Investigation of reward quality-related behaviour as a tool to assess emotions

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Abstract

Animals are likely to appraise events as positive or negative based on their subjective perception, current state and past experiences. We tested the effects of anticipating positive (food anticipation), negative (inaccessible food) and neutral (clicker sound) events on behavioural and physiological responses of 30 goats. The experimental paradigm involved the presentation of a conditioned stimulus (CS) followed by an unconditioned stimulus (US) after a delay. The following parameters were measured at three different time points over 11 test sessions (2 trials / session total of 22 trials): activity, head movements, vocalisations, ear positions, structure of vocalisations produced, and physiological activity. In the positive condition, goats were more active, had increased head movements and call rate, longer durations of ears positioned forward and higher heart rates compared to the other conditions. In the control condition, goats kept their ear backwards for longer compared to the negative condition. No differences were found in vocal parameters and heart-rate variability across conditions. Overall, goats showed different behavioural and physiological responses to positive compared to negative and neutral events, suggesting that the anticipatory response paradigm may be used as a valid tool to capture the affective state of an individual.

Key words: Anticipatory behaviours, goats, positive animal welfare, reward-related behaviour, wellbeing.

1. Introduction

In recent years, the importance of rendering an animal's life "worth living", in which eliminating negative experiences is as important for welfare as promoting positive experiences, has been increasingly emphasized (Wathes, 2010; Dawkins, 2015; Webster, 2016; Mattiello et al., 2019). However, what constitutes a positive or a negative event depends on the subjective perception of the individual and can be based on its current emotional and motivational state as well as its past experiences (Spruijt et al., 2001; van der Harst and Spruijt, 2007; Lawrence et al., 2019).

One of the current definitions of wellbeing describes this state as a balance between positive and negative events (Spruijt et al., 2001; van der Harst and Spruijt, 2007). This definition takes into account the interaction between the evaluation process of the individual's current state and the selection of the most appropriate response that is mediated by the reward and stress systems in the brain. Based on this definition of wellbeing, the balance between positive and negative events can be affected and modified. For example, repeated negative events can lead to increased sensitivity to rewards (Luo et al., 2019; van der Harst and Spruijt, 2007; Spruijt et al., 2001) . Likewise, negative experiences, could be counteracted by exposing an individual to positive situations and stimulating the reward system (van der Harst et al., 2005).

The effects of negative and positive experiences on behaviour have been investigated using the anticipatory behaviour paradigm (van der Harst et al., 2003; van der Harst et al., 2003; van der Harst et al., 2005; Dudink et al., 2006; Chincarini et al., 2018). According to this paradigm, anticipatory behaviour is prompted through classical conditioning, consisting in an animal learning to associate a stimulus (e.g. a light or a sound) with a reward (Craig, 1918). When the association has been established, the sole presentation of the stimulus can evoke anticipatory behaviour. The behavioural response (e.g. activity level and frequency of behavioural transitions) to the stimulus can be assessed when a delay is added before the arrival of the reward. For instance, rats (*Rattus norvegicus*) exposed to poor housing conditions exhibit higher levels of anticipation behaviour compared to animals experiencing enriched housing conditions (van der Harst et al., 2003). In addition, socially stressed rats presented with regular food reward after a chronic period of social isolation and defeat do not develop symptoms of depression (van der Harst et al., 2005). Similarly, in pigs (*Sus scrofa domesticus*), the presentation of a cue associated with a positive event (i.e. enriched enclosure) induces an increase in play behaviour and reduces stress-related weaning (i.e. aggression; Dudink et al., 2006). These findings suggest that previous or current experiences can modulate anticipatory behaviour.

Anticipatory behaviour can also be used to assess an animal's perception of the reward properties of a stimulus (van der Harst and Spruijt, 2007). In rats, the anticipatory response to positive conditions (i.e. locomotion and exploration) differs from the response to negative and control conditions, supporting the hypothesis that responses are affected by the valence of the stimuli (van der Harst et al., 2003). In mink (*Neovison vison*), a general increase in activity level was observed when anticipating a food reward, while an increase in freezing behaviour was observed when anticipating being trapped in a cage (Hansen and Jeppesen, 2004). By contrast, in chicks (*Gallus gallus domesticus*), recent work on anticipatory behaviour in response to different reward properties (i.e. food, soil substrate, and no reward) found that these animals were more active regardless of the nature of the stimuli (McGrath et al., 2016).

Overall, although most findings indicate that anticipation can be quantified by using levels of activity and total occurrence or transition of behavioural elements displayed, some might be specific to the species under consideration (Spruijt et al., 2001; van den Bos et al., 2003; Boissy et al., 2007). For this reason, it is important to map the specific behaviours that the species investigated display in response to negative and positive events, in order to evaluate the potential use of the anticipatory behaviour paradigm to capture their emotional states. In addition, the assessment of more than one parameter to measure anticipatory responses (e.g. behaviours, as well as physiological indices and vocalisations) allows a better identification of the subjective perception of the events (Mendl et al., 2010; Briefer et al., 2015; Perry et al., 2016).

Goats (*Capra hircus*) represent an ideal model to investigate anticipatory behaviour. They have the essential cognitive prerequisites to show this kind of behaviour, such as object permanence and the ability to associate two events temporally (Nawroth et al., 2015). Goats also have excellent visual discriminative abilities and long term memory for complex tasks (Langbein et al., 2004; Briefer et al., 2014). The behaviours, physiology and vocalisations of goats are affected by contexts differing in emotional valence (i.e. positive and negative) and arousal (higher and low intensity; Briefer et al., 2015). Moreover, recently, it was shown that goats are able to discriminate calls with different valences, as displayed by their behavioural and physiological reaction to these calls (Baciadonna et al., 2019). The aim of this study was to investigate the behavioural, physiological and vocal responses of goats when anticipating positive, negative and neutral events in order to determine the key parameters that allow us to identify different emotions.

2. Methods

2.1. Subjects and experimental set-up

The study was carried out at Buttercups Sanctuary for Goats, Kent, UK (www.buttercups.org.uk). In total, 30 adult goats (15 females and 15 castrated males) that had been at the sanctuary for at least one year were tested from May to September 2014. The animals at the sanctuary are habituated to human presence. Employees and volunteers provide routine care for the animals. During the day, all goats are released together into one of two large fields. At night, they are kept indoors in individual or shared pens with straw bedding, within a larger stable complex. Goats have ad libitum access to hay, grass (during the day) and water, and are also fed with commercial concentrate in quantities related to their health condition and age. Animals receive fruits and vegetables on a daily basis.

The experimental enclosure was set up in an open field, which is part of the normal daytime range of the goats. It consisted of an arena 7 m long and 5 m wide (**Figure 1**). Access to the arena was via a door placed in the middle of the waiting pen partition. The waiting pen was used to prepare the goats for the testing procedure (i.e. placing and adjusting the device to record physiological activity on the thorax of the subject and checking that the ECG trace was clearly visible on a laptop). A small partition was built within the waiting pen, on the right side, in order to provide space for Experimenter 1. Experimenter 2 remained outside the arena on the left side.

2.2. Equipment used for data collection

Physiological measures (heart rate and heart-rate variability) were recorded using a wireless, non-invasive device, fixed to a belt attached around the goat's thorax (MLE120X BioHarness Telemetry System, Zephyr Technology Corporation, Annapolis, MD, USA.). All tests were video-recorded using a Sony DCR-SX50E camcorder for behavioural analyses. Vocalizations were continuously recorded during the tests using a Sennheiser MKH-70 directional microphone (frequency response 50 - 20 000 Hz; max SPL 124 dB at 1 kHz), connected to a Marantz PMD-661 recorder (sampling rate: 44.1 kHz).

2.3. Habituation

The day before starting the habituation phase, a small patch of hair (approx. 7 cm X 15 cm) was clipped in order to increase the contact between the skin and the electrodes and thus improve the quality of the signal. To familiarise the animals with the experimental enclosure, each goat was individually placed in the arena twice over two consecutive days (Baciadonna et al., 2016). The experimenter approached the goats in the waiting pen and fixed the BioHarness belt around their thorax, before letting them freely explore the arena for 10 min.

2.4. Conditions and procedure

A classical conditioning paradigm was used to associate a conditioned stimulus (CS) to an unconditioned stimulus (US). In order to measure the conditioned response (CR) between the end of the CS and the onset of the US, the delay between the CS and US was gradually increased over a period of 11 days, from 20 s to 5 min (**Table 1**). Subjects were tested twice per day (i.e. two consecutive trials for each time delay), in order to strengthen the association between the CS and US. Before starting the association procedure, the behaviour and physiology of the goats were recorded for 5 min. These measurements served as a baseline, within each condition, for which no association between the US and CS was yet established.

Goats were allocated to three different condition groups of ten subjects each. In the control condition, goats received only the CS, which was not paired with either positive or negative US. In the positive condition, a rectangular plastic box with highly palatable food (mix of apple and carrots; approx. 70-80 g) was provided at the end of the delay. In the negative condition, a transparent plastic box of unreachable food (mix of apple and carrots; approx. 70-80 g) was shown. In this condition, goats could smell the food through small holes created on the lid surface, but could not access it.

During testing, goats were individually placed inside the waiting pen in order to attach the BioHarness belt and ensure that a clear ECG trace could be obtained. Access to the central arena was then provided by opening a sliding manual operating door. After 1 min inside the central arena, one experimenter (Experimenter 2) whistled and made two click noise using a dog training clicker (WhizzClick™). During the positive and negative conditioning, after the planned delay (range between 20 s and 5 min), a second experimenter (Experimenter 1; concealed behind a screen at the far end of the waiting pen) slotted inside the arena a small rectangular plastic box containing the accessible or inaccessible food, according to the test condition. In the positive condition, the goats then had the time to eat all the food from the container, and in the negative condition, enough time was allocated to give the opportunity to the subject to approach and smell the inaccessible food. At the end of the first daily trial, the goat was guided towards the waiting pen and prepared for the following trial (same delay time interval as the previous trial). The BioHarness belt was readjusted and the ECG trace was checked again (time interval less than 2 min). Afterwards, the experimenter opened the sliding manual operating door again to provide access to the central arena and the same procedure previously described was repeated. At the end of the second trial, the goat was guided back to the waiting pen, the BioHarness was removed and the subject was released back to the rest of its herd. Because the suitable testing time at the sanctuary is limited to 5-hour periods each day, the subjects in the positive condition and half of sample in the control condition were tested in the first 14 days. Subjects in the negative condition and the other half of the sample in the control condition were tested in the following 14 days.

2.5. Physiological measures

The continuous ECG trace was visualised, transmitted and stored in real time to a laptop (ASUS S200E). LabChart software v.7.2 (ADInstrument, Oxford, U.K.) was used to visualise and analyse the data, i.e. to extract the heart rate and heart-rate variability (root mean square of successive interbeat interval differences; RMSSD). When a good-quality signal of the heartbeat was clearly visible on the ECG trace, heartbeats over three 10 s sections (beginning, i.e. after the whistle and clicker sounds; middle; and end, i.e. when the plastic box was slotted inside the arena) were extracted and analysed for each trial. The mean ± SE duration of analysed sections for all conditions were: control, 10.37 ± 0.05 s; negative, 10.49 ± 0.06 s; and positive, 10.50 ± 0.07 s. The software provided the averages of the heart rate (beats/min). RMSSD was then calculated from the extracted individual intervals between heartbeats (ms).

2.6. Behavioural measures

The behavioural measures selected were based on those shown by previous studies to be clearly linked to emotions in goats (Briefer et al., 2015). The following measures were scored from the start of the sound to the end of the planned time: activity time (i.e. at least two legs moving) number of rapid head movements (i.e. < 1 s in any direction). The time spent with the ears forwards (i.e. tip of the ear pointing forwards), backwards (i.e. tip of the ear pointing backwards), horizontal (i.e. ears in parallel) or asymmetrical (i.e. right and left ears positioned in a different way) was recorded. Behaviours were scored using CowLog software (Hänninen and Pastell, 2009).

2.7. Call rate and vocal parameters

The calls produced between the start of the clicker and the end of the planned time were scored. Furthermore, vocalizations were imported into a computer at a sampling rate of 44.1 kHz and saved in WAV format at 16-bit amplitude resolution. Analyses were conducted using PRAAT (Boersma and Weenink, 2009). Each call was visualized on spectrograms using the following settings: Fast Fourier Transform (FFT) method, window length = 0.03 s, time steps = 1000, frequency steps = 250, Gaussian window shape, dynamic range = 60 dB. All good-quality calls recorded during each condition were selected (total: 145 calls; 103 for the positive condition, 13 for the negative condition and 29 for the control condition). Non-consecutive calls produced by individuals were selected to avoid pseudoreplication(Briefer et al., 2015).

The vocal measures selected were based on a previous study (Briefer et al., 2015). Using a custom-built program in PRAAT, vocal measures linked to both the source and the filter were extracted (Reby and McComb, 2003; Charlton et al., 2009). The settings to extract the acoustic analyses must be adjusted individually(Briefer and McElligott, 2011), because contact calls produced by goats show considerable variation, especially for the parameters linked to the fundamental frequency (F0). For this reason, the settings were adapted to each subject. Source-related vocal parameters were measured by extracting the F0 contour of each call using a cross-correlation method ([Sound: To Pitch (cc) command], time step: 0.01 s, pitch floor: 90 - 200 Hz, pitch ceiling: 200 - 350 Hz). The following vocal parameters were extracted from each F0 contour: the mean F0 across the call (F0mean), the frequency at the start (F0start) and at end (F0end) of the call, the minimum (F0min) and the maximum (F0max) F0 across the call. To characterize F0 variation along the call, the mean peak-to-peak variation of each F0 modulation (FMextent) was extracted. Filter-related vocal parameters (formants) were measured by extracting the contour of the first four formants of each call using linear predictive coding analysis (LPC [Sound: To Formant (burg) command]: time step: 0.01 s, maximum number of formants: 4 - 5, maximum formant: 3000 - 5500 Hz, window length: 0.05 s). Each LPC output computed with PRAAT was visually inspected along with the spectrogram to control whether the formants were precisely detected. Spurious values were deleted and we corrected for octave jumps, when necessary. For each call, the mean values of the formants (F1, F2, F3 and F4mean) were then calculated. The intensity characteristics were examined by extracting the intensity contour of each call [Sound: To Intensity command]. Mean peak-to-peak variation of each amplitude modulation was considered (AMextent). Finally, the duration of the call was computed directly from the spectrogram.

2.8. Data analyses

The baseline, the two trials in which the delay between the US and CS was of 2:30 min (Middle phase), and the two trials in which the delay between the US and CS was of 5 min (End phase), were selected for the physiological and behavioural analyses. Because the Middle and End phase consisted of two trials, an average between the two trials was computed. The duration of data collection was not identical amongst the Baseline (5 min), Middle (2.30 min) and End (5 min) phases. For this reason, activity time and the time spent with the ears forwards, backwards, horizontal or asymmetrical (i.e. right and left ears positioned in a different way) were calculated and expressed in sec/min. The number of rapid head movements and call rate were calculated and expressed as events/min. For the vocal parameters, a different approach was necessary. Due to the small number of vocalisations spontaneously emitted, vocalisations were combined and analysed regardless of the phases during which they were produced.

Physiological, behavioural and vocal data were analysed using linear mixed-effects models (LMM; lmer function, lme4 library; Pinheiro & Bates 2000) in R 3.0.2 (R Development Core Team, 2013). The models based on physiological data included heart rate or RMSSD as a response variable, and condition (Control, Positive and Negative), section (part selected from the ECG trace: at the beginning, central and end of the trial), phase (Baseline, Middle and End), sex, and interaction between condition and phase as fixed factors. The identity of the goats was included as random factor, to control for repeated measurements of the same subjects between sections and phases. The sex and the interaction effect between condition and phase were not retained during the model selection.

The models used to analyse the behavioural data included the behaviour (i.e. activity, head movements, vocalisations, and ear positions) as a response variable. Condition (Control, Positive and Negative), phase (Baseline, Middle, End), sex and the interaction between condition and phase were included as fixed factors. The identity of the goats was included as random factor, to control for repeated measurements of the same subjects between phases. In order to meet the model assumptions, activity time and call rate were square-root transformed, while head movement, ears backwards, ears asymmetrical and ears horizontal were log-transformed.

The models used to analyse the vocal data included the acoustic parameter (F0mean, F0start, F0end, F0min, F0max, FMexten, F1-F4mean, AMextent and Duration) as a response variable, and condition, phase (Baseline, Middle, End) and sex as fixed factors. The identity of the goats was included as random factor, to control for repeated measurements of the same subjects between phases. The interaction between condition and phase was, this time, not considered, because it was not statistically meaningful (e.g. often, only one call was available in each phase of each condition). In order to meet the model assumptions, call duration, F0end, FMextent and AMextent were log-transformed. F0max was square-root transformed.

Non-significant factors were removed one by one from the models if this did not cause any significant reduction in goodness of fit, using a standard model simplification procedure. P values were extracted by comparing the two models with and without each term, both fitted with the maximum likelihood method (ML), using a likelihood ratio test. The results are presented after model simplification. When a significant interaction effect was found, further post-hoc comparisons were performed using a Tukey test. The significance level was set at alpha = 0.05.

2.9. Ethical Note

Animal care and all experimental procedures were conducted in accordance with the guidelines of the Association for the Study of Animal Behaviour (2019). The study was approved by the Animal Welfare and Ethical Review Board of Queen Mary University of London (001/2015AWERBqmul). The tests were non-invasive and did not cause any distress behaviour (goats were monitored throughout the tests using the ECG trace displayed in real time). None of the goats had to be removed from the study because of distress.

3. Results

3.1. Physiology

Heart rate was affected by the interaction between the test condition (control, negative and positive) and the phase (delay between sound and reward; Baseline, Middle and End; $\chi^2_{(4)} = 28.14$, $p < 0.0001$; **Figure 2a**). Post hoc analyses revealed a reduction in the heart rate from the Baseline (mean bpm: 115.63 ± 2.76) to the Middle phase (mean bpm: 107.74 ± 2.59; $z = -3.68$, $p = 0.005$) and from the Baseline to the End phase (mean bpm: 102.86 ± 1.49; $z = -5.87$, $p < 0.001$) in the control condition. Within the negative condition, heart rate decreased from the Baseline (mean bpm: 104.83 ± 2.45) to the End phase (mean bpm: 94.74 ± 1.95; $z = -4.45$, $p < 0.001$). Post-hoc analyses also revealed that the heart rate was higher in the End phase of the positive condition ($z = -3.97$, $p = 0.001$) compared to the End phase of the negative condition. All the other two-by-two comparisons were not significant (*p*

> 0.16). An effect of sex was also found ($\chi^2_{(1)}$ = 6.66, p = 0.009). Females had higher heart rates (mean bpm: 111.11 ± 10.06) compared with males (mean bpm: 101 ± 1.37). The sections selected to analyse the heart rate (10 s at the beginning, middle and end of the ECG trace during each session), did not differ ($\chi^2_{(2)}$ = 5.13, p = 0.07). Heart-rate variability (**Figure 2b**) was not significantly affected by condition ($\chi^2_{(2)}$ = 4.58, p = 0.10), phase ($\chi^2_{(2)}$ = 1.09, p = 0.57), or section ($\chi^2_{(2)}$ = 1.32, p = 0.51).

To summarise, heart rate in the control and negative conditions decreased over the phases, whereas this measure remained stable in the positive condition. When comparing the negative and positive conditions, the heart rate differed only in the End phase, with higher values observed in the positive condition.

3.2. Behaviour

The analysis of activity time revealed an effect of the phase ($\chi^2_{(2)}$ = 12.92, p = 0.0015; **Figure 3a**). Post-hoc analyses showed that activity time decreased from the Baseline (mean sec/min: 8.60 ± 1.09) to the End phase (mean sec/min: 5.08 ± 0.65; z = -3.72, p < 0.001), across all conditions. Activity time also decreased from the Middle phase (mean duration per min: 7.21 ± 0.76) to the End phase (mean sec/min: 5.08 ± 0.65; z = 2.65, p < 0.021). Differences between the Baseline and Middle phase were only marginally significant (z = - 1.09, p = 0.053). In addition, an effect of condition was found ($\chi^2_{(2)}$ = 20.78, p < 0.0001; **Figure 3b**). Post-hoc analyses showed that activity time was higher in the positive (mean sec/min: 9.57 ± 0.96) than the control condition (mean sec/min: 5.03 ± 0.64; z = 4.48, p < 0.001) and negative condition (mean sec/min: 6.32 ± 0.85; z = 2.94, p = 0.008). By contrast, the activity level did not differ between the control and negative conditions (z =

1.39, p > 0.05). All other comparisons included in the post-hoc analyses were not significant ($p \geq 0.35$). Activity level differed between males and females ($\chi^2_{(1)} = 5.82$, $p = 0.015$). Females were more active (mean sec/min: 7.99 ± 0.63) compared with males (mean sec/min: 6.18 ± 0.77). To summarise, goats were less active in the End phase compared with the Baseline and Middle phases. Furthermore, goats in the positive condition were more active compared with the control and negative conditions.

The analysis of rapid head movement showed a significant interaction effect between condition and phase ($\chi^2_{(4)} = 19.22$, $p < 0.0001$, **Figure 3c**). Post-hoc analyses revealed that the rate of rapid head movements increased from Baseline (mean event/min: 0.56 ± 0.12) to Middle phase (mean event/min: 1.28 ± 0.16; $z = 3.13$, $p = 0.043$) within the negative condition. Within the positive condition, the rate of rapid head movements increased from Baseline (mean event/min: 0.68 ± 0.20) to Middle phase (mean event/min: 2.90 ± 0.47; $z = 6.94$, $p < 0.001$) and from Baseline to End phase (mean event/min: 2.20 ± 0.17; $z= 5.68$, $p < 0.001$). Post-hoc analyses also revealed a higher rate of rapid head movements in the Middle phase of the positive condition (mean event/min: 2.90 ± 0.47; $z = 4.65$ $p < 0.001$) compared with the Middle phase of the control condition (mean event/min: 1.28 ± 0.39). Similarly, goats displayed a higher rate of rapid head movements in the End phase of the positive condition mean event/min: 2.20 ± 0.17) compared with the End phase of the control condition (mean event/min: 1.01 ± 0.20; $z = 3.80$, $p < 0.01$). Finally, goats showed a higher rate of rapid head movements in the Middle phase of the positive condition (mean event/min: 2.90 ± 0.47) compared to the Middle phase of the negative condition (mean event/min: 1.28 ± 0.16; $z = 3.79$, $p < 0.01$). All other comparisons included in the post-hoc analyses were not significant ($p \geq 0.15$). The number of rapid head movements performed differed between males and females ($\chi^2_{(1)} = 5.38$, $p = 0.02$). Females displayed more rapid head movements (mean event/min: 1.52 ± 0.16) compared with males (mean event/min: 1.18 ± 0.15).

To summarise, rapid head movements increased in the negative and positive conditions from the Baseline to the Middle phase, and also to the End phase for the positive condition. In addition, rapid head movements in the positive condition were higher in the Middle phase compared to the negative and control conditions, and were also higher in the End phase compared to the control condition. No difference was found between the control and negative conditions for rapid head movements.

The analysis of ears positioned forward revealed an interaction effect between condition and phase ($\chi^2_{(4)} = 18.15$, $p = 0.001$; **Figure 4a**). Post-hoc analyses showed an increase in the time spent with the ears forwards from the Baseline (mean sec/min: 10.77 ± 4.71) to the End phase (mean sec/min: 34.50 ± 4.38; $z = 7.25$, $p < 0.001$) and from the Middle (mean sec/min: 17.68 ± 3.99) to the End phase ($z = -5.14$, $p < 0.001$), within the positive condition. In addition, post-hoc analyses showed a longer time spent with the ears forward in the Middle phase of the positive condition (mean sec/min: 17.68 ± 3.99) compared with the Middle phase of the control condition (mean sec/min: 3.17 ± 1.16; $z = 4.11$, $p = 0.0012$). The time spent with the ears forward was also longer in the End phase of the positive condition (mean sec/min: 34.50 ± 4.38) compared with the End phase of the control condition (mean sec/min: 7.94 ± 2.20; $z = 7.02$, $p < 0.001$), and with the End phase of the negative condition (mean sec/min: 14.88 ± 4.96; $z = 4.41$, $p < 0.001$). All other comparisons included in the post-hoc analyses were not significant ($p \geq 0.11$). The time spent with the ears in forward position did not differ between males and females ($\chi^2_{(1)}$ = 2.21, *p* = 0.13).

To summarise the goats kept the ears positioned forward longer in the positive condition and the duration of this behaviour increased over the phases. In addition, there was a longer time spent with the ears forward in the Middle and End phases of the positive condition compared with the Middle and End phases of the control condition and with the Middle phase of the negative condition. No differences were found between control and negative conditions.

The analysis of ears positioned backwards revealed an effect of condition ($\chi^2_{(2)}$ = 7.44, *p* = 0.024; **Figure 4b**). Post-hoc analyses, showed that the time spent with the ears positioned backwards was longer in the control (mean sec/min: 5.09 ± 1.34) compared with the negative condition (mean sec/min: 1.16 ± 0.44; *z* = - 2.71, *p* < 0.018). No differences in this parameter were found between the control and positive conditions (mean sec/min: 2.72 ± 3.11; *z* = -1.83, *p* = 0.15), and between the negative and positive conditions (*z* = 1.02, *p* = 0.55). The analyses also showed no difference between phases ($\chi^2_{(2)}$ = 2.18, *p* = 0.33) and no interaction effect between condition and phase ($\chi^2_{(4)}$ = 2.32, *p* = 0.67). Additionally, there was no difference between males and females in the duration of time spent with the ears in backward position ($\chi^2_{(1)}$ = 0.18, *p* = 0.66). To summarise, in the control condition, goats had their ears positioned backwards for longer compared with the negative condition. No differences between control versus positive and between negative and positive conditions were found.

The analysis of ears positioned horizontally revealed an interaction between condition and phase ($\chi^2_{(4)}$ = 11.25, *p* = 0.023; **Figure 4c**). Post-hoc analyses showed that the time spent with the ears horizontal decreased from the

Baseline (mean sec/min: 1.58 ± 0.82) to the End phase (mean sec/min: 0.19 ± 0.09; z = -4.37, p < 0.001) of the negative condition. All the other comparisons included in the post-hoc analyses were not significant (p ≥ 0.26). Additionally, there was no difference between males and females in the duration of time spent with the ears in horizontal position ($\chi^2_{(1)}$ = 0.07, p = 0.78). To summarise, in the negative condition, the duration of time spent with the ears positioned horizontally decreased between the Baseline and the End phase.

The analysis of ears positioned asymmetrically revealed an effect of phase ($\chi^2_{(2)}$ = 7.49, p = 0.023; **Figure 4d**). Post-hoc analyses showed that this behavioural measure increased, across all conditions, from the Baseline (mean sec/min: 0.80 ± 0.22) to the End phase (mean sec/min: 1.79 ± 0.45; z = 2.84, p = 0.012). By contrast, it did not differ between the Baseline and the Middle phase (mean sec/min: 1.29 ± 0.36; z = 1.41, p = 0.33) and between the Middle and End phases (z = -1.42, p = 0.32). The analysis showed no difference between conditions ($\chi^2_{(2)}$ = 2.09, p = 0.35), and no interaction between condition and phase ($\chi^2_{(4)}$ = 6.81, p = 0.14). Additionally, there was no difference between males and females in the duration of time spent with the ears in asymmetrical position ($\chi^2_{(1)}$ = 0.31, p = 0.57). To summarise, the duration of ears positioned asymmetrically was similar across conditions, but increased between the Baseline and End phase.

3.3. Vocal parameters and call rate

The analyses of the vocal parameters did not reveal any differences between conditions. All the descriptive statistics and the results for the main factors included in the models corresponding to each parameter are reported in **Table 2**. The analyses of the call rate revealed an interaction effect between condition and phase ($\chi^2_{(4)}$ = 18.08, *p* = 0.001, **Figure 3d**). Post-hoc analyses revealed an increase in the rate of calls emitted from the Baseline (mean event/min: 0.10 ± 0.10) to the Middle phase (mean event/min: 1.56 ± 0.74; *z* = 3.69, *p* = 0.006) and from the Baseline to the End phase (mean event/min: 2.42 ± 1.09; *z* = 5.76, *p* < 0.001), within the positive condition. Goats also emitted more calls in the Middle phase of the positive condition (mean event/min: 1.56 ± 0.74) compared to the Middle phase of the control condition (mean event/min: 0.26 ± 0.26; *z* = 4.15, *p* = 0.001). Similarly, goats emitted more calls in the End phase of the positive condition (mean event/min: 2.42 ± 0.09) compared with the End phase of the control condition (mean event/min: 0.04 ± 0.03; *z*= 6.07, *p* < 0.001). Post-hoc analyses also indicated that the call rate was higher in the End phase of the positive condition (mean event/min: 2.42 ± 1.09) compared with the End phase of the negative condition (mean event/min: 0.07 ± 0.04; *z* = 4.60, *p* < 0.001). All the other comparisons included in the post-hoc analyses were not significant (p ≥ 0.46). The rate of calls emitted did not differ between males and females ($\chi^2_{(1)}$ = 2.02, *p* = 0.15). To summarise, the number of calls emitted in the positive condition increased over the phases, whereas in the control and negative conditions, the number of calls remained stable. The rate of calls emitted was higher in the Middle and End phases of the positive condition compared with the Middle and End phases of the control condition and with the Middle phase of the negative condition. No differences were found between the control and negative conditions.

4. Discussion

The aim of this study was to examine the physiological, behavioural and vocal responses of goats to the presentation of positive, negative and neutral events. At the physiological level, goats anticipating a positive event had higher heart rates compared with the control and the negative conditions, while no difference in heart rate variability was found. Accordingly, in the positive condition, we found a general increase over the phases in activity time, rapid head movements and vocalisation rate. In addition, the duration of ears positioned forward was longer compared to the control and negative conditions. Finally, in the control condition, ears were kept backwards for longer than in the negative condition. These physiological and behavioural responses suggest that the positive condition was perceived differently than the negative and control conditions. By contrast, the anticipatory response of the goats did not seem to differ when expecting a negative outcome compared to the control condition. Despite the challenges in measuring positive emotional states, which are often less intensely expressed than negative emotions (Boissy et al., 2007), the paradigm used in the present study thus appears to be effective in discriminating anticipation of a positive compared to a negative or control event. This supports the use of paradigms involving the assessment of cognitive processes influenced by emotional stimuli, such as cognitive bias and expectation of events, to measure emotional valence in animals (Kremer et al., 2020; Mattiello et al., 2019; Chincarini et al., 2018; Baciadonna and McElligott, 2015; Greiveldinger et al., 2011; Paul et al., 2005; Spruijt et al., 2001).

We used heart rate and heart-rate variability (HRV) to assess the arousal level related to anticipatory behaviour of goats that had been trained to associate a sound to a positive (palatable food), or mildly negative (inaccessible palatable food) outcome, compared to a control condition. Heart rate was higher in the positive compared to the negative condition in the End phase, when the association between the sound and the outcome was supposed to be at maximum in both conditions, following repetition over time. Therefore, the physiological data corroborate the behavioural responses that indicate higher arousal level in the positive condition compared to the negative ones. In the control and negative conditions, heart rates decreased between the Baseline and the End phase. In addition, no differences were found between these two conditions. Heart-rate variability did not show any difference in relation to the specific conditions tested. These results partly support the physiological profiles observed in horses (*Equus caballus*) anticipating a positive reward (Peters et al., 2012). Horse heart rates increased between baseline and cue presentation, whereas no differences were observed in heart-rate variability (Peters et al., 2012). However, the findings of this study are quite difficult to interpret, because the heart-rate parameters were detected using a naturalistic set-up (horses learned spontaneously to associate the caregiver with food) and therefore do not follow the systematic procedure that is normally used in an anticipatory behaviour paradigm (Peters et al., 2012). In addition, it is not possible to disentangle whether the increased heart rate observed in horses was due to the expectation of food or to the presence of the caregiver. Overall, our results confirm that heart rate is more indicative of emotional arousal than emotional valence (von Borell et al., 2007; Reefmann et al., 2009b; Briefer et al., 2015).

Heart-rate variability (measured here using RMSSD) has been suggested to be a good indicator of valence (Reefmann et al., 2009c; Zebunke et al., 2011; Quintana and Heathers, 2014; Coulon et al., 2015; Zupan et al., 2015). However, this is debated, especially when the emotional arousal of the situations faced by the animals is not controlled (Briefer et al., 2015; Travain et al., 2016). In studies where the arousal of the situations has been controlled (e.g. by comparing situations of opposite valence but similar arousal), heart-rate variability appeared not to be affected by the valence of the situations, but only by the arousal, similarly to the heart rate (Reefmann et al., 2009b; Briefer et al., 2015). Accordingly, in our study, heart-rate variability did not differ between the conditions, which were characterised by opposite valences. This lack of differences in RMSSD between conditions also suggests that the control condition induced similar arousal levels in goats as the negative condition.

Our findings suggest that activity is the most obvious parameter that can be identified in response to the announcement of a positive reward or of a mild negative event. We found that the general activity level decreased over time, and that goats in the positive condition were overall more active compared to the control and negative conditions. No difference between control and negative conditions was found. Similarly, rapid head movements and call rate were higher in the positive condition, and no differences were found between the control and negative conditions. This suggests that these are more linked to emotional arousal (higher in the positive condition) than to the valence. Based on these behavioural parameters, it is not possible to discriminate the effects of the control and negative conditions.

The position of the ears has been previously linked to the expression of emotions and suggested as a promising indicator of the perceived valence of various stimuli (Boissy et al., 2007; Reefmann et al., 2009a; Reimert et al., 2013; Proctor and Carder, 2014). In this study, the most informative position that showed differences between conditions was the duration of ears positioned forward. Goats expecting palatable food, especially towards the end of the treatment period, kept their ears positioned forward for longer than goats in the control and negative conditions. The lack of differences in this measure between the control and the negative conditions suggests, in line with a previous study on goats (Briefer et al., 2015), that ears forward in this species indicates emotional arousal more than valence. The forward position of the ears could thus indicate a general level of activity across emotional situations, or attention linked with the expectation of a reward. The duration of ears positioned backwards was longer in the control condition compared with the negative one. This particular position has been associated with discomfort and signs of negative states (Reefmann et al., 2009a; Reimert et al., 2013; Proctor and Carder, 2014). However, foxes (*Vulpes vulpes*) trained to receive positive predictable and positive unpredictable food and negative reward (i.e. being captured), showed longer duration of ears positioned backwards in the unpredictable positive and in the negative reward conditions (Moe et al., 2006). This could suggest that ears positioned backwards indicate a state of uncertainty, more than a negative emotion. However, it is important to highlight that this hypothesis is drawn upon the comparison between two rather different species, goats and foxes. The horizontal and asymmetrical position of the ears did not show any difference between conditions and therefore did not appear to be informative to establish the anticipatory profile of the goats. Whether the asymmetric ear position indicates emotion valence or more likely arousal, as the current findings suggest, is still under debate (Chincarini et al., 2018).

One of the main aims of this study was to investigate if goats would show different vocal responses to the anticipation of putative positive reward or negative outcomes compared with the control condition (Briefer et al., 2015). However, none of the vocal parameters differed between conditions. This is surprising, because goats tested in a feeding situation (i.e. positive, high arousal) that simulated anticipatory training, showed specific vocal parameters linked with emotional valence and arousal (Briefer et al., 2015). For example, the F0 range was smaller and the calls had smaller frequency modulations in the positive (anticipation of a reward) compared with the negative conditions (social isolation and food frustration). The F0mean, F0End, Q25%, Q50%, Q75% and the F1mean were more linked to arousal than valence and increased with this emotional dimension (Briefer et al., 2015). Several reasons could explain why we did not replicate these results. First, in order to have an adequate sample size of good quality calls, we selected all the calls produced during the experiment. This did not allow us to control for the effect of phase in the statistical analyses. In addition, the number of calls produced in each condition varied largely (total number of calls used for the acoustic analyses: 145 calls; 103 for the positive condition, 13 for the negative condition and 29 for the control condition) and were emitted by few goats (positive condition: six goats out of 10 and two of them emitted 84 calls out of 103; negative condition: five goats out of 10 and one goats emitted six calls out of 13; control condition: three goats out of 10 and one goats emitted 17 calls out of 29). However, while it seems that the different treatments did not affect the structure of calls, they had, as mentioned above, a very strong effect on the goats call rate.

Overall, our results suggest that it is essential to assess whether the conditions designed to induce an emotional change are effective in inducing such change, and whether they trigger emotions of different valence based on the assessed parameters. Our results suggest that it is important to note that designing a control situation that does not induce a fluctuation of the core affect space is a challenge. Assessing emotions in non-human animals is still difficult and requires using an array of strategies to ensure detection of all their components and reliability. Furthermore, the use of "iceberg indicators" (i.e.

different measurers of welfare; Collins et al., 2015) has been suggested as a good way to improve the overall welfare assessments and its practicability. The validation of experimental protocols for the detection and mapping of the different components of emotions is crucial to promote a good welfare balance that takes into account the life history of an individual (Spruijt et al., 2001; Boissy et al., 2007; van der Harst and Spruijt, 2007).

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Conflict of interest

The authors declare that they have no conflict of interest

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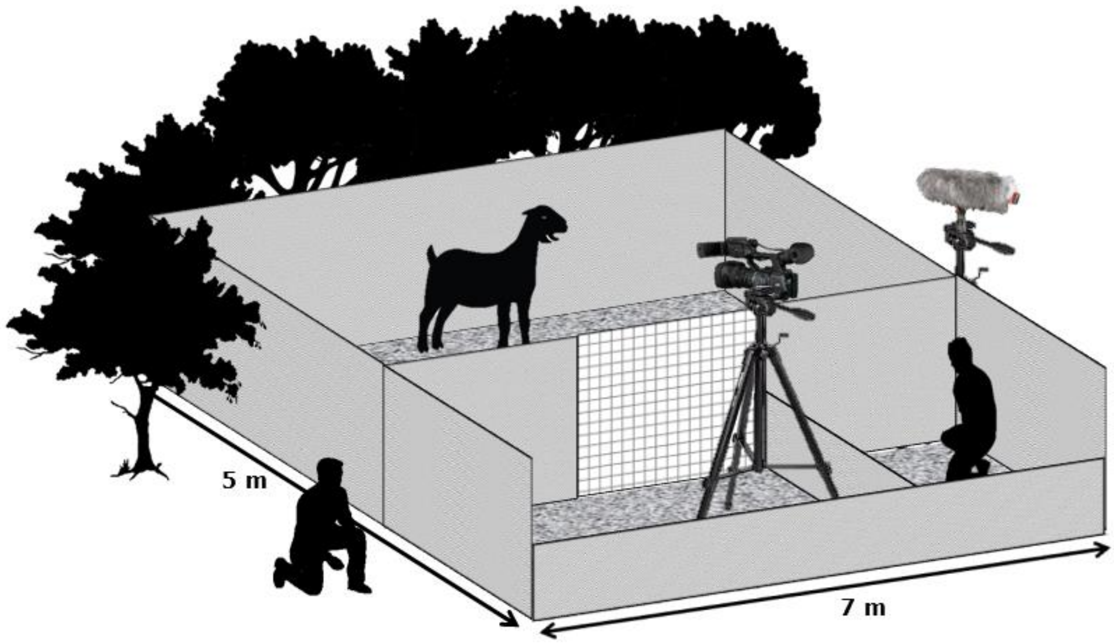

Figure 1. Graphical representation of the experimental enclosure. The experimental apparatus used (7 m x 5 m) consisted of a waiting pen and a central arena. A manually operated sliding door provided access from the waiting pen to the central arena. Experimenter 1 was outside on the left side of the arena to make the whistle and clicker sounds. Experimenter 2 was positioned in a partition built in the waiting pen. Experimenter 2 was responsible for slotting a transparent box filled with food (positive condition) or a box filled with food but inaccessible to consume (negative condition) inside the central arena, and check the ECG trace displayed on a laptop. The entire experiment was recorded using a camcorder placed in the waiting pen. Vocalisations emitted were also recoded using a microphone placed on a tripod outside the arena on the right side.

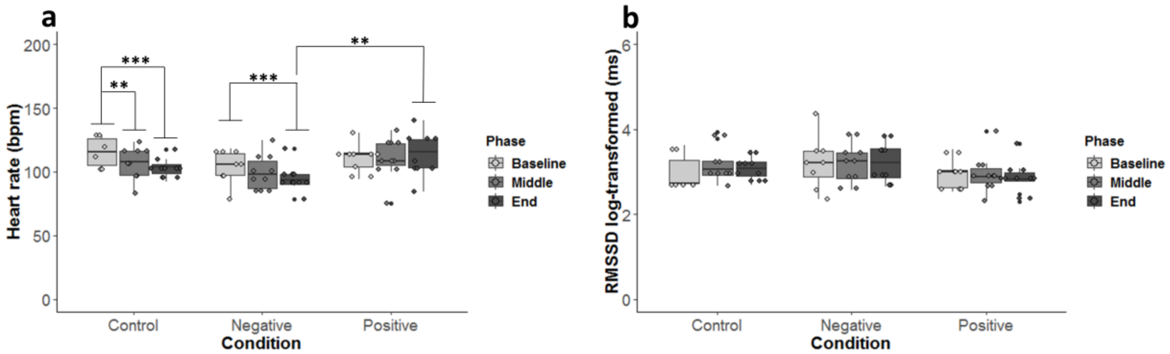

Figure 2. Heart rate and heart-rate variability. (**a**) Hear rate and (**b**) RMSSD as a function of the conditions and phases; box plot: the horizontal line shows the median, the box extends from the lower to the upper quartile and the whiskers to the interquartile range above the upper quartile (max) or below the lower quartile (min); solid diamonds indicate each individual goats and black solid dots indicate outliers. Heart rate (**a**) was affected by the condition and phase ($\chi^2_{(4)}$ = 28.14, p < 0.0001). Heart-rate variability (**b**) did not differ between condition ($\chi^2_{(2)}$ = 4.58, p = 0.10) and phase ($\chi^2_{(2)}$ = 1.09, p = 0.57). *** p < 0.001; ** p < 0.01; * p < 0.05.

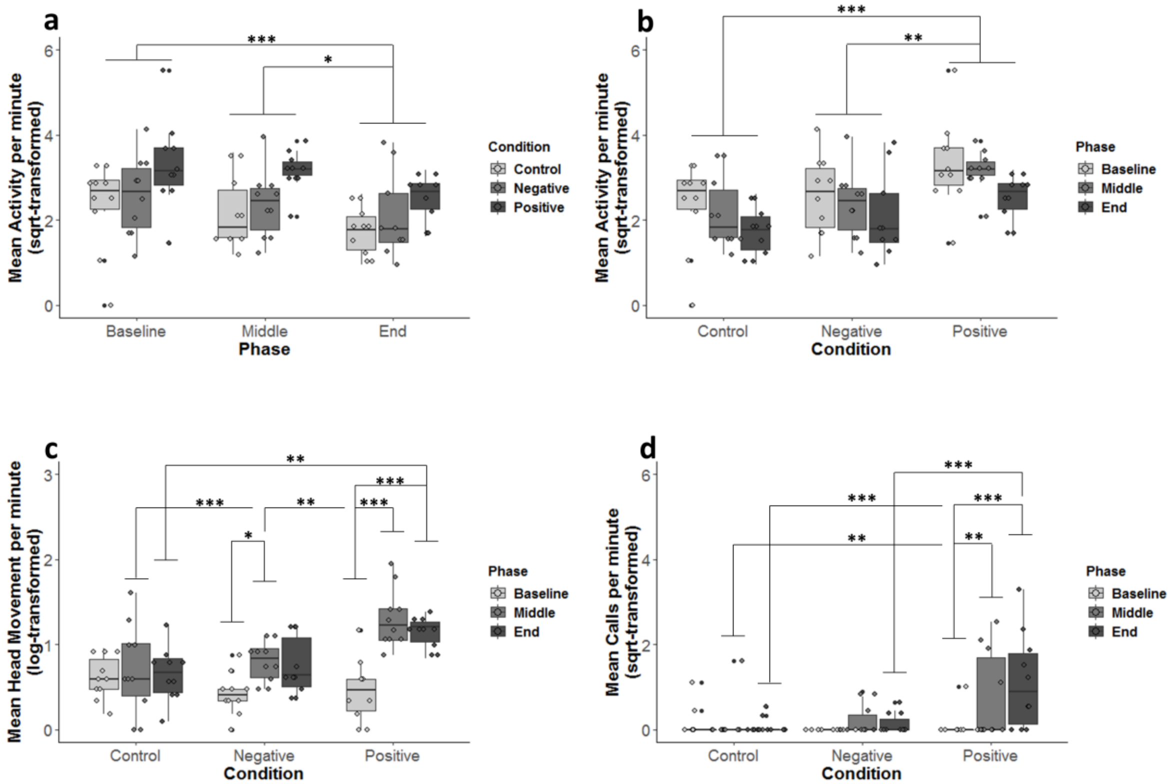

Figure 3. Activity, rapid head movement and call rate. (**a, b**) Activity, (**c**) Head movements, and (**d**) Call rate as a function of the conditions and phases; box plot: the horizontal line shows the median, the box extends from the lower to the upper quartile and the whiskers to the interquartile range above the upper quartile (max) or below the lower quartile (min); solid diamonds indicate each individual goats and black solid dots indicate outliers. The time spent in activity differed between phases ((**a**) $\chi^2_{(2)}$ = 12.98, *p* = 0.0015); and across conditions ((**b**) $\chi^2_{(2)}$ = 20.78, *p* < 0.0001); The rate of rapid head movements differed between conditions and phases ((**c**) interaction effect: ($\chi^2_{(4)}$ = 19.22, *p* < 0.0001); (**d)** The call rate differed between conditions and phases ((**d**) interaction effect: $\chi^2_{(4)}$ = 18.08, *p* = 0.001). *** *p* < 0.001; ** *p* < 0.01; * *p* < 0.05.

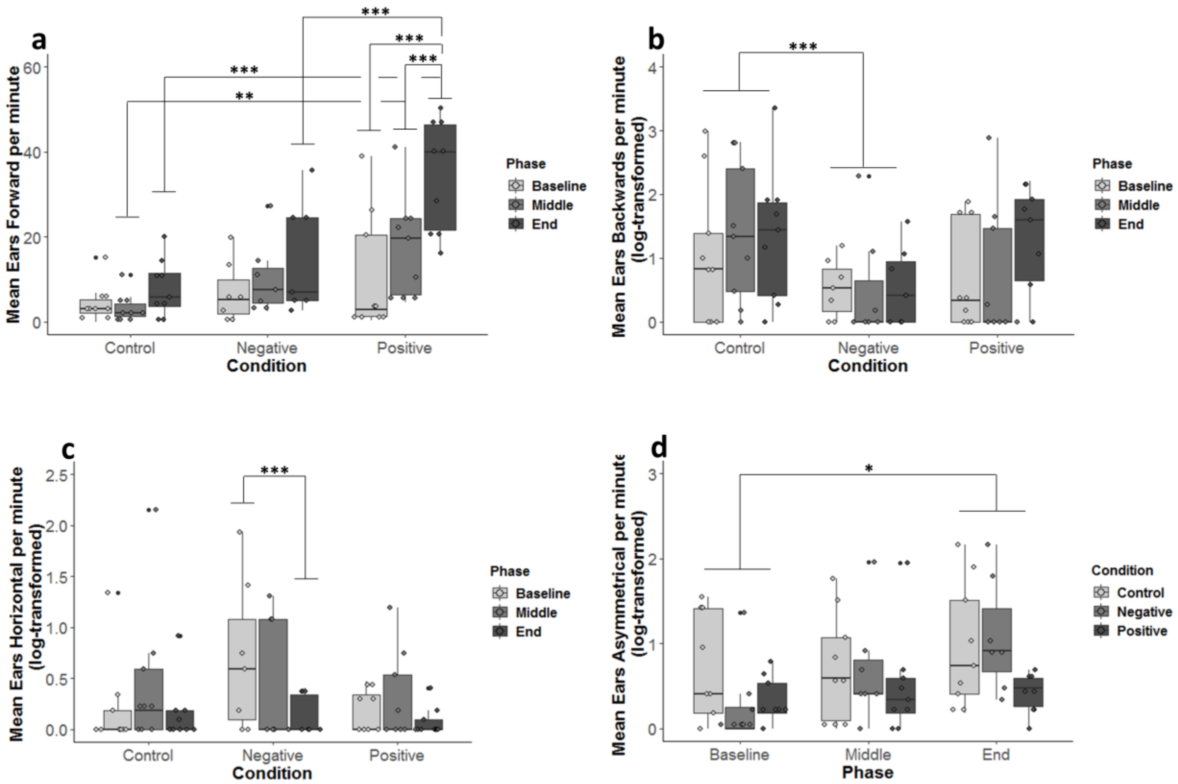

Figure 4. Ear positions. (**a**) Ears forwards, (**b**) Ears backwards, (**c**) Ears horizontal, and (**d**) Ears asymmetrical as a function of the conditions and phases; box plot: the horizontal line shows the median, the box extends from the lower to the upper quartile and the whiskers to the interquartile range above the upper quartile (max) or below the lower quartile (min); solid diamonds indicate each individual goats and black solid dots indicate outliers. The time spent with the ears positioned forward differed between conditions and phases ((**a**) interaction effect: $\chi^2_{(4)} = 18.15$, $p = 0.001$); The time spent with the ears positioned backwards differed between conditions ((**b**) $\chi^2_{(2)} = 7.44$, $p < 0.024$); The time spent with the ears horizontal differed between conditions and phases ((**c**) interaction effect: $\chi^2_{(4)} = 11.42$, $p = 0.023$); The time spent with the ears asymmetrical differed between phases ((**d**) $\chi^2_{(2)} = 7.49$, $p = 0.023$). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

Table 1. Anticipatory behaviour procedure. Cases in bold and grey (Baseline, Middle and End) indicate the trials used for the statistical analyses. Trail 0 (Baseline) was not repeated whereas Trial 1 to Trial 11 were repeated twice within a day to strengthen the association between the sound and the type of reward.

No association between US[1] and CS[2] (no repetition)	Delay between US and CS (each trial repeated twice on the same day)										
Trial	Trial										
0	1	2	3	4	5	**6**	7	8	9	10	**11**
Baseline						**Middle**					**End**
5 min	20 s	40 s	60 s	1.3 min	2 min	**2.3 min**	3 min	3.3 min	4 min	4.3 min	**5 min**

[1] US Unconditioned stimulus
[2] CS Conditioned stimulus

Table2. Descriptive statistics and results of each vocal parameter considered.

Acoustic Parameters						
		Condition				
	Control	**Negative**	**Positive**			
	Mean ±ES	**Mean ±ES**	**Mean ±ES**	**Factor**	X^2	***P***
F0mean (Hz)	216.91 ± 9	241.35 ± 18.26	275.99 ± 4.22	Phase	0.85	0.36
				Condition	0.18	0.91
				Sex	0.15	0.69
F0start (Hz)	204.47 ± 6.90	214.15 ± 12.99	253.20 ± 5.48	Phase	0.21	0.63
				Condition	0.05	0.97
				Sex	0.70	0.40
F0end (Hz)	210.01 ± 10.56	237.23 ± 17.14	262.81 ± 4.60	Phase	0.09	0.75
				Condition	0.14	0.93
				Sex	3.32	0.06
F0min (Hz)	189.28 ± 8.30	205.98 ± 13.50	241.89 ± 4.95	Phase	0.67	0.41
				Condition	0.09	0.95
				Sex	2.77	0.09
F0max (Hz)	236.69 ± 9.91	261.15 ± 19.86	292.65 ± 4.01	Phase	0.67	0.41
				Condition	0.19	0.90
				Sex	0.01	0.89
FMextend (Hz)	28.72 ± 2.50	30.93 ± 5.06	32.37 ± 1.95	Phase	0.005	0.94
				Condition	2.05	0.35
				Sex	0.27	0.59
F1mean (Hz)	765.65 ± 10.27	770.59 ± 24.19	725.03 ± 7.78	Phase	1.12	0.28
				Condition	2.28	0.31
				Sex	3.39	0.06
F2mean (Hz)	1469.42 ± 18.69	1545.03 ± 38.99	1505 ± 9.76	Phase	0.54	0.46
				Condition	1.69	0.42
				Sex	1.21	0.26
F3mean (Hz)	2546.20 ± 10.25	2510.25 ± 18.94	2513.36 ± 10.01	Phase	1.16	0.20
				Condition	0.14	0.92
				Sex	2.18	0.

Measure						
		Condition				
	Control	**Negative**	**Positive**			
	Mean ±ES	**Mean ±ES**	**Mean ± ES**	**Factor**	X^2	***P***
F4mean (Hz)	3312.21 ± 13.39	3327.30 ± 31.16	3399.30 ± 10.62	Phase	0.38	0.53
				Condition	2.68	0.26
				Sex	2.69	0.10
AMextent (dB)	8.24 ± 0.78	11.95 ± 0.82	15.24 ± 0.75	Phase	0.23	0.62
				Condition	4.30	0.11
				Sex	0.05	0.82
Duration (s)	0.84 ± 0.03	0.75 ± 0.01	0.70 ± 0.03	Phase	0.16	0.68
				Condition	5.2	0.07
				Sex	0.35	0.54