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Animal welfare indicators for sheep during sea transport: The effect of voyage day and time of day

Renee S. Willis *, Patricia A. Fleming, Emma J. Dunston-Clarke, Anne L. Barnes, David W. Miller, Teresa Collins

College of Science, Health, Engineering and Education, Murdoch University, 90 South St, Murdoch, WA, 6150, Australia

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ABSTRACT

Ensuring the well-being of animals during transport is becoming an increasingly important societal concern. The Australian livestock export industry recognises the need for comprehensive monitoring and reporting on animal welfare during sea transport. It is predicted that pen-side assessments of sheep can be used to monitor environmental conditions, resource access, and animal health and behavioural outcomes throughout a sea voyage. Pen-side assessments by observation are non-invasive and practical to apply in an industry setting. This study monitored sheep using a pilot list of welfare indicators during two sea voyages from Australia to the Middle East, in contrasting seasons. Sheep behaviour, environment and resources were recorded three times daily via pen-side observations of six pens of Merino wethers (castrated males), repeated over three decks for each voyage. Behavioural outcomes were examined for the effect of sampling frequency on group assessments. The number of behavioural measures were reduced via Principal Component (PC) analysis. The primary three PC factors were tested against the time of sampling and pen location after accounting for the effect of environmental- and resource-based predictor variables. PC 1 (24.0 % of the total variance) described activity levels, with sheep on Voyage B being more active in the morning and resting or recumbent in the middle of the afternoon and evening. PC 2 (14.7 %) reflected heat responses with the majority of the variation in these data accounted for by changes in Wet Bulb Globe Temperature (WBGT) and manure pad moisture. The heat responses described by PC 2 also varied by voyage day ($p < 0.001$) and time point ($p < 0.001$). PC 3 scores (9.5 %) reflected flight distances and feeding behaviour and strongly correlated to WBGT and pellet consumption per head per day. Feeding behaviour generally became more competitive, and flight distances reduced as both voyages progressed. Results indicate that a comprehensive welfare monitoring protocol requires repeated daily sampling throughout a voyage. The findings of this study are pertinent for developing a sampling strategy to assess sheep welfare during sea transport.

1. Introduction

The welfare of sheep exported from Australia by sea is an important issue for all stakeholders, specifically for the Australian public, industry and importing countries (Meat and Livestock Australia Ltd., 2018; Department of Agriculture and Water Resources., 2020). In 2019, Australia exported 1.1 million live sheep (Meat and Livestock Australia Ltd., 2020), and a substantial duty of care is required for these animals. The need to improve animal welfare monitoring beyond current regulatory requirements has been recognised by the livestock export industry (McCarthy, 2018) and sought by advocates opposing the trade (Foster and Overall, 2014). Improved animal welfare monitoring can increase industry transparency (Wickham et al., 2017; McCarthy, 2018; Australian Livestock Exporters' Council., 2019), allow the implementation of informed risk mitigation strategies (Colditz et al., 2014; Inspector--General of Live Animal Exports., 2020) and help to avoid unacceptable risk. Over 95 per cent of live sheep exported from Australia are transported by sea to the Middle East, where freshly slaughtered sheep meat is a requisite commodity (Meat and Livestock Australia Ltd., 2020). During a voyage, sheep are managed on-board livestock carrier vessels for an average of 21 days (Collins et al., 2018b). The development of a comprehensive animal welfare monitoring protocol requires not only the identification and validation of suitable measures, but also the testing of a feasible sampling protocol.

* Corresponding author.
E-mail address: reneeswillis@gmail.com (R.S. Willis).

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Pen-side assessments are an effective method used for the appraisal of animal welfare outcomes in intensive livestock production systems. They have been included in animal welfare monitoring protocols such as Welfare Quality® (Blokhuis et al., 2010) and Animal Welfare Indicators Network (AWIN) (Ruiz and Dwyer, 2015) for use on farms; by Llonch et al. (2015) for use in abattoir facilities; and Messori et al. (2015) for assessing livestock after road transport. Animal welfare is a multidimensional concept, and pen-side assessments can be used to gauge welfare outcomes by considering a combination of measures regarding access to resources, environmental conditions, and animal-based outputs relating to behaviour and health (Colditz et al., 2014; Caroprese et al., 2016; Mellor, 2016). Behavioural measures can be used to record livestock activity, and to assess their affective state. Behavioural observations, including descriptors of demeanour, have been shown to correlate with physiological markers during on-farm welfare and land transport studies (Stockman et al., 2012; Wickham et al., 2015; Fleming et al., 2016). However, unlike physiological markers, behavioural observations are non-invasive, can be time efficient, and are practical to apply in an industry setting (Messori et al., 2015; Dunston-Clarke et al., 2020). Demeanour terms are frequently used by stock handlers to describe the behavioural expression of animals and how livestock are coping with their surrounding environment. A list of standardised and clearly defined demeanour terms can be scored to capture dynamic aspects of animal behaviour and body language. These scores can provide welfare insight in addition to recording individual ethogram behaviours in isolation (Fleming et al., 2016). Qualitative measures of behavioural expression can identify positive or negative mental states and are currently recognised as a fundamental component of welfare assessments aiming to evaluate well-being (Andreasen et al., 2013; Wemelsfelder and Mullan, 2014).

A pen assessment provides a snapshot of welfare at a point in time; therefore, the schedule of assessments may influence their outcomes due to changing shipboard conditions and fluctuations in sheep behaviour. Importantly, we need to determine whether behavioural outcomes vary according to the frequency of monitoring and the time of day to demonstrate whether the frequency of recording influences the overall impression of animal welfare. Health outcomes, such as the incidence of disease and mortality, are likely to be cumulative throughout a voyage. However, behavioural observations may vary due to changes in environmental influence (Zhang et al., 2017), resources, the work schedules of the crew (Collins et al., 2018a), and circadian patterns in animal physiology and behaviour (Piccione et al., 2008). Livestock may also become less responsive to their environment as they habituate to their pens and adapt to intensive management during the voyage (Hemsworth and Coleman, 2011). Environmental factors, such as sea swell and ambient temperatures, are likely to vary during a 24 -h cycle. If harsh environmental conditions are encountered, or resource access limited, this may result in the ongoing summation of stress, or transient periods of stress and respite (Phillips and Santurtun, 2013; Collins et al., 2018a). Furthermore, examining daily periods of activity and rest can indicate whether animals are adapting to their pen environment. Understanding how sheep respond to changing conditions during sea transport can inform risk mitigation and decisions regarding voyage planning, animal management, and livestock selection (McCarthy and Banhazi, 2016). Once a standardised monitoring protocol is adopted, and significant data analysed, the application of welfare thresholds may be determined.

It is hypothesised that sheep behaviour during a voyage will fluctuate as animals respond to varying conditions on the vessel. This paper forms part of a wider study to develop a welfare assessment protocol for sheep during sea transport and specifically examines variations in the recorded behavioural components of a welfare protocol when assessments are performed at different stages of the voyage. We aimed to identify the behavioural traits of sheep during two sea voyages and investigate how they varied by voyage day and time of day. This study may determine the optimal timing and frequency of welfare assessments for livestock monitoring and reporting during sea voyages.

2. Materials and methods

This study utilised a welfare monitoring protocol based on the four welfare principles defined for the Welfare Quality® (WQ) project (Blokhuis, 2008) and further developed for the livestock export industry (Dunston-Clarke et al., 2020; Fleming et al., 2020). Measures were adapted to incorporate pen-side observations routinely used by on-board veterinarians and stock persons (Jubb and Perkins, 2019). The proposed welfare assessment protocol was piloted on two commercial sheep voyages. Data were collected from Merino wethers (castrated males), representing the most common class and type of Australian sheep transported by sea. Data were analysed from pens located in different areas of the vessel.

2.1. Livestock and pen selection

Data were collected by the same single observer (RW) on-board two commercial livestock voyages that transported sheep from Fremantle, Australia to Muscat, Oman in May 2018 (Voyage A, 13 days at sea) and November 2018 (Voyage B, 14 days at sea). Two livestock carrier vessels of the same size and design were used and the selected sheep were loaded on decks 1−5. These decks had between 40–70 pens with 14–55 sheep per pen. For each voyage, six pens of between 31–51 Merino wethers (castrated males) were selected across three decks, with two pens per deck (**Table S1**). Pens were chosen to capture a range of environmental conditions between decks, but not between pens on the same deck. Pen selection involved consideration of the loading plan of the vessel in combination with the researcher and crew's prior knowledge of the expected environments across that vessel. Due to differences in stowage and stocking density, it was not practical to sample the same pens on both voyages. All sheep had a fleece length < 25 mm, were of body condition score 3.5–4 (Department of Agriculture and Food Western Australia., 2018), and were evenly drafted by bodyweight. The wethers selected on Voyage A were of mixed ages (date of birth: 2015–2016) and averaged 49 kg, while on Voyage B they were predominantly one year of age (date of birth: 2017) and averaged 54 kg.

Pen assessments were carried out at three time points (TP) on days 2–13/14: TP 1 morning (06:30–10:00 h), TP 2 afternoon (14:00–16:00 h), and TP 3 after evening feeding (17:00–21:00 h). Assessment times were chosen to work alongside the daily schedule of the ship and varied slightly between voyages due to logistical constraints. Exceptions: pen assessments were not made on Day 1 and at some TPs due to the handling of livestock as stocking densities, troughs and pens gates were adjusted, or due to ship factors such as a temporary power supply interruption to the cargo holds (**Table S2**).

2.2. Pen assessment technique

A list of environmental-, resource-, and animal-based measures (Tables 1 and 2) were loaded onto a data collection platform using the mobile phone application Kizeo Forms (Kizeo, 2017), which had been pre-tested by assessing sheep on land. Group level (pen-side) recordings were made by point sampling as the one observer stood stationary in the vessel's alleyway at the front of each of six focal pens. On board a livestock carrier vessel, sheep are frequently exposed to humans as they are monitored by crew throughout the day and night, fed 2−4 times daily, and the water troughs are regularly cleaned. Furthermore, many pens are situated in areas adjacent to service points for crew activities, increasing the frequency of human exposure. The impact of the pen-side observer on the behaviour of animals is unavoidable, but it is also indicative of the animal experience during a sea voyage where continuous human activity in close proximity occurs. Observations were made over a period of approximately 5–8 minutes at each TP. Initially, static data regarding the time, date, location, voyage day and pen ID were recorded as the livestock settled; subsequently, four categories of measures were recorded. Animals were scored for eight behavioural

Table 1
Pen-side assessment measures: animal-based outputs (behaviour and heat stress). * Panting scores of 3 and 4 were infrequent; however, the observations are important indicators of welfare (Collins et al., 2018b; HSRA Technical Reference Panel, 2019). To achieve better comparisons for heat response, the percentages of the pen at panting scores 2, 3 and 4 were combined to produce the variable "panting score 2 or above (%)".

Measures	Method
(a) Quantitative behaviour	
1. Flight distance	Distance (m) the observer approached the pen before the animals moved away (Llonch et al., 2015; Ruiz and Dwyer, 2015)
2. Recumbent	Percentage (%) of the pen lying down
3. Eating	Percentage (%) of the pen eating
4. Drinking	Percentage (%) of the pen drinking
5. Resting	Percentage (%) of the pen resting (in a state of sleep or minimal activity and engagement with their environment, posture can be either standing or lying down)
6. Ruminating	Percentage (%) of the pen chewing their cud
7. Exploring the Environment	Percentage (%) of the pen performing seeking or searching behaviour, or physically interacting with or manipulating an object in their environment (licking, chewing, pushing etc)
8. Antagonistic Behaviour	Percentage (%) of the pen performing antagonistic behaviour (riding, head butting, exerting pressure by pushing against another animal)
9. Panting Scores*	Percentage (%) of the pen at each panting score. 0: no panting; 1: slight panting; 2: fast panting, open grin; 3: open mouth panting; 4: open mouth panting, tongue out. (Jubb and Perkins, 2019)
(b) Qualitative behaviour	
Scored on a continuous sliding scale bar (scores of 0–100) where the observer indicated their perception to what degree the animals in the pen matched this description, where 0 = none of the animals matched the description, and 100 = all the animals fully matched this description. Therefore, the score accounted for both the proportion of animals displaying the demeanour, and the extent that it was displayed (Wemelsfelder et al., 2001; Wemelsfelder, 2007; Fleming et al., 2016).	
1. Anxious	Showing worry, nervousness or unease; increased vigilance behaviour
2. Settled	Quiet, calm, relaxed and not tense
3. Active	Energetic, lively, characterised by busy or lively activity (body movement and actions)
4. Uncomfortable	Showing signs of physical discomfort, unease or irritation
5. Alert	Wide awake, fully aware, attentive, (how engaged the animals are with their surrounding environment)
6. Lethargic	Lacking interest, dispirited, apathetic, slow moving, listless, dull
7. Inquisitive	Showing a positive interest or curiosity towards surroundings

measures, and evidence of heat stress using panting score (Jubb and Perkins, 2019) (Table 1**a**). Using the Kizeo data platform, the modal panting score for each pen of sheep was recorded, and subsequently a record of any individual animals with panting scores that differed from the modal score. This improved recording efficiency as the observer was not counting the number of sheep at each panting score, only the number of sheep presenting outside the modal score. The sheep were then scored against seven descriptive terms selected from a list of terms outlined by Dunston-Clarke et al. (2020) relating to demeanour (Table 1**b**). Finally, resource-based data regarding the feeding regimen and access to water (Table 2**a**) were recorded along with environmental variables (Table 2**b**).

Some measures required retrospective information (e.g. clean water availability, fodder ration, feeding regimen, access to roughage and feeding behaviour scores); these observations were made by the researcher throughout each day and by and stockpersons and crew who were monitoring the deck environment at all times. Sheep behaviour at feeding was assessed on a five-point scoring system, hereinafter referred

Table 2
Pen-side assessment measures: resource- and environment-based measures.

(a) Resource-based measures	
1. Clean water availability[a]	Hours of access to fresh clean water from at least one watering point in the last 24 h
2. Water contamination[a]	A description of water trough contamination - 1: clean; 2: mild contamination; 3: moderate contamination; 4: marked contamination
3. Watering points contaminated[a]	Percentage (%) of the watering points that were contaminated
4. Water Consumption	Water consumption for the livestock decks/heads (L/hd/day)
5. Pellet consumption	Amount of pellets fed as approximate % of body weight (BW) per head per day (total pellet consumption for the livestock decks for 24 h /total heads/ average bodyweight for B wethers x 100)
6. Feeding regimen [a]	A description of the feeding regimen - 1: increased roughage/reduced pellets; 2: restricted fodder; 3: maintenance; 4: above maintenance; 5: *ad lib*
7. Roughage feeding[a]	Grams of long fibre roughage fed per head per day
8. Roughage access	Number of feeds to include long fibre roughage in the previous 24h
9. Amount of feed in troughs[a]	Amount of fodder in troughs at the time of observation - 0: empty troughs, 1: some crumbs left; 2: $^{1}/_{4}$ full; 3: ½ full; 4: ¾ full; 5: troughs full
10. Feed trough contamination [a]	A description of the fodder in the troughs at the time of observation - 1: clean; 2: some fines; 3: majority fines; 4: some faeces/saliva/mould; 5: marked faeces/saliva/mould
11. Feeding behaviour score	Behaviour of sheep when fresh feed delivered at the most recent feeding time prior to the pen assessment - 1: disinterested (no animals attending troughs); 2: some interest (some animals eating, trough space available); 3: keen (no trough space available and animals waiting to attend troughs); 4: jostling (no trough space available, animals pushing to attend troughs; 5: smothering (no trough space available, some animals pushing and climbing or lunging to attend troughs)
(b) Environment-based measures	
1. Sea swell score	Scale based on the height of the swell at the time of undertaking pen assessments - 1: no swell, 2: low swell (<2 m), 3: moderate swell (2–4 m), 4: heavy swell (>4 m), 5: phenomenal/confused swell
2. Manure pad moisture score	Visual perception of the moisture content of the manure pad at the time of pen assessment - 1: dry and dusty; 2: firm; 3: tacky; 4: high moisture; 5: sloppy; 6: flooded
3. Manure pad depth[a]	Visual perception of the average depth (cm) of the manure pad at the time of pen assessment - 1: 0–5 cm; 2: 6–10 cm; 3: 11–15 cm; 4: 16–20 cm; 5: >20 cm
4. Dry bulb temp (^{o}C) 5. Wet bulb globe temp (^{o}C) 6. Relative Humidity (%)	A hand-held Extech HT 30 Heat Stress WBGT Meter was used to measure dry bulb temperature (DBT), wet bulb globe temperature (WBGT) and relative humidity (RH); readings were taken from shoulder height at arm's length into the pen from the point of observation immediately following the behavioural observations.

[a] Insufficient variability in these measures across the two voyages – data were recorded but have not been included in the GLMM analysis due to lack of variation.

to as 'feeding behaviour score' (Table 2**b**). This measure was designed to assess social competition at feeding and reflects how environment and management factors may impact appetite (Dunston-Clarke et al., 2020). This measure was observed during the most recent feeding time prior to the pen-side observations listed in Table 1. The design of shipboard pens means that it is not possible for all animals to attend the feed troughs simultaneously, and, *ad lib* feeding is not always feasible due the finite reserves of fodder loaded for each voyage. The amount of pellets and roughage fed were calculated from the exporter's voyage instructions document, the Chief Officer's daily records, and the Australian Standards for the Export of Livestock (ASEL) daily reports (Department of Agriculture Fisheries and Forestry., 2011).

2.3. Statistical analysis

Multiple aspects of behaviour contribute to the overall welfare state of an animal, and these components are not independent of each other (Webster, 2005; Mellor, 2016). Therefore, Principal Components (PC) analysis (Statistica., 2018) was used as a data reduction tool to simplify all behavioural variables in the dataset. PC analysis identified the behavioural dimensions that captured the majority of the data variation. PC factors derived from mean standardised behaviour variables that had eigenvalues >1.5 were further analysed. The factors were described using the strongly loaded variables (factors with >75 % of the absolute value of the largest positive or negative correlation coefficient) on either end of the PC dimension axis. Some resource- and environment-based measures were omitted from covariate comparisons if they had insufficient variation within these voyages (Table 2). Wet Bulb Globe

Table 3

a) Principal components analysis results. Variables that were >75 % of the highest absolute correlation coefficient were highlighted on either end of each PC factor axis **b)** F Values listed for generalised linear mixed modelling (GLMM) results comparing the effect of several environmental or management measure on each PC factor. F Values are also listed for GLMM results comparing voyage, voyage day, time point, deck and pen group effect. Significant variations are highlighted in bold (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

	a) PCA Variables	PC 1	PC 2	PC 3
	Flight zone (m)	-0.098	-0.161	**-0.786**
	Recumbent (%)	**0.798**	0.262	0.087
	Eating (%)	-0.200	-0.277	0.098
	Drinking (%)	-0.110	-0.198	0.265
	Ruminating (%)	0.065	0.300	-0.209
	Resting (%)	**0.861**	0.048	-0.012
	Exploring Environment (%)	-0.247	0.078	0.172
	Antagonistic Behaviour (%)	-0.174	-0.030	0.290
	Anxious	-0.356	-0.064	-0.145
	Settled	0.455	**0.689**	0.048
	Active	**-0.770**	0.038	0.162
	Uncomfortable	0.371	**-0.817**	-0.041
	Alert	**-0.798**	-0.203	-0.111
	Lethargic	0.365	**-0.732**	-0.052
	Inquisitive	**-0.794**	0.091	0.036
	Panting Score 2 or Above (%)	0.260	**-0.665**	0.299
	Feeding Behaviour Score	-0.043	0.054	**0.768**
	Eigenvalue	4.07	2.49	1.61
	Total Variance (%)	24.0	14.6	9.5
	b) GLMM results – F values and their significant variations			
Voy A&B	WBGT (°C)	**46.97*** **	**71.62*** **	**179.20*** **
	Pellet Consumption (% BW/head/day)	3.80	**18.89*** **	**89.18*** **
	Roughage Access	0.70	**6.72** **	2.31
	Sea Swell Score	0.29	0.29	**6.09* **
	Manure Pad Moisture Score	**14.44*** **	**48.70*** **	**6.68* **
	(1) Voyage Day	**1.90* **	**3.61*** **	**20.73*** **
	(2) Time Point	**11.72*** **	**6.49** **	**6.72** **
	(3) Voyage	**4.40* **	0.05	**35.20*** **
	(4) Deck	**25.40** **	**16.24* **	2.57
	(5) Pen Group	0.19	0.58	1.31
Voy A	WBGT (°C)	**44.73*** **	**31.17*** **	**175.16*** **
	Pellet Consumption (% BW/head/day)	**5.08* **	2.68	**19.89*** **
	Roughage Access	0.68	2.20	**32.47*** **
	Sea Swell Score	3.19	0.01	0.95
	Manure Pad Moisture Score	2.12	2.83	2.13
	(1) Voyage Day	1.57	**3.96*** **	**10.51*** **
	(2) Time Point	0.79	**10.28*** **	2.55
	(3) Deck	**78.51*** **	**44.31** **	**9.35* **
	(4) Pen Group	0.17	0.26	1.10
Voy B	WBGT (°C)	**6.47* **	**4.67* **	**241.65*** **
	Pellet Consumption (% BW/head/day)	0.03	**70.90*** **	2.36
	Roughage Access	3.26	0.59	**5.98* **
	Sea Swell Score	0.13	**4.26* **	**7.75** **
	Manure Pad Moisture Score	0.17	0.65	**4.02* **
	(1) Voyage Day	**2.07* **	**6.81*** **	**6.74*** **
	(2) Time Point	**15.80*** **	**23.32*** **	**12.87*** **
	(3) Deck	2.05	1.99	3.32
	(4) Pen Group	0.18	2.09	1.01

Temperature (WBGT) was selected as the representative covariate for climatic variation as it considers dry bulb temperature, relative humidity and radiant heat.

Generalised linear mixed modelling (GLMM) (Statistica., 2018) was used to test each PC factor (as a separate dependent variable) against independent factors: (1) voyage day, (2) voyage and (3) deck, with pen group included as random factor to account for repeated measures. Six covariates were also included: (4) WBGT, (5) pellet consumption, (6) roughage access, (7) sea swell score, and (8) manure pad moisture score. The factorial design allowed one degree of interaction between factors.

PC factors that had an eigenvalue >1.5 were graphed against covariates, voyage day, time point and by deck and pen group. Where a significant effect was detected by voyage day, time point, deck or pen group, a Tukey's post hoc test was used to identify where differences occurred.

Data relating to the incidence of disease and mortality were omitted from this study as these variables were cumulative and showed insufficient variability across the time frame of comparisons used in the statistical analyses.

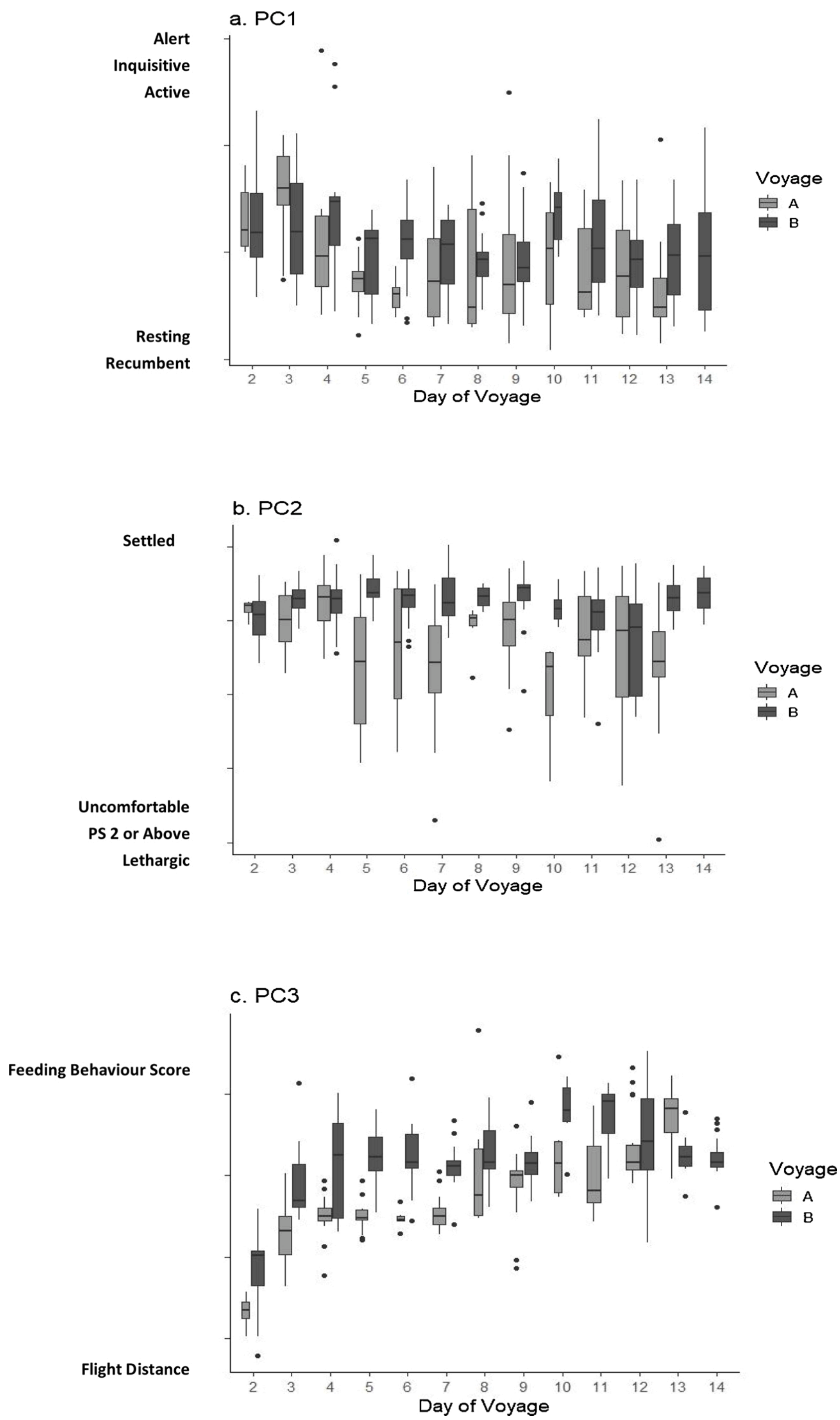

Fig. 1. PC 1 (a) and PC 2 (b) by voyage day, all three TP combined per day. Note: width of box indicates sample size (where a smaller width indicates fewer samples).

3. Results

3.1. Principal components analysis

Principal components analysis identified three PC factors that had eigenvalues >1.5 (Table 3). PC 1 explained 24.0 % of the total variance and described sheep behaviour consistent with terms related to activity *vs.* rest. Descriptive terms 'alert', 'inquisitive' and 'active' strongly loaded at one end of the PC 1 axis, while the quantitative scores for resting and recumbent strongly loaded on the other end. The second factor, PC 2, accounted for 14.6 % of the total variance and was associated with demeanour and behaviour reflecting a response to heat. These variables include the descriptive terms 'uncomfortable' and 'lethargic', and sheep being scored at panting score 2 or above loaded on one end of the axis, with the term 'settled' strongly loaded on the other end. PC 3 accounted for 9.5 % of the total variance, and scores associated with flight distance strongly loaded on one end of the axis, with feeding behaviour scores loaded on the other end.

3.2. Voyage, voyage day and time point effects

After accounting for the influence of covariate measures, PC 1 and PC 3 varied significantly between voyages, but there was no significant variation between voyages for PC 2 (Table 3).

The behavioural responses of sheep showed variation attributed to voyage day on PC 1, 2 and 3 for the assessment of combined voyage data (Table 3). PC 1 scores for Voyage B showed a relatively weak effect of voyage day, ($F_{12,189} = 2.07$, $p = < 0.05$) on GLMM but no individual voyage day effect was detected on a Tukey's pairwise comparison (Fig. 1a). PC 2 scores indicated day-to-day variability for both voyages. Voyage A results showed a significant shift in scores towards heat response variables from Day 5 onwards, while Voyage B results showed

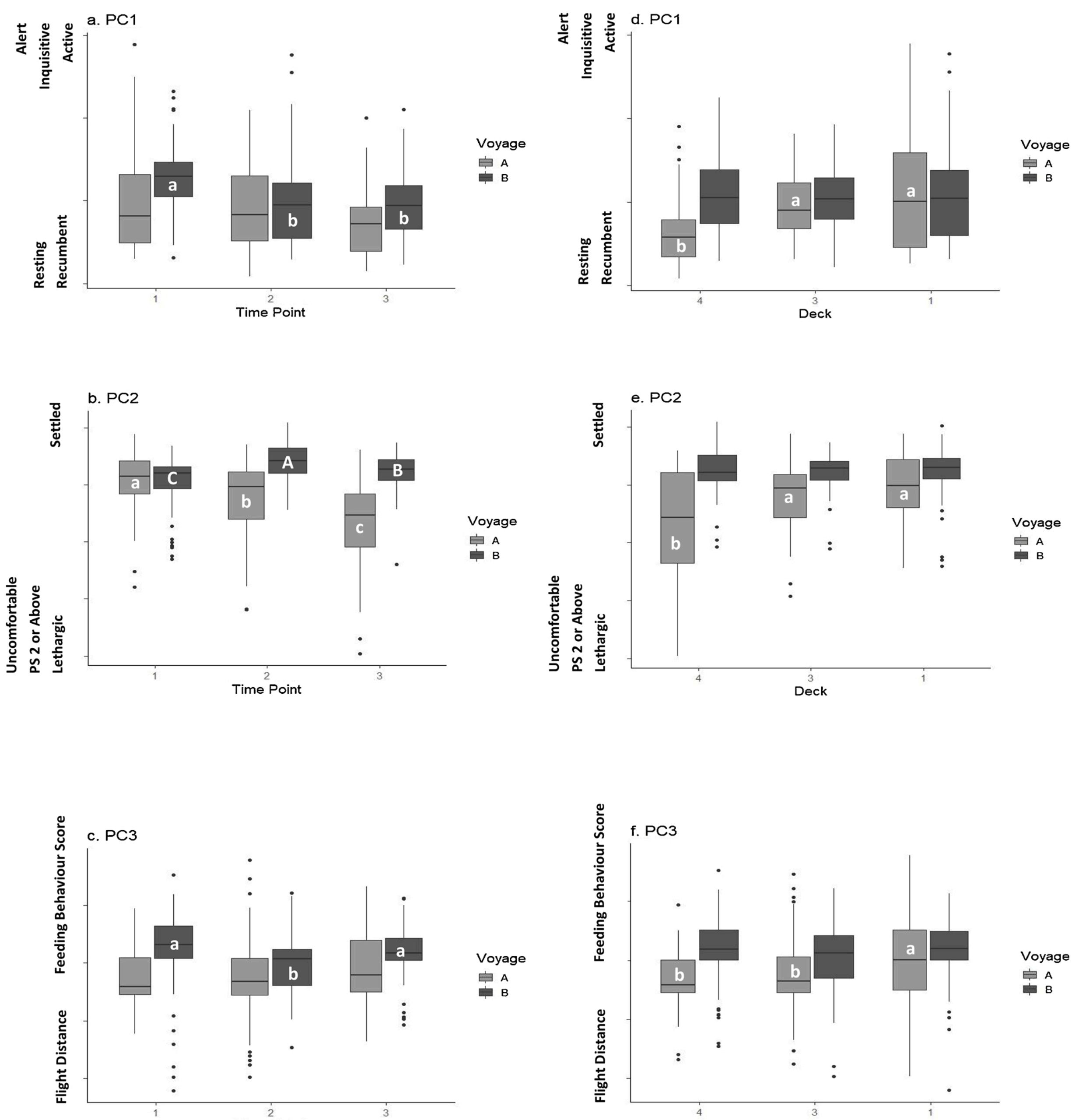

Fig. 2. PC 1 **(a)**, PC 2 **(b)** and PC 3 **(c)** by time point; PC 1 **(d)**, PC 2 **(e)** and PC 3 **(f)** by deck for all days combined. Note: width of box indicates sample size (where a smaller width indicates fewer samples). Letters indicate time points and decks that were significantly different to each other at $p < 0.05$.

a shift towards heat responses on Day 12 (Fig. 1b). PC 3 scores also indicated day-to-day variability; there was a tendency for reduced flight distances and more competitive feeding behaviour as both voyages progressed (Fig. 1c).

The time of day when scoring was performed had a significant effect on PC 1, 2 and 3 for combined voyage data (Table 3). PC 1 data for Voyage A did not change significantly; however, Tukey's pairwise comparisons on Voyage B data showed that sheep were significantly more likely to be described as 'alert', 'inquisitive' or 'active' at TP 1 and scored as 'resting' or 'recumbent' at TPs 2 and 3 (Fig. 2a). The distribution of PC 2 scores differed between the two voyages. Voyage A scores shifted from 'settled' towards heat response variables between TP 1, TP 2 and TP 3 progressively; however, Voyage B scores showed greater heat response at TP 1 with scores tending more towards 'settled' at TPs 2 and 3 (Fig. 2b). PC 3 scores also varied by time point; Voyage B sheep scored higher for competitive feeding behaviour and lower for flight distance measures at TPs 1 and 3 than at TP 2 (Fig. 2**c**).

3.3. Deck and pen group effects

Results of GLMM comparisons for random factors detected a significant effect of deck but not pen. A significant deck effect was detected for PC 1 and 2 for combined voyage data. When the voyages were compared separately, Voyage A data showed a significant deck effect for all three PC factors while no deck effects were detected from Voyage B data (Table 3). GLMM comparisons on pen groups showed no significant variance for any PC factors when voyages were assessed together or separately (Table 3). Tukey's pairwise comparisons on Voyage A data showed that for PC 1 and PC 2, Deck 4 varied significantly from Deck 3 and 1, and there was no significant variation between Deck 3 and 1 (Fig. 2**d, e**). Sheep on Deck 4 were more likely to be 'resting' or 'recumbent' (PC 1), or more likely to be described as 'uncomfortable', 'lethargic' or at 'panting score 2 or above' (PC 2) than sheep on Decks 1 and 3 (Fig. 2**d, e**). Voyage A PC 3 results showed that sheep on Deck 1 were more likely to have lower flight distances and higher feeding behaviour scores than sheep on Decks 3 and 4 **(**Fig. 2f**)**.

3.4. Environmental and management effects

WBGT had a significant effect on all PC factors when voyage data were assessed both in combination and separately (Table 3). PC 1 showed that sheep were less active and more likely to rest as temperatures increased (Fig. 3a). The PC 2 relationship with WBGT was not linear. Sheep scored most strongly for the term 'settled' in the mid-range of temperatures. At the upper end of the WBGT range, sheep were more likely to be described as 'uncomfortable', 'lethargic' and a greater proportion were scored at panting score 2 or above (Fig. 3b). PC 3 scores showed that flight distance measures were lower at lower WBGTs, and feeding behaviour scores increased as WBGT increased (Fig. 3c). Higher WBGTs were recorded on Voyage A than Voyage B (Fig. 3a**, b, c**) and average WBGTs were not consistent between decks (Table 4). For Voyage A, WBGTs varied between 20.4–32.7 °C with Deck 4 pens recording higher average temperatures than those on Decks 1 and 3 (Table 4). During Voyage B, WBGT's varied between 19.3–31.7 °C with Deck 1 recording higher temperatures than Decks 3 and 4 (Table 4).

Feed type and availability showed significant correlations to behavioural outputs. Pellet consumption had a significant effect on PC 2 and 3 for combined voyage data (Table 3). During Voyage A, PC 1 showed that sheep were described as more 'alert' 'inquisitive' and 'active' when pellet consumption was higher (Fig. 3d). Heat response variables on PC 2 increased as pellet consumption increased during Voyage B, and on combined data (Fig. 3e). PC 3 scores on Voyage A showed increased flight distance and lower feeding behaviour scores with higher pellet consumption (Fig. 3f). Roughage access did not have a significant effect on PC 1 (Table 3, Fig. 3g). However, it was linked to heat response variables on PC 2 for the combined analysis with sheep more likely to be described as settled when receiving more roughage feed (Table 3, Fig. 3h). Flight distance and feeding behaviour (PC 3) were correlated to roughage feeds for separate Voyage A and B analyses (Table 3, Fig. 3i); however, results from each voyage did not show the same trend and a combined voyage effect was not detected. More pellets were fed as a percentage of body weight on Voyage A (3.4 %) than B (3.01 %), and four more roughage feeds were given on Voyage B than A (Table 4; Fig. 3g**, h, i**).

Sea swell scores affected PC 3 for the combined voyage analysis and affected PC 2 and 3 for Voyage B when analysed separately (Table 3). Average sea swell scores varied between each voyage (Voyage A: 1.15 and Voyage B: 1.58) (Table 4); however, there was minimal overall variation in sea swell score with results remaining between score 1 (no swell) and 2 (low swell <2 m) for both voyages. Consequently, there were limited associations between sea swell and behavioural changes (Fig. 3***j*****, k, l**).

PC 1, 2 and 3 varied significantly with manure pad moisture score for combined analysis (Table 3). During Voyage A manure pad moisture scores ranged between 1 (dry and dusty) – 4 (high moisture), and during Voyage B, score 2 (firm) was the highest and most predominant score (Table 4; Fig. 3m**, n, o**). Combined voyage data for manure pad moisture scores show that as pad moisture increased, sheep were less likely to be described as 'alert', 'active' or 'inquisitive' (PC 1) and more likely scored as 'resting' and 'recumbent' (Fig. 3m). Sheep were less likely to be described as 'settled' and more likely to be 'uncomfortable', 'lethargic' or have higher panting scores (PC 2) as pad moisture increased (Fig. 3n). For PC 3, flight distances reduced at low manure pad moisture, and feeding behaviour scores increased as manure pad moisture increased (Fig. 3o)

4. Discussion

Animal welfare monitoring during sea transport should capture sheep responses to environmental- and resource-based challenges, including behavioural adaptations. Sheep health and behavioural responses can then be appraised against criteria relating to good health and appropriate behaviour to gauge the welfare state of animals. During the two shipments studied, there were measurable differences in sheep outcomes between voyages, day of the voyage, time of day, and deck location. These findings suggest that evaluating the behavioural components of a welfare assessment protocol must occur at multiple times of the day, on each voyage day, to achieve a representative report on animal outcomes.

4.1. Behavioural findings

Changes in sheep activity and rest contributed to the majority of behavioural variation detected during the voyages. Activity and rest are important indicators of good welfare as they can signal balanced and appropriate behaviour and biological rhythms (Piccione and Caola, 2002; Maloney et al., 2013; Richmond et al., 2017). Sheep who are described as alert, active and inquisitive are likely to be showing active engagement with their environment, which may indicate an affective state that is positive (Ruiz and Dwyer, 2015; Mellor, 2016). Similarly, resting and recumbency are essential measures of welfare, because it is imperative that sheep have the opportunity to lie down and have periods of rest (Ruiz and Dwyer, 2015; World Organisation for Animal Health (OIE)., 2018). Observations of sheep lying and resting indicate the provision of a suitable resting surface and the allowance of sufficient space (Ruiz and Dwyer, 2015; Richmond et al., 2017). When assessed by a pen-side observer, high resting and recumbency percentages can also indicate a reduced fear of humans, as animals continued to rest despite the presence of an observer (Waiblinger et al., 2006; Hemsworth and Coleman, 2011; González-Pech et al., 2018). It is informative that a welfare monitoring protocol can indicate periods of activity and rest, reflect habituation to humans, and record any disruptions to such

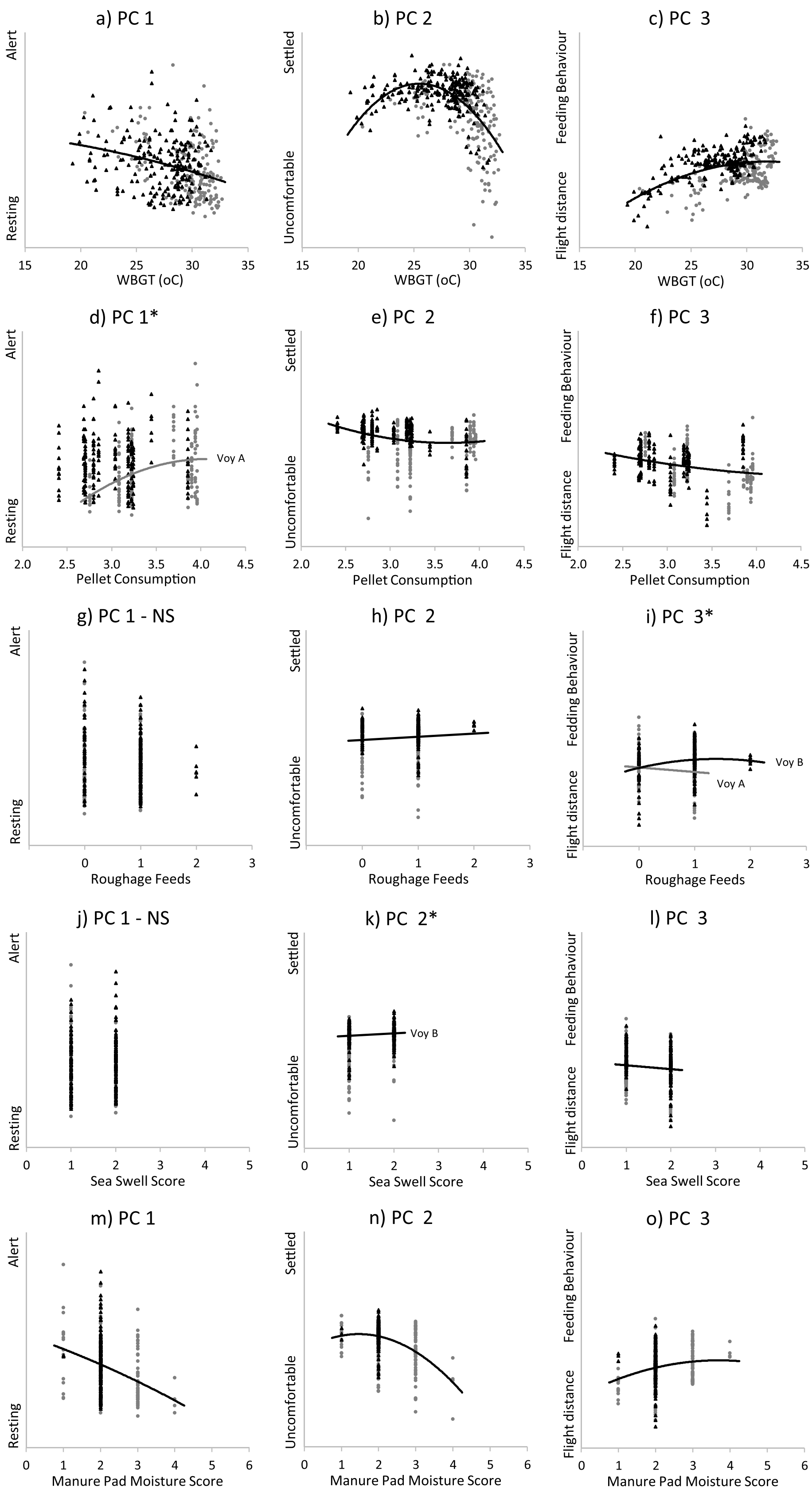

Fig. 3. a–n PC factor scores for PC 1, PC 2 and PC 3 plotted against **(a, b, c)** Wet Bulb Globe Temperature (^{o}C), **(d, e, f)** Pellet Consumption, **(g, h, i)** Roughage Access **(j, k, l)** Sea Swell Score and **(m, n, o)** Manure Pad Moisture Score where • indicates Voyage A data and ▲ indicates Voyage B data. A trendline is displayed for PC factors shown to have a significant correlation to the covariant. Not Significant (NS) is displayed where there was no significant effect. *Where no significant effect was found on combined voyage data, trendlines represent the significant effect per voyage.

Table 4
Covariate values for each voyage and deck.

	Ave. WBGT °C (Range)	Ave. Pellet Consumption (% BW/head/day)	No. of Roughage Feeds	Ave. Sea Swell Score	Ave. Manure Pad Moisture Score (Range)	No. of Pen Assessments
Voyage A	29.8 (20.4–32.7)	3.40	7	1.15	2.3 (1−4)	163
Deck 4	30.0 (23.4–32.7)	3.45	7	1.14	2.4 (1−4)	56
Deck 3	29.6 (23.3–32.4)	3.44	7	1.15	2.2 (1−3)	54
Deck 1	29.8 (20.4–32.4)	3.43	7	1.15	2.2 (1−3)	53
Voyage B	27.2 (19.3–31.7)	3.01	11	1.58	2.0 (1−2)	221
Deck 4	27.0 (19.3–30.7)	3.01	11	1.58	2.0 (1−2)	72
Deck 3	27.0 (19.9–30.8)	3.01	11	1.58	2.0 (2)	72
Deck 1	27.7 (19.3–31.7)	3.01	11	1.57	2.0 (2)	68

behaviour.

The second predominant behavioural domain identified was correlated with demeanour and behaviour that indicated a heat response. Sheep primarily use respiratory means to shed heat load; therefore, panting scores are recognised as a sensitive observation for measuring response to heat (Collins et al., 2018b). PC 2 scores loaded descriptors of demeanour typical of those seen in hot conditions ('lethargic', 'uncomfortable') and elevated panting scores (HSRA Technical Reference Panel, 2019). Heat stress is a well-documented welfare concern for Australian sheep transported by sea (Collins et al., 2018b; McCarthy, 2018), therefore detecting and recording behaviour and affective states associated with a heat challenge are essential for a successful welfare monitoring protocol for live export (Wickham et al., 2017; HSRA Technical Reference Panel, 2019). Comparing the scores for heat response behaviour against environmental conditions and resources can provide insight into risk factors that affect animal outcomes during a heat event.

The third behavioural domain related to feeding behaviour and habituation to humans. It is advantageous for sheep to habituate to human interaction when being managed intensively. Reactive animals are challenging to inspect and handle, and fearful behaviour is also an indicator of stress (Grandin and Shivley, 2015). As the voyage progressed, sheep became less responsive to the presence of an observer and competitive feeding behaviour increased. These results likely reflect sheep becoming more accustomed to the presence of people in close proximity (Hemsworth and Coleman, 2011; González-Pech et al., 2018), and the manual delivery of feed by crew (Jubb and Perkins, 2019). Highly competitive behaviour at feeding indicates inadequate access to feed (Jubb and Perkins, 2019), therefore, feeding behaviour scores reflect the ability of sheep to adapt to management, and the adequacy of nutritional provision.

4.2. Time of sampling

Behavioural responses differed by voyage day, after accounting for covariate factors. This indicates that repeated daily observations can capture adaptations by sheep not directly correlated to changing environmental conditions and resource access. These adaptations might include the habituation of animals to their pen environment, or cumulative responses to possible stressors encountered during the transport process (Hemsworth and Coleman, 2011; Phillips and Santurtun, 2013; Collins et al., 2018a). The ASEL currently require a daily report on shipboard conditions (Department of Agriculture Fisheries and Forestry., 2011). Our data confirmed that reporting sheep responses to sea transport requires sampling of data on consecutive voyage days.

A welfare assessment protocol designed to detect positive and negative welfare states must capture indicators of appropriate behaviour. Sheep on the first voyage showed minimal variation in activity levels across the day; however, sheep on the second voyage were more active in the morning compared to the afternoon and evening. These findings suggest that sheep on Voyage B exhibited diurnal behaviour by displaying periods of positive engagement with their environment, while also having the ability and inclination to lie down and rest (Piccione and Caola, 2002; Piccione et al., 2008). Our results indicate that daily observations in the morning and either afternoon or evening were sufficient to capture whether diurnal activity and resting patterns are occurring.

Sheep demeanour and behaviour associated with heat response varied by voyage day, and time of day, for both voyages. Voyage A presented more heat challenge than Voyage B, and more pronounced variations in heat responses were recorded. Sheep on Voyage A showed higher heat responses at afternoon assessments (TP 2 and TP 3), likely reflecting that they accumulated heat from the morning to the afternoon (Stockman et al., 2011). Conversely, Voyage B sheep showed more heat responses in the morning (TP 1) and were more settled later in the day (TP 2 and TP 3). The higher activity levels at TP 1 observed during Voyage B may have increased sheep panting scores at this time (Al-Dawood, 2017). Voyage B sheep were more likely to be described as settled in the afternoon and evening, suggesting that, on average, Voyage B sheep did not accumulate heat as the day progressed. Determining if heat accumulation is occurring over the day, or if animals can shed heat in respite periods, provides pertinent insight into the impact of heat challenges on sheep behaviour (Collins et al., 2018b; McCarthy, 2018; HSRA Technical Reference Panel, 2019).

A welfare monitoring protocol should identify trends in heat load accumulation and respite. Although recording responses across all three time points will provide a more accurate depiction of animal response to periods of heat, we propose that recording responses at TP 1 and TP 3 is sufficient to determine if animals are able to shed heat over the day. Reporting on-board livestock carrier vessels has not previously accounted for the time of day when sampling animal outcomes (Caulfield et al., 2014). A structured pen sampling technique will improve accuracy when comparing between voyages, and reduce errors in interpretation of animal responses to heat load. Reviewing combined voyage day and time point data has the capacity to describe whether animals are shedding or accumulating heat overnight and for consecutive days of the voyage.

Feeding behaviour and flight distances varied by voyage day and time point. As each voyage progressed, flight distances reduced, and competitive feeding behaviour increased. This suggests that sheep initially habituated to the feeding regimen and their environment (González-Pech et al., 2018), and that feed access was then sub-optimal in the later stages of the voyage due to the finite availability of fodder reserves on the vessel. Scores for sheep on Voyage A did not vary by time point for these behaviour variables; however, sheep on Voyage B had lower scores for feeding behaviour in the afternoon (TP 2) compared to the morning and evening assessments (TP 1 and TP 3). This trend may be explained by the palatability of fodder fed at different times of the day (Baumont et al., 1990). Feeding behaviour scores at TP 1 and TP 3 reflected activity when sheep were most recently fed pellets; however, at TP 2, the most recent feed had often been roughage (chaff). It is proposed that this effect was detected only on Voyage B as more chaff was fed during this journey. The association between observation time and feeding behaviour may be related to the sheep's preference for consuming pellets over chaff.

4.3. Covariate correlations and pen location

This study is a component of a larger study which includes detailed analysis of animal responses to changing conditions. Discussion of co-variate measures is beyond the scope of this paper; however, the results presented show that significant responses were associated with all five environmental- and resource-based factors, indicating that the measures and method of behavioural scoring applied were sufficiently sensitive to environmental variability. Furthermore, sheep behavioural responses around activity and rest, heat challenge, habituation to humans and access to feed aligned with expected outcomes based on documented welfare concerns during sea transport (Collins et al., 2018a). Similarly, behavioural responses were also variable by deck location, indicating that the pens sampled were representative of the range of conditions expected between areas of the ship.

4.4. Limitations and summation

This study represents an initial step to build animal welfare transparency in the live export industry. It did not intend to empirically score the welfare of sheep transported from Australia by sea, as this would require much larger sample sizes and human resources than were available. The study compares data from two voyages, not all covariates measured showed sufficient variability to influence health and behaviour, and neither voyage encountered extreme conditions. However, the findings were important for piloting a welfare assessment protocol during commercial conditions. Additional studies comparing pen-side scoring of demeanour and activity made in situ with scoring using video assessments by a panel of experts or using Qualitative Behavioural Analysis would further validate the proposed protocol. Additional studies can investigate whether the full assessment protocol is required at two time points each day, or if the protocol could be applied once daily, with a smaller subset of key measures taken at other time points. The latter may detect changes in the variables most sensitive to environmental, resource or circadian fluctuations while reducing sampling time.

5. Conclusion

The development of a practical, yet comprehensive, welfare monitoring protocol for the Australian livestock export industry requires an understanding of the effect of sampling frequency. The behavioural outputs of Merino wethers transported by sea from Australia to the Middle East were assessed using a novel welfare protocol. Sheep behavioural responses to varying wet bulb globe temperatures, feed access, sea swell and manure pad moisture are described. Behaviour varied significantly by voyage day and time of the day, suggesting that repeated assessments must be taken to record responses to voyage conditions. In addition, measures should be taken across multiple deck locations if the deck environment or resources vary. This study provides a blueprint for a practical welfare protocol which after further testing could be utilised by industry when sheep are transported by sea.

Declaration of Competing Interest

The author wishes to draw the attention of the Editors to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. The presenting author was contracted to provide veterinary services as an Australian Government Accredited Veterinarian during the sea transport of the animals assessed in this study. Co-authors Teresa Collins and Anne Barnes undertake occasional advisory roles for the Australian Government Department of Agriculture, Water and the Environment, and all authors have received funding from Meat and Livestock Australia /Livecorp.

The author confirms that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

The author confirms that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

The author further confirms that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.applanim.2021.105304.

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