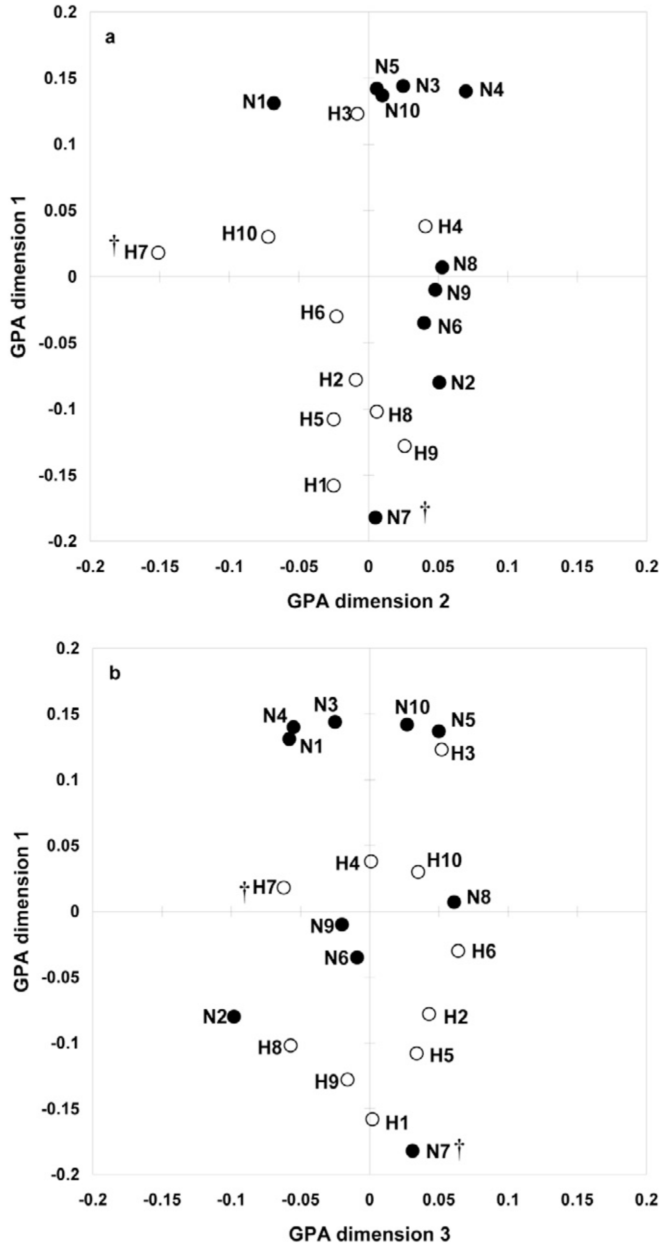

Figure 1. Positions of individual sheep (represented by ID numbers) on Generalized Procrustes Analysis (GPA) dimensions 1 and 2 (a) and 1 and 3 (b). Each sheep is represented twice, once as naïve (N) and secondly as habituated (H). By contrast with the pattern of greater energy and arousal for naïve compared with habituated treatments for all other animals, a single individual († ID #7) showed decreased energy and greater arousal naïve compared with habituated.

White blood cell count was 8% greater for the naïve compared with the habituated event (treatment: $P = 0.009$) whereas basophils were 23% less (treatment: $P = 0.007$); however significant treatment × time interactions (WBC: $P = 0.007$; basophils: $P = 0.017$) reflected an increase in WBC and basophil count over the naïve event contrasting with a decrease during the habituated event. Eosinophil values were 30% greater for the naïve com-

Table 2. Mean (± 1 SD) physiological variables for treatment (naïve vs. habituated) and sampling time (before transport vs. during/after transport). Significant effects of the 2-way repeated-measures ANOVA are indicated by bold *P*-values. Spearman's correlation coefficients for the relationship between each physiological measure and the Generalized Procrustes Analysis (GPA) dimensions are shown (r_{18} for all factors except r_{12} for heart rate and heart rate variability) and values with *, ** or *** are statistically significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively[1]

Item	Naïve		Habituated		Two-way repeated-measures ANOVA: Treatment (naïve vs. habituated)	Time (before vs. after)	Treatment × time interaction	Spearman's correlation coefficients: GPA dimension 1	GPA dimension 2	GPA dimension 3
	Before	After	Before	After	Results of RM ANOVA $F_{1,18}$, *P*-values			Correlation coefficients		
Plasma hormone concentration[2]										
ACTH, pgml^{-1}	387.2 ± 245.2	126.7 ± 50.3	200.6 ± 236.3	63.9 ± 51.2	<0.001	<0.001	0.773	0.027	-0.093	-0.143
Cortisol, ngml^{-1}	51.2 ± 27.1	50.0 ± 13.9	40.1 ± 24.3	21.4 ± 11.3	0.003	0.097	0.043	-0.152	0.022	-0.114
IGF-1, ngml^{-1}	31.4 ± 10.5	27.2 ± 10.7	40.7 ±13.8	36.8 ± 12.4	<0.001	0.008	0.882	-0.385 *	-0.001	-0.189
Insulin, μUml^{-1}	5.87 ± 1.75	6.36 ± 1.09	5.82 ± 1.19	5.75 ± 1.60	0.218	0.543	0.339	0.278	-0.157	-0.082
Leptin, ngml^{-1}	1.19 ± 0.25	1.18 ± 0.24	1.62 ± 0.28	1.39 ± 0.22	<0.001	<0.001	<0.001	0.034	0.261	-0.163
Plasma metabolites										
β-OH, mmol · L^{-1}	0.28 ± 0.06	0.28 ± 0.09	0.26 ± 0.06	0.24 ± 0.06	0.067	0.619	0.744	0.129	0.282	-0.099
Glucose, mmol · L^{-1}	4.76 ± 1.15	5.32 ± 1.35	3.91 ± 0.36	4.37 ± 0.66	<0.001	0.133	0.866	0.044	-0.012	0.217
Hematological variables										
Hematocrit	0.39 ± 0.02	0.36 ± 0.02	0.35 ± 0.02	0.32 ± 0.02	<0.001	<0.001	0.583	-0.174	0.336	-0.017
Red blood cell, × 10^{12} · L^{-1}	10.12 ± 0.66	9.40 ± 0.65	9.58 ± 0.51	8.77 ± 0.46	<0.001	<0.001	0.571	-0.293	0.348	0.020
White blood cell, × 10^9 · L^{-1}	8.10 ± 1.73	8.84 ± 1.79	8.04 ± 1.58	7.59 ± 1.60	0.009	0.438	0.007	-0.067	0.115	-0.108
Eosinophils, ×10^9 · L^{-1}	0.33 ± 0.13	0.25 ± 0.11	0.24 ± 0.14	0.16 ± 0.10	<0.001	0.002	0.845	-0.124	0.104	-0.325
Monocytes, × 10^9 · L^{-1}	0.11 ± 0.04	0.09 ± 0.03	0.24 ± 0.17	0.16 ± 0.10	0.002	0.065	0.371	0.262	-0.409 *	-0.089
Basophils, × 10^9 · L^{-1}	0.026 ± 0.013	0.035 ± 0.012	0.042 ± 0.016	0.039 ± 0.014	0.007	0.323	0.017	-0.223	-0.296	-0.445 *
Neutrophils, × 10^9 · L^{-1}	2.85 ± 1.67	4.80 ± 1.68	2.22 ± 0.82	2.90 ± 1.34	<0.001	<0.001	0.002	-0.193	0.513 *	-0.384 *
Lymphocyte, × 10^9 · L^{-1}	4.91 ± 0.98	4.13 ± 1.06	5.70 ± 1.17	4.99 ± 1.22	<0.001	0.002	0.635	0.338	-0.542 **	0.138
Neutrophil: Lymphocyte	0.60 ± 0.37	1.24 ± 0.57	0.40 ± 0.15	0.62 ± 0.34	<0.001	<0.001	<0.001	-0.320	0.673 ***	-0.399 *
HR, bpm	65.8 ± 7.29	112.9 ± 32.7	72.6 ± 18.5	77.2 ± 11.1	0.059	0.002	0.008	0.755 ***	0.163	0.056
HRV (SDNN), ms	61.58 ± 31.23	84.56 ± 40.64	62.25 ± 26.79	47.52 ± 24.69	0.133	0.728	0.120	0.766 ***	-0.219	-0.038
Tcore average, °C	39.29 ± 0.17	40.00 ± 0.24	39.36 ± 0.16	39.50 ± 0.17	<0.001	<0.001	<0.001	0.417 *	0.390 *	-0.125
Tcore maximum, °C	39.36 ± 0.18	40.16 ± 0.24	39.48 ± 0.19	39.62 ± 0.15	0.001	<0.001	<0.001	0.430 *	0.425 *	0.209

[1]For hormones, metabolites and hematological variables, blood samples were collected immediately 'before' loading and 'after unloading. Heart rate (HR) and heart rate variability (HRV) were measured either 'before' transport (10 to 5 min. before departure) or 'during' transport (5 to 10 min. after departure, at the same time as video footage analyzed in this study). Body temperature (Tcore) was measured either 'before' transport (averaged over 40 min. at same time of day on a non-transport day), or 'during' transport (0 to 40 min after departure).

[2]β-OH = beta-hydroxybutyrate; ACTH = adrenocorticotrophic hormone; HR = heart rate; HRV = heart rate variability; Tcore = core body temperature; GPA = Generalised Procrustes Analysis.

pared with the habituated event (treatment: $P < 0.001$), and decreased by 33% during transport (time: $P = 0.002$). Monocyte numbers were 29% less for the naïve compared with the habituated event (treatment: $P < 0.002$). The neutrophil: lymphocyte ratio had significant treatment × time interactions ($P < 0.001$); the neutrophil: lymphocyte ratio doubled over the duration of the naïve event (compared with a 50% increase for the habituated event).

Heart Rate and Heart Rate Variability

Heart rate was measured continuously throughout transport (Figure 2) but due to lost contact and interference, reliable data was not obtained for all animals for both naïve and habituated events; n = 5 (naïve event), n = 9 (habituated event). Transport resulted in significantly greater HR (bpm) for naïve sheep compared with their habituated event (treatment × time interaction: $P = 0.008$; Table 2). Measurements during 5 to 10 min after departure were 42% greater than a similar 5-min interval before departure. By contrast, in sheep habituated to transport, HR increased only 6% during the 5 to 10 min after departure compared with the same 5-min period before departure. Values before departure for both treatments were not statistically different (Table 2; post hoc analyses not shown). In addition to greater HR, HRV increased by 137% (5 to 10 min after departure) over pre-departure values in the naïve sheep and decreased

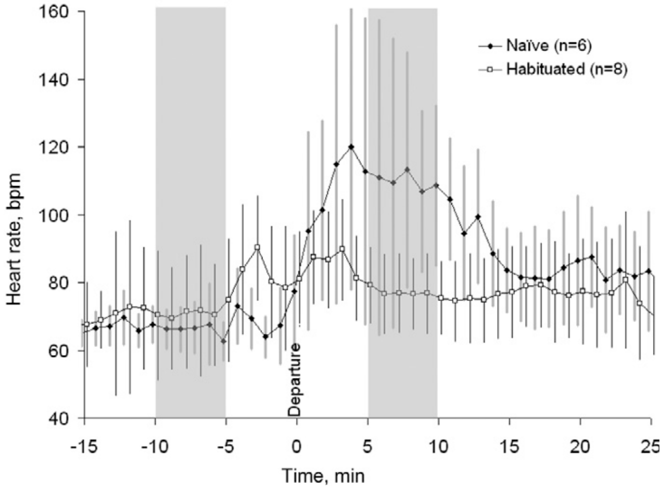

Figure 2. Mean (± 1 SD) heart rate (HR; beats per min, bpm) of sheep during the naïve and habituated transport events. Shaded areas show the 2 time periods where statistical comparisons were made: −10 to −5 min (before transport), and 5 to 10 min (during transport).

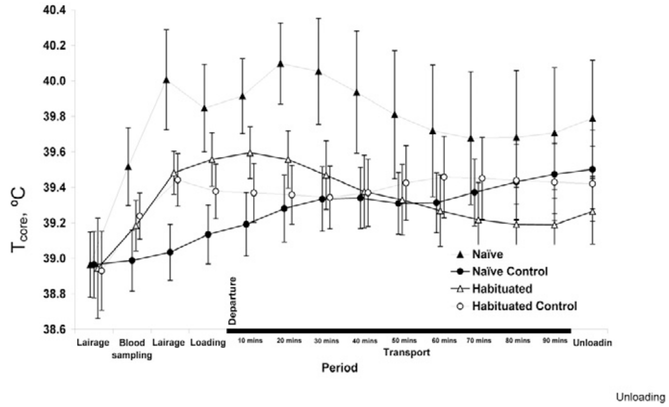

Figure 3. Mean (± 1 SD) core body temperature (Tcore) of sheep during the naïve and habituated transport events. Mean Tcore 3 d before the naïve event (naïve control) and 3 d after the habituated event (habituated control) is shown for comparison.

by 15% in habituated sheep (although these data were too variable to achieve statistical significance).

Core Body Temperature

Core body temperature (Figure 3) was significantly greater in naïve sheep compared with when they had become habituated to transport (treatment: $P < 0.001$ for both average and maximum Tcore) and was greater during transport compared with naïve and habituated control (non-transport) days (time: $P < 0.001$ for both average and maximum Tcore). Average Tcore during the first 40 min of the naïve event was 0.5°C greater than for sheep during the first 40 min of the habituated event ($P < 0.01$) and 0.7°C greater than the naïve control ($P < 0.01$; Table 2; post hoc analyses not shown). Maximum Tcore during the naïve event was 0.5°C and 0.8°C greater than the habituated event and naïve control period, respectively. This greater Tcore during the naïve event did not reflect ambient temperatures; the environmental temperature was actually cooler for the naïve compared with the habituated event (Figure 4).

Correlation of Physiology to Behavior

Significant correlations were observed between the GPA scores and IGF-1 concentrations, neutrophil: lymphocyte ratios, monocyte and basophil numbers, HR, HRV, and Tcore. Heart rate, HRV ($P < 0.001$ for each) and average and maximum Tcore ($P < 0.05$ for each) were positively correlated with GPA dimension 1 (Table 2) and were therefore greater for animals also described as more ‘anxious’, ‘nervous’, and ‘worried’. By contrast, IGF-1 concentration was negatively correlated with GPA dimension 1 ($P < 0.05$) and IGF-1 concentration was therefore greater for sheep also described as more ‘calm’, ‘relaxed’, or ‘bored’. Body temperature was also positively correlated with GPA dimension 2 scores ($P < 0.05$ for each) and was greater for sheep that were also described as more ‘alert’, ‘anxious’, and ‘aware’. The neutrophil: lymphocyte ratio was positively correlated ($P < 0.001$) and monocyte numbers were negatively correlated ($P < 0.05$) with GPA dimension 2 scores, indicating that sheep described as more ‘alert’, ‘anxious’, and ‘aware’ also had increased numbers of neutrophils and decreased numbers of lymphocytes and monocytes. The neutrophil:lymphocyte ratio was also negatively correlated ($P < 0.05$) with GPA dimension 3, along with basophil numbers ($P < 0.05$). Therefore, on GPA dimension 3, sheep described as ‘frightened’, ‘agitated’, and ‘afraid’ had reduced basophil and neutrophil numbers and greater lymphocyte numbers.

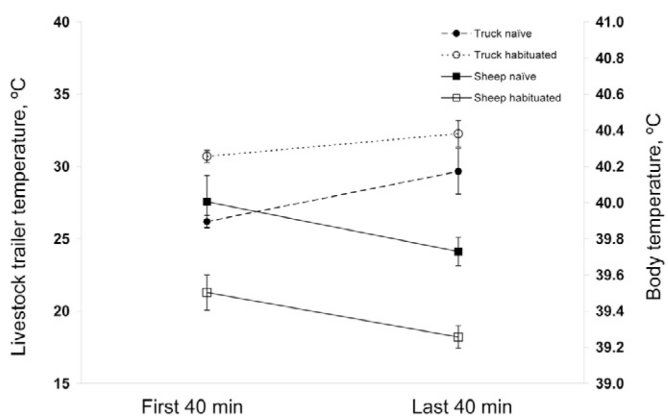

Figure 4. Mean (± 1 SD) core body temperature (Tcore) of sheep and environmental temperature (livestock trailer temperature) during transport for the naïve and habituated transport events.

DISCUSSION

Despite a limited repertoire of behavior exhibited by the animals during transport (given the confined environment), observers using QBA were able to reach consensus in their assessment of the behavioral expression of sheep, distinguishing between video footage of the same animals collected when they were either naïve or habituated to transport. Furthermore, the scores attributed to individuals were significantly correlated with physiological variables in a meaningful manner. This study supports the use of QBA as an assessment method during transport, which is a challenging environment in which to carry out real time welfare assessments under commercial conditions.

Although there are no previous published QBA studies in sheep, research has shown that observers can reliably and repeatedly assess the behavioral expression of pigs (Wemelsfelder et al., 2000; 2001; Temple et al., 2011a,b), cattle (Rousing and Wemelsfelder, 2006; Stockman et al., 2011), horses (Napolitano et al., 2008; Minero et al., 2009), poultry (Wemelsfelder, 2007) and dogs (Walker et al., 2010). In these published studies, there has been significant agreement between observers in their use of terminology to qualitatively score the behavioral expressions of the animals. We similarly recorded consensus between observers in how they scored the behavioral expression of transported sheep, with the GPA consensus profile explaining over half the variation between the observer scores.

From the GPA consensus profile, it was possible to identify distinct clusters of words with similar meanings on the 3 GPA dimensions. For example, ‘anxious’, ‘nervous’, and ‘worried’ are terms that indicate a similar body language and are distinct from ‘calm’, ‘relaxed’, and ‘bored’. We note, however, that some terms may appear in more than 1 cluster of words. For example the term ‘alert’ was associated with both high values for GPA 2 and low values for GPA 3. Some observers applied alert to sheep that were also described as ‘curious’ and ‘interested’ in their surroundings, whereas others used it to describe sheep that were also scored highly for the terms ‘worried’ and ‘fearful’. This suggests that the term alert is not helpful in deciding the overall welfare status of sheep when used on its own because its meaning is quite ambiguous and does not clearly indicate a positive or negative welfare state. Stockman et al. (2011) found a similar level of potential ambiguity with the term alert. We have not edited out terms that may appear on multiple lists because this would introduce subjectivity (as to which list from which to remove the term), but the reader should be mindful that each individual observer had a unique list of terms and therefore how he or she scored the same term may to some degree be influenced by the remaining terms in his or her repertoire.

An important aspect of validating QBA as a welfare assessment method is ensuring that the technique is sufficiently sensitive to discriminate between groups of animals. A significant finding of this study was that the relative position of sheep on GPA dimension 2 differed according to the treatment imposed, with the majority of naïve animals scored as being more ‘alert’, ‘anxious’, and ‘aware’, but habituated animals were scored as more ‘comfortable’, ‘tired’, and ‘confident’. This supports observations that animals (including sheep) can become habituated to transport (Grandin, 1997; Kent, 1997; Jacobson and Cook, 1998). The scores for GPA dimension 1 were also informative, with naïve sheep being generally more ‘anxious’, ‘nervous’, and ‘worried’. However, there was 1 individual that did not follow the same pattern as others (#7). Analysis of the data excluding this individual revealed that the relative position of naïve and habituated sheep on GPA dimension 1 would be statistically significantly different. The video footage available to show to observers for this individual was limited during the habituated event; the sheep had his head down for the majority of the first 15 min (the time frame within which the video footage was selected), and the clip subsequently available to show to observers was of short duration. This raises the important issue of collecting representative and informative video footage for analysis, which needs to be given careful consideration in future studies.

These findings support previous studies in cattle and horses, demonstrating that QBA can be used to distinguish between treatment groups. Stockman et al. (2011) found that observers distinguished between video footage of cattle recorded when they were either naïve or habituated to transport; transport-naïve cattle were described as more ‘agitated’, whereas transport-habituated were described as more ‘calm’. Observers were also able to distinguish between the behavior of the same horses before and after handling, with yearlings characterized as ‘suspicious/nervous’ and ‘impatient/reactive’ before any handling, and as more ‘explorative/sociable’ and ‘calm/apathetic’ after 1 mo handling (Minero et al., 2009). Therefore, QBA can be sufficiently sensitive to detect subtle differences in the behavioral expression of animals.

Another important finding of this study was that the scores attributed under QBA were significantly correlated with meaningful physiological variables. The physiological variables measured in this study included those that are established as indicators of a stressful environment for sheep. For example, both short- and long-term responses in WBC were recorded. In terms of the acute response, the total numbers of WBC were significantly greater after the naïve event, but were reduced after the habituated event. Over the longer term, significant differences in numbers of WBC types between the naïve and habituated events were recorded (decreased eosinophil numbers and increased neutrophil: lymphocyte ratio, basophil and monocyte numbers), suggesting a prolonged physiological response to the experimental process (intensive and repeated handling and 7 transport events over an 8-d period). The normal neutrophil: lymphocyte ratio should be 0.5:1 in adult sheep but reaches 2 to 3:1 (i.e., increase in neutrophil proportion and a decrease in lymphocyte proportion) when animals exhibit indicators of stress (Cole et al., 1997; Jones and Allison, 2007). The neutrophil: lymphocyte ratio was 0.6:1 and 0.4:1 for naïve and habituated sheep (respectively) before transport, but increased to 1.2:1 and 0.6:1 for naïve and habituated sheep (respectively) after transport. Therefore, transport resulted in an increase in the neutrophil:lymphocyte ratio (also recorded by other authors: Murata et al., 1987; Kegley et al., 1997; Kannan et al., 2000; Stockman et al., 2011), a response that was most pronounced when the animals were first exposed to the transport environment. These changes in blood cell variables indicate that the 90-min transport event was associated with substantial physiological changes in the sheep. The WBC changes were also significantly correlated with the QBA scores. For example, the neutrophil:lymphocyte ratio was correlated positively GPA dimension 2, with sheep described as more 'alert', 'anxious', and 'aware' also demonstrating the greatest neutrophil:lymphocyte ratio.

Transport-naïve sheep had increased HR when they were first exposed to transport, and their HR were greater during the initial 15 min of transport than before the animals were loaded onto the livestock trailer. This increased HR was less evident when the animals were habituated to transport. This suggests a reduction in stress associated with transport as a consequence of a learning process (habituation) and is supported by Stockman et al. (2011) in adult cattle and Jacobson and Cook (1998), who found that mean HR of 6-wk-old calves reduced with transport repetition. Heart rate variability was not statistically different between treatments or before and during transport and this is possibly due to the large inconsistency in these data coupled with an inability to use more powerful repeated-measures analyses due to missing data. However, HR and HRV were significantly correlated with QBA scores, with greater, more variable HR in animals that were also described as more 'anxious', 'nervous', and 'worried'.

Increased HCT and RBC count may reflect increased splenic contractions in response to novel or threatening processes (a sympathetic response). In the present study, greater HCT and RBC count were recorded for the naïve compared with the habituated event, and both variables decreased during transport. Other authors have similarly reported that habituation to transport resulted in decreases in hematocrit (Broom et al., 1996; Cockram et al., 1996; Stockman et al., 2011). Stockman et al. (2011) and Knowles et al. (1993) also report decreases in hematocrit after transport compared with before, which can be attributed to recovery after an initial increase due to handling. Because handling effects could not be isolated from the data, it is likely that both acute (i.e., greater pre-transport values due to handling) and longer term responses (i.e., habituation to transport and therefore reduced sympathetic response) in HCT and RBC count were occurring.

Increased body temperature may also represent a sympathetic response to the challenges of being transported. Core body temperature was greater in sheep during their naïve event compared with the habituated event, and was greater during transport than on non-transport control days (even though the environmental temperature decreased over the duration of the transport event). This is consistent with other studies for sheep (Parrott et al., 1999) and cattle (Steinhardt and Thielscher, 1999; Stockman et al., 2011) which show an increase in body temperature during transport. The cause of this increased temperature could be either psychological or physical, or both. Stress hyperthermia has been demonstrated for sheep during shearing (Beatty et al., 2008) or exposure to a novel environment (Villalba et al., 2009). Increased Tcore during transport could also reflect a physical reaction to maintaining balance as the vehicle moves; consistent with this hypothesis, animals showed greater movement in the livestock trailer during the naïve transport event compared with the habituated event (although this was not quantified). However, although the challenges of physical exertion are predicted to be maintained while the vehicle is still moving, Tcore initially increased, but subsequently decreased over each transport event. Other studies have similarly found that Tcore increases immediately after the start of transport, but can decrease (i.e., return to normal) during a single journey (Warriss et al., 1995; Lay et al., 1996; Pettiford et al., 1998). There were correlations between Tcore and the QBA scores, with increased Tcore in animals that were also recorded as more 'anxious', 'nervous', and 'worried' (GPA dimension 1) or 'alert', 'anxious', and 'aware' (GPA dimension 2).

Correlations between the physiological measurements and GPA dimensions were calculated using the change in the physiological measurement for each animal due to transport (i.e., after compared with before values for each event). If all individuals demonstrated a similar change in a physiological variable but different behavior, then there would be no correlation between these measures. Similarly, individual responses may preclude treatment effects while there are still significant correlations between physiological variables and GPA scores. Significant correlations with behavioral expression (GPA scores) were therefore more likely for those variables when individuals have marked differences in their physiological response to a challenge (e.g., HR responses are often different between individuals of different temperament; Visser et al., 2002).

A number of important physiological variables demonstrated treatment or transport effects, but there was no significant correlation with the QBA scores. Responses of the hypothalamic-pituitary-adrenal-axis may be an important indicator of stress. Previous studies have reported increased plasma cortisol and ACTH concentrations during the initial minutes of transport (Lay et al., 1996; Smith and Dobson, 2002), whereas cortisol concentrations decrease later in transport or after journey completion (Cole et al., 1988; Broom et al., 1996; Knowles et al., 1996, 1997; Lay et al., 1996; Smith and Dobson, 2002). Handling before loading, the length of journey and the time at which samples are collected will therefore influence these responses. In the present study, ACTH concentration was less for samples taken after 90-min transport compared with before, consistent with previous studies; greater ACTH values before transport may reflect the 10 to 15 min of handling when animals were moved through yards into a race to wait in line to be blood sampled. Concentration of ACTH decreased over the habituated transport event, but remained high over the naïve event, suggesting a sustained response for the naïve event. Cortisol concentration measured after transport was greater than before, but paradoxically, cortisol concentration was greater both before and after the habituated transport event than the naïve event. This cortisol response may reflect a greater production of cortisol earlier (e.g., when animals were first yarded and handled), even before blood sampling. The timing of blood sampling may therefore not accurately reflect activation of the HPA response (Djordjevic et al., 2003; Miller et al., 2007).

Plasma glucose concentrations did not increase significantly during transport but were significantly less in sheep habituated to transport. Increased glucose concentrations reflect the breakdown of glycogen in the liver and could reflect more movement of the sheep in the trailer during the naïve transport event compared with the habituated event. There were no significant differences in β-OH between the naïve and habituated transport events or before and after transport. Beta-hydroxybutyrate is usually present when glucose does not meet the energy requirements of the animal. For example, β-OH concentrations are more likely to increase in animals that are experiencing starvation (Leng and Annison, 1964; Vandermeerschen-Doise et al., 1983). In this study, it is likely that the energy requirements of the sheep during the 90-min transport events were met and therefore it is not unexpected that there was no change in β-OH concentration. Stockman et al. (2011) report a similar finding of no treatment effects of transport on plasma β-OH concentration.

In summary, observers reached consensus in how they scored the behavioral expression of sheep filmed during road transport, when the animals were restricted in their movements and could only demonstrate a fairly limited repertoire of behavior. In addition, there were statistically significant differences in the behavioral expression scores attributed to sheep exposed to different treatments, suggesting observers were able to detect extremely subtle differences in behavioral expression. Finally, there were meaningful correlations between behavioral expression scores and physiological variables (including those that have been interpreted as indicating stress in various animal species). Similar meaningful correlations between physiology (Tcore, HR, plasma glucose, and the neutrophil:lymphocyte ratio) and QBA scores have been recorded in cattle exposed to transport (Stockman et al., 2011). In conclusion, this study supports the application of QBA as a tool to assess sheep behavioral responses under transport conditions. Further development of this method as an objective measure of animal welfare is recommended.

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