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Acoustic markers of negative arousal in lambs: evidence from behavioural and eye thermal profiles

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Abstract

While vocalizations are widely recognized as indicators of emotional arousal across animal species, concurrent validation of changes in vocalisation structure using physiological and behavioural measures recorded simultaneously is still lacking in numerous species including sheep. Here, we examined the vocal expression of negative emotional arousal in sheep using both stress-related behaviours to quantify bodily activation and eye temperature measured via infrared thermography, as non-invasive indicators of arousal. To this aim, twenty lambs underwent a short-term isolation test with two phases, aimed at eliciting different levels of arousal in response to separation from conspecifics: partial isolation, where lambs maintained visual, acoustic and tactile contact with conspecifics through a fence, and full isolation, with complete separation. During full isolation, lambs exhibited increased bodily activation—spending more time running, jumping, and changing state behaviours—and produced more open-mouthed bleats (321 *vs* 27) than in partial isolation, validating higher arousal. Eye temperature also increased from partial to full isolation (however only in small lambs compared to large ones). Calls emitted in full isolation were characterised by higher frequencies, noisiness (higher chaos) and lower durations. When combining behavioural and physiological assessment of arousal and test they impact on spectro-temporal structure of vocalizations, we found that bodily activation, but not eye peak temperature, impacted the frequency distribution and noisiness of the calls. Call duration increased with eye temperature, but only in lambs exhibiting a bodily activation high enough and the mean of the second formant increased with eye temperature in smaller, but not in larger, lambs. Hence, lamb vocalizations indicate arousal and are correlated with bodily activation, while co-variations with physiological measures depended on behaviour and individual traits.

1. Introduction

Emotions are defined as short-term multi-component responses resulting from rewarding or punishing stimuli or events (Mendl & Paul, 2020). Among the theories developed to quantify emotions in non-human animals (hereafter 'animals'), the dimensional theory is one of the most widely used. According to this theory, emotions are characterised by their valence, which can be positive (pleasant) or negative (unpleasant), and their arousal (i.e., bodily activation) (Russell, 1980; Mendl et al., 2010; Bradley et al., 2001). To assess these two dimensions in animals, several indicators can be used, such as behavioural (e.g., ears and tail postures in sheep, *Ovis aries*, Reefmann et al., 2009a; body postures in cows, *Bos taurus*, de Oliveira & Keeling, 2018; head movements, moving,

ears and tail postures and vocalizations in goats, *Capra hircus*, Briefer et al., 2015a; tail and ears movements in pigs *Sus scrofa*, Rius et al., 2018), physiological (e.g., cardiorespiration and body surface humidity in sheep, (Reefmann et al., 2009b), nasal temperatures in dairy cows, Proctor & Carder, 2015, heart rate in laying hens, Davies et al., 2014), and cognitive indicators (e.g.,; judgements bias in goats, Baciadonna et al., 2016).

Infrared thermography is a technique used to indirectly assess the temperature of a subject by measuring the intensity of infrared radiation it emits. In recent years, the potential use of thermography to assess animal emotions in a non-invasive manner has been increasingly investigated (Travain & Valsecchi, 2021). These studies have revealed that temperature changes can be very useful to assess arousal, but cannot be generalised to all areas of an animal's face and body, as they exhibit different thermal behaviours. Indeed, increasing the level of arousal leads to a decrease in nasal temperature in marmosets (Ermatinger et al., 2019), a decrease in tail and paw temperature in rats (Vianna & Carrive, 2005), and an increase in eye temperature in dogs (Rigterink et al., 2018). Species specific knowledge is thus required when interpreting measures based on infrared thermography.

Alongside physiological responses, vocalizations have been shown to be highly relevant for assessing animal emotions non-invasively in various species, from birds to mammals (Villain & Briefer, in production; Briefer, 2012; Briefer, 2020). In particular, some vocal features change similarly with varying arousal across species, while vocal correlates of valence might be more species specific (Briefer, 2020). In high arousal contexts, calls are often louder, with higher frequencies and a more variable fundamental frequency (hereafter 'f_o'). The existence of these common rules linking variation in vocal characteristics with arousal across species makes vocalisations a good candidate as a universal indicator of emotions (Briefer, 2020).

Sheep (*Ovis aries*) are a gregarious and prey species, and separation from conspecifics is therefore perceived as a stressful and fear-provoking event (Cockram, 1994). The behavioural reactions of sheep to social stress have been described in the literature and consist of changes in body and ear posture, mobility, and rate of vocal activity (Ligout et al., 2011; Engeldal et al., 2013; Boissy et al., 2011). In addition, previous studies have shown that thermography can be used in sheep to evaluate negative emotions such as pain, fear, and stress. Transport stress has been found to increase core temperature in these animals, while social isolation causes a drop in ear pinna temperature due to vasoconstriction. Several studies demonstrated an increase in eye temperature in response to different stressors (i.e., tethering, shearing, and fear-inducing stimuli; e.g., opening an

umbrella; Cannas et al., 2018; Arfuso et al., 2022; Comin et al., 2024; Ingram et al., 2002; Lowe et al., 2005).

Sheep vocalisations serve several functions, such as reinforcing maternal bonding (Sèbe et al., 2007) and mutual recognition (Papadaki et al., 2021), as well as group cohesion (contact calls) and to signal stress (Dwyer, 2008; chapter 3. Behaviour and the Welfare of the Sheep). Sheep produce low-pitched bleats (emitted with the mouth closed) and high-pitched bleats (emitted with the mouth open) as well as mixed bleats (starting with a closed mouth and finishing with opened mouth). Low-pitched bleats or "rumble" sounds are emitted by the mother in the presence of her lamb, but also by rams during courtship (Dwyer, 2008; chapter 3. Behaviour and the Welfare of the Sheep). High-pitched bleats are produced in high-arousal positive contexts, such as waiting for or receiving food, but also in some high-arousal negative contexts, such as isolation from conspecifics and separation of lambs from their mothers (Dwyer, 2008; chapter 3. Behaviour and the Welfare of the Sheep; chapter 8: The Impact of Disease and Disease Prevention on Welfare in Sheep). Therefore, the production of open-mouthed bleats seems to occur in situations characterised by a high level of arousal (Papadaki et al., 2021). Overall, little is known about how the spectro-temporal structure of vocalizations within call-types produced by sheep changes with emotional valence and arousal. With regard to acoustic features, the few studies on this species show an increase in f_o contour features (Sèbe et al., 2012) and a lower energy distribution (i.e., energy quartiles; Sèbe et al., 2012; Engeldal et al., 2013), with increasing negative emotional arousal, assessed through behavioural and physiological measures (i.e., cardiac activity and cortisol assay). In addition, using isolation tests in ewes, Papadaki et al., (2021) found an increase in fundamental frequency-related features (min and mean f_o and f_o disturbance; Papadaki et al., 2021), as well as an increase in frequency distribution when ewes had no lambs under their care (dry period) compared to when they had lambs under their care (suckling period), although it could be linked to both higher levels of stress or variation of hormonal levels. With the exception of Sèbe et al. (2012), other studies did not include indicators of emotional arousal, and therefore, the current scientific evidence on vocal correlates of this emotional dimension in sheep requires further investigation.

This study aimed to fill knowledge gaps on the use of vocalization as potential indicators of negative emotional arousal in sheep using behavioural and physiological indicators. Lambs were subjected to a two-phase isolation test (partial *vs* full), designed to trigger clear changes of arousal within the negative valence dimension. The aim was to validate vocal expressions of negative emotional arousal by using behavioural and physiological measures as proper indicators of arousal. This was also achieved by considering how all the indicators co-varied. Based on previous studies

summarised above, when going from partial to full isolation, we predicted an increase in bodily activation and a higher vocal rate, with bleats composed of higher frequency and duration, and an increase in eye temperature. In addition, if bleat structure varied with emotional arousal, we expected a relationship between vocal descriptors and behavioural and thermal proxies of arousal regardless of the lamb biometry.

2. Materials and methods

2.1. Animals and housing conditions

The study was carried out on a commercial meat sheep farm in North Zealand, Denmark, from April to May 2023. The flock was composed by 110 sheep (*Ovis aries*) of different breeds (Frisian milksheep, Lacaune, Charolais, Zwartbles, Texel, Spelsau Lleyn Suffolk and Dorset). Among these sheep, 58 were lambs aged between four and five months at the start of the experiment, and 52 were ewes up to eight years of age. All the animals were housed in the same stable of 200 m^2 on a deep-litter straw bedding and had daily access to pasture from 8 am to 4 pm, accompanied by the farmer and sheepdogs. The pasture was composed of two areas of 15,100 m^2 and 36,200 m^2. All animals were managed in the same way in terms of feeding, which was calibrated to the nutritional requirements of adult sheep at approximately 320 g of barley per animal, divided in two daily administrations at 8.30 am and 3 pm. All sheep had *ad libitum* access to water.

The animals were naive to any experiment and were used for two subsequent experiments. In the other experiment (not described here), sheep were exposed to a playback experiment in the field, involving no previous manipulation. This playback experiment occurred after the tests presented in this study.

2.2. Experimental procedure

2.2.1 Individual identification and habituation

All sheep were marked using specific sheep spray (Agrihealth Quickdry Sheep Marker Spray) according to a unique and specific colour code applied to the back and flanks of each animal. For the need of the other experiment running at the same time, the flock was divided into two homogeneous groups in terms of numbers, keeping the lambs together with their mothers. The barn was divided in two equal parts and each group was assigned to a pasture field. A habituation phase of two days was adopted to let the animals familiarize themselves with the division of the flock, the location of pasture field they were assigned to, and the position of the feeders in the fields. The sheep were also habituated to the presence of two experimenters, who walked for 10 min twice a

day in the fields for two days, maintaining approximately a distance of thirty meters from the animals. The sheep were also habituated to the equipment: a hide (supplementary material, figure S1), which was installed in the barn to allow the experimenters to observe the sheep without visual disturbance, and a metal pen.

2.2.2 Isolation tests

The isolation tests started two days after the habituation phase. A total of twenty randomly selected lambs (n=11 females; n=7 males, n=2 unknown sex) were involved in the study. Based on the STRANGE framework, the study subjects appeared to be representative of the target population, considering that their behaviour is influenced by their genetic makeup, as well as their experiential and social background (Webster & Rutz, 2020). The tested lambs were isolated from conspecifics in the morning (08:21 (hh:min) ± 00:12) before the flock was moved to the pasture. For each isolation test, a randomly selected lamb was gently restrained by two experimenters, and placed in a metal pen within the stable where the animals were normally housed. The pen was shaped like an irregular polygon, approximately 1.43 m high and with a surface area of 2 m^2 (supplementary material, figure S1). The isolation test was divided into two phases: 1) the lamb had visual, vocal and physical access through the grid of the pen with its group members (partial isolation); 2) the group was guided toward the pasture field by one experimenter, while the tested lamb remained in the isolation pen in the absence of physical, visual or vocal contact with group members (full isolation). Each phase lasted 4 minutes, for a total duration of 8 minutes. At the end of the test, the subject was allowed to reunite with the herd at pasture. Each animal was tested only once.

2.3. Behavioural recording and scoring

Behavioural observations were made indirectly by video recording, using a GoPro Hero9 Black (GoPro Inc, CA, USA). Videos were scored later on by a trained observer continuously during the 8 min test duration using an ethogram created using Boris software (Friard & Gamba, 2016), which included both state and point events (Table 1). Ten percent of the videos were scored twice to assess intra-observer reliability, which was quantified using Cohen's Kappa and ranged from 0.731 to 0.836.

Table 1. Ethogram of the behaviours and postures scored during the isolation tests and their description (based on Boissy et al., 2011).

Scored behaviour	Behaviour type	Description
Bodily activation		**Static (standing + lying down) and dynamic (walking + running) behaviours**
Standing	State	The lamb stands on its four legs and remains still for at least two seconds
Walking	State	The lamb slowly moves forward or backward
Running	State	The lamb moves forward with a fast gait, lifting one limb before the other is supported
Head movements	Point	All changes (i.e., fast and slow) in the position of the lamb's head in any direction
Bar head touches	Point	The lamb sticks its head between the bars of the pen or hits them
Jump/rearing on the hind legs	Point	The lamb is off the ground with all legs raised/the lamb is standing on its hind legs
Lying down	State	The lamb is in sternal or lateral decubitus (abdomen in contact with straw bedding)
Ears position[1]		**The position of the lamb's ears in relation to the orientation of the auricles**
Open	State	The inner and outer sides are not visible, the inner side is parallel to the floor, the observer can see the full ears
Flat	State	The inner sides are visible by an observer placed in front of the animal
Hidden	State	The inner sides are not visible, the outer sides are visible at the root of the ear, the ears cannot be fully seen by the observer
Asymmetric	State	One inner side visible, one not
Unidentifiable	State	The position of the lamb’s ears cannot be classified because at least one of them is not visible
Tail position		**The position of the lamb's tail**
Upward	State	The lamb raises its tail up above a 45-degree angle
Down	State	The lamb has its tail down, below a 45-degree angle

Arched upward	State	The lamb keeps its tail arched over its rump
Shaking	State	The lamb moves its tail rapidly in any direction
At 45 degrees	State	The lamb has its tail raised at a 45-degree angle
Unidentifiable	State	The position of the lambs' tail is not visible
Vocalisation		**The lamb is bleating**
Open mouth	Point	The lamb bleats with its mouth open or begins to bleat with its mouth closed and ends with its mouth open
Closed mouth	Point	The lamb bleats with its mouth closed
Excreting behaviours		**The lamb is urinating or defecating**
Urinate	Point	The lamb is urinating
Defecate	Point	The lamb is defecating

1: adapted from Boissy et al., 2011

The percentage of time spent performing states, and the rate of occurrence of point events, was computed by dividing the behaviour duration (for state behaviours) or the number of occurrences (for point events) by the time during which the behaviour was visible. Video recordings were used also to obtain the height of the lambs, calculating the proportion with a known-height element of the isolation pen, which served as a reference. This information was used as a proxy of body weight and was useful to account for biometric differences that can affect both the acoustic parameters (Taylor & Reby, 2010) and the thermoregulatory process (Kleiber, 1947). Lambs were considered "small" if their height was less than 52 cm and "large" if their height was greater than 52 cm.

2.4 Thermal profiles

Thermal images of the lamb in partial and full isolation were taken with a Hikmicro G60 handled thermal camera by using the following parameters: emissivity = 0.98, reflected temperature = 25°C and distance = 4 m. To avoid influencing the subject's emotional response, the experimenter took the pictures from the hide, through a small opening. The thermal images (n = 3010) were manually inspected to determine their quality, resulting in the selection of 707 high-quality pictures (435were acquired during partial isolation and 272 during full isolation). Eye areas from these pictures (one or two eyes per picture, depending on the lamb position, and consequently on eye visibility) were manually defined in the Hikmicro software. Outliers, located outside the mean +-3sd range, were removed, as they likely resulted from false calculations of eye temperature, resulting in 755 eye areas. The maximum temperature of each eye was used for statistical analysis to avoid the impact of how the area boundaries were defined on the computation of the mean temperature (Mason, 2023). Finally, a delta time value was calculated between the time when the thermal images were taken and the start of each phase of the test, in order to have an information about the effect of the time on thermal eye profiles.

2.5 Acoustic recordings and analyses

Vocalizations produced by the lambs during the isolation tests were recorded with an omnidirectional microphone (Sennheiser MKH20) placed at approximately 3.4 m in front of and 2.4 m above from the isolation pen, mounted on a metal rod attached to the barn structure. The microphone was connected to a recorder (Marantz PMD661, sampling frequency: 44100 Hz and 16-bit WAV file format). Vocalizations were manually labelled according to the call type (low or high bleats) and checked for sound quality: only clean calls, *i.e.* having no overlapping sounds and a correct Signal to Noise Ratio (SNR) were retained for analysis (*get_SNR_dir()* function, *SoundChunk* R-package, (Villain & Renaud-Goud, 2023). The inspection of the Signal to Noise Ratio (SNR) revealed that calls (n = 464) were louder by 10.1 ± 0.56 dB compared to background noise. Only High Bleats (HB) were included in the statistical analysis, as the incidence of low bleats was very low (e.g.

1.56% of total recorded) vocalizations. After call selection, acoustic parameters were extracted on the remaining clean HB (n = 348; 17.4 ± 9.8 calls per individual) (*extract_chunk_features_dir()* function, *Soundchunk* R, *tuneR* and *Seewave* R packages, (Ligges et al., 2023; Sueur et al., 2008; Table 2). The following automatic computations were applied after inspection of the frequency range of interest, frequency range of the fundamental frequency (f_o) and number of putative formants on averaged mean spectra (supplementary figure S2). The sound was bandpass filtered between 150 and 6000 Hz using "hanning" window type (FFT window length = 1024 pts and overlap = 50%). The duration of the calls was determined using an amplitude threshold of 3% on the smoothed envelop (window length = 4000 pts, overlap = 90%). The extracted acoustic features related to the mean spectrum were the Median, Inter Quartile Range (IQR) and the Wiener entropy (sfm). The Dominant Frequency (figure 1A) was computed on each sliding temporal window (FFT window length = 1024 pts and overlap = 50%) and detected with a 10% amplitude threshold, by searching the loudest frequencies in each window. The average (Dominant frequency mean) and the standard deviation (Dominant frequency sd) were extracted as descriptors of the dominant frequency and its variability. The fundamental frequency (f_o) was detected by filtering the calls with a frequency range of 150-350 Hz and by searching for the loudest frequencies within this band (figure 1A). The average (f_o mean) and the standard deviation (f_o sd) were extracted as descriptors of the fundamental frequency and its variability. Finally, formant analysis was performed by searching for three main formants between 350 and 6000 kHz (figure 1B). Based on the detection of three peaks on a mean spectrum computed with FFT window length of 256 pts, the relative amplitude of formants F1 and F2 were extracted. Three frequency intervals were then computed to extract the frequency in each frequency interval (Relative Amp. F2, F3), corresponding to the three putative formants. For each frequency interval, the calls were filtered and the average of dominant frequencies computed over the duration of the call was extracted (mean F1, F2, F3).

Table 2. Acoustic features extracted from the high bleats and used for acoustic analysis

Acoustic feature	**Unit**	**Description**
Duration	s	Total duration of the vocalisation
The Median	Hz	Frequency below which 50 % of the energy is contained (also termed 'Q50%' or 'spectral centre of gravity')
Inter Quartile Range (IQR)	Hz	Frequency range between the third and the first quartile (indicates the bandwidth)
Wiener entropy (sfm)	-	Ratio of the geometric mean to the arithmetic mean of the spectrum (0: pure tone; 1: random noise)

Dominant frequency mean	Hz	Mean of the dominant frequency, computed per FFT window over the duration of the vocalization
Dominant frequency sd	Hz	Standard deviation of the dominant frequency, computed per FFT window over the duration of the vocalization
Relative Amp. F2*	dB	Relative amplitude of the second formant
Relative Amp. F3*	dB	Relative amplitude of the third formant
Mean F1	Hz	Mean frequency value of the first formant
Mean F2	Hz	Mean frequency value of the second formant
Mean F3	Hz	Mean frequency value of the third formant
f_o mean	Hz	Mean of the fundamental frequency of the vocalization over the duration of the vocalisation, computed per FFT window over the duration of the vocalization
f_o sd	Hz	Standard deviation of the fundamental frequency of the vocalization, computed per FFT window over the duration of the vocalization

** relative amplitude= normalized amplitude (the amplitude of the bleats has been reduced or scaled so that they fall between 0 and 1; for each sound, the loudest amplitude has been set to 1 and the other sounds have been normalised between 0 and 1)*

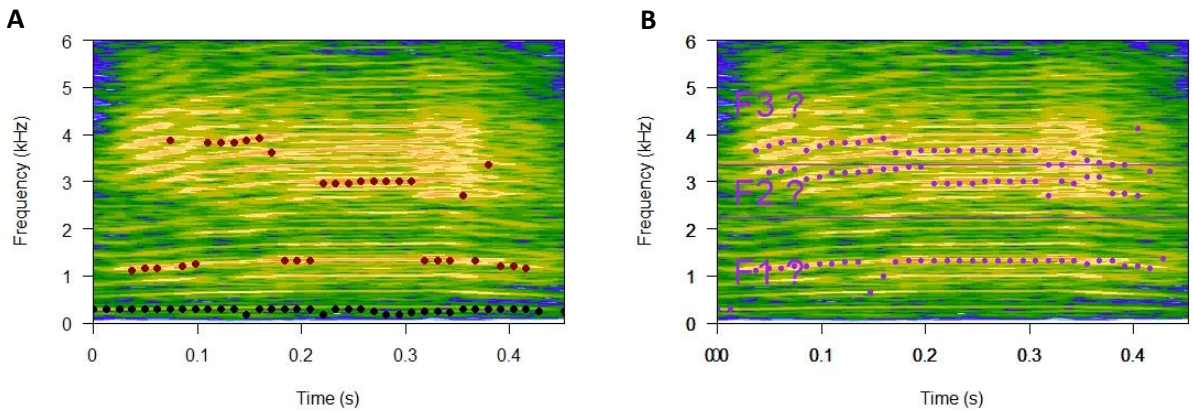

Figure 1A. Spectrogram of a high bleat (computed with FFT wl = 2048 and ov = 90) with illustration of detection of dominant frequencies (red dots) and fundamental frequency (fo; black dots), both *computed over the duration of the call in each sliding time window of the FFT computation (wl =1024 and ov = 50%, using either the frequency range of interest (150-6000Hz) or the frequency range expected for fo (150-350Hz) respectively for the dominant frequencies and the fundamental frequencies. Figure 1B. Spectrogram of a high bleat (computed with FFT wl = 2048 and ov = 90) with illustration of detection of the three main formants between 350 and 6000 kHz (purple dots). Each dot illustrates the dominant frequency computed over the duration of the call in each sliding time window of the FFT computation (wl =1024 and ov = 50%) after filtering the call within the frequency range of each formant.*

2.6 Statistical analysis

All statistical analyses were all performed in R 4.4.2.

2.6.1 *Behavioural response to full isolation*

After inspection of zero inflated data on rates and percentages of time spent expressing the scored behaviour, the ear and tail positions were excluded from further behavioural analysis, due to the low percentage of time they were visible during both phases of the isolation tests (15.1 ±19.0% and 33.1±21.9% for ears and tail, respectively). Furthermore, excreting behaviours were not included in the analysis due to their rare occurrence. Two behavioural variables were calculated from the catalogued behaviours, i.e., state behaviours changes, namely the number of transitions between standing, walking, running and lying down behaviours per time unit, and the percentage of time being in static posture (aggregating standing and lying down behaviours). Therefore, the behavioural variables used for analysing the behavioural response were the rate of jumps, state behaviours changes, bar head touches, head movements, vocalisations, and percentage of time spent walking, running or being static (Dray & Dufour, 2007). After inspection of symmetrical distributions of behavioural variables and transformations when needed (see Table 3), a Principal Component Analysis (PCA) was performed on the above-mentioned behavioural variables to generate composite behavioural scores and reduce the number of response variables (dudi.pca() function, 'ade4' R package). All principal components (PCs) with an eigenvalue greater than one were retained for further analyses and used as behavioural composite scores (behPCs).

The following fixed effects were used to explain the lamb's behavioural scores (behPC1, behPC2), used as response variables in a Linear Mixed-Effects Model (lmer() function,'lme4' R package; Bates et al., 2015): the 'Phase of the test' (two levels: partial *vs* full), the size of the lamb (two levels: 'small' *vs* 'large') and the interaction between these two factors. The identity of the lamb was used as random factor to take into account the within subject design.

*Model 1: behPC1/behPC2 ~ Phase of the test*Lamb size + (1|Subject)*

2.6.2 Thermal response to full isolation

Considering natural within-subject physiological variations in eye temperature, the effect of full isolation on eye maximum temperature was assessed by computing a coefficient of reaction (CR) during the full isolation compared to the partial isolation of the same individual, used as the baseline. The CR was computed as follows:

temperatureCR=(T - baseline)/baseline

where:

T = maximum eye temperature in sample

baseline = average of maximum eye temperature in partial isolation

Using this computation, positive or negative *temperatureCR* during the full isolation phase would be the result of, respectively, increasing or decreasing temperature compared to individual baseline values during partial isolation. The larger the absolute value of the *temperatureCR*, the stronger the variation from partial to full isolation. Model 2 was built with the aim to test the effect of the phase of the test on *temperatureCR*, while using the size of the lamb as interacting fixed effect. In addition, the centred and reduced delta time to the start of the phase (Zdelta) was also added as interactive covariate to the phase of the test to assess the effect of time within each phase on thermal eye profile. Lastly, the identity of the subject was included in the model as a random effect to control for repeated measurements.

Model 2: temperatureCR ~ Phase of the test (lamb size + Zdelta) +(1|Subject) data = all eye max temperature data points*

2.6.3 Acoustic response to full isolation

Similarly to the behavioural variables, composite acoustic scores were computed to describe the general structure of the vocalisations while decreasing the number of variables. A PCA was performed on the set of acoustic features listed in Table 2 ('dudi.pca' function, 'ade4' R package, Dray & Dufour, 2007), after inspecting their symmetrical distribution. All principal components with an eigenvalue greater than one were retained to be used as descriptors of the spectro-temporal structure of lamb bleats (acPCs). Furthermore, to align the direction of the acoustic feature loadings with the conventional representation of arousal in the dimensional theory of emotions (Mendl et al., 2010)—i.e., increasing from bottom to top—the signs of the first two principal components were inverted.

The comparison between the calls produced in partial and in full isolation was investigated with a Linear Mixed-Effects Model (LMER) ('lmer', 'lme4' R package; Bates et al., 2015). The following fixed factors were included the model: the phase of the test (partial or full) and the lamb size (small or large), as well as their interaction (Model 3). The identity of the subject was included in the model as a random effect to control for repeated measurements. Since not all lambs produced calls during the partial isolation, this analysis was performed twice, considering all the lambs and only considering the lambs that had produced calls in both phases (6 animals: N = 27 calls during partial isolation, and N = 128 calls during the full isolation). Since the statistics using both the entire dataset and the subset yielded similar results, the analysis of the entire dataset is presented in the results and used for further analysis (see Table S10 in supplementary material for statistical results referred to the subset).

*Model 3: acPCs ~ phase of the test*lamb size + (1|Subject), data = all calls*

2.6.4 Impact of behavioural and physiological indicators on vocalisations

To estimate co-variation between the acoustic structure of high bleats (acPCs), the behavioural response (behPC1) and the thermal profiles (eye maximum temperature) in response to isolation, a full model was built using each composite acoustic scores (acPCs) as response variables (Model 4) and the first behavioural score (behPC1), describing the bodily activation of the lamb in response to full isolation (see model 1) as a proxy of arousal, as continuous fixed effect. The size of the lamb, which affected both the acoustic scores and the eye thermal profile, was used as factorial fixed effect (see models 2 and 3). Considering the increase in eye maximum temperature in response to full isolation (see model 3) and the effect of time during the phase on temperature variation (see model 3), the peak eye temperature per lamb and per phase was used as a further continuous fixed effect in the model. Since the number of temperature points per phase and lamb ranged from one to 46, and since 90% of the data was composed of four or more temperature points per phase and subject, we decided to average the four highest temperatures per lamb and per phase to have a more representative value of the eye peak temperature than using only one point per subject and phase. The interaction between behPC1 and eye peak temperature, as well as the interaction between lamb size and eye peak temperature were also included to test for co-variation. Lastly, the individual identity of the subject was included in the model as a random effect to control for repeated call measurements.

*Model 4: acPCs~ peak eye temperature * (behPC1 + lamb size) + (1|Subject), data = all calls.*

2.6.5 Model fit and extraction of p - values (for all models)

After inspection of the distribution of the residuals of the models, a type-II analysis-of-variance was performed on each model ('Anova' function, 'car' R package, Fox & Weisberg, 2018). When significant interactions between fixed effects were found, pairwise comparisons were computed using 'emmeans' and 'emtrends' function from 'emmeans' R package (Lenth, 2016), with Tukey correction for factors and 'emtrends' function for interacting covariates. When significant interactions were found in model 4, comparisons were carried out by fixing some values of the covariate behPC1 (mean, mean - sd, mean + sd).

3. Ethical statement

This study was approved by the Animal Ethics Institutional Review Board at the Faculty of Health and Medical Sciences of the University of Copenhagen (official number: 023-03-BehaVEco-007A). As social isolation induces a negative affective state in the lambs, both isolation phases lasted only 4 minutes each. The isolation took place in a familiar environment, and any negative

effect of this procedure on the sheep was not expected to last beyond the end of the test. Five isolation trials were interrupted because the lamb being tested escaped.

4. Results

4.1 Behavioural response to full isolation

The Principal Component Analysis (PCA) performed on the behavioural variables resulted in two principal components with eigenvalues > 1. The first principal component (behPC1, 58.4% of explained variance) was characterized by high loadings of call rate, time spent running, rate of body position changes, jumps and bar head touches, and low loadings of time spent in static behaviours (standing + lying). In other words, behPC1 may be interpreted as bodily activation. The second principal component (behPC2, 14.9% of explained variance) was negatively correlated with the percentage of time spent walking.

Table 3. Results of the Principal Component Analysis performed on the behavioural dataset, showing the parameters loadings on PC1 and PC2 (relative contribution for each behaviour to the PCs). The high parameters' loadings (i.e., > 50 or < -50) are in bold

Behavioural parameters	**PC1**	**PC2**
Jump rate[1]	**70.7**	0.01
Percent of total time in in static behaviours[2]	**-65.5**	-0.01
Percent of total time walking[2]	5.08	**-88.3**
Percent of total time running	**76.7**	11.5
Rate of state behaviours changes	**74.3**	-2.17
Vocal dynamic[1]	**80.1**	4.91
Rate of bar head touches	**52.0**	0.58
Rate of head movements	43.2	-12.0

1 Square root-transformed variable

2 Log1-transformed variable

BehPC1 significantly increased from partial to full isolation (Chisq = 96.3; df = 1, p<0.001; figure 2), indicating an increase in bodily activation from partial to full isolation (Table 3). No effect of the size of the lamb on behPC1 was observed (Chisq = 0.13; df = 1, p = 0.71; supplementary Table 1). No effect of the phase of the test nor of the size of the lamb was found on behPC2 (Chisq = 2.34; df = 1, p = 0.13; Chisq = 2.92; df = 1, p = 0.09, respectively; supplementary Table 1). Only behPC1 was therefore used as proxy of bodily activation for further analysis.

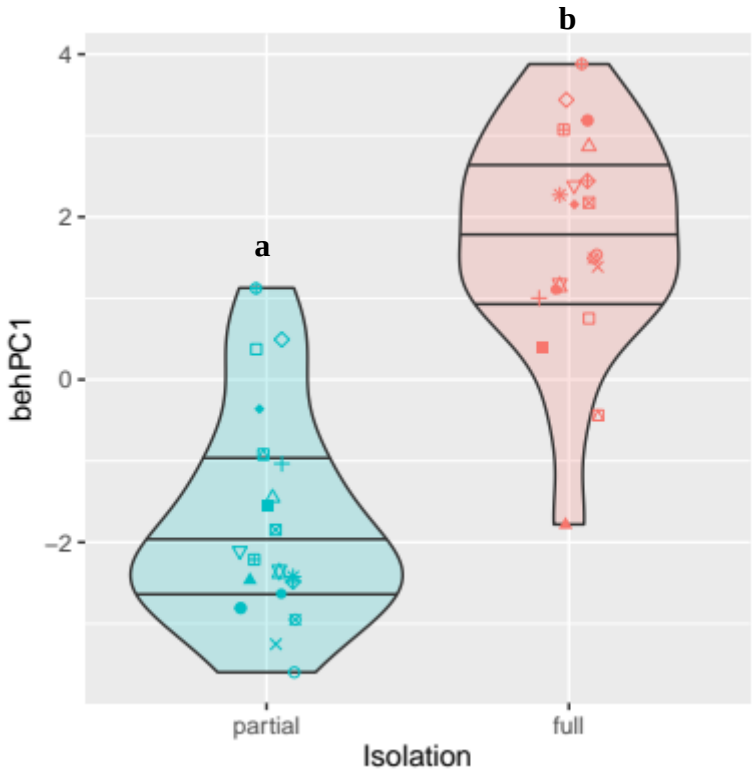

Figure 2. Violin plot representing the distribution of lambs' behavioural response scores (behPC1) in partial and full isolation. Individual subject points in partial and full isolation are depicted by shapes. Horizontal lines represent first, second and third quartiles of data distribution (Anova Table and model estimates are available in supplementary Tables S1, S2, S3 respectively, model 1)

4.3 Thermal eye profile from partial to full isolation

The eye max temperature Coefficient of Reaction (temperatureCR) was significantly affected by the interaction between the phase of the test and lamb size (LMM main effect, Chisq = 38.6; p = <0.001; figure 3): from partial to full isolation, temperatureCR increased (i.e., became positive) in small lambs (partial *vs* full, t.ratio = 8.82, p <0.001, figure 3A), but not in large ones (partial *vs* full, t.ratio = 0.42, p = 0.68, figure 3A), indicating that the maximum eye temperature of lambs increased in full isolation compared to partial isolation in small lambs. In addition, the delta from the start of the phase also affected temperatureCR (LMM main effect, Chisq = 9.26; p = 0.002; figure 3B: the higher the delta, the higher the temperatureCR (slope estimate[95%CI] = 0.58 [0.21:0.96]; supplementary Tables S4 -S6)).

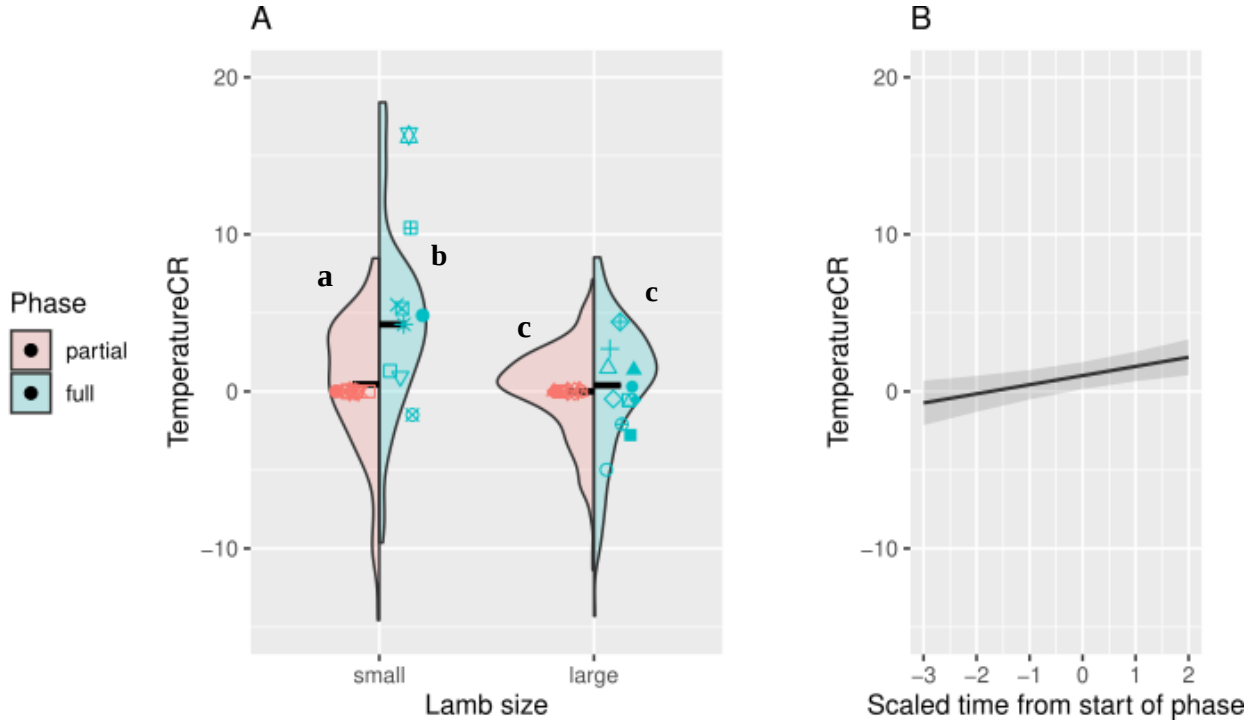

Figure 3. Lambs' thermal response score (TemperatureCR) of small and large lambs in partial and full isolation (4A) and depending on the delta to the start of the phase (4B). Violin plot represents the contour of data distribution and mean and standard error of the groups are depicted with horizontal lines. Individual subject points of small and large lambs are depicted by shapes (Anova Table and model estimates are available in supplementary Tables S4 - S6).

4.2 Variation of high bleats acoustic structure from partial to full isolation

The Principal Component Analysis performed on the acoustic data (348 high bleats, 321 produced during the full isolation and 27 during the partial isolation) showed four components describing the spectro-temporal features of high bleats: -acPC1 to acPC4, respectively explaining 37%, 15%, 13% and 9% of data variance. According to the loadings of the acoustic features on the PCs (Table 4), higher -acPC1 scores indicated calls with a higher median, a higher Wiener entropy, a higher dominant frequency mean and sd, a higher mean of the second and third formants and, marginally, a higher fundamental frequency. Therefore, as -acPC1 scores increased, calls became noisier and were characterised by higher frequencies. -acPC2 was characterized by high loadings of the second formant of higher frequency. Higher values of acPC3 indicated calls that were characterized by a higher value of Inter Quartile Range (higher frequency bandwidth). Finally, higher values of acPC4 indicated calls with longer duration and, marginally, less variability in fundamental frequency.

Table 4. Results of the Principal Component Analysis performed on the acoustic dataset giving the features' loadings on -acPC1, -acPC2, ac PC3 and acPC4 (relative contribution of each parameter to the PCs). The highest contribution of each acoustic feature appears in bold letters.

	-acPC1	**-acPC2**	**acPC3**	**acPC4**
Inertia	4.8	1.9	1.7	1.2
Cumulative percent of variance explained	37.1	52.0	65.1	74.3
Duration	-0.7	1.9	.7	**68.9**
Median	**86.2**	-2.8	-1.0	.0
IQR	4.0	-2.6	**78.8**	.0
Wiener entropy (sfm)	**50.9**	-6.4	11.3	-6.0
Dominant frequency mean	**76.5**	-1.4	-12.4	.1
Dominant frequency sd	**60.3**	-8.9	2.0	1.5
Relative Amp. F2	25.1	-2.1	-29.0	-.9
Relative Amp. F3	**40.4**	-36.1	-2.5	.1
Mean F1	32.2	37.8	-1.8	.8
Mean F2	32.9	**50.3**	-.9	.1
Mean F3	**42.2**	29.5	1.8	.0
fo mean	**27.7**	0.0	18.9	13.6
fo sd	3.7	13.1	9.5	**-27.8**

The LMMs showed that -acPC1 was significantly higher in full compared to partial isolation (LMM main effect, Chisq = 8.23; df = 1, p = 0.004, figure 4A), and was affected by the size of the lamb (LMM main effect, Chisq = 8.43; df = 1, p = 0.004, figure 4A). In addition, acPC4 was significantly lower in full compared to partial isolation (LMM main effect, Chisq = 11.58; df = 1, p < 0.001, figure 4C). No evidence of any effect of the phase nor the size of the lamb was found on -acPC2 (Chisq = 1.94; df = 1, p = 0.16; Chisq = 0.75; df = 1, p = 0.39, respectively) and acPC3 (Chisq = 2.38; df = 1, p = 0.12; Chisq = 0.45; df = 1, p = 0.50, respectively; supplementary Table S7 - S9). Hence, considering the loadings of acoustic features on the PCs (Table 4), this indicates that, compared to high bleats produced in partial isolation, bleats produced in full isolation were noisier, of shorter durations, and composed of higher frequencies.

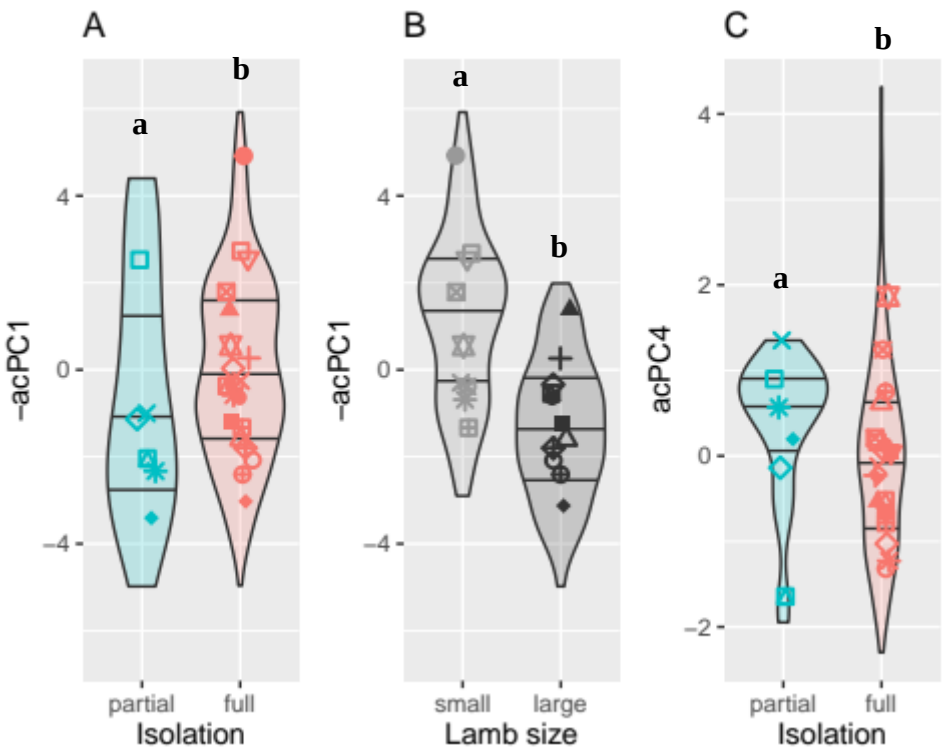

Figure 4. Violin plots representing the distribution of lambs' acoustic response scores (-acPC1 and acPC4) in partial and full isolation and in small and large lambs (-acPC1). Individual subject points in partial and full isolation are depicted by shapes. Horizontal lines represent first, second and third quartiles of data distribution. (Anova Table and model estimates are available in supplementary Tables S7-S9, model 2)

4.4 Co-variation of acoustic, thermal and behavioural profiles

-AcPC1 was significantly affected by behPC1: the higher behPC1, the higher -acPC1 (LMM main effect, Chisq =3.87; df = 1; p = 0.05; figure 5; Table S11). As behPC1 was found to be a descriptor of bodily activation, according to the loadings of the acoustic parameters on acoustic PCA, our results indicate that the higher the arousal of the lambs were during the test, the higher the frequencies and noisiness in their calls. -AcPC1 was significantly higher for small than for large lambs (LMM main effect, mean estimates [95% confidence interval] = 1.01[-0.21:2.23] and -1.21[-2.44:0.03], respectively Table S12). However, no evidence of any effect of the peak eye temperature was found on -acPC1 (LMM main effect, Chisq = 0.05 p = 0.82, Table S11). AcPC4 was significantly affected by the interaction between behPC1 and the peak eye temperature (LMM main effect, Chisq = 7.17, p = 0.007, figure 5; Table S11). Posthoc tests investigating differences between slope trends showed that acPC4 significantly increased with eye peak temperature, but only for lambs having higher behPC1 scores (slope estimates [95% confidence interval] = -0.04[-0.24:0.17], 0.20[-0.08:0.49] and 0.44[0.01:0.88] respectively for low, medium and high behPC1; Table S16). According to the loadings of the acoustic parameters on acoustic PCA, this indicate that eye peak temperature during the test was positively correlated with call duration; however, this was true only when lambs reached a sufficiently high level of bodily activation. No effect of lamb size was found on acPC4 (LMM main effect, Chisq = 2.0 p = 0.16, Table S11). AcPC3 was

significantly affected by the interaction between behPC1 and eye peak temperature (LMM main effect, Chisq = 4.8 p = 0.03, Table S11); however, posthoc tests did not reveal significant slopes nor significant difference between them (Table S17). AcPC3 and -acPC2 were significantly affected by the interaction between lamb size and eye peak temperature (LMM main effect, Chisq = 4.2 p =0.04 and Chisq = 4.0 p =0.04 respectively; Tables S11), yet, posthoc tests only revealed a slightly significant slope for -acPC2, which increased with eye peak temperature in small lambs compared to large lambs (t.ratio = -2.00, p = 0.05 (slope estimates [95% confidence interval] = -0.37[-0.01:0.75], -0.09[-0.51:0.33] respectively for small and large lambs; Tables S13 and S17).

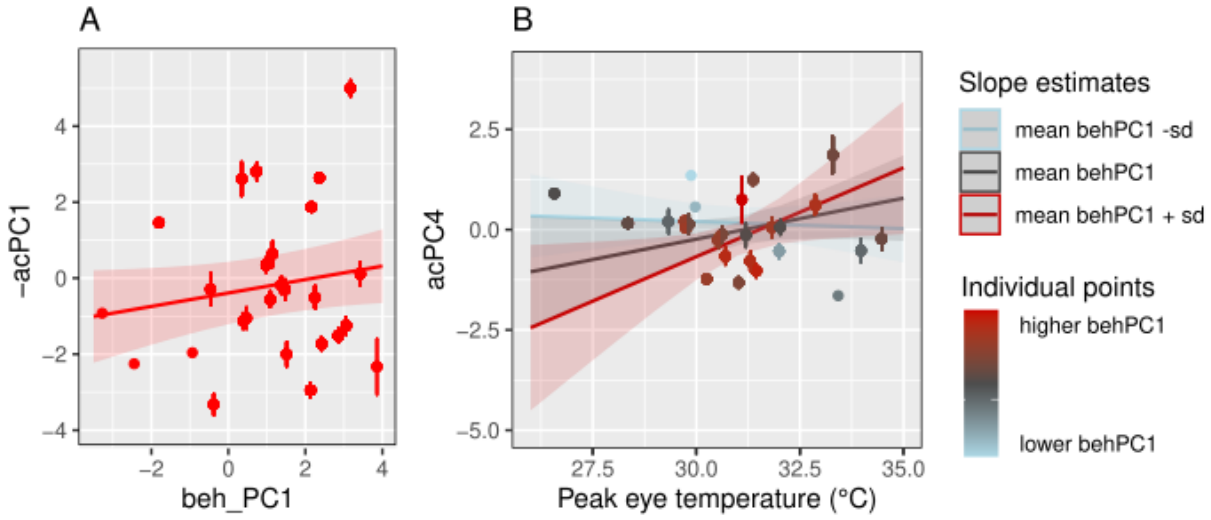

Figure 5. Co-variation of -acPC1 (A) and acPC4 (B) as a function of behPC1 (A) and eye peak temperature (B). Slopes depict model estimates and confidence interval. Points and segments depict individual mean and standard error. (Anova Table, model estimates and posthoc tests are available in supplementary Tables S11 – S17, model 4)

5. Discussion

We investigated how lambs express emotional arousal through vocalizations in response to experimentally induced negative situations of varying intensity (i.e., partial and full isolation). To validate our findings, we used behavioural and physiological indicators. We found behavioural, vocal and thermal differences between the two experimental phases. When examining the relationships between bodily activation and eye peak temperature with vocal acoustic features, we found that vocalizations co-varied with the level of bodily activation, showing higher frequencies and increased noisiness (chaotic structure) when bodily activation increases. Call duration also increased with eye peak temperature however only when bodily activation was high enough. We discuss below the validation of our test phases to trigger changes in potential indicators, the range of co-variation between them, their sensitivity and limits.

5.1 Bodily activation as a proxy for negative arousal in response to full isolation.

In this study, during full isolation lambs spent more time running and less time in static behaviours (i.e., standing and lying), performed more jumps, changes of state behaviours and bar head touches, and produced more calls than during partial isolation, suggesting a higher arousal level. An increase in locomotor activity can be seen as a behavioural response to fear and anxiety induced by social isolation in sheep (Vandenheede et al., 1998, Engeldal et al., 2013), resulting in escape attempts (Siebert et al., 2011). The fight-or-flight response is enacted by individuals in negative situations characterised by high levels of arousal, because it allows them to respond quickly to threats to their survival and fitness (Dawkins, 1998). Therefore, an increase in the individuals' bodily activation would be an indication that they are in a state of high emotional arousal. Increases in locomotor activity (head movements, moving; Briefer et al., 2015a) and escape attempts (jumping and rearing (i.e., standing on hind legs; Siebert et al., 2011) have also been observed in goats in situations of increased emotional arousal. Other studies in goats and sheep have shown that passing from partial to full isolation reduces locomotor activity, indicating a lower level of arousal (Siebert et al., 2011; Engeldal et al., 2013). However, these results are not directly comparable with those of the present study, as duration of the isolation test was longer (15 min in Engeldal et al., 2013 and 30 min in Siebert et al., 2011). The prolonged complete absence of contact during full isolation may have reduced the animals' reactivity, resulting in an attenuated behavioural response, leading to resignation on the part of the animals. The predominant production of high (open-mouth) vocalisations has been previously observed in sheep in response to stress due to separation from offspring and social isolation (Dwyer, 2008; chapter 3. Behaviour and the Welfare of the Sheep; Papadaki et al., 2021), similarly to our findings. We observed an increased production of calls per time unit (faster rate), which can be attributed to increased respiratory rate with increasing levels of emotional arousal (Briefer, 2020). The emission of more calls per time unit with increasing arousal levels has been observed in several species during negative situations, including other livestock (e.g., cattle, Thomas et al., 2001; pigs; Marchant et al., 2001), as well as in positive situations (Villain et al., 2020), and is therefore possible to consider it as valid indicator of this emotional dimension (Briefer, 2020). In the present study, it was not possible to investigate ear and tail postures as behavioural indicators of emotional arousal, as they were not visible for most of the time. Although there is some evidence in the literature that ear and tail postures reflect the emotional state of sheep (ears: Boissy et al., 2011; Reefmann et al., 2009a and Vögeli et al., 2015; tail: Reefmann et al., 2009a), existing studies have often examined only a single emotional dimension (either valence or arousal), without controlling for the other. Consequently, it is currently not possible to identify specific ear or tail postures as indicative of valence and arousal. Further research on this topic is therefore needed.

5.2 Increase in maximum eye temperature in response to full isolation

We found positive and increasing individual coefficient of reaction from partial to full isolation, indicating that the maximum eye temperature increased with arousal. Interestingly, this phenomenon was only found in small lambs. Several studies that have used infrared thermography to assess physiological response to negatively valenced situations in sheep, including stress, have measured the temperature of the eye (Stubsjøen et al., 2009; Arfuso et al., 2022), or part of it, such as the lacrimal caruncle (Cannas et al., 2018; Comin et al., 2024), lateral canthus, central cornea and medial canthus (Arfuso et al., 2022). Eye temperature and the aforementioned eye regions were shown to increase in response to fear (Comin et al., 2024) and stress (Cannas et al., 2018; Arfuso et al., 2022), similarly to what was observed in our study for small lambs. Here, we extracted the thermal matrices that covered the entire ocular area. Arfuso et al. (2022) found that the medial canthus ocular region of sheep was the most reliable for monitoring stress, as it is affected by vasodilation and heat dissipation due to exposure of an animal to a stressor. The authors also showed that medial canthus temperature was positively correlated with serum cortisol concentration in ewes after the exposure to the stressor and with the rectal temperature in ewes before and after stressor exposure. We showed that increasing temperature in response to full isolation depends on lamb size. In mammals, body size can influence the metabolic rate and thus the thermoregulatory process (Kleiber, 1947). For instance, in Standardbred trotting horses, a higher basal temperature was observed in young animals (2-3 years) compared to adult horses (> 3 years; Negro et al., 2018). In our case, we can hypothesise that larger lambs might have higher basal eye temperature, and that no further increase was found due to a ceiling effect. Last, ocular surface temperature increased progressively over time (i.e., delta from phase start), independently of the experimental phase. This trend may influence the choice of methodology for extracting a thermal proxy during testing: extracting the peak eye temperatures as a general proxy to arousal might indeed be a more relevant choice than averages.

5.3 Variation of vocal structure of high bleats in response to full isolation

Based on the loadings of the PCA describing the acoustic structure of high bleats, we found that in full isolation lambs produced bleats that were characterised by higher frequencies, formants, fundamental frequency, and dominant frequency than in partial isolation. An increase in frequencies with increases in emotional arousal has been observed in many species (goats; Briefer et al., 2015a; piglets, Linhart et al., 2015; horses, Briefer et al., 2015b). Briefer et al., 2015a observed an

increase of the f_o mean, but also of other f_o contour features (i.e., f_o end) in goats experiencing high arousal situations (i.e., anticipation of a food reward and food-related frustration) *vs* low arousal situations, i.e., control and isolation from conspecifics). The increase of the fundamental frequency with arousal was also observed by Sèbe et al. (2012) in ewes exposed to separation from their offspring. An increase in f_o with the intensity of an emotional state may be due to increased sub-glottal pressure as a result of changes in respiration or laryngeal muscle action/tension or to an increased vocal fold tension (Titze, 1994; Fant, 1960). In the present study we also observed a change of bleats energy distribution towards higher frequencies with increasing arousal (median and dominance frequency), as observed in several other species (Briefer, 2012; Briefer, 2020). In goats, the aforementioned changes were proposed to be associated to a reduced laryngeal retraction and/or increased pharyngeal constriction with increased arousal (Briefer et al., 2015a). The only available study on the variation of these acoustic features of sheep bleats with arousal level showed opposite results to the present study, i.e., a reduction of energy quartiles (i.e., Q25%, Q50% and Q70%; Sèbe et al., 2012). Engeldal et al. (2013) also found a reduction in energy distribution in vocalizations of sheep subjected to partial than in full isolation (however, as discussed above, the duration of partial and full isolation phases in Engeldal et al. (2013) were longer than in the present study and may have triggered opposite reactions in terms of arousal). Finally, increased pharyngeal constriction and tension of the vocal tract walls, in addition to reduced salivation, are also predicted to lead to higher formant frequencies (Scherer, 1986), which could explain our findings (mean F3).

We also found that lamb calls became more chaotic as the level of arousal increased, as indicated by an increase in Wiener entropy (sfm). This finding is in line with what was observed in goats by Siebert et al. (2011), who recorded lower entropy in goat calls in response to 30 min of complete isolation, which induced a condition of lower emotional arousal, probably due to the prolonged absence of sensory contact and reduced motivation for social reunion. Furthermore, in pigs, entropy increased during anticipation regardless of valence (Villain et al., 2020), although how tonality/chaos vary with arousal seem to depend on the call type in this species (Linhart et al., 2015). Overall, an increase in chaos with arousal is rather common across species, as shown also in dog puppies (Massenet et al., 2025), marmots (Blumstein, 2025), and bonobos (Pisanski et al., 2022). As expected, the increase in frequencies, fundamental frequency, formants and noisiness of calls observed in small lambs compared to large lambs in the present study was due to anatomical differences rather than a difference in the level of arousal experienced by the lambs. In fact, larger animals produce calls characterized by lower frequencies which is coherent with the source filter theory of vocal production (Taylor & Reby, 2010).

Based on the loadings of the acoustic PCA, passing from the partial to the full isolation seems to be correlated with decreasing call duration. The duration of vocalisations is the only acoustic feature that changes relatively consistently with emotional valence across species, and has therefore been suggested as an indicator of this emotional dimension more than of arousal (Briefer, 2020). In several species, negative situations lead to calls with longer duration than positive ones (Sèbe et al., 2007; Briefer, 2020; Villain & Briefer, 2020, (Lefèvre et al., 2025). Call duration also tends to increase with arousal, but considerable variation exists between species and call types, with notably alarm calls becoming shorter with arousal (i.e., danger in this case) due to their function (Briefer, 2012; Zimmermann et al. 2013). In our study, the emotional arousal elicited by full isolation may have led to the production of calls of shorter duration as they are quicker to produce and can be repeated rapidly, as demonstrated by the increase in vocal rate. A trade-off might thus exist between producing calls at faster rate and longer duration.

5.4 Vocal expression of emotional arousal correlates with behavioural and physiological indicators

The results from the combined model indicate that acoustic features of lamb vocalisations are modulated by bodily activation (i.e., behavioural arousal) and, to a limited extent, by their interaction with body size and/or physiological arousal. Specifically, an increase in the frequency and noisiness (chaos) of the calls was associated with higher levels of bodily activation. While peak eye temperature alone was not related with the frequencies and the noisiness of the calls, it interacted with the bodily activation to influence call duration, which increased with temperature only in highly activated lambs. This could suggest that physiological arousal primarily leads to changes in vocal duration when behavioural arousal is sufficiently high. Nevertheless, it should be noted that the way in which call duration changed was inconsistent when vocalisations were considered alone, as opposed to when they were combined with other indicators of arousal. This could be explained by the fact that the analysis of the vocalisations considered the test phase and, therefore, the small number of lambs that produced calls even during partial isolation could have influenced the results. Furthermore, about the relationship between the mean of the second formant and eye peak temperature depending on lamb size, we found a positive relationship between the acoustic score and the eye peak temperature, which tended to increase in small lambs but not in large ones. Although this difference did not reach significant threshold, this trend is in line with the hypothesis we raised earlier, namely that the absence of evidence of increasing peak eye temperature in large lambs may be due to ceiling effects. We can hypothesize that larger lambs have higher baseline eye temperatures, which could limit further physiological increases deriving from a high arousal

level. In other words, these findings underline that, similarly to acoustic features, potential sensitivity issues for validating the use of thermography may arise when not considering the biometry of the considered animal and we encourage future studies to take body size into account.

Overall, the findings highlight that vocal expression of emotional arousal in lambs reflects a complex interplay between behavioural and physiological indicators, while the role of biometric characteristic needs to be further investigated. Vocalizations seem to provide a more robust and accessible proxy for emotional arousal in lambs in response to negative situations.

6. Conclusion

In this study we have identified promising non-invasive vocal indicators of negative arousal in lambs. Vocalizations showed to be valid indicators of arousal levels in lambs and to be correlated with bodily activation, with higher frequencies and increased noisiness (chaos) observed as arousal increases. Co-variations with physiological measures, however, depended on both behavioural activation and the animal's biometric characteristics. Our study provides a method to study the interplay between behavioural, physiological and vocal responses to emotional arousal. These findings highlight the need for a multi-disciplinary approach to accurately assess emotional arousal in animals.

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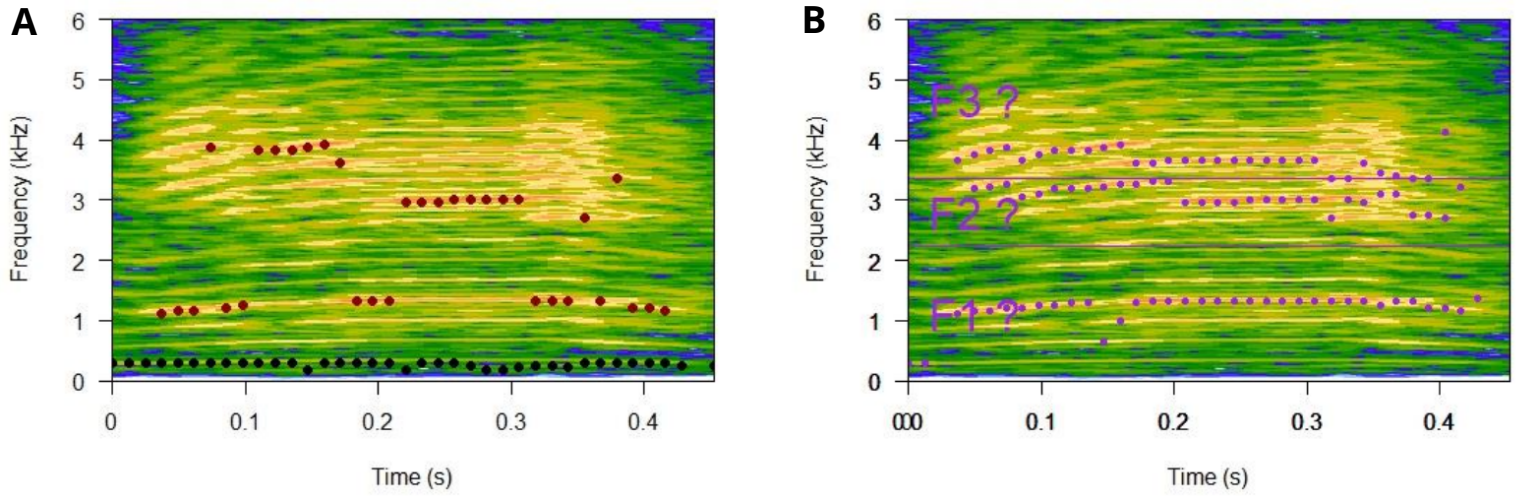
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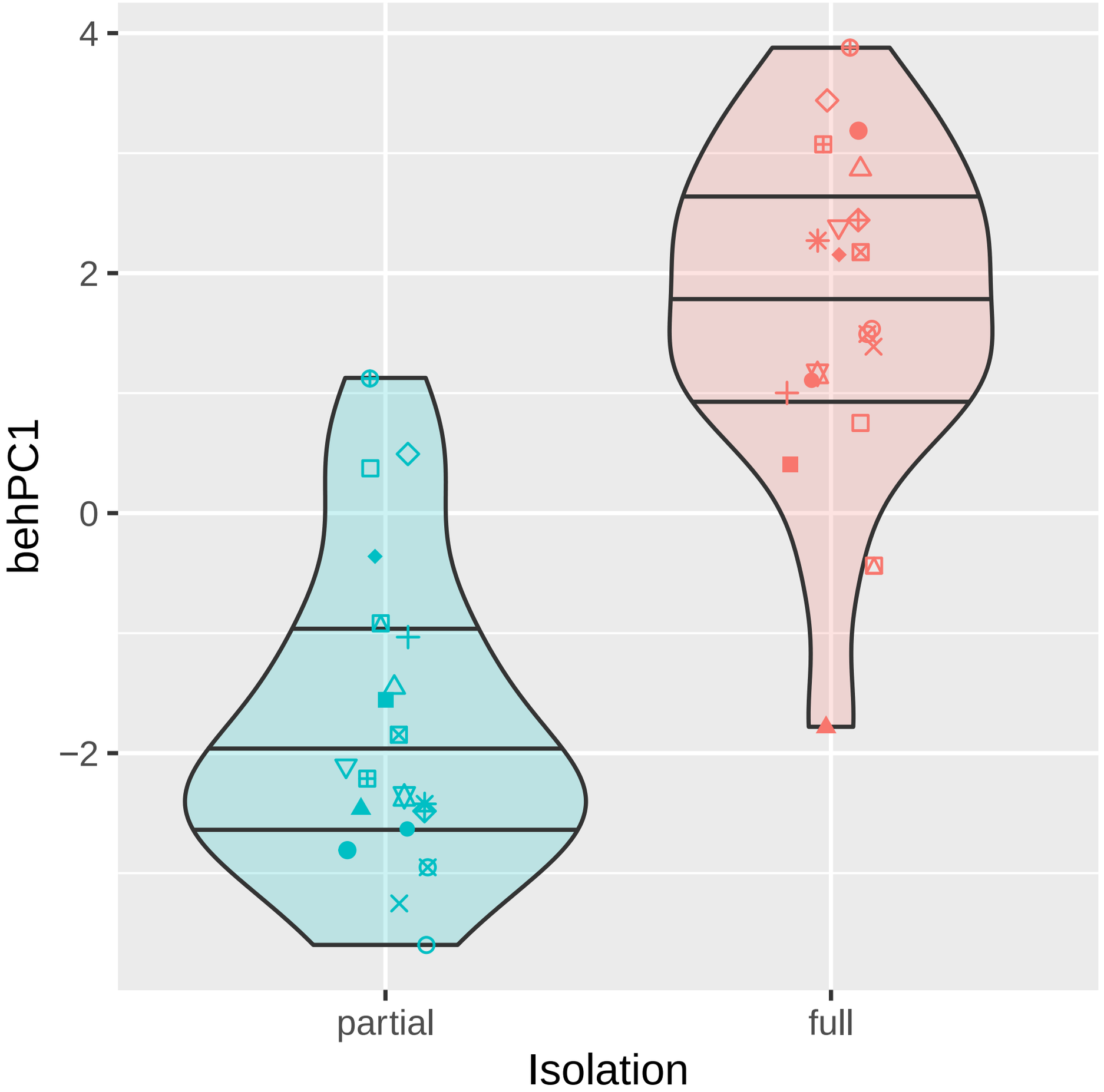
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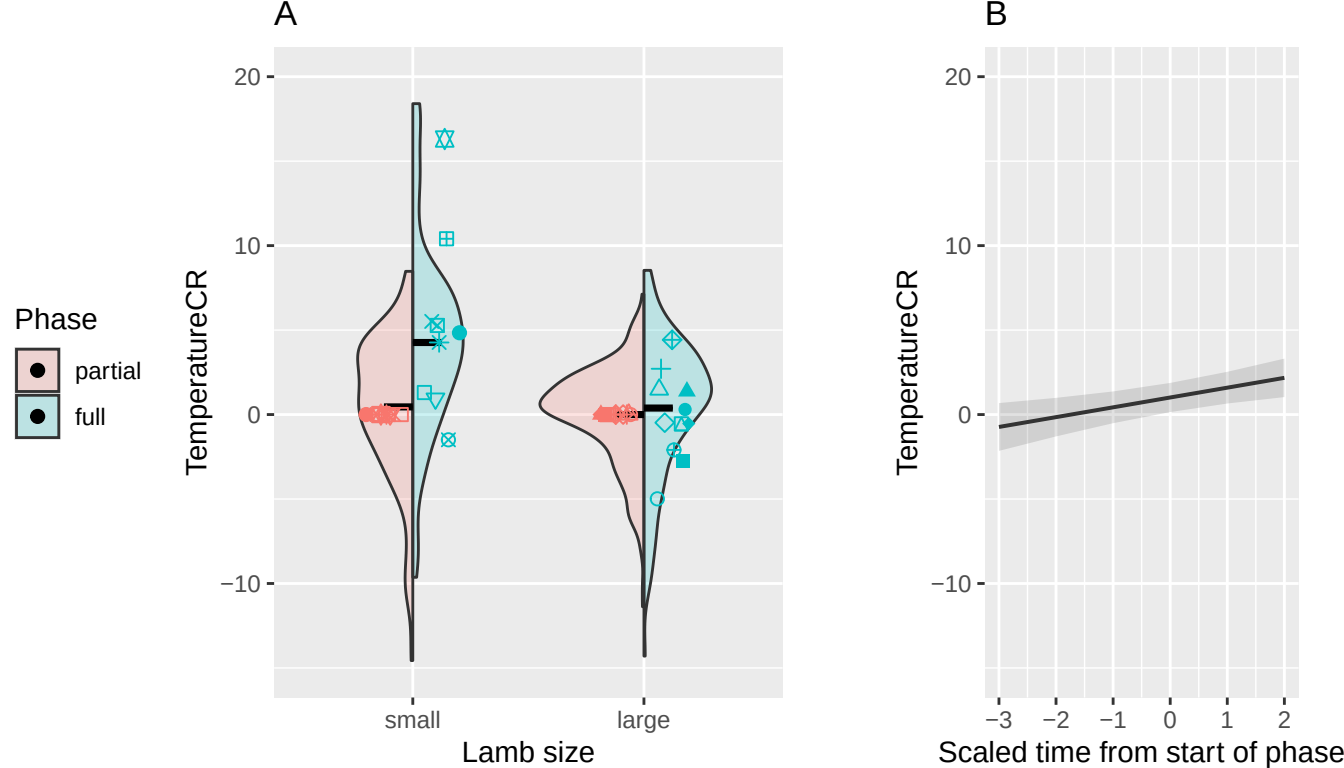
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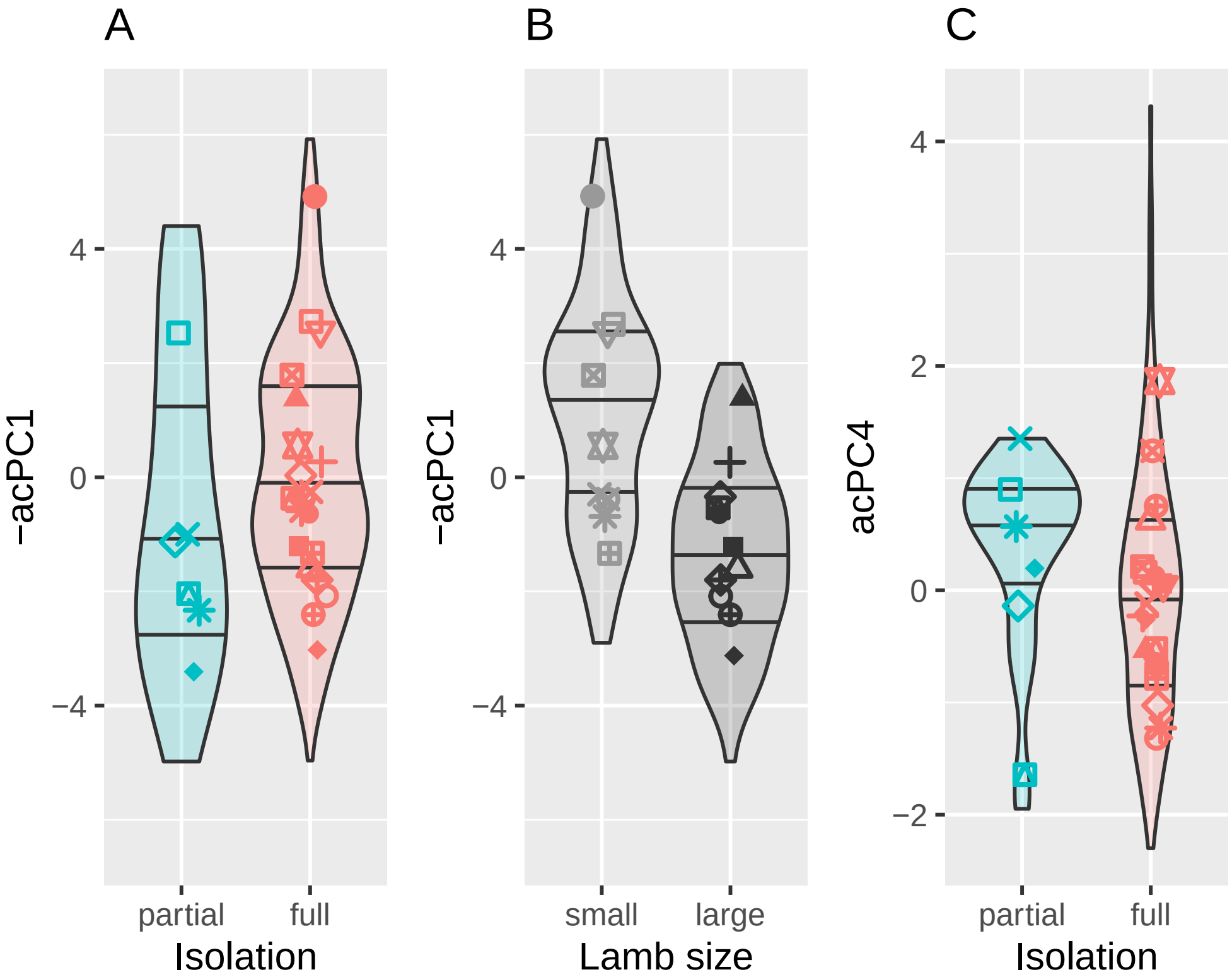
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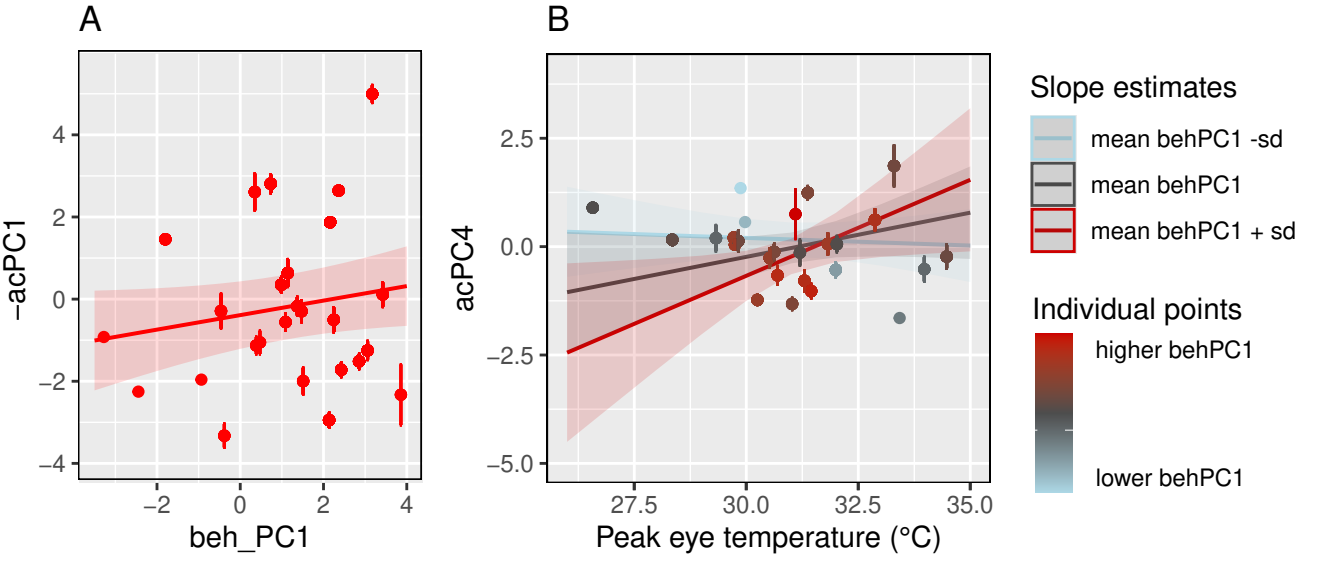
A
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Peak eye temperature (°C)
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mean behPC1 -sd
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Individual points
higher behPC1
lower behPC1