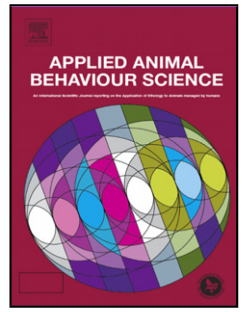
Accepted Manuscript



Title: The effects of regularity of simulated ship motions on the behaviour and physiology of sheep

Authors: Eduardo Santurtun, Clive J.C. Phillips

PII: S0168-1591(18)30115-1
DOI: https://doi.org/10.1016/j.applanim.2018.03.005
Reference: APPLAN 4615

To appear in: *APPLAN*

Received date: 7-6-2017
Revised date: 2-3-2018
Accepted date: 12-3-2018

The effects of regularity of simulated ship motions on the behaviour and physiology of sheep

Eduardo Santurtun* and Clive J.C. Phillips

Centre for Animal Welfare and Ethics, School of Veterinary Sciences, The University of Queensland, Gatton, Queensland, 4343, Australia

*Corresponding author and present address: Departamento de Etología, Fauna Silvestre y Animales de Laboratorio, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Mexico City, Mexico; e-mail: esanturtun@gmail.com

Highlights

- Irregular motion causes stress, imbalance and affiliative behaviour in sheep. Close stocking during irregular motion increases instability and stress in sheep. Sheep that can interact show more stepping and agonistic behaviours

Abstract

Floor movement influences sheep responses to transport, but the importance of movement regularity and interactions between sheep are unknown. To test this, sheep were restrained in pairs in a crate mounted on a moveable, programmable platform for 60 min periods, changing treatments over 12 consecutive days. In an initial experiment a repeated speed of movement and change in angle (regular movement) was compared to variable angles and speeds (irregular movement) of roll, pitch or combined movements, for sheep behaviour, heart rate and feed and water intake responses. Feed intake was increased by irregular roll + pitch motion (P=0.04). During irregular sequences sheep affiliated more, with their heads one above the other (P= 0.001) and supported themselves against the crate (P < 0.001) or kneeling (P = 0.03). Irregular sequences and combined roll and pitch synergistically increased stepping behaviour, indicating loss of balance, and heart rate, possibly indicating stress (P < 0.001). Heat rate data demonstrated that the RMSSD band was reduced during irregular movement (P = 0.04), and LF/HF ratio increased during irregular sequences of roll + pitch (P = 0.007), suggesting less parasympathetic nervous system activity. In a second experiment, we investigated the effects of these floor motion patterns with and without a barrier to separate the sheep. With no barrier or irregular motion, sheep stepped more to avoid loss of balance (P < 0.001) and were again more affiliative. During irregular motion they supported themselves more against the crate (P < 0.001). With no barrier there was more agonistic behaviour (body pushing (P = 0.02), butting (P = 0.02) and evading the other sheep (P = 0.001) and less rumination (P = 0.02), which together with a reduction in RMSSD and NN50 suggested that sheep welfare was reduced by the close proximity of the other sheep. The ratio of low to high frequency beats was highest (P = 0.005) and the RMSSD and NN50 were lowest (P < 0.001) during irregular motion and no barrier. Evidence is provided that sheep were both more stressed in this combination of treatments and also exercising more, through stepping behaviour. Thus irregular sequences and combined roll and pitch caused stress and increased activity to correct loss of balance, as well as increased affiliative behaviour. Separating sheep during irregular motion reduced body instability and stress, suggesting that close stocking is detrimental to their welfare.

Keywords: Behaviour; Interactions between sheep; Regularity; Motion; Sheep; Ship

1. Introduction

During transport, livestock continuously try to avoid contact with other individuals and the vehicle (Broom, 2003; Jones et al., 2010). Balance is maintained by stepping movements and support from vehicle structures (Broom and Fraser, 2007). Some research describes increased stress under loose stocking (Hall et al. 1998; Jones et al., 2010), however tightly stocked livestock may fall down when trying to avoid a fallen animal (Cockram et al., 1996; Das et al., 2001; Jones et al., 2010).

Unpredictable situations are likely to surpass animals' regulatory capacity, resulting in stress (Johannesson and Ladewig, 2000; Bassett and Buchanan-Smith 2007; Koolhaas et al., 2011). In particular, the regularity of floor motions may determine the stress impact if animals cannot predict or habituate to them, particularly if the time delay between exposures is significant (Johannesson and Ladewig, 2000; Abeyesinghe et al., 2001; Phillips and Rind, 2001).

We developed a moving platform to simulate ship motion and to monitor sheep responses to movement variables (Santurtun et al., 2014). With this, we demonstrated from heart rate evidence that a roll (side to side) motion stressed sheep, as well as requiring them to make regular posture changes (Santurtun et al., 2015). Pitch (end to end) motion did not evince such responses, but in combination with roll may increase the unpredictability of the movements.

The hypotheses of this study were that irregular sequences of movement, a combination of different movement types, and contact with other sheep would be stressful as sheep would be less able to cope with the changes of motion. The specific objectives of this study were twofold. First we examined the effects of regular and irregular sequences of roll and pitch on feed intake, behaviours, heart rate, and body posture. Second, to examine whether the effects were dependent on the ability of sheep to move around, we compared sheep that were either isolated or paired within a crate.

2. Materials and methods

Two experiments were conducted at the University of Queensland, Australia (27.3° S, 152.2° E) with approval from the University's Animal Ethics Committee (SVS/315/12).

2.1 Animals, housing and management

The design of novel methodology for exposing sheep to floor movement, including the programming of a movement platform, heart rate monitoring and video recording of behaviour, have been described in full elsewhere (Santurtun et al., 2014). Six merino cross wethers, approximately 34 months of age, weighing (mean ± SEM) 44.2 ± 0.1 kg and shorn over the front half of the body to facilitate heart rate monitor placement, were acquired from the University's flock. Before and after each trial, sheep were kept in a small paddock with ad libitum water and wheaten chaff and access to the experimental rooms. During the trials, sheep were restrained in pairs in a crate (Figure 1) made with 3 tubular steel bars (0.87 m wide × 1.2 m long × 0.95 m high), divided in 2 by a removable barrier. The crate was covered with a sheet to reduce visual stimulation. Aluminium bowls and plastic bottles were attached to the outside of the crate for the first experiment only. A small external mesh barrier was placed to prevent sheep eating from their companion's bowl.

2.2 Regular and irregular roll and pitch motions

The motion platform was programmed to move in both regular and irregular sequences for roll and pitch independently or in combination, using two variables, amplitude and period. An irregular sequence programme was constructed from thirty separate amplitude and period values that were randomly selected by the software Microsoft Visual Studio Solution C++ Express 2008. Regular roll and pitch sequences were programmed as the mean amplitude (4.3º) and period (235 ms) of the irregular roll and pitch sequence. A detailed explanation of the methods to obtain both regular and irregular sequences, including the programming commands, as well as the characteristics of the motion platform used to produce roll and pitch movements independently and in combination, is available in Santurtun et al. (2014).

2.3 Experimental protocols

Before the start of each experiment, over a period of 32 d sheep were habituated to the experimental conditions to minimise the confounding effects of other potential stressors preceding and during experimental trials. Potential stressors identified were handling of the sheep, use of a ramp to get them into the crate, drinking from a water bottle, adjustment to a new environment in the research facility, heart rate monitoring, the researchers' presence and a pelleted diet. The first step involved the reduction of fear of researchers by offering high quality pellets by hand as a positive reinforce for the sheep in triads every two hours a day for 10 d (Photo 4). The next stage involved different, simultaneous training procedures, including loading and unloading into the crate using a ramp (8 d), clipping the area of skin where the heart rate monitor electrodes would be placed (10 d), attaching the heart rate monitor (7 d), and 3-4 hours inside the research facility for feeding, resting and use of crate

(20 d). The training stopped when there were no obvious fear behaviours and the heart rate mean was close to resting rate.

During the initial Motion Regularity Experiment, sheep were exposed in pairs to six treatments with two factors: regular and irregular sequences of pitch, roll, and combined roll and pitch. During the second experiment, Interactions between Sheep in Irregular and Regular Motion, sheep were again exposed in pairs in a 2 factor design: factor 1, Regular or Irregular sequences of a combined pitch and roll motion, or a Control treatment with no motion, and factor 2, with or without a barrier between the two sheep.

Each treatment was applied to the sheep in the crate for a 60 min period in a 6 × 6 Latin Square with one repetition, lasting 12 consecutive days (see Table 1 for Experiment 1 design). In total, each sheep was exposed to 12 treatment periods. Sheep experienced treatments in 6 pairs (1+2, 3+4, 5+6, 1+4, 3+6 and 2+5), with pair effects evaluated. During the Motion Regularity trials, sheep had ad libitum access to water and a container with 1.5 kg of lucerne pellets (® Lockyer Lucerne Products PTY. Ltd, Queensland, Australia).

2.4 Behaviour and feed and water recording

Sheep behaviour was recorded continuously in real time by 3 video cameras/sheep (Kobi CCD Video Camera, Model K-32HCVF, Ashmore, QLD, Australia) during exposure to treatment. A digital video recorder (Kobi H.266, Model XQ-L 900H, Ashmore, QLD, Australia) was used to record the images, and the video data were then analysed using a continuous recording of each animal and Cowlog 2.0 behaviour software for coding of behaviours (Hänninen and Pastell, 2009), according to a prescribed ethogram. The duration of time spent in the following states was continuously recorded: standing (and whether the sheep was ruminating only for the Interactions between Sheep Experiment as it was not observed in the first experiment), body unsupported or supporting their body against the crate; lying (also only for Interactions between Sheep Experiment); kneeling; the duration of various head positions, which were recorded as up (for the Motion Regularity Experiment), level (with withers) or down (below withers), above or under the companion sheep's head or body, and looking towards or opposite the companion sheep; time spent drinking, eating and licking the bowl feeder (only for the Motion Regularity Experiment). Stepping, pawing, butting, slipping, pushing the companion sheep with the body or moving to evade touching the other sheep (only for the Interactions between Sheep Experiment) were recorded as events. Slipping was too rare to include in treatment analyses, but was included in across treatment analyses outlined below. At the end of each exposure of a pair of sheep to a treatment in the Motion Regularity Experiment, food and water intake were measured. After each exposure to treatment, sheep were taken to an adjacent room, and sheep were continuously video recorded for 30 min to determine residual effects on standing, walking and lying behaviours.

2.5 Heart rate variability

Heart rate monitors (Polar S810i, Kempele, Finland) were attached to each sheep for detection of heart rate and inter-beat intervals (IBI) throughout the 60 min exposure to treatment in the crate. Eight sections of 512 beats were randomly extracted from each treatment application for time and frequency domain analysis using the following variables: IBI mean (RR mean); Root Mean Square of Successive Differences (RMSSD), the number of pairs of intervals in normal to normal beats differing by > 50 ms (NN50), power in high and low frequency bands in normalised units [HF and LF, respectively, calculated as HF or LF/(Total power –very low frequency power) x 100, and the ratio of these (LF/ HF)].

2.6 Statistical analysis

During analyses, all data were checked for normal distribution of residuals using the Anderson-Darling test. For data not satisfying the Anderson-Darling test, $\log_{10}$ transformations were made and back-transformed means are reported in addition to transformed data.

Preliminary analyses by the Mood median test determined that sheep within pair was a significant predictor of behaviour results, and therefore the mean of paired sheep behaviours were the units of analysis. Heart rate data were analysed using individual sheep. For the Interactions between Sheep Experiment, 4 sections of 6 minutes with a 12 min interval (min 0-6, 18-24, 36-42, 54-60) were analysed. For the Motion Regularity Experiment, the entire 60 minutes were analysed. The proportion of time spent in each behaviour, frequency of behaviour, heart rate data and Fast Fourier transformed heart rate variability data were analysed using a general linear model of the orthogonal Latin Square. For Experiment 1, the model had the equation:

$$Y_{ijklm} = \mu + D_i + S_j + M_k + R_l + R_l\, M_k + P_m$$

Where Y_{ijklm} = the observed value of one of the replicates of sheep behaviour measurement during a treatment application

D_i = Day

S_j = Sheep

M_k = Motion: roll, pitch or roll + pitch

R_l = regularity of motion

P_m = Random effect of sheep Sj within $D_i + S_j + M_k + R_l$

For experiment 2, the model had the equation:

$Y_{ijklm} = \mu + D_i + S_j + M_k + B_l + B_lM_k + P_m$

Where Y_{ijklm} = the observed value of one of the replicates of sheep behaviour measurement during a treatment application

D_i = Day

S_j = sheep

M_k = motion: regular or Irregular sequences of a combined pitch and roll motion, or a Control treatment with no motion, and factor 2, with or without a barrier between the two sheep

B_l = barrier

P_m = Random effect of sheep Sj within $D_i + S_j + M_k + B_l$

In both experiments, an additional factor ‘Section’, was added for analysis of heart rate variables.

Stepwise multiple regressions explored relationships between the heart rate variables that differed between treatments and all of the behaviour variables, using mean values of all observations for individual animals. Alpha value to enter and leave the equation was set at 0.05. Variance Inflation Factors were verified as less than 5 to protect against correlation between behaviour variables.

The statistical package Minitab (2010) was used for the data analysis. For all tests, probability levels are two-tailed and are considered significant when $P < 0.05$. Post hoc Tukey’s tests were used to identify which means were significantly different from each other.

3. Results

3.1 Experiment 1: Motion regularity

3.1.1 Feed intake and behaviour

Irregular Roll and Pitch sequences increased feed intake compared to the same combined motion with Regular sequences (P = 0.04) (Table 2), and this tended to be reflected in a longer time spent eating (P=0.06). However, sheep spent more time drinking (P <0.001) and consumed more water (P = 0.001) when experiencing Regular sequences (Table 3).

During Irregular sequences, sheep spent more time with their heads under /above the other sheep (P= 0.001), and orientated down (P = 0.001), and spent less time with their head up (P = 0.04) or level (P <0.001) compared with during Regular sequences (Table 3). Sheep also spent more time with their head down when experiencing Roll or the combination of Roll and Pitch, compared with just Pitch (P=0.007), and tended to keep their head level most during Pitch (P = 0.08) (Table 4). During Irregular sequences sheep also spent more time supported on the crate (P <0.001) and kneeling (P = 0.03) (Table 3), and conversely during Regular sequences they spent more time standing without support (P=0.001). Motion type did not affect body position (Table 4). Stepping behaviour was increased during Irregular Roll and Pitch sequences, and Regular Roll had the least stepping (P= 0.001) (Table 2). During combined Roll and Pitch motions sheep pawed most (P=0.02) and tended to spend less time butting (P = 0.06) (Table 4). Bowl licking was less common (P = 0.01) and butting tended to be more common during Irregular sequences (P = 0.08) (Table 3).

Post-treatment analysis showed no differences in walking or standing following different sequences (Table 3) or motions (Table 4). However, sheep tended to lie less ($P = 0.08$) following Irregular sequences (Table 3).

3.1.2 Heart Rate Variability

Sheep in combined Roll and Pitch had increased heart rate if the sequences were Irregular ($P < 0.001$) (Table 2). Regardless of sequence, heart rate was lower for Roll than Pitch. The inter-beat interval showed the reverse pattern. In Regular sequences, RMSSD, NN50 and HF were all increased ($P \leq 0.04$) and LF and LF:HF decreased ($P < 0.001$), compared with Irregular sequences (Table 3). These differences were most evident for the Roll and Pitch treatment (Table 2).

3.1.3 Relationships between key heart rate variables and sheep behaviour

Stepwise regressions, with units for variables provided in the tables, indicated that RMSSD was negatively related with feed intake and slipping:

RMSSD = 172.9 - 0.22 Feed intake ($P < 0.0001$) + 23.0 Slipping ($P = 0.002$); $r^2 = 98.1\%$

Equation 1

NN50 was negatively related to feed intake and positively related to standing against the crate:

NN50 = 580 - 0.86 Feed intake ($P < 0.0001$) + 0.52 standing against the crate ($P = 0.005$); $r^2 = 98.9\%$

Equation 2

Low frequency beats were also negatively related to feed intake:

Low Frequency = 9.3 + 0.11 feed intake ($P = 0.01$); $r^2 = 74.6$

Equation 3

A biplot of two principle components (Figure 2) demonstrated that feed intake had a similar variance to low frequency beats. Spearman rank correlations also indicated that RMSSD and NN50 and Low frequency beats were all negatively related to feed intake (CC = -0.83, 0.83 and 0.89, P = 0.04, 0.04 and 0.02, respectively).

3.2 Experiment 2: Interactions between Sheep in Irregular and Regular Motion

3.2.1 Behaviour

There were few significant interactions between motion and barrier presence on behaviour (Table 5). Sheep in the Control treatment without the barrier spent longer with their head level than sheep with the barrier in irregular motion (Table 5). Sheep without the barrier spent more time with their head under or above the other sheep (P = 0.03) and less time looking towards their companion (Table 6). Sheep in irregular motion spent more time with their head looking down, compared with control sheep (P = 0.02) (Table 7). Sheep in either regular or irregular motion (P <0.001) spent more time with their heads under or above the other sheep, compared to control sheep (Table 7).

Sheep in irregular motion also spent longer supporting themselves against the crate (P< 0.001) and did more stepping (P< 0.001) compared with regular motion and control sheep (Table 7). Those with the barrier stood without support most if the motion was regular (P = 0.004) (Table 5), and they did less stepping (P < 0.001) and had a tendency for less kneeling (P = 0.06) than those without the barrier (Table 6).

The barrier decreased the prevalence of pushing (P = 0.02), butting (P = 0.02), evading the other sheep (P = 0.001) (Table 6), whereas motion type did not affect agonistic behaviours (Table 7). Sheep spent more time ruminating (P = 0.02) when the barrier was in place (Table 6).

Post treatment sheep that had been in irregular or regular motion on the platform lay down for longer than those that had been in the Control treatment (P = 0.03) (Table 7).

3.2.2 Heart Rate Variability

With the barrier present sheep in Irregular motion had increased heart rate, decreased interbeat interval (both P = 0.03), and increased RMSSD (P < 0.001) and NN50 (P < 0.001), compared with Regular motion (Table 5). Without the barrier these differences in heart rate and RMSSD were not evident, and the NN50 was less in the Irregular treatment. With the barrier present LF/HF values were lower and NN50 values higher (P < 0.001) for Control sheep compared to sheep experiencing regular motion (Table 5).

3.2.3 Relationships between key heart rate variables and sheep behaviour

Stepwise regressions, with units for variables as provided in the tables, indicated that heart rate was positively associated with sheep having their head down, standing against the crate, pawing and evading:

HR mean = 75.8 + 0.032 Head Down (P < 0.001) + 0.0076 Standing against crate (P = 0.03) + 0.14 Pawing (P = 0.03) + 0.81 Evading (P = 0.003); r^2 = 17%

Equation 1

NN50 was negatively related to head down, and positively related to Standing without support, lying down and stepping:

NN50 = 56.1 - 0.21 H_Down ($P = 0.003$) + 0.20 No support ($P = 0.03$) + 0.40 Lying ($P < 0.001$) + 1.8 Stepping ($P = 0.02$); $r^2 = 17\%$

Equation 2

Finally the ratio of Low to High frequency beats was related to head under, Head down, the absence of ruminating and stepping

LF/HF = 3.0 + 0.025 Head under/above ($P < 0.0001$) + 0.013 Head Down ($P < 0.0001$) + 0.0057 Not ruminating ($P = 0.008$) - 0.16 Stepping ($P < 0.0001$)

Equation 3

A biplot of two principle components (Figure 3) demonstrated that lying and to a lesser extent ruminating had a similar variance pattern to the heart rate variables, RMSSD, NN50 and High Frequency beats. Spearman rank correlation also showed that there was a positive correlation between lying and high frequency beats (Correlation coefficient, CC, 0.83; $P = 0.04$). Head down, under/above and standing against the crate had similar variance to Low Frequency beats (Figure 1).

4. Discussion

The hypotheses that irregular sequences of a combination of Roll and Pitch, with sheep able to interact with each other, would be most stressful to sheep appears to have been confirmed, depending on the definition of stress used. Stress is often considered to essentially have negative impacts on welfare, specifically 'an environmental effect on an individual which overtaxes its control systems and results in adverse consequences, eventually reduced fitness' (Broom, 2001). Here we regard stress to be potentially more moderate, specifically a threat to an animal's homeostasis (Moberg, 2000; Charmandari et al., 2005). Under this definition stress can demonstrably be proven to have been confirmed for the situations described at the start of this paragraph. In this discussion we focus on the evidence for stress, compared with alternative hypothesis of increased activity to explain the observed effects.

4.1 Experiment 1: Motion Regularity

The results suggest that firstly, Irregular sequences and secondly, the combination of Roll and Pitch, had a negative impact on sheep welfare. Good sheep welfare, as outlined by either the Five Domains or Five Freedoms models (Mellor, 2016), includes avoidance of restrictions on behaviour. The high levels of stepping behaviour in Irregular combinations of Roll and Pitch presumably functioned to maintain the sheep's balance and are evidence of reduced welfare, as the sheep were no longer able to maintain a steady footing. The specific combination of roll and pitch is known to adversely affect humans during sea transport (Wertheim et al., 1998). Waves are also usually irregular during severe sea conditions (Clauss and Kühhnlein, 1997), which aggravates motion sickness in humans (Li et al., 2012). Although this study did not replicate sea and wave conditions precisely, heart rate responses suggest that irregular and combined roll and pitch simulated ship motions appears to have stimulated stress. Heart rate was increased, NN50 reduced and LF/HF ratio increased, suggesting that the parasympathetic/sympathetic nervous system balance was adversely affected. Sheep also ate more pellets in this treatment. This may be to reduce negative emotions, an ovine equivalent of comfort/emotional eating observed in humans, rats and companion animals (Ortolani et al., 2011; McMillan, 2013), arising out of the need to have secure feed sources in stressful conditions. Alternatively, it may derive from reduced feelings of malaise when the rumen is full (it has been suggested that sheep experience motion sickness, Santurtun and Phillips, 2015) or that extra physical effort may have increased their appetite.

The analysis of the relationship between heart rate variables and feed intake/behaviour variables clearly connected the increased feed intake with reduced heart rate variability. We have previously observed that sheep exposed to an even more stressful motion, heave (up and down motion), ate more in the observation period after exposure to treatment (Santurtun et al., 2015). The positive correlation between RMSSD and slipping suggests that slipping may have occurred in some sheep that did not engage in adaptive behaviour, such as stepping, which reduced heart rate variability. In contrast to feed intake, water intake and time spent drinking were reduced during Irregular sequences, probably because it was more difficult for sheep to walk towards the dispenser and maintain a fixed body position and pressure with their tongue on the dispenser to obtain water.

Irregular sequences and the combination of Roll and Pitch had a synergistic effect on the sheep's body posture. During Irregular sequences, sheep positioned their head above or below the companion sheep's body, a behaviour that we have observed previously in sheep's response to simulated heave of a ship (Santurtun et al., 2015) and which we presume functions to offer comfort. They also positioned their head more downwards, which lowers the centre of gravity, whereas during Regular sequences sheep appeared able to maintain balance with a level or raised head. The affiliative behaviour, and head down during the Irregular sequences, coincided with an increased heart rate and reduced heart rate variability, and a tendency for increased butting, all related to stress (von Borell et al., 2007; Gougoulis et al., 2010). Sheep positioning their head down has been associated with stress during transport (Hall et al., 1998), but the increased affiliation suggests increased gregariousness during stress, with consequent implications for preferred stocking density. Additional evidence of an unstable body posture is provided by the fact that sheep stepped more in the combined Irregular Roll and Pitch treatment, and pawed more during the combination of Roll and Pitch compared to Pitch. These behaviours have been linked to stressful environments in sheep (Cockram et al., 1994) and nervousness and anxiety in cattle (Wenzel et al., 2003). In our previous observations (Santurtun et al., 2015), sheep stepped more during roll motion than pitch, heave or no motion. Despite this, an observed swaying response of the sheep to Roll and reduced heart rate and increased RMSSD, compared with Pitch, indicate that Roll may have had either a calming effect or required less effort to adapt to than Roll and Pitch combined, especially when the latter was in Irregular sequences. In Roll and Pitch, Irregular sequences increased the Low:High Frequency ratio, compared with Regular sequences, demonstrating increased sympathetic and reduced parasympathetic nervous system activity. An increase in LF:HF ratio has been associated with poor welfare, it has been observed in humans exposed to flight simulator movement (Chu et al., 2013) and in sheep infected with footrot (Stubsjoern et al., 2015).

As well as frequent stepping behaviour, standing against the crate and kneeling, both increased in Irregular sequences, indicated a departure from normal behaviour and possibly a reduced level of control over their environment, which may be emotionally stressful. In a previous study (Santurtun et al., 2015), sheep supported themselves more against the crate during heave motion, which had the most extreme heart rate responses of any motion. The increased time spent standing without support in Regular motion and lowest level of stepping in Regular Roll suggests that sheep could anticipate the platform motion and did not need side support to avoid stepping.

Sheep tended to spend more time lying following Irregular sequences. This suggests an impact on sheep fitness and a need to rest following physical exertion, in particular the increased stepping. The approximately doubling of stepping frequency during Irregular sequences of Roll and Roll/Pitch may have caused fatigue in sheep over the one hour period, requiring a longer lying period post-treatment and providing evidence of a negative emotion.

There was evidence too of adaptive behaviour, sheep in Irregular movement bracing themselves against the crate and kneeling to lower their centre of gravity are evidence of responses that assisted sheep to cope with the environmental conditions. Sheep during Roll increasing their lying after treatment suggests a rebound behaviour indicative of fatigue. During Roll, sheep putting their head down may not have been related to stress, it may have been an adaptive behaviour to improve balance.

4.2 Experiment 2: Interactions between Sheep

The results of the second experiment suggest that irregular motion and the interactions between sheep allowed by removal of the barrier both caused behavioural evidence of loss of balance, and produced body position adjustment behaviours (head down, stepping, supporting themselves against the crate). As mentioned above, lowering their head also lowers their centre of balance, helping to protect against slipping. Agonistic behaviours, which are potentially useful welfare measures during transport (Broom, 2003), were particularly observed when sheep had no barrier and hence greater opportunities to be in contact with their partner. This suggests that agonistic intent may have been greater than observed in our earlier experiment with this apparatus, in which a barrier was always in place (Santurtun et al., 2015). Agonistic behaviours present threats (particularly of loss of balance) to animals to which they are directed, making them fearful. Fear is a central tenet of several welfare assessment protocols, especially the Five Freedoms. Although agonistic behaviour is a natural component of animals' behaviour in the wild, it is usually minimised to avoid threats to the wellbeing of these animals, often by ritualising aggressive interactions. Under field conditions, overt aggression is relatively rare between sheep, e.g. 0.8 interactions/h (Lynch et al., 1985), compared with an average of about 20 interactions/h across these two experiments. However, extrapolation of these results to commercial sea transport conditions should be done carefully, as the space allowance used was significantly higher than that recommended by the Australian government (DAFF, 2011).

Accompanying the behavioural responses to the removal of the barrier, there was a decreased RMSSD and NN50, providing physiological evidence of stress and a possible reduction of the parasympathetic system (Langbein et al., 2004; von Borell et al., 2007). In addition, the Control sheep had low LF/HF values and high NN50 with the barrier, both suggesting either low stress or exercise levels. As well as stress, exercise can increase LF:HF ratio, at least in humans (Shi et al., 2017), and the absence of the need to step as frequently or brace their body against the sides may have affected heart rate and its variability. As the response occurred in both HF and LF components, we cannot tell whether it was primarily due to altered activity in the parasympathetic or sympathetic function (Marchant-Forde, 2010).

Despite this apparent increase in stress when there was no barrier, there was greater affiliative behaviour as well as agonistic behaviour. Sheep spent almost twice as long with their head under or above their partner when the barrier was removed, whereas when the barrier was in place they doubled the time spent looking towards their companion. A desire for emotional support from their companion is suggested. Removal of the barrier reduced

NN50 in this experiment, as did Irregular movement in experiment 1. As expected, when there was no barrier there was evidence from the NN50 measure that either irregular motion was more stressful or required more adaptive movements by the sheep, or both, than regular motion. Evidence that both were involved is presented in the regression equation for NN50, which was negatively related to both head down, a stress response, and stepping, a movement response.

Rumination was reduced when sheep had the opportunity to interact with their companion. This could be because the sheep had to face additional potential stressors, such as having more contact with the companion sheep, which resulted in more agonistic behaviours and a prolonged effort to avoid the other sheep. Stress during road transport conditions also reduces time small ruminants spend ruminating (Das et al., 2001; Cockram, 2004).

Irregular motion and absence of the barrier had major effects on body posture and balance, especially that sheep positioned their head above or below their companion's head or body. This behaviour was again perhaps a result of a stress response to motion, because of the increased LF/HF ratio during irregular motion, and a reduction of RMSDD and NN50 (von Borell et al., 2007) in sheep without the barrier. As in Experiment 1, sheep spent more time with their head down during irregular motion, associated with stress situations (Hall et al., 1998) and a submissive posture (Nowak et al., 2008). Additional evidence of an unstable body posture was that the sheep stepped more when they had no barrier and were in irregular motion. Conversely when there was no barrier, sheep tended to spend more time kneeling, which may have increased stability or protected them from aggressive interactions with their companion. Crucially, when there was no barrier and the sheep had the opportunity to be in contact with their companion sheep, there was no record of them ever supporting each other. This coincides with a previous study of road transport in which sheep did not support each other even if they had space to do so (Jones et al., 2010). It is commonly believed that 'animals should be loaded tightly enough to give each other mutual support' (Wythes, 1994), but this study shows that the uncertainty surrounding other animals' reactions during motion mitigates against any benefit being derived from mutual support.

The increased lying time of sheep that had experienced motion, as opposed to the Control treatment, supports residual period responses observed in Experiment 1, and demonstrates that physical exertion during the 60 min treatment periods was sufficient to increase the motivation to rest. It has been observed (Phillips, 2008) that sheep at high stocking densities are reluctant to lie down during the early stages of the voyage, probably because of the fear of other animals closing over them, and this work suggests that the motion experienced will increase their need for rest.

5. Conclusions

Irregular motion increased stepping behaviour and affiliative behaviour in paired sheep, which supported themselves more on the sides of their crate than sheep in regular motion. Allowing the sheep to interact increased their stepping behaviour and led to agonistic behaviour and evidence of physiological stress from heart rate parameters. There was no evidence that sheep voluntarily or involuntarily supported each other, which leads us to conclude that high stocking densities during transport are not defensible on the grounds that sheep support each other.

Conflict of interest

None.

Funding

The authors acknowledge financial assistance from the Humane Slaughter Association and the Humane Society International.

Acknowledgements

The authors would like to thank all persons who keenly gave their time to this project. In particular to Dr. Ali Ramin for his assistance during experiment trials, and the support of animal technician Andrew Kelly.

References

Abeyesinghe, S. M., Wathes, C. M., Nicol, C. J., Randall, J. M. 2001. The aversion of broiler chickens to concurrent vibrational and thermal stressors. Appl. Anim. Behav. Sci. 73:199-215. doi:10.1016/S0168-1591(01)00142-3.

Bassett, L., Buchanan-Smith, H. M. 2007. Effects of predictability on the welfare of captive animals. Appl. Anim. Behav. Sci. 102: 223-245. doi:10.1016/j.applanim.2006.05.029.

Broom, D.M. 2003. Transport stress in cattle and sheep with details of physiological, ethological and other indicators. Dtsch. Tierarztl. Wochenschr. 110: 83-89.

Broom, D.M. 2001. Coping, stress, and welfare. In: Broom, D.M. (ed.) Coping with Challenge: Welfare in Animals, Including Humans. Dahlem University Press, Germany, pp. 1-9.

Broom, D.M., Fraser, A.F. 2007. Domestic Animal Behaviour and Welfare. CAB International, Wallingford, pp. 180–207.

Chu, H., Li, M-H., Huang, Y-C and Lee, S-Y. 2013. Simultaneous transcutaneous electrical nerve stimulation mitigates simulator sickness symptoms in healthy adults: a crossover study. BMC Compl. Alt. Med. 13: 84 https://doi.org/10.1186/1472-6882-13-84

Clauss, G. F., Kühhnlein, W. L. 1997. Simulation of design storm wave conditions with tailored wave groups. In: Proc. 7th Int. Offshore Polar Eng. Conf. Honolulu, USA, p. 25-38.

Cockram, M. S., Ranson, M., Imlah, P, Goddard, P.J., Burrells, C., Harkiss, G.D. 1994. The behavioural, endocrine and immune responses of sheep to isolation. Anim. Sci. 58:389-399. DOI: http://dx.doi.org/10.1017/S0003356100007339.

Cockram, M. S., Kent, J. E., Goddard, P. J., Warran, N. K., McGilp, I. M., Jackson, R. E., Muwanga, G. M., Prytherch, S. 1996. Effect of space allowance during transport on the behavioural and physiological responses of lambs during and after transport. Anim. Sci. 62: 461-477. doi: http://dx.doi.org/10.1017/S1357729800015009.

Cockram, M. S. 2004. A review of behavioural and physiological responses of sheep to stressors to identify potential behavioural signs of distress. Anim. Welf. 13: 283-291.

DAFF. 2011. Australian standards for the export of livestock (Version 2.3) and Australian position on the export of livestock. Commonwealth of Australia, Canberra.

Das, K. S., Srivastava, B. B., Das, N. 2001. Standing orientation and behaviour of goats during short-haul road transportation. Small Rumin. Res. 41:91-94. doi: 10.1016/S0921-4488(01)00187-0.

Gougoulis, D. A., Kyriazakis, I., Fthenakis, G.C. 2010. Diagnostic significance of behaviour changes of sheep: a selected review. Small Ruminant Res. 92:52-56. doi:10.1016/j.smallrumres.2010.04.018.

Hall, S. J. G., Kirkpatrick, S. M., Lloyd, D. M., Broom, D. M. 1998. Noise and vehicular motion as potential stressors during the transport of sheep. Anim. Sci. 67:467-473. doi:10.1017/S1357729800032884.

Hänninen, L., Pastell, M. 2009. CowLog: Open-source software for coding behaviours from digital video. Behav. Res. Methods 41:472-476. doi:10.3758/BRM.41.2.472.

Johannesson, T., Ladewig, J. 2000. The effect of irregular feeding times on the behaviour and growth of dairy calves. Appl. Anim. Behav. Sci. 69:103-111.

Jones, T. A., Waitt, C., Dawkins, M.S. 2010. Sheep lose balance, slip and fall less when loosely packed in transit where they stand close to but not touching their neighbours. Appl. Anim. Behav. Sci. 123: 16-23. doi:10.1016/j.applanim.2009.12.006.

Koolhaas, J.M., Bartolomucci, A., Buwalda, B., de Boer, S.F., Flugge, G., Korte, S.M. Meerlo, P., Murison, R., Olivier, B., Palanza, P., Richter-Levin, G., Sgoifo, A., Steimer, T., Stiedl, O., van Dijk, G., Wohr, M, Fuchs, E. 2011. Stress revisited: A critical evaluation of the stress concept. Neurosci. Biobehav. Rev. 35: 1291-1301. doi:10.1016/j.neubiorev.2011.02.003

Langbein, J., Nurnberg, G., Manteuffel, G. 2004. Visual discrimination learning in dwarf goats and associated changes in heart rate and heart rate variability. Physiol. Behav. 82: 601-609. DOI: 10.1016/j.physbeh.2004.05.007.

Li, J. R., Zhu, L., Yuan, W., Jin, G. R., Sun, J.J. 2012. Habituation of seasickness in adult during a long voyage. Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 47:642-645.

Lynch, J.J., Wood-Gush, D.G.M., Davies, H.I. 1985. Aggression and nearest neighbours in a flock of Scottish Blackface ewes. Biol. Behav. 10: 212-225.

Marchant-Forde, R. 2010. Heart rate variability. In: Mills, D., Marchante-Forde, J., McGreevy, P.D., Morton, D.B., Nicol, C.J., Phillips, C.J.C., Sandøe, P. and Swaisgood, R.R. (ed.) Encyclopaedia of Applied Animal Behaviour and Welfare. CAB International, Wallingford. Pp 308-310.

McMillan, F. D. 2013. Stress-induced and emotional eating in animals: A review of the experimental evidence and implications for companion animal obesity. J. Vet. Behav. 8:376-385. doi:10.1016/j.jveb.2012.11.001.

Mellor, D. J. 2016. Updating animal welfare thinking: moving beyond the “Five Freedoms” towards “A LifeWorth Living. Animals 6, 21; doi:10.3390/ani6030021

Minitab. 2010. [Minitab 17 Statistical Software (2010). State College, PA: Minitab, Inc. (www.minitab.com)]

Nowak, R., Porter, R. H., Blache, D., Dwyer, C.M. 2008. Behaviour and the welfare of the sheep. In: C. M. Dwyer (ed.) The Welfare of Sheep. Pp 81-122. Springer Science, Netherlands.

Ortolani, D., Oyama, L. M., Ferrari, E. M., Melo, L. L., Spadari-Bratfisch, R. C. 2011. Effects of comfort food on food intake, anxiety-like behaviour and the stress response in rats. Physiol. Behav. 103:487-492. doi:10.1016/j.physbeh.2011.03.028.

Phillips, C. J. C., Rind, M. I. 2001. The effects of frequency of feeding a total mixed ration on the production and behaviour of dairy cows. J. Dairy Sci. 84:1979-1987. doi:10.3168/jds.S0022-0302(01)74641-3.

Phillips, C.J.C., 2008. The welfare of livestock during sea transport. In: Long Distance Transport and Welfare of Farm animals. CAB International, Wallingford, UK, pp. 137-154.

Santurtun, E., and Phillips, C.J.C. 2015. The impact of vehicle motion during transport on animal welfare. Res. Vet. Sci. 100: 303-308.

Santurtun, E., Moreau, V., Phillips, C. J. C. 2014. A novel method to measure the impact of sea transport motion on sheep welfare. Biosyst. Eng. 118:128-137. doi:10.1016/j.tvjl.2013.01.007.

Santurtun, E., Moreau, V., Marchant-Forde, J. N., Phillips, C. J. C. 2015. Physiological and behavioural responses of sheep to simulated sea transport motions. J. Anim. Sci. 93: 1250-1257. doi:10.2527/jas2014-8037.

Shi, P., Hu, S. and Yu, H. 2017. Recovery of heart rate variability after treadmill exercise analyzed by lagged Poincaré plot and spectral characteristics. Med. Biol. Eng. Comp. 56: 221-231.

Stubsjoen, S.M., Knappe-Poindecker, M., Langbein, J., Fjeldaas, T. and Bohlin, J. 2015. Assessment of chronic stress in sheep (part II): Exploring heart rate variability as a non-invasive measure to evaluate cardiac regulation. Small Rum. Res. 133, 30-35.

von Borell, E., Langbein, J., Despres, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Marchant-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I. 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals. A review. Physiol. Behav. 92:293-316. doi:10.1016/j.physbeh.2007.01.007.

Wenzel, C., Schönreiter-Fischer, S, Unshelm, J. 2003. Studies on step-kick behaviour and stress of cows during milking in an automatic milking system. Livest. Prod. Sci. 83:237-246. doi:10.1016/S0301-6226(03)00109-X.

Wertheim, A. H., Bos, J. E., Bles, W. 1998. Contributions of roll and pitch to sea sickness. Brain Res. Bull. 47:517-524. doi:10.1016/S0361-9230(98)00098-7.

Wythes, J.R., 1994. Literature review of welfare aspects and carcass quality effects in the transport of cattle, sheep and goats: Part A, Handling and Transport of Beef Cattle. Meat Research Corporation Project LMAQ.011, Final Report, Meat Research Corporation, Sydney, pp. 36

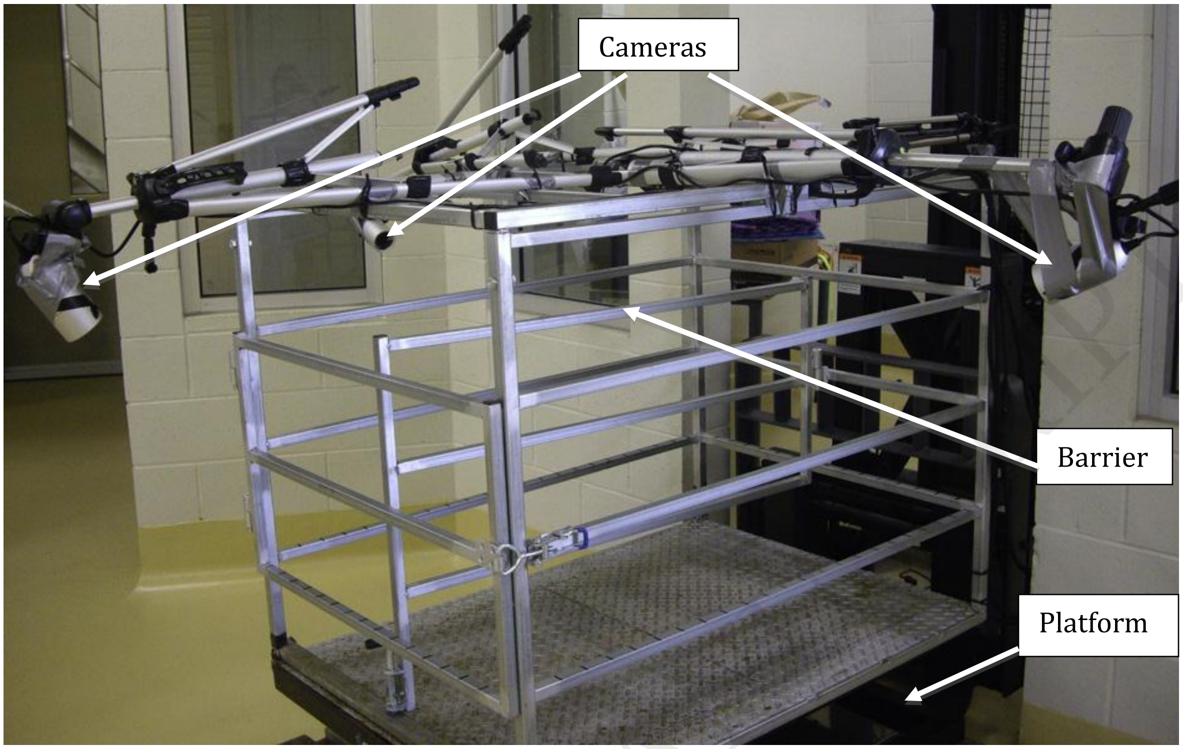


Figure 1. Crate positioned on the platform showing the barrier between the two sides and camera positions depicted

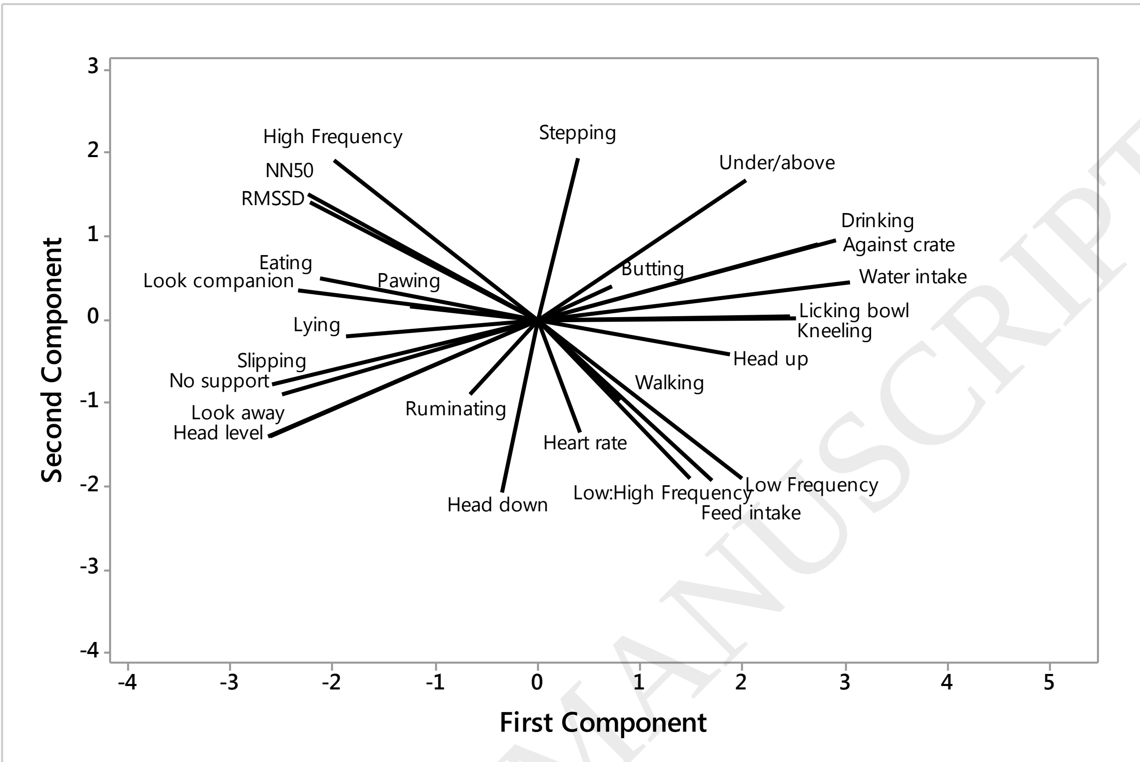


Figure 2. Biplot of components 1 and 2 in a principle components analysis of heart rate and behaviour variables in Experiment 1. RMSSD = Root Mean Square of Successive Differences; NN50 = Number of pairs of intervals in normal to normal beats differing by > 50 ms

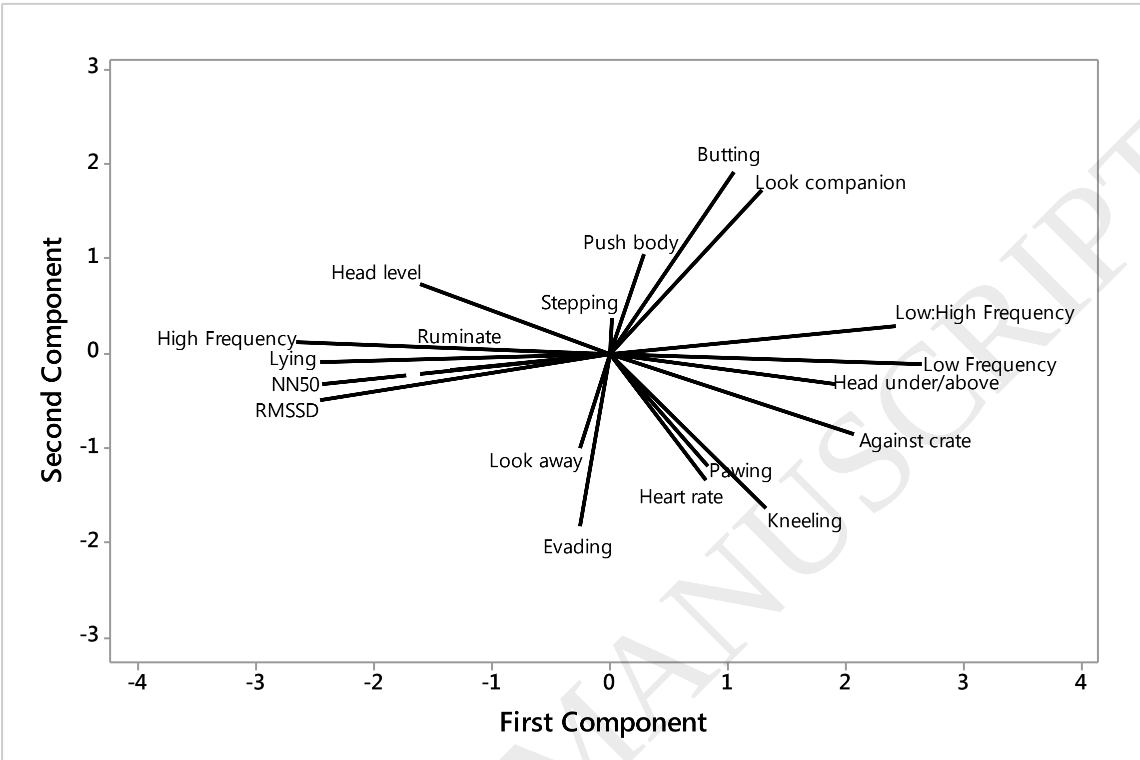


Figure 3. Biplot of components 1 and 2 in a principle components analysis of heart rate and behaviour variables in Experiment 2. F, Freq = frequency; Comp. = companion; RMSSD = Root Mean Square of Successive Differences; NN50 = Number of pairs of intervals in normal to normal beats differing by > 50 ms.

Table 1. The two factor factorial design for Experiment 1, with of sequence (regular or irregular) and motion type (roll, pitch or a combination of the two) as the two factors

Day	1 – 2	3 - 4	5 - 6	Day	1 - 4	3 - 6	2 - 5
1	Reg. Roll	Irreg. Pitch	Reg. Comb.	2	Reg. Pitch	Irreg. Comb.	Irreg. Roll
3	Irreg. Pitch	Reg. Pitch	Reg. Roll	4	Irreg. Roll	Reg. Comb.	Irreg. Comb.
5	Reg. Pitch	Irreg. Roll	Irreg. Pitch	6	Irreg. Comb.	Reg. Roll	Reg. Comb.
7	Irreg. Roll	Irreg. Comb.	Reg. Pitch	8	Reg. Comb.	Irreg. Pitch	Reg. Roll
9	Irreg. Comb.	Reg. Comb.	Irreg. Roll	10	Reg. Roll	Reg. Pitch	Irreg. Pitch
11	Reg. Comb.	Reg. Roll	Irreg. Comb.	12	Irreg. Pitch	Irreg. Roll	Reg. Pitch

Reg. = Regular sequence; Irreg. = Irregular Sequence; Comb.= Roll and pitch combined

Table 2. Significant or close to significant effects on least square mean values for feed intake, eating time, stepping and heart rate variables, as a result of interactions between type of sequence (regular or irregular) and motion type (roll, pitch or a combination of the two) in Experiment 1.

	Regular sequences			Irregular sequences					
	Roll	Pitch	Roll & Pitch	Roll	Pitch	Roll & Pitch	F value (df)	SED[2]	P-value
Feed intake, g/h	632^{ab}	548^{ab}	463^{b}	599^{ab}	569^{ab}	714^{a}	3.55	107.6	0.04
Eating time, s/h	643	657	531	690	589	820	2.85	138.5	0.07
Stepping, n/h	118^{c}	145^{bc}	142^{bc}	208^{b}	190^{b}	315^{a}	8.49	28.5	0.001
	Heart rate measurements								
Heart rate, $\log_{10}$ (beats/min)[1]	1.919^{cd} (83.0)	1.937^{ab} (86.5)	1.889^{e} (77.4)	1.906^{d} (80.5)	1.928^{bc} (84.7)	1.946^{a} (88.3)	50.49	0.0188	<0.001
Interbeat interval, ms	726^{bc}	706^{cd}	777^{a}	751^{ab}	714^{cd}	692^{d}	41.57	31.9	<0.001
NN50[3], count	162^{ab}	154^{abc}	171^{a}	157^{abc}	138^{bc}	136^{c}	3.23	29.9	0.04
High Frequency $\log_{10}$ (n.u.)[1]	1.39^{a} (24.5)	1.35^{a} (22.4)	1.41^{a} (25.7)	1.33^{ab} (21.4)	1.32^{ab} (20.9)	1.26^{b} (18.2)	5.26	0.102	0.005
Low Frequency, n.u.	69.5^{cd}	72.3^{bcd}	68.0^{d}	75.5^{ab}	73.6^{abc}	77.1^{a}	4.70	5.55	0.01
Low:High frequency ($\log_{10}$)[1]	0.44^{bc} (2.75)	0.49^{bc} (3.09)	0.41^{c} (2.57)	0.54^{ab} (3.46)	0.53^{abc} (3.39)	0.62^{a} (4.17)	5.03	0.134	0.007

[a,b,c,d,e] Least square means within rows with different superscripts are significantly by Tukey's test ($P < 0.05$)

[1] Statistical analysis performed on $\log_{10}$ transformed data. Least squares mean were calculated and then back transformed. Degrees of freedom: 2

[2] Standard Error of the Difference between two means

[3] Number of pairs of intervals in normal to normal beats differing by > 50 ms

Table 3. Effects of Regular and Irregular motion sequences on on least square mean values for feeding, head position, position, residual behaviour, and heart rate variability in sheep (n = 6) exposed for 60 min in Experiment 1.

	Regular	Irregular	F value	SED	P-value
	Feeding				
Feed intake, g/h	548	628	2.73	107.6	0.10
Water intake, ml/h	203	101	12.89	64.31	0.001
Eating time, s/h	610	699	2.09	138.5	0.15
Drinking time, s/h	611	249	22.3	172.2	<0.001
	Head position				
Under/above, s/h	173	467	12.95	183.9	0.001
Up, $\log_{10}$ (s/h)[1]	0.49 (3.1)	0.27 (1.9)	4.58	0.237	0.04
Level, s/h	965	683	14.39	166.9	<0.001
Down, s/h	713	1028	11.47	208.7	0.001
Looking					
Towards companion, $\log_{10}$ (s/h)[1]	2.29 (195)	2.21 (162)	0.72	0.199	0.40
Towards side bars, s/h	143	150	0.10	52.01	0.76
	Body position				
Standing					
No support, $\log_{10}$ (s/h)[1]	3.52 (3311)	3.45 (2818)	12.01	0.0467	0.001
Against crate, s/h	162	624	25.0	207.1	<0.001
Kneeling, $\log_{10}$ (s/h)[1]	0.075 (1.2)	0.340 (2.2)	4,70	0.277	0.03
	Other behaviour				
Stepping, no./h	135	238	64.6	28.54	<0.001
Pawing, $\log_{10}$ (no./h)[1]	0.68 (4.79)	0.87 (7.41)	2.25	0.277	0.14
Butting, $\log_{10}$ (no./h)[1]	0.12 (1.32)	0.26 (1.82)	3.08	0.0522	0.08
Licking bowl, s/h	79.2	22.5	7.27	15.08	0.01
	Post-treatment behaviour				
Walking, s/30 min	38	48	2.64	14.24	0.11
Standing, s/30 min	1013	1188	2.73	236.7	0.10
Lying, s/30 min	749	564	3.01	72.1	0.09
	Heart rate measurements				
Heart rate, $\log_{10}$ (beats/min)[1]	1.91 (81.3)	1.92 (83.2)	12.51	0.0188	<0.001
Interbeat interval, ms	734	719	11.24	31.9	0.001
RMSSD[3], $\log_{10}$ (ms)[1]	1.70 (50.1)	1.68 (47.9)	4.53	0.0434	0.03
NN50[4], count	162	143	16.32	3.4	<0.001
High frequency, $\log_{10}$ (n.u.)[1]	1.38 (24.0)	1.31 (20.4)	21.01	0.102	<0.001
Low frequency, n.u.	70	75	35.76	5.55	<0.001
Low:High frequency, $\log_{10}(ms^2)$[1]	0.45 (2.8)	0.56 (3.6)	27.2	0.134	0.001

[1]Statistical analysis performed on $\log_{10}$ transformed data. Least squares mean were calculated and then back transformed. Degrees of freedom: 1

[2]Standard Error of the Difference between two means

[3]Root Mean Square of Successive Differences

[4] Number of pairs of intervals in normal to normal beats differing by > 50 ms

Table 4. Effects of Roll, Pitch and their combination on least square mean values for feeding, head position, position, residual behaviour, and heart rate variability in sheep (n = 6) exposed for 60 min in Experiment 1.

	Roll	Pitch	Roll & Pitch	F value	SED[2]	P-value
	Feeding					
Feed intake, g/h	615	558	588	0.49	107.6	0.61
Water intake, ml/h	136	146	174	0.56	64.31	0.57
Eating time, s/h	666	623	676	0.20	138.5	0.82
Drinking time, s/h	338	444	508	1.86	172.2	0.17
	Head position					
Under/above, s/h	365	401	194	1.77	183.9	0.18
Up, $\log_{10}$ (s/h)[1]	0.369 (2.34)	0.416 (2.60)	0.365 (2.32)	0.07	0.237	0.93
Middle, s/h	753	955	763	2.55	166.9	0.09
Down, s/h	951[a]	618[b]	1044[a]	5.50	208.7	0.007
Looking						
Towards companion, $\log_{10}$ (s/h)[1]	2.28 (190.6)	2.25 (177.8)	2.23 (169.8)	0.10	0.199	0.91
Towards side bars, s/h	150	179	110	1.99	52.01	0.15
	Body position					
Standing						
No support, $\log_{10}$ (s/h)[1]	3.47 (2951)	3.48 (3020)	3.50 (3162)	0.59	0.00467	0.56
Against crate, s/h	445	456	277	1.21	207.1	0.31
Kneeling, $\log_{10}$ (s/h)[1]	0.265 (1.84)	0.143 (1.39)	0.216 (1.64)	0.34	0.277	0.71
	Other behaviour					
Stepping, no./h	163[b]	167[b]	228[a]	9.12	28.54	<0.001
Pawing, $\log_{10}$ (no./h)[1]	0.78[ab] (6.0)	0.49[b] (3.1)	1.04[a] (11.0)	4.18	0.277	0.02
Licking bowl, s/h	62.9	53.2	36.5	0.53	15.08	0.59
Butting, $\log_{10}$ (no./h)[1]	0.15 (1.4)	0.34 (2.2)	0.08 (1.2)	2.88	0.0589	0.06
	Post-treatment behaviour					
Walking, s/30 min	37	46	46	1.07	14.24	0.35
Standing, s/30 min	1064	1133	1105	0.16	236.7	0.85
Lying, s/30 min	699	621	649	0.21	81.5	0.81
	Heart rate measurements					
Heart rate, $\log_{10}$ (beats/min)[1]	1.913[b] (81.8)	1.932[a] (85.5)	1.918[b] (82.8)	9.58	0.0188	<0.001
Interbeat interval, ms	739[a]	710[b]	734[a]	9.95	31.9	<0.001
RMSSD[3], $\log_{10}$ (ms)[1]	1.706[a] (50.8)	1.675[b] (47.3)	1.699[ab] (50.0)	3.39	0.0434	0.04
NN50[4], count	159	146	153	2.59	3.8	0.08
High Frequency, $\log_{10}$ (n.u.)[1]	1.361 (23.0)	1.338 (21.8)	1.334 (21.6)	1.20	0.102	0.30
Low Frequency, n.u.	72.5	72.9	72.5	0.08	5.55	0.92
Low:High Frequency ($\log_{10}$(ms^2)[1]	0.49 (3.1)	0.51 (3.2)	0.52 (3.3)	0.68	0.134	0.50

[a,b] Least square means within rows with different superscripts are significantly ($P < 0.05$) different by Tukey's test.
[1] Statistical analyses performed on $\log_{10}$ transformed data. Least squares mean were calculated and then back-transformed. Degrees of freedom: 2
[2] Standard Error of the Difference between two means
[3] Root Mean Square of Successive Differences
[4] Number of pairs of intervals in normal to normal beats differing by > 50 ms

Table 5. Significant interactions between motion type and barrier presence on least square mean values for behaviour and heart rate measurements in Experiment 2.

Barrier	✓			X					
Motion	Irregular	Regular	Control	Irregular	Regular	Control	F value	SED[2]	P-value
	Head position								
Level, s/min	13.5^{b}	24.0^{ab}	19.6^{ab}	20.4^{ab}	18.9^{ab}	25.2^{a}	3.28	39.06	0.04
Standing									
No support, s/min	39.8^{b}	54.8^{a}	41.5^{b}	48.5^{ab}	47.8^{ab}	50.5^{ab}	5.85	39.80	0.004
	Heart rate measurements								
Heart rate, log_{10}(beats/min)[1]	1.913^{a} (81.8)	1.890^{b} (77.6)	1.895^{ab} (78.5)	1.902^{ab} (79.8)	1.903^{ab} (80.0)	1.907^{ab} (80.7)	3.55	0.0164	0.03
Interbeat interval, ms	740^{b}	777^{a}	774^{ab}	762^{ab}	760^{ab}	758^{ab}	3.43	26.98	0.03
RMSSD[3], log_{10} (ms)[1]	1.68^{a} (47.9)	1.59^{b} (38.9)	1.71^{a} (51.3)	1.58^{b} (38.0)	1.66^{ab} (45.7)	1.63^{ab} (42.7)	8.92	0.0659	<0.001
NN50[4], count	142^{ab}	107^{cd}	149^{a}	102^{d}	131^{abc}	119^{bcd}	11.25	22.64	<0.001
High frequency, n.u.	25.2^{ab}	23.2^{b}	30.7^{a}	22.3^{b}	27.3^{ab}	26.0^{ab}	5.03	4.49	0.007
Low frequency, n.u.	74.3^{ab}	76.6^{a}	69.0^{b}	77.5^{a}	72.5^{ab}	73.7^{ab}	4.95	4.56	0.008
Low:High frequency log_{10} (ms^2)[1]	0.52^{ab} (3.31)	0.59^{a} (3.89)	0.41^{b} (2.57)	0.59^{a} (3.89)	0.44^{ab} (2.75)	0.48^{ab} (3.0)	5.44	0.119	0.005

[1] Statistical analyses performed on log_{10} and square root transformed data. Least squares mean were calculated and then back transformed to s/min for presentation in parentheses. Degrees of freedom: 1, 118 for behaviour variables, 204 for heart rate variables.
[2] Standard Error of the Difference between two means
[3] Root Mean Square of Successive Differences
[4] Number of pairs of intervals in normal to normal beats differing by > 50 ms

Table 6. Effects of a barrier on least square mean values for head and body position, agonistic and residual behaviour, and heart rate measurements in sheep (n = 6) exposed for 60 min in Experiment 2.

Barrier	✓	X	F value	SED[2]	P-value
Head position					
Under/above, (√s/min)[1]	3.47 (12.1)	4.6 3(21.4)	4.73	1.69	0.03
Level, s/min	19.0	21.5	1.41	39.06	0.24
Down, s/min	16.2	18.7	1.47	1.40	0.23
Looking					
towards companion, s/min	7.3	4.0	18.18	14.20	<0.001
away from companion, (√s/min)[1]	5.0 (25.0)	4.7 (22.1)	0.47	1.30	0.49
Body position					
Standing					
No support, s/min	45.3	48.8	2.85	39.80	0.09
Against crate,(√s/min)[1]	4.6 (21.2)	4.7 (22.1)	0.07	0.69	0.80
Lying, s/min	7.7	4.8	1.36	23.2	0.26
Kneeling, (√s/min)[1]	0.36 (0.13)	1.26 (1.59)	4.08	0.502	0.06
Stepping, (√no./min)[1]	2.07 (4.28)	2.90 (8.41)	27.95	0.501	<0.001
Other behaviour					
Push body,(√no./min)[1]	0.34 (0.12)	0.81 (0.66)	6.30	0.295	0.02
Butting, no./min	0.04	0.21	6.68	0.613	0.02
Evading, no./min	0.065	0.383	14.17	0.811	0.001
Pawing, no./min	0.63	0.78	0.14	3.92	0.71
Ruminating, s/min	31	24	5.30	57.55	0.02
Post-treatment behaviour					
Walking, s/30 min	29	32	0.92	9.10	0.53
Standing, s/30 min	1697	1687	0.07	26.0	0.80
Lying, (√s/30 min)[1]	5.6 (31.4)	4.8 (23.0)	0.16	4.99	0.69
Feed intake, g/ 30 min	802	840	0.61	109.3	0.44
Water intake, ml/30 min	645	574	0.27	300.3	0.61
Heart rate measurements					
Heart rate, $\log_{10}$ (beats/min)[1]	1.89 (77.6)	1.90 (79.4)	1.05	0.0164	0.31
Interbeat interval, ms	763	760	0.28	26.98	0.60
RMSSD[3], $\log_{10}$ (ms)[1]	1.66 (45.7)	1.62 (41.7)	5.46	0.0659	0.02
NN50[4], count	132	117	7.41	22.64	0.007
High frequency, n.u.	26.5	25.2	1.27	4.49	0.26
Low frequency, n.u.	73	75	1.34	4.56	0.25
Low:High frequency, $\log_{10}$ (ms^2)[1]	0.508 (3.22)	0.500 (3.16)	0.66	0.119	0.78

[1] Statistical analyses performed on $\log_{10}$ and square root transformed data. Least squares mean were calculated and then back transformed to s/min for presentation in parentheses. Degrees of freedom: 1, 118 for behaviour variables, 204 for heart rate variables.
[2] Standard Error of the Difference between two means
[3] Root Mean Square of Successive Differences
[4] Number of pairs of intervals in normal to normal beats differing by > 50 ms

Table 7. Effects of Irregular, Regular and control motion treatments on least square mean values for head and body position, agonistic and residual behaviour, and heart rate measurements in sheep (n = 6) exposed for 60 min in Experiment 2.

Motion	Irregular	Regular	Control	F value	SED[2]	P-value
Head position						
Under/above, (√s/ min)[1]	5.78[a] (33.4)	4.65[a] (21.6)	1.72[b] (2.97)	15.87	1.69	<0.001
Level, s/min	17.0	21.5	22.3	2.43	39.06	0.09
Down, s/min	21.0[a]	17.8[ab]	13.5[b]	3.91	1.40	0.02
Looking						
towards companion, s/min	5.8	6.2	5.0	0.75	14.20	0.48
away from companion, (√s/min)[1]	4.8 (23.0)	5.2 (27.0)	4.6 (21.2)	0.72	1.30	0.49
Body position						
Standing						
No support, s/min	44.2[b]	51.3[a]	46.0[ab]	4.10	39.80	0.02
Against crate, (√s/min)[1]	7.39[a] (54.7)	4.18[b] (17.5)	2.41[b] (5.81)	19.39	0.69	<0.001
Lying, s/min	4.8	3.8	10.3	2.11	23.2	0.15
Kneeling , (√s/min)[1]	1.05 (1.10)	0.81 (0.66)	0.57 (0.32)	0.32	0.502	0.73
Stepping, (√no./min)[1]	3.06[a] (9.34)	2.41[b] (5.79)	2.00[b] (4.00)	12.94	0.501	<0.001
Other behaviour						
Push body, (√no./min)[1]	0.38 (0.14)	0.66 (0.44)	0.67 (0.45)	0.99	0.295	0.39
Butting, no./min	0.06	0.17	0.13	1.11	0.613	0.35
Evading, no./min	0.15	0.29	0.23	1.06	0.811	0.36
Pawing, no./ min	0.92	0.53	0.70	0.31	3.92	0.74
Ruminating, s/min	26.2	24.3	31.8	1.71	57.55	0.18
Post-treatment behaviour						
Walking, s/30 min	31.9	27.7	32.3	0.50	9.10	0.61
Standing, s/30 min	1628[b]	1687[ab]	1761[a]	3.25	82.3	0.05
Lying, (√s/30 min)[1]	8.52[a] (72.6)	6.61[ab] (43.7)	0.49[b] (0.24)	3.61	4.99	0.03
Feed intake, g/30 min	802	790	872	0.82	109.3	0.45
Water intake, ml/30 min	639	727	462	1.05	300.3	0.36
Heart rate measurements						
Heart rate, $\log_{10}$(beats/min)[1]	1.907 (80.7)	1.896 (78.7)	1.901 (79.6)	2.58	0.0164	0.08
Interbeat interval, ms	751	768	766	2.46	26.98	0.09
RMSSD[3], $\log_{10}$ (ms)[1]	1.628 (42.5)	1.625 (42.2)	1.671 (46.9)	2.37	0.0659	0.10
NN50[4], count	122	119	134	1.96	22.64	0.14
High frequency, n.u.	24[b]	25[ab]	28[a]	3.89	4.49	0.02
Low frequency, n.u.	75.9[a]	74.5[ab]	71.4[b]	3.91	4.56	0.02
Low:High frequency, $\log_{10}$ (ms^2)[1]	0.55[a] (3.55)	0.51[ab] (3.23)	0.44[b] (2.75)	3.55	0.119	0.03

[1] Statistical analyses performed on $\log_{10}$ and square root transformed data. Least squares mean were calculated and then back transformed to s/min for presentation in parentheses. Degrees of freedom: 2, 118 for behaviour variables, 204 for heart rate variables.
[2] Standard Error of the Difference between two means
[3] Root Mean Square of Successive Differences
[4] Number of pairs of intervals in normal to normal beats differing by > 50 ms