



Behavioural and physiological assessment of positive and negative emotion in sheep

Nadine Reefmann^{a,b,*}, Beat Wechsler^a, Lorenz Gyga^a

^a Centre for Proper Housing of Ruminants and Pigs, Federal Veterinary Office, Agroscope Reckenholz-Tänikon Research Station ART, Tänikon

^b Department of Behavioural Biology, University of Münster, Germany

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The assessment of emotional states in animals, particularly positive ones, remains a scientific challenge. We investigated differences in behavioural and physiological measures recorded in sheep, *Ovis orientalis aries*, during situations likely to coincide with negative, intermediate and positive emotional valence. Reactions of 15 sheep were observed during separation from group members (negative valence), standing in the feeding area (intermediate valence) and being voluntarily groomed by a familiar human (positive valence). Several ear postures, relative eye aperture, cardiorespiration and body surface humidity and temperature were recorded continuously for up to 4 min in each experimental situation. Data were analysed using linear mixed-effects models, and correlations were calculated between ethophysiological measurements. Groomed sheep showed few ear posture changes, low proportions of forward ear postures, low relative eye aperture and a low variance in body surface humidity. The values of most of these measures increased linearly towards standing in the feeding area and further towards separation from group members. Conversely, groomed sheep showed high proportions of axial ear postures, long mean interheartbeat intervals and high heart rate variability, with values declining linearly towards the negative situation. In addition, behavioural and cardiac measurements correlated moderately to strongly. In conclusion, emotional valence in sheep could be differentiated by both behavioural and physiological measures. Based on our data, it may be possible to replace some physiological measures with observations of ear postures. The findings provide valuable insights for assessing both negative and positive emotion in animals, which can help to promote positive experiences in captive housing conditions.

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The objective assessment of emotional states in animals is an ongoing scientific challenge (Curtis & Stricklin 1991; Botreau et al. 2007), owing to the subjective nature of emotions (Watanabe 2007). Emotions are internal psychological states (Cabanac 2002) deriving from the reactions of an individual to various stimuli, situations or expectations (Sloman 2001). As a working definition, emotional states can be described by their valence (ranging from negative to positive) and arousal (low to high; Russell 1980; Posner et al. 2005). In addition, they can coincide with subjective, cognitive, physiological and behavioural reactions (Dantzer & Mormède 1983; Paul et al. 2005; Désiré et al. 2006). A key question in current research is how to distinguish positive from negative emotional states (Boissy et al. 2007). Well-known indicators of emotion in human psychophysiological research (Hubert & de Jong-Meyer

1990; Collet et al. 1997) include facial expressions (Bradley & Lang 2000), amount of eyewhite (Whalen et al. 2004) and pupil size (Granholm & Steinhauer 2004), as well as cardiorespiratory measures (Rainville et al. 2006; Brotman et al. 2007) and electrodermal activity (Turpin & Grandfield 2007). These autonomic measures have also been applied in several animal species to identify stimuli that are likely to be stressful or perceived as emotionally negative; common examples are tests for fear and anxiety (Stiedl et al. 2004; Forkman et al. 2007), including separation from group members in the case of gregarious animals (Baldock & Sibly 1990; Sandem & Braastad 2005).

Despite an increasing amount of research, there is still a lack of comprehensive experimental knowledge on how ethophysiological measures can be used to describe positive emotional states and unambiguously distinguish them from negative ones (Boissy et al. 2007). In particular, research is complicated by the fact that inter- and intraindividual variability in response to positive stimuli is likely to be greater than the variability in reaction to negative stimuli. Negative emotional situations essentially demand an immediate reaction because they often directly threaten survival

* Correspondence: N. Reefmann, Centre for Proper Housing of Ruminants and Pigs, Federal Veterinary Office, Agroscope Reckenholz-Tänikon Research Station ART, Tänikon, CH-8356 Ettenhausen, Switzerland.

E-mail address: Nadine.Reefmann@gmx.ch (N. Reefmann).

and fitness (Dawkins 1998). Individual responses to positive stimuli can vary more, since fitness may be enhanced in such opportunity situations, although it is not necessarily decreased if no response is shown (Fraser & Duncan 1998). Thus, one challenge that arises in the study of positive emotion is to find a stimulus that is always perceived as positive by all or at least a majority of individuals, and that elicits a reliably measurable response.

Situations of presumed positive emotional valence include play and gustatory pleasure during and after feeding, at least in mammals (Burgdorf & Panksepp 2006). For many animal species from rats, *Rattus norvegicus* (Burgdorf & Panksepp 2001) to rhesus monkeys, *Mocaca mulatta* (Taira & Rolls 1996), touch and grooming also possess inherently rewarding properties, and are thus likely to be perceived as positive (Bertenshaw & Rowlinson 2008). In sheep, *Ovis orientalis aries*, the presumably positive emotional state when animals are fed is distinguishable from presumed negative states (e.g. separation from group members and frustration of feeding expectations) by fewer ear posture changes (Reefmann et al. 2009a), lower heart and respiration rates, and a lower variance in bodysurface humidity (Reefman et al. 2009b). In a recent study, long duration of hanging ears and low heart rate were used as indicators of positive emotion when cattle, *Bose taurus*, were stroked on preferred body areas (Schmied et al. 2008). The question remains as to whether these results can be replicated and applied to other situations likely to be associated with negative and positive emotional states.

For practical purposes, such as the on-farm application of measurement methods (Edwards 2007), it is desirable to identify an optimal number of necessary but sufficient measures for differentiating emotional states reliably and with the smallest effort possible. However, any single correlate of emotional valence appears to be ambiguous (Wilhelm et al. 2006), at least in some cases, since responses can overlap for presumably negative and positive states (Dawkins 1998). By using a combination of several behavioral and physiological measures, interpretation could be more reliable, although increasing work effort at the same time. With the identification of an essential number of measures, the increased effort of using several methods simultaneously could be minimized to facilitate on-farm application. Moreover, behavioral observations are more easily applicable in such a setting than physiological measures, since they do not require devices to be attached to the animals. The question therefore arises whether physiological variables can be replaced by behavioral observations.

A further important methodological point to consider is the duration of the measurements made to assess animals' emotional states. In experimental situations, short-term reactions are commonly assessed in measurement periods of approximately 5 min, as recommended for example for heart rate (Kim et al. 2004; Sztajzel 2004). Short-lived emotional states may only last a few seconds, however, and 5 min seems a rather long time period for detecting such ephemeral emotional reactions. At least for the interheartbeat interval, a 10 s assessment period has been used successfully to judge subjective states in animals (Désiré et al. 2004; Greiveldinger et al. 2007).

Our aim was to test hypotheses on how behavioural and physiological measures in sheep, relating to both the sympathetic and parasympathetic nervous systems, react to positive emotional states compared with negative ones. Nineteen sheep were subjected to three situations likely to induce states of negative, intermediate and positive emotional valence: separation from group members (Baldock & Sibly 1990; Sandem & Braastad 2005), standing in the feeding area, and being voluntarily groomed by a familiar human (Bertenshaw & Rowlinson 2008), respectively. Given the evidence from previous experiments, we expected that positive emotional states would coincide with infrequent ear

posture changes and a high proportion of axial ear postures compared to a negative emotional situation. In addition, we predicted low relative eye aperture, cardiorespiratory deceleration and reduced electrodermal activity during the situation presumed to be positive. To investigate the possibility of replacing physiological measurements with behavioural ones, we tested measures for correlation. To address whether measures can be assessed in short measurement durations, we compared results based on different time periods used for analysis (10 s versus several minutes).

METHODS

Animals, Housing and Husbandry

A total of 19 nonreproducing and nonlactating female sheep of two different breeds (nine Swiss White Alpine and 10 Lacaune ewes) were used for the experiment. These had been acquired in two lots in autumn 2005 and early summer 2006 at an approximate age of 4 months from a Swiss shepherd who had raised them all as lambs. At the start of the experiment, all animals (62 ± 15 kg) had been housed together as a group for 8 months at the Agroscope Reckenholz-Tänikon Research Station ART, Tänikon, Switzerland. The open-front pen (58 m^2) consisted of an area with deep-litter straw bedding (42 m^2) and a feeding area with a solid concrete floor and a hayrack, 7 m long, maintained under natural temperature and lighting conditions. Adjacent to the pen was a commercially available squeeze (patura, STALLAG, Stanssad, Switzerland). During dry weather, animals were given access to an additional outdoor exercise yard (18 m^2). Water and hay were available ad libitum and the hayrack was refilled twice daily before 0730 and at around 1600 hours. From spring to autumn, sheep were on pasture at times when no experiments were being conducted. The study was approved by the Federal Veterinary Office (Switzerland) and licensed by the county office (Frauenfeld, Thurgau, Switzerland, F2/06).

Experiment

The experiment was conducted from February to March 2007 in the animals' home pen and the adjacent squeeze. The experimental situations of (1) separation from group members in the squeeze, (2) standing in the feeding area of the home pen while ruminating or feeding, and (3) being voluntarily groomed in the home pen by a familiar human were assumed to coincide with emotional states of negative, intermediate and positive emotional valence, respectively. To describe correlates of emotional states, several behavioural measures (ear postures, eye aperture) and physiological variables (cardiorespiration, body surface humidity and temperature) were recorded over a 5 min period in each situation.

Experimental procedure

Three weeks prior to the experiment, sheep were habituated to wearing the physiological measurement equipment (for details see below). The equipment was attached to each sheep at least five times for a longer period each successive time, starting with 5 min and working up to 2 h. Once a day, the sheep were also familiarized with the general procedure of walking from the feeding area into the squeeze, into the exercise yard and back into the litter area, with doors being opened and closed after each animal. In addition, sheep were groomed by the experimenter (N.R.) in the feeding area once a day if they showed themselves willing. Animals that approached the human experimenter of their own volition on the first 2 days of the habituation period and stayed for at least 1 min of grooming were used for the experiment (15 of 19). The sheep were not tied in any way and remained free throughout the grooming to move away.

Some ewes directed various attention-seeking behaviours at the experimenter (e.g. nibbling at her clothes, establishing body contact with her, and placing their heads under her hand), presumably in an attempt to solicit grooming. Some even tried to push away other sheep that were being groomed by the experimenter.

Each animal was observed once in each experimental situation, with the exception of four animals (see above). One to four days before its experimental day, the sheep in question was shorn and depilated on the relevant body areas (see Physiological measures below). The experimental animals were selected according to a schedule balanced for breed (Swiss White Alpine or Lacaune) and time of day (0930 or 1330 hours). Half the sheep of each breed underwent one of the two possible test sequences of 'standing in the feeding area' and 'being voluntarily groomed' first. All were tested last in the 'separation from group members' situation. This was done to avoid a carryover effect of negative valence and arousal to the intermediate and the positive situations, as the responses to the negative situation were expected to be stronger than those to the positive and intermediate situations. For the experiment, the selected sheep and three companion animals were herded into the feeding area of the pen, while all other sheep remained in the litter area. The experimental animal was then equipped with all of the physiological devices, with the experiment proper beginning after a rest period of 10 min following attachment of the devices.

For the intermediate-valence emotional state, during which sheep were not expected to show a strong negative or positive emotional reaction, animals were observed while standing in the feeding area of the home pen and feeding or ruminating. For the positive emotional situation of being voluntarily groomed by a human, the experimenter (N.R.) entered the feeding area and sat down at the feed rack. If the sheep approached the experimenter's outstretched hand of its own volition within 30 s, she groomed the sheep in the standardized way described below. If the animal did not approach the experimenter, she slowly approached the sheep in a crouched position and attempted to establish physical contact. If the sheep moved away, the intended positive situation of voluntary grooming did not occur for that particular animal, which was the case for six of the 15 experimental animals. If the sheep allowed body contact and grooming, however, the experimenter gently stroked the animal approximately once every second on the breast muscle. If the sheep moved slightly, it was groomed on the body area that was then beneath the experimenter's hand. After measurements were taken during the situations of standing and grooming, the doors to the squeeze were opened. If the experimental sheep did not voluntarily walk into the squeeze within 1 min, the experimenter gently moved the animal into it. As soon as the sheep was on its own in the squeeze, measurement recordings were started. Following this separation from group members, the trap door to the exercise yard was lifted. Here, the physiological equipment was detached from the sheep, and the animal was allowed to return to the litter area to rejoin the rest of the herd.

Behavioural measures

The focal animals' ear postures were observed during each emotional situation via continuous recording (Martin & Bateson 2007). For the separation from group members, a video camera (CCD b&w camera, C-Pro Electronics Co Ltd, Surgnam, South Korea) was installed at the front above the squeeze to monitor the ear postures of the experimental sheep while no other sheep or human were in sight. Relayed images were recorded with a videocassette recorder (Panasonic Time Lapse Recorder AG-6040 E) and later analysed from videotape once and in real time using the Etho data collection software (R. Weber, Agroscope Reckenholz-Tänikon Research Station ART, Tänikon, Switzerland). Ear postures while standing in the feeding area were observed directly from outside the pen by

means of the Etho data collection software as with the video observations. Data on the ear postures during the voluntary grooming period were directly recorded by the experimenter as with the observation while standing in the feeding area but using a dictaphone. We primarily chose these slightly differing means of recording because we wanted to obtain the most accurate data possible in each situation, and avoid loss of data owing to the experimental sheep's ears being hidden behind the companion sheep or experimenter while sheep were standing in the feeding area or being groomed. In terms of the data produced, we expected no methodological differences in our approaches since we were interested in recording all ear movements and resulting postures, and did not use the exact time of each posture. The following ear postures were recorded separately for the right and left ears according to the definitions of Reefmann et al. (2009a): axial ear (perpendicular to the head-rump axis), forward ear (tip of the ear towards the front at an angle of more than 30° from the perpendicular), backward ear (tip of the ear towards the back at more than 30° from the perpendicular). For analysis, the total number of ear posture changes of both ears from forward, axial and backward to another of these ear postures was calculated. Axial and forward ear postures were calculated from the recorded data as the proportion of all observed ear postures within the analysed measurement period.

The relative eye aperture was determined from pictures of the sheep's eyes. These were taken with two finger cameras (DV-2000B Weatherproof, CCD b&w finger camera, Conrad Electronics, Bach, Switzerland, diameter 20 mm, 69 mm long, 32 g), which were fixed with a halter to the sheep's head. The necessary battery pack was fastened on a leather belt around the sheep's chest behind the forelegs. Image data were transferred via radio frequency from the camera to a stationary receiver (15-1200VR, CL-Electronics GmbH, Milpitas, CA, U.S.A.). Alternating between the two eyes at 1 s intervals, image sequences were recorded on a video recorder (Panasonic Time Lapse Recorder AG-6040 E) and later digitized (V-Mate, SanDisk, Littau, Switzerland). Vigorous head movements of sheep changed the preset focus of the cameras in several instances, resulting in out-of-focus images for the rest of the observation period, in particular for one of the two eyes. Images were available for three, two and one of the situations for one, four and four sheep, respectively. For analysis of video sequences with recognizable eye boundaries, one snapshot was made every 1 s from the digitized images. The eye aperture was determined by measuring the length of the widest line from the bottom to the top eyelid on the pictures. The area of the completely visible eye was digitally measured by superimposing a polygon on each snapshot that was built up by adding the corners of the polygon one after the other using on-screen mouse clicks (R-package rpanel; Bowman et al. 2006). The relative eye aperture was then calculated by dividing the length of the line from the top to the bottom lid by the square root of the area of the completely visible eye.

Physiological measures

The electrocardiogram of the sheep was continuously measured with a Holter recorder commonly used with humans (Modular Digital Holter Recorder, Lifecard CF, DelMar Reynolds GmbH, Alpnach Dorf, Switzerland; 130 g). The device was fixed on a leather belt worn around the chest of the sheep behind its forelegs. Three electrodes (Red Dot 2560, 3 M, Rüschlikon, Switzerland) were attached to shorn skin on the caudal part of the left scapula, right beside the sternum and on the left loin. Mean inter-heartbeat interval (mean R-R interval), which is an inverse measure of heart rate, and RMSSD (root mean square of successive interheartbeat interval difference) as a measure of heart rate variability were calculated using the Pathfinder software (DelMar Reynolds GmbH).

Body surface humidity as a measure of perspiration and body surface temperature of the sheep were recorded by sensors

detecting the relative humidity (%) and temperature (°C) on a commercial device (MSR145 W, Modular Signal Recorder, Electronics GmbH, Henggart, Switzerland; 16 g). This logger was attached to the sheep's depilated skin at the last rib on the left body side 8 cm from the spine by means of a breathable bandage (Fixomull stretch, BSNmedical GmbH, Solothurn, Switzerland). Previous validation had shown that a microclimate developed beneath this adhesive tape within 10 min, so that the measured local relative humidity and temperature reflected the values of the sheep's skin. The logger recorded the humidity and temperature once every 1 s. For each sheep, the median and the variability of all measurements within the analysed time period of each of the situations were calculated.

Respiration rate was measured with an extensible belt (1132 Pneumotrace II, UFI, Morro Bay, CA, U.S.A.) fixed with a Velcro strap around the abdomen and in front of the hindlegs of the sheep. The belt generated a continuous signal for its relative extension during inhalation–exhalation cycles. The signal was saved at a rate of 10 Hz by the logger used for body surface humidity and temperature recordings. The respiration rate was determined from the signal on the basis of the strongest frequency in a smoothed spectrogram of the time series of the signal (in S-PLUS, Version 7.0 for Windows, Tibco Software Inc., Palo Alto, CA, U.S.A.).

Analysis

We analysed recorded measures for the first 4 min of each experimental situation, and for the central 10 s of these 4 min. Each R-peak of the electrocardiogram recordings was visually checked for correct detection by the Pathfinder software. In some cases, the quality of the recordings during the central 10 s was poor, and good-quality recordings following on most closely in time were chosen for analysis. If only a few R-peaks were not identified correctly, then the heartbeats in question were omitted, and the omitted time period added on directly after the time period to be analysed. Given that data recorded during the longer measurement periods were more likely to be of low quality, a few values for mean R–R interval and RMSSD had to be excluded from the analysis, and the remaining sample size for each variable and situation is indicated in the figures. We analysed ear postures for the same 10 s as the physiological data, and for a period of 1 min, starting at the same time as for the 10 s analyses. For these data, periods of 1 min instead of 4 min were chosen as ear posture changes were very frequent in the situation of 'separation from group members' and, as a consequence, analysis of videos was time consuming. Since the evaluation of the relative eye aperture was also a labour-intensive task, it was only performed for the 10 s period. Owing to software failure, six animals were tested twice; the analysis is based on the second data set.

Statistical analysis was performed in R (version 2.6.1; *R Development Core Team 2007*), with one data point per individual and valence situation. Each response variable (total number of ear-posture changes, proportion of forward ear postures, proportion of axial ear postures, relative eye aperture, mean R–R interval, RMSSD, respiration rate, body surface humidity, body surface temperature, and the variance in humidity and temperature) was modelled separately using a linear mixed-effects model (*Pinheiro & Bates 2000*). Explanatory variables were valence (coded as a continuous and equally distanced variable with 1 = separation from group members, 2 = standing in the feeding area and 3 = being voluntarily groomed by a human) and valence squared (valence^2). Together, these two variables correspond to the coding of valence as an ordered factor, with the advantage of being able to remove the term valence^2 from the model if it did not reach significance ($\alpha > 0.05$). Removing this quadratic term indicates that changes in

data values across the three investigated situations were not statistically different from linearity. A random effect for individual sheep was included to reflect the repeated measurements of the same individuals in the different experimental situations. The assumptions of normal distribution and homoscedasticity of the errors of the models were checked by graphical analysis of the residuals. To satisfy the assumptions, we used log transformations for RMSSD, respiration rate, variance in body surface humidity, variance in body surface temperature (4 min), and a logit transformation for the relative eye aperture and forward ears. Owing to large differences in the variance of data between the valence situations, a term accounting for this heteroscedasticity was included for the proportion of axial ear postures (1 min) and RMSSD (10 s). The residuals of the total number of ear posture changes analysed for 10 s did not follow the model assumptions despite the use of transformation. A generalized linear mixed model of the Poisson family using a penalized quasi-likelihood (PQL) technique was therefore applied. The proportions of forward and axial ear postures analysed for 10 s were not evaluated statistically, since the residuals followed neither the assumptions of the model based on the normal distribution nor those of a model using a dichotomized response variable. This was possibly because of the short time period of the analysis, during which these ear postures rarely occurred.

To test for the possibility of replacing physiological with behavioural measures, we calculated Spearman rank correlations between a selection of behavioural variables (total number of ear posture changes/1 min, proportion of axial ear postures/1 min, relative eye aperture/10 s) and physiological measurements that differed in terms of valence (mean R–R interval, RMSSD and variance of body surface humidity analysed for 10 s). To test whether variables can be assessed within 10 s, we correlated the different lengths of time analysed (1 min and 10 s for total number of ear posture changes; 4 min and 10 s for mean R–R interval, RMSSD, respiration rate and variance of body surface humidity). All correlations were calculated across the three situations, resulting in a maximum sample size of $N = 45$ (one data point for each of the three situations with 15 individuals; as indicated in *Tables 1, 2*) The observed values of the variables were not used directly for any of these correlations, since repeated measurements of the individuals led to dependency in the data set. Instead, we calculated (generalized) linear mixed-effects models as described above using the same transformation but with only an intercept as the fixed effect. The residuals of these models represent the measurement values corrected for individual differences, and these were correlated.

All mean values given in the text are based on model estimates.

RESULTS

Behavioural Reactions to Emotional Valence

The total number of ear posture changes (changes/min; *Fig. 1a*) observed in sheep differed between the three valence situations (valence²: $F_{1,20} = 94.66$, $P < 0.001$). It was much higher during separation from group members (estimated mean: 24.4) than while standing in the feeding area (1.3). In addition, posture changes were slightly more frequent when sheep were groomed than while standing in the feeding area (2.7). This pattern was also similar for the 10 s analysis (separation: 4.6; stand: 0.5; groom: 0.5; valence²: $t_{18} = 2.16$, $P = 0.045$). The proportion of forward ear postures (*Fig. 1b*) tended to decline linearly from separation from group members (0.33) towards standing in the feeding area (0.27) and further towards being groomed (0.21; $F_{1,16} = 4.06$, $P = 0.06$). Conversely, the proportion of axial ear postures increased (separation: 0.05; stand: 0.31; groom: 0.58; $F_{1,16} = 17.71$, $P < 0.001$; *Fig. 1c*). The relative eye aperture of the sheep was highest during

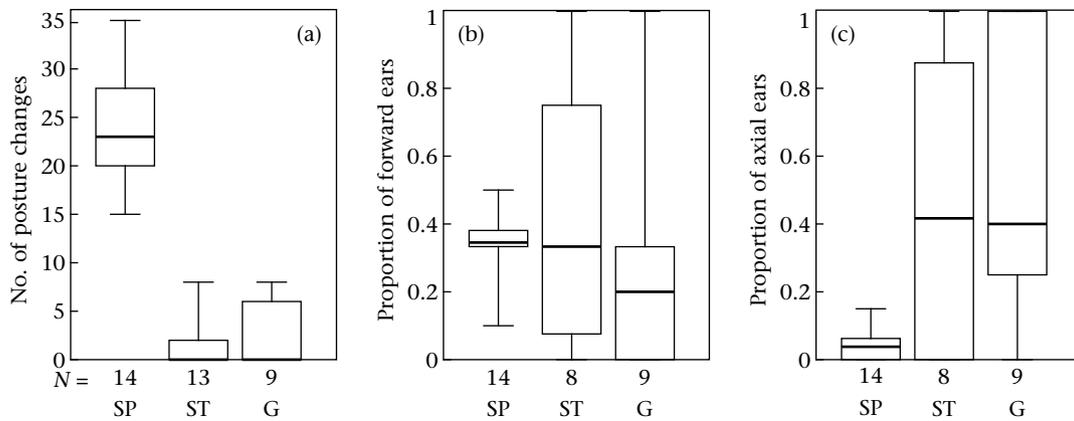


Figure 1. Ear postures observed in sheep during separation from group members (SP, negative valence), standing in the feeding area (ST, intermediate valence) and being voluntarily groomed by a human (G, positive valence). (a) The total number of ear posture changes, (b) and (c) the proportion of all observed forward and axial ear postures, respectively, within 1 min. Box plots show the observed median, first and third quartiles and absolute range of data.

separation from group members (Fig. 2). The animals' eyes were less wide open while they were standing in the feeding area, and even less while they were being groomed by a human (separation: 0.81; stand: 0.78; groom: 0.74; $F_{1,5} = 13.17$, $P = 0.02$).

Physiological Reactions to Emotional Valence

The reactions of the sheep to the three situations differed in terms of cardiac measures, variance in body surface humidity and the 4 min analysis of the variance in body surface temperature, while respiration rate and the other electrodermal measures were not significantly influenced by presumed emotional valence. The mean R–R interval was shortest during separation from group members and increased linearly towards standing in the feeding area and towards being groomed, for both the 4 min (separation: 512 ms; stand: 551 ms; groom: 590 ms; $F_{1,19} = 27.79$, $P < 0.001$ Fig. 3a) and 10 s analyses (separation: 490 ms; stand: 548 ms; groom: 606 ms; $F_{1,20} = 35.79$, $P < 0.001$; Fig. 3e). RMSSD also increased from separation from group members towards standing

in the feeding area, and further towards being groomed. The analysis over 4 min was nonsignificant (separation: 41 ms; stand: 46 ms; groom: 51 ms; $F_{1,19} = 2.67$, $P = 0.12$; Fig. 3b), while the 10 s analysis of this pattern was significantly linear (separation: 27 ms; stand: 40 ms; groom: 58 ms; $F_{1,20} = 30.02$, $P < 0.001$; Fig. 3f).

Respiration rate (breaths/s) appeared to decrease from separation from group members towards standing in the feeding area, towards being groomed. This decrease, however, was not significant for either the 4 min (separation 0.77; stand: 0.75; groom: 0.73; $F_{1,13} = 0.46$, $P = 0.51$; Fig. 3c) or the 10 s analysis (separation: 0.98; stand: 0.87; groom: 0.78; $F_{1,20} = 1.71$, $P = 0.21$; Fig. 3g).

Neither the median of body surface humidity (4 min: separation: 42.5%; stand: 43.1%; groom: 43.8%; valence: $F_{1,17} = 2.53$, $P = 0.13$; 10 s: separation: 42.9%; stand: 43.2%; groom: 43.5%; valence: $F_{1,20} = 0.166$, $P = 0.69$) nor the median of body surface temperature (4 min: separation: 24.84 °C; stand: 24.89 °C; groom: 24.94 °C; valence: $F_{1,19} = 0.06$, $P = 0.81$; 10 s: separation: 24.87 °C; stand: 24.88 °C; groom: 24.89 °C; valence: $F_{1,21} = 0.002$, $P = 0.97$) were influenced by situation. Values for variance in body surface humidity of the sheep were highest during separation from group members, and declined linearly towards standing in the feeding area and from there towards being groomed, both during the 4 min (separation: 0.015; stand: 0.009; groom: 0.005; $F_{1,19} = 8.82$, $P = 0.008$; Fig. 3d) and 10 s analyses (separation: 0.006; stand: 0.003; groom: 0.001; $F_{1,21} = 18.26$, $P < 0.001$; Fig. 3h). The 4 min analysis of variance in body surface temperature was high both for separation from group members and for being groomed, and lower for standing in the feeding area (separation: 3.5×10^{-4} ; stand: 2.8×10^{-4} ; groom: 3.4×10^{-4} ; valence²: $F_{1,18} = 8.22$, $P = 0.01$). The pattern for the 10 s analysis was more linear, but did not differ significantly in terms of situation (separation: 3.0×10^{-4} ; stand: 2.9×10^{-4} ; groom: 2.9×10^{-4} ; $F_{1,19} = 0.10$, $P = 0.75$).

Correlations of Behavioural and Physiological Measures

Since behavioural variables differed across valence situations in a similar manner to physiological variables, we tested the data for correlation (Table 1). The total number of ear posture changes analysed for 1 min correlated negatively with the mean R–R interval and RMSSD, and positively with the variance in body surface humidity. The opposite pattern was found for the proportion of axial ear postures. Correlations for relative eye aperture followed the same direction as those for ear posture changes, with moderate to strong correlations observed with the mean R–R interval and RMSSD. Correlation with the variance in body surface humidity did not reach statistical significance.

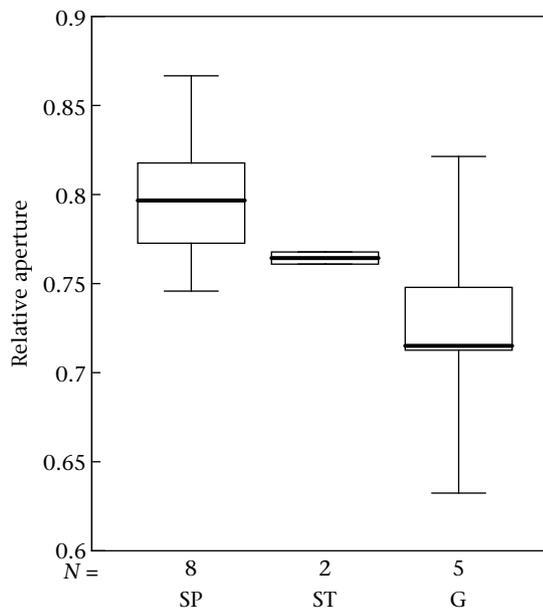


Figure 2. Relative eye aperture observed in sheep for 10 s during separation from group members (SP, negative valence), standing in the feeding area (ST, intermediate valence) and being voluntarily groomed by a human (G, positive valence). Box plots show the observed median, first and third quartiles and absolute range of data.

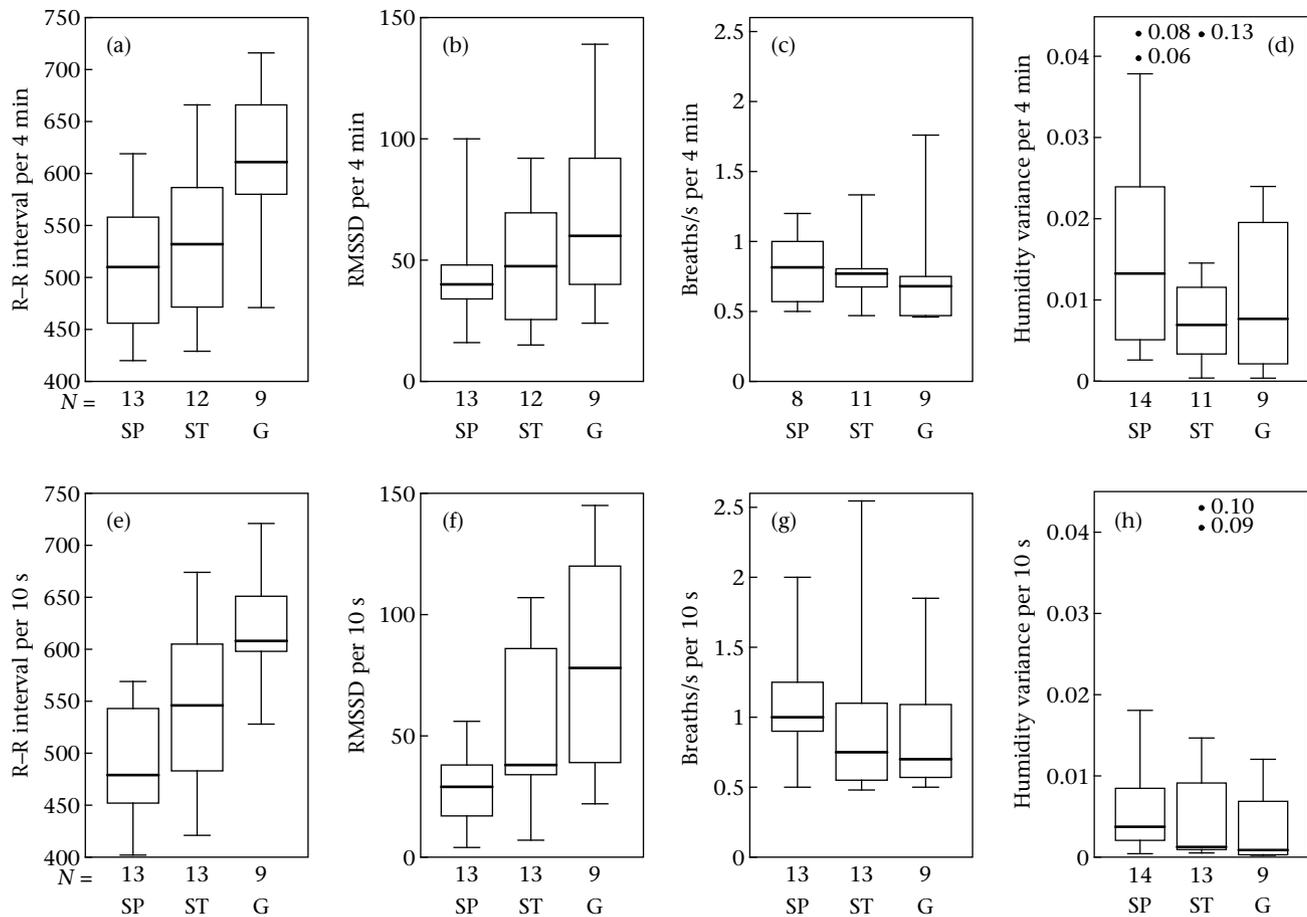


Figure 3. Physiological measures recorded in sheep and analysed for both 4 min and 10 s during separation from group members (SP, negative valence), standing in the feeding area (ST, intermediate valence) and being voluntarily groomed by a human (G, positive valence); (a) and (e) mean length of the interheartbeat R–R interval (ms); (b) and (f) root mean square of successive beat-to-beat interval difference (RMSSD, ms); (c) and (g) respiration rate (breaths/s); (d) and (h) variance in body surface humidity. Box plots show the observed median, first and third quartiles and absolute range of data. A few outlines are also indicated in (d) and (h).

Length of Measurement Period

Strong positive correlations between 10 s of analysis and longer time periods were found for the total number of ear posture changes and mean R–R interval (Table 2). The different lengths of measurement period for RMSSD and variance in body surface humidity reached lower but none the less significant positive correlations. Respiration rate did not correlate significantly in a comparison of the 10 s and 4 min analyses.

DISCUSSION

Differentiation of Emotional Valence

For each of the analysed behavioural measures, the animals' responses differed in respect to emotional valence. During the presumably positive situation of being groomed, sheep showed

relatively few ear posture changes, as well as small relative eye aperture, a low proportion of forward and a high proportion of axial ear postures, in contrast to the presumably negative situation of being separated from group members. Comparable results in terms of ear postures were obtained in a previous study on the same sheep population during presumably positive feeding situations (Reefmann et al. 2009a). Axial ear postures in the present study may actually have coincided with passively hanging ears observed in that study, but the latter were not specifically noted in the current experiment. With cattle, long durations of hanging ears were observed when the animals were stroked on body areas of social licking (Schmied et al. 2008), the latter being likely to coincide with positive emotion.

In respect of physiological measures, the mean R–R interval and RMSSD were highest while the variance in body surface humidity was lowest during the presumably positive emotional situation as opposed to the negative situation. These data are in line with

Table 1
Spearman rank correlations between behavioural measures and physiological variables (analysed for 10 s) across all three situations for assessing emotional reactions in sheep

	Total no. of ear posture changes/1 min			Proportion of axial ear postures/1 min			Relative eye aperture/10 s		
	r_s	<i>N</i>	<i>P</i>	r_s	<i>N</i>	<i>P</i>	r_s	<i>N</i>	<i>P</i>
Mean R–R interval (ms)	–0.44	32	0.013	+0.43	27	0.025	–0.63	11	0.044
RMSSD (ms)	–0.60	32	<0.001	+0.55	27	0.003	–0.76	11	0.010
Variance in body surface humidity	+0.46	35	0.005	–0.40	31	0.024	+0.48	12	0.133

RMSSD: root mean square of successive interheartbeat interval difference.

Table 2

Spearman rank correlations between behavioural and physiological variables for different durations of analysed measurement periods (comparing a period of 10 s with the indicated duration)

	Time period (min)	r_s	N	P
Total no. of ear posture changes	1	+0.78	35	<0.001
Mean R–R interval	4	+0.74	34	<0.001
RMSSD	4	+0.44	34	0.009
Respiration rate	4	+0.20	28	0.308
Variance in body surface humidity	4	+0.39	34	0.023

RMSSD: root mean square of successive interheartbeat interval difference.

studies in various animal species. In rhesus macaques, *Macaca mulatta* (Aureli et al. 1999), horses, *Equus caballus* (Feh & Mazières 1993) and cattle (Schmied et al. 2005), for example, grooming coincides with decreased heart rate (Hargreaves & Hutson 1990), which is equivalent to the lengthened mean R–R interval observed in the present experiment. From amphibians (Cabanac & Cabanac 2000) to mammals (Cockram et al. 2000; da Costa et al. 2004), negative emotional states are characterized by increased heart rate compared to intermediate or positive emotional states. In addition, our results support the evidence that high RMSSD, an indicator of parasympathetic nervous system activity (Task Force 1996; Sztajzel 2004), may coincide with positive emotional states, while parasympathetic deactivation may occur during negative emotion. This is postulated to be the case in humans (McCraty et al. 1995; Neumann & Waldstein 2001; Wong et al. 2007), and has also been hypothesized for farm animals by Boissy et al. (2007). The direct influence of the sympathetic nervous system on the sweat glands of the skin additionally suggests that the variance in body surface humidity is likely to reflect solely the activation waves of the sympathetic nervous system (Kim et al. 2004; Nagai et al. 2004; Harrison et al. 2006). The observed decrease in variance in body surface humidity would hence suggest sympathetic deactivation during positive emotional states in addition to parasympathetic activation.

Values of the majority of the assessed variables increased (proportion of axial ear postures, mean R–R interval, RMSSD) or decreased (proportion of forward ear postures, relative eye aperture, variance in body surface humidity) linearly from the negative towards the presumably intermediate and the positive situations. Such a pattern might suggest the gradual activation and deactivation of the parasympathetic and sympathetic branches of the autonomic nervous system, respectively, for positive emotional states (and vice versa for negative emotional states). Studies other than this one investigating such a regulation of the autonomic nervous system during positive emotional states appear to be lacking in the literature. Patterns regarding the activity of the sympathetic and parasympathetic branches in terms of negative valence can be derived from stress research (Johnson et al. 1992; Porges 1995; Brotman et al. 2007), which confirms our current findings.

When studying emotion, it is important to consider the possible interaction of emotional valence with arousal. Negative emotional states often coincide with high arousal, for example during the separation from group members. From the adaptive perspective, high arousal coincides with increased attention and mobilization of energy to cope with an adverse (i.e. negative) situation, mainly to facilitate fight-or-flight responses (Dawkins 1998). Positive emotional states, by contrast, are often characterized by lower arousal, as may have been the case for the sheep being groomed by a familiar human. The third experimental situation, that is standing in the feeding area, is likely to be characterized by intermediate emotional valence and low arousal. Hence, our data may not reflect the influence of emotional valence irrespective of arousal. Given that animals were always tested last in the situation of separation

from group members, increasing arousal over the experiment may partly explain our results. However, since sheep were exposed to familiar, non-negative stimuli preceding separation, this seems unlikely, and would add little extra arousal to that experienced because of separation. Findings from two previous experiments imply that physiological and behavioural data reflect a dimension of emotional valence in addition to arousal. In the first of these experiments (Reefmann et al. 2009a), the sheep's ear postures during rumination (presumed intermediate valence) and feeding on fresh hay (presumed positive valence) were observed. Although feeding is likely to produce greater arousal than rumination (which involves e.g. less overt locomotor activity), the total number of ear posture changes was lower during feeding than during rumination. This contrasts with the increase in ear posture changes with increasing arousal in the current study (during separation compared to the other two situations), suggesting that there is not a simple linear relationship between ear posture changes and arousal. In the second experiment, the heart rate of sheep offered an unexpected type of feed increased in both the presumed negative (unpalatable feed) and positive (enriched feed) treatments, indicating possible arousal (Reefmann et al. 2009b). Despite this, the valence of the negative and positive treatments was clearly differentiable owing to both the behaviour (i.e. ear postures; Reefmann et al. 2009a) and physiological reactions of the sheep, such as respiration rate and variance in body surface humidity. Based on these findings, it is likely that the variables measured in the present experiment mainly reflect emotional valence.

Selection of Measures

Patterns observed for the different parameters describing the ear postures resembled those identified for physiological measurements. Given the correlations of the total number of ear posture changes with physiological measures, observations of these changes may be used in future to replace physiological measures such as the mean R–R interval or RMSSD with the aim of differentiating situations of varying valence. The pattern for the proportion of axial ear postures was similar to that found for RMSSD, and inverse to the pattern of variance in body surface humidity. For on-farm studies, observations of ear postures would be more feasible than measuring physiological correlates of emotion, since no devices have to be attached to the animals. Given that correlations in this experiment were only moderate, however, more extensive validation is necessary.

Correlations of the relative eye aperture with cardiac measures were even stronger than those of the ear postures. From the pattern observed for RMSSD, relative eye aperture might be regulated by parasympathetic deactivation. To date, however, it seems that eyelid muscle control has only been investigated in terms of sympathetic regulation (Patel et al. 2008), while our results on the variance in body surface humidity would also suggest sympathetic activation for increased eye aperture. Apparently, this measure has not yet been used as a correlate of emotional states in either humans or animals. Although sophisticated eye trackers are available for measuring pupil size during emotional activation in humans (Bradley et al. 2008), no such device is available for animals. In cattle, a high percentage of visible eye white seems to indicate negative emotional states such as frustration or fear, and a low percentage of visible eye white appears to indicate positive emotional states such as presumed contentedness and satisfaction (Sandem et al. 2002, 2006). Since this measure has not yet been found to be applicable in sheep (Reefmann et al. 2009b), relative eye aperture might be a more useful indicator of emotional states in these animals. Given that observations performed according to the methods used in the present experiment are fairly labour intensive,

however, it may be more convenient for on-farm applications to assess eye aperture according to the categories of, for examples, 'wide open' and 'half closed'.

The absolute body surface humidity and temperature were not helpful in differentiating emotional valence in sheep in this experiment and this result is in line with one of our previous studies (Reefmann et al. 2009b). Therefore, excluding these two variables from the wide range of measurement methods would appear to be a sensible step. The 4 min analysis of variance in body surface temperature was similarly high for the presumed negative and positive situations. As with, for example, pupil size (Bradley et al. 2008), this may have reflected autonomic activation during emotional stimulation irrespective of valence. For the 10 s analysis, the pattern was more linear but nonsignificant. Given these results, variance in body surface temperature does not appear to be useful for distinguishing negative and positive emotional states in sheep either.

Although respiration rate appeared to decrease from the negative to the positive emotional situation, in contrast to other studies (Brouk et al. 2003; Reefmann et al. 2009b), we found no significant influence of situation. Both heart and respiration rates are controlled by the sympathetic and parasympathetic nervous systems, but changes in these autonomic branches may occur without observable changes in respiration (McCraty et al. 1995). Despite this, the combination of electrodermal or cardiac measures with respiration may be valuable for assessing emotional valence in some situations (Collet et al. 1997; Rainville et al. 2006).

Given the evidence published by von Borell et al. (2007) and our own findings obtained to date, heart rate, RMSSD and variability in body surface humidity, or their potential behavioural substitutes assessed in this study (i.e. ear postures and eye aperture), appear to be the most useful measures for distinguishing negative and positive emotional valence in farm animals under experimental conditions. These may all be necessary, since RMSSD and variance in body surface humidity reflect the activity of the parasympathetic and sympathetic nervous systems, respectively, while heart rate gives a combined impression of both branches. For future applications, it remains to be shown whether these measures are sufficient for the unambiguous differentiation of emotional valence in more varied experimental settings and other animal species.

Length of Measurement Period

Most measures followed a similar pattern, irrespective of whether data were collected over 10 s or over longer analysis periods (1 and 4 min). Although absolute values of the variables differed according to the length of the time period analysed, moderate to strong correlations were found. Shortening the measurement period to 10 s would therefore seem to be a sensible approach for measuring mean R–R interval and RMSSD. Given that values obtained from the 10 s analysis for the variance in body surface humidity did not decline significantly from the presumed negative towards the positive situation, longer measurement periods should be considered for this measure. Although the two different lengths of analysis period correlated strongly for the total number of ear posture changes, we cannot recommend shortening the observation period. Over 10 s, only a few different ear postures were observed in our study and, consequently, the observation of the sheep's ears over such a short time period is not sufficient to judge the valence of a situation from the sheep's perspective. In order that differences between situations may be identified, the analysed observation periods may have to be at least 1 min long. The correlation of RMSSD between the 4 min and 10 s analysis periods was only moderate, although the patterns followed the same direction. Considering that heart rate and heart rate

variability are regulated comparatively quickly and may already vary within 10 s (von Borell et al. 2007; Vila et al. 2007), shorter analysis periods may well be more sensitive for assessing negative and positive emotional reactions. Analysis of respiration rate did not appear to be useful, unlike in our previous study, where values were high during the negative and low during the positive situation (Reefmann et al. 2009b). In the present experiment, the breathing rates of the sheep varied considerably throughout the 4 min analysis period, and shorter intervals may be more appropriate for assessing the animals' responses. On the other hand, given that one breath may last for several seconds, a 10 s assessment may not produce sufficient data while the animals are breathing slowly. Based on a post hoc evaluation (data not shown), we would recommend 1 min periods to assess the animals' respiration.

In summary, our findings suggest that negative and positive emotional states in sheep can be differentiated using behavioural and physiological measures. To date, mean R–R interval, RMSSD and variance in body surface humidity would appear to be the most useful measures. In addition, it may be possible to replace these physiological measures with observations of ear postures, such as the number of ear posture changes or the proportion of axial ear postures for comparing the valence of emotional situations relative to each other. According to our results, cardiac measures can be assessed in 10 s periods, since these parameters are subject to quick regulation, and such short measurement durations may be even more appropriate for assessing emotional reactions than longer ones. In conclusion, our findings provide valuable data for understanding the assessment of both negative and positive emotional states in sheep that lay the basis for assessing emotions in other animal species.

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