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# ACOUSTIC CHARACTERISTICS OF VOCALISATIONS EMITTED BY THE DOMESTIC RABBIT (*ORYCTOLAGUS CUNICULUS*) DURING COPULA EJACULATION AND ELECTRO-EJACULATION WITH OR WITHOUT ANAESTHESIA

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**Abstract:** Vocalisations can be used as reliable indicators of pain, but little information is available in rabbits, where acoustic tools for farming environments can be used for welfare judgements. The aim of this study was to compare vocalisations produced during copula ejaculation and electro-ejaculation (EE), with or without general anaesthesia, in domestic rabbits. Vocalisations of nine New Zealand white adult males were digitally recorded. The number of males vocalising and vocal characteristics including high, low, maximum and fundamental frequencies and duration of the vocalisations were analysed. There were no differences in the number of males vocalising or any vocalisation parameter between the 1st and 2nd ejaculation while copulating, even though the fundamental frequency increased in all males in the 2nd ejaculation ($P$=0.008). More males vocalised while mating than while being electro-ejaculated ($P$=0.03), and all vocalisation parameters were greater during EE than while mating ($P$=0.004). The use or not of anaesthesia during EE did not modify any of the parameters evaluated. It was concluded that: 1) more males vocalised during copula ejaculation than while being electro-ejaculated; 2) bio-acoustic analysis allowed us to identify aversive utterance vocalisations, which are characterised with higher frequencies, that those from non-aversive stimulus; and 3) at least with the anaesthetic combination and the responses studied, anaesthesia had no effect on the acoustic characteristics of the vocalisation emitted during EE in rabbits.

**Key Words:** animal welfare, calls, painful procedures, copulation, mating, rabbit.

## INTRODUCTION

Electro-ejaculation (EE) involves the application of electrical stimuli through an electrode inserted into the rectum connected to a current modulator controlling the intensity of voltage, stimulating the muscles and the male sex glands and encouraging the ejaculation. In several species, this procedure has been reported to cause pain and stress (rams: Orihuela *et al.,* 2009a, b; Damián and Ungerfeld, 2011; bucks: Abril-Sánchez *et al.,* 2018). In humans, EE without anaesthesia is painful (Ohl, 1993), so its application to animals raises welfare concerns. The use of general anaesthesia in rams (Orihuela *et al.,* 2009a) or sedation in bucks (Abril-Sánchez *et al.,* 2018) are recommended to decrease the welfare concerns.

Among other pain indicators, vocalisations seem to be reliable signs, particularly those induced by EE (Damián and Ungerfeld, 2010, Aguirre *et al.,* 2015). Moreover, Fumagalli *et al.* (2015) reported that pampas deer (*Ozotoceros bezoarticus*) males vocalise during EE even under general anaesthesia, and those vocalisations are closely associated with application of electrical pulses. On the other hand, rabbits vocalise during mating, vocalisations that are consequence of non-aversive emotions. Therefore, it may be expected that the characteristics of the sound differ in both situations, even if those differences cannot be easily perceived by the human ear.

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The study of the spectrogram of animals' vocalisations is a reliable method to identify and differentiate some characteristics of animal communication (Seyfart and Cheney, 2003). In this sense, acoustic signals might be indicators of stress or comfort (Dawkins, 1998), as has been studied in other farm animals (Watts and Stookey, 2000, Manteuffel *et al.,* 2004, da Silva Cordeiro *et al.,* 2013). However, to the best of our knowledge there is no similar information in rabbits. Furthermore, current tools to assess pain in rabbits are poor and more reliable methods are required; the study of bio-acoustic analysis may thus be an interesting alternative.

Considering all this information, the aim of this study was to compare the vocalisations produced during copula ejaculation and EE, with or without general anaesthesia, in the domestic rabbit.

## MATERIAL AND METHODS

All animals were handled humanely throughout the study, and their care and all experimental treatments complied with norm NOM-062-ZOO-1999 of Mexico's Department of Agriculture, Ranching, Rural Development, Fishing and Alimentation, for animal-based experimentation. The study was approved by the Internal Committee for the Care and Use of Experimental Animals at the Universidad Autónoma del Estado de Morelos, México (UAEM).

The study was conducted at the UAEM, Mexico, located at 18° 37' N and 99° 19' W, with an altitude of 1965 m above sea level. All animals were housed in individual commercial wire cages (60×90×40 cm), with controlled light (14 h light: 10 h dark) and natural temperature (18-28°C), and fed with a commercial concentrate with 15.5% of protein (Purina rabbit pellets, Mexico, Mexico) and water *ad libitum*.

### *Animals*

Nine sexually mature (10-12 mo old) sexually active, New Zealand white male rabbits (body weight: 3.0±0.5 kg; body mass index: 2.5±0.1, mean±standard error), were used. All males were tested on three experimental dates separated by 8 d. Each experimental day, three rabbits were tested in each treatment, overcrossing them in the following testing date, so that each rabbit was finally tested in the three different conditions. All tests were conducted in the morning, between 08:00 and 10:00 h, with a temperature ranging within 20 and 26°C.

### *Copula ejaculation*

Rabbits were assessed individually in random order in a circular wire mesh pen (1.5 m diameter), with concrete floor, with a female in oestrus that was changed for each male. A male was placed in the mating arena 5 min before introducing the sexually receptive female. Each test lasted until males achieved two successive ejaculations. The pen was washed between tests to eliminate odours. All tests were audio-recorded locating the microphone at approximately 20 cm from the pen and 10 cm above the floor.

### *Electro-ejaculation*

Electro-ejaculation was performed with or without anaesthesia. The anaesthesia was applied according to Donovan and Brown (1998): 50 mg/kg of ketamine (Anesket, Pisa Agropecuaria, Mexico, Mexico) and 10 mg/kg of xylazine (Procin, Pisa Agropecuaria, Mexico, Mexico) intra-muscular 10 min before EE. Each rabbit was checked every 2 min to assure deep anaesthesia, and then was moved to an area located 50 m away from the rest of the animals to keep them out of sight and hearing from the rest of the animals.

A CGS electro ejaculator, model 500 MI (Ratek Instruments Pty Ltd., Thornton Cr, Mitcham, Vic 3132. Victoria, Australia) sine-wave equipment was used throughout the experiment, operating at approximately 18 Hz with a fully controlled output voltage from 0 to 15 V root mean square (RMS, DC equivalent of sinusoidal waveform). The rectal probe was 15 cm long and 0.8 cm wide, and had two longitudinal electrodes separated by an angle of approximately 100° of arc on the body of the probe. Carboxymethyl cellulose (O.B. Lube, Agrilabs, St Joseph, MO, USA) was applied to the probe and to the anal sphincter before insertion to minimise trauma. Pulses were applied with the manual control, allowing the operator to modify the voltage applied to the animal. Electrical stimulation was applied for intervals of 3-5 s, alternated with rest periods of similar duration. The voltage was gradually increased after each

stimulation until achieving ejaculation. The entire procedure was performed in less than 1 min. Audio recording started immediately before inserting the probe in the rectum of the animal, and finished when it was withdrawn, identifying the moment of ejaculation.

Copula ejaculations and electro-ejaculations tests were conducted in an isolated and quiet room to minimise background noise. Spectrograms were generated with adobe audition 3.0 software (Adobe Systems Inc., 2007); best quality vocalisations were selected (greatest difference in dB between the signal of interest and the background noise) of each recording. These fragments were extracted and analysed automatically using active sound analysis software (Raven pro version 1.4). A spectrogram example is included in Figure 1.

### *Analysis of vocalisations*

Vocalisations were recorded using a Telinga Twinscience Pro 5W parabolic microphone and a Fostex FR2-LE digital audio recorder. All files were recorded in uncompressed wave format (.wav) with a sampling rate of 44.1 hHz and 16-bit precision (i.e., standard CD audio quality).

High, low, maximum and fundamental frequencies were analysed, and the duration of the vocalisations was also calculated. High and low frequencies were considered the most frequent frequencies of the upper and lower part of the spectrogram, respectively, while maximum frequency was considered the most frequent frequency in the peak of the spectrogram. The fundamental was defined as the lowest frequency of a periodic waveform and the duration refers to the length of the vocalisation. All measurements were made automatically using interactive sound analysis software (Raven Pro version 1.4).

### *Statistical analysis*

Different analyses were performed: first *vs.* second ejaculation during mating; first ejaculation during mating *vs.* EE without anaesthesia; and ejaculation with *vs.* without anaesthesia. The proportion of males that vocalised was compared with the Fisher exact probability test, and when they corresponded, the number of males in which each variable increased was compared with a sign rank test. High, low, maximum and fundamental frequencies and the duration of the vocalisation were tested for normal distribution with the Shapiro-Wilk test, and then compared with a mixed model, including the treatment as the main effect, and the day as a random effect into each treatment.

## RESULTS

There were no significant differences in any parameter between the first and second ejaculation while males copulated (Table 1), but the fundamental frequency increased in all males in the second ejaculation (sign test, $P = 0.008$). More

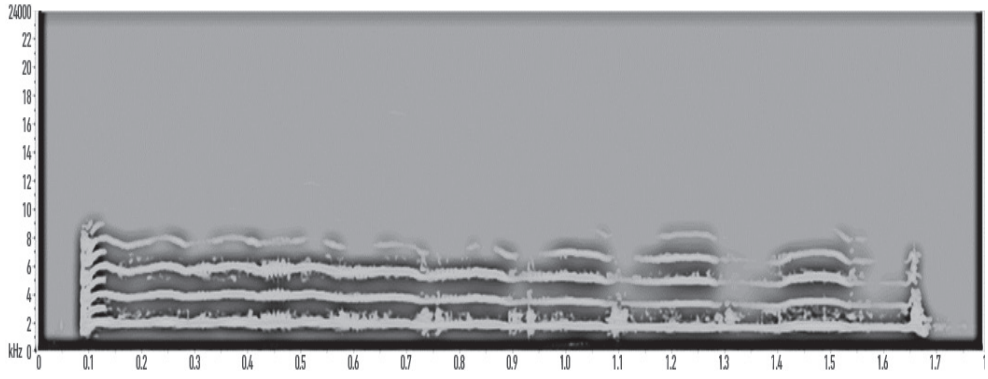


Figure 1: Example of spectrogram of rabbit vocalisation during copulation. Milliseconds and kHz are represented in axis "x" and "y", respectively.

Table 1: Number of males that vocalised, number of calls of different frequencies and duration of vocalisations (mean±standard error of mean) of rabbits during two consecutive copula ejaculations.

| | Ejaculation | | |
|---|---|---|---|
| | First | Second | Significance |
| Number of males that vocalised | 9/9 | 7/9 | NS |
| High frequency (kHz) | 4.1±0.5 | 3.6±0.6 | NS |
| Low frequency (kHz) | 0.09±0.01 | 0.08±0.02 | NS |
| Maximum frequency (kHz) | 0.35±0.11 | 0.23±0.13 | NS |
| Fundamental frequency (kHz) | 0.37±0.09 | 0.35±0.10 | NS |
| Duration (s) | 0.81±0.15 | 0.59±0.17 | NS |

mean±standard error of mean were based on the number of rabbits that vocalised.
NS: no significant.

males vocalised while mating than while being electro-ejaculated, and all the characteristics of the vocalisations, except duration (Figure 1), were significantly higher while males were electro-ejaculated than while males copulated (Table 2). The use or not of anaesthesia during EE did not modify any of the parameters evaluated (Table 3).

## DISCUSSION

More males vocalised during copula ejaculation than during EE both with or without anaesthesia, limiting the usefulness of vocalisations to study negative responses to EE in rabbits. Although almost all the characteristics studied of the vocalisations differed in both situations, the fact that many males did not vocalise during EE restricts the usefulness of the analysis to only those males that did it. However, it is interesting that the vocalisations emitted during the EE had higher frequencies in all the parameters studied than during copula ejaculations; thus, the characteristics of the vocalisations might be used as indicators of negative welfare situations. This research has practical implications, indicating that bio-acoustic analysis allows us to identify pain utterance vocalisations in rabbits, which are characterised with higher frequencies than those from other origins, offering applicable acoustic tools for farming environments where non-invasive techniques for welfare judgements are urgently needed.

All the characteristics of the vocalisations emitted during ejaculation and during EE differed, demonstrating that the vocalising pattern differs according to the factor that provoked it. When an animal becomes injured and is obviously suffering from acute pain, the respective vocalisation may reasonably be termed a pain utterance (White *et al.,* 1995; Watts and Stookey, 1999), as occurred during EE. Coinciding with the higher frequencies recorded during EE, Morton`s Motivation-Structural Rules highlighted that fearful calls are high-pitched tonal utterances (Morton, 1977; Jürgens, 1979). Even though fewer than 50% of the males vocalised during the EE, it has been demonstrated that vocalisations and other physiological stress responses are directly related to the administration of the electric pulses (Orihuela *et al.,* 2009b; Fumagalli *et al.,* 2015). It remains to be studied whether the lack of vocalising of those males during EE is related to a less common use of vocalisations to show pain in this species, or a greater tolerance of it to EE.

Table 2: Number of males that vocalised, number of calls of different frequencies and duration of vocalisations (mean±standard error of mean) of rabbits during copula ejaculation and electro-ejaculation.

| | Copula ejaculation | Electro-ejaculation | *P*-value |
|---|---|---|---|
| Number of males that vocalised | 9/9 | 4/9 | 0.029 |
| High frequency (kHz) | 4.1±0.5 | 7.2±0.7 | 0.004 |
| Low frequency (kHz) | 0.09±0.07 | 0.80±0.10 | <0.0001 |
| Maximum frequency (kHz) | 0.35±0.14 | 1.83±0.21 | <0.0001 |
| Fundamental frequency (kHz) | 0.37±0.11 | 1.83±0.16 | <0.0001 |
| Duration (s) | 0.81±0.16 | 1.23±0.24 | NS |

mean±standard error of mean were based on the number of rabbits that vocalised.
NS: no significant.

Table 3: Number of males that vocalised, number of calls of different frequencies and duration of vocalisations (mean±standard error of mean) of rabbits during electro-ejaculation with or without anaesthesia.

| | Electro-ejaculation | | |
|---|---|---|---|
| | With anaesthesia | Without anaesthesia | Significance |
| Number of males that vocalised | 4/9 | 4/9 | NS |
| High frequency (kHz) | 7.6±0.5 | 7.4±0.5 | NS |
| Low frequency k(Hz) | 0.74±0.24 | 0.80±0.24 | NS |
| Maximum frequency kHz) | 1.57±0.17 | 1.83±0.17 | NS |
| Fundamental frequency (kHz) | 1.76±0.15 | 1.83±0.15 | NS |
| Duration (s) | 1.15±0.19 | 1.23±0.19 | NS |

mean±standard error of mean were based on the number of rabbits that vocalised.
NS: no significant.

An unexpected result was the lack of any difference in the characteristics of the vocalisations emitted during EE in anesthetised or non-anesthetised males. However, we should be cautious in drawing conclusions from this, as few males vocalised during EE. The number of animals that and the frequencies of their vocalisations were similar; therefore, at least the anaesthetic combination used in this work did not block the rabbits' ability to vocalise and did not modify the characteristics of the vocalisations. There is almost no information available on the emission of vocalisations and the possible effects of anaesthesia during EE. However, it seems that the response is highly species-specific. While rams and bucks frequently vocalise when EE is applied without anaesthesia (Damián and Ungerfeld, 2011, Abril-Sánchez *et al.,* 2017), vocalisations are not emitted if the animals are anaesthetised (Abril-Sánchez *et al.,* 2018), or even subjected to epidural anaesthesia (Damián and Ungerfeld, 2010). On the other hand, pampas deer males vocalised during all the EE procedures even under general anaesthesia, and the latencies to the emission and the length and number of vocalisations were related to the voltage applied (Fumagalli *et al.,* 2015), suggesting that anaesthesia was not able to block this response.

Vocalising during mating seems to be extremely frequent in rabbits, as all of them vocalised in the first ejaculation, and most did it also during the second ejaculation. However, it is striking that there is scarce information available in this subject in this species. During ejaculation there is an acute release of dopamine (Giuliano and Allard, 2001) and serotonin (Cools *et al.,* 2008) in male rats, and both neuro-transmitters reinforce behaviours involved in non-aversive emotions (Hull *et al.,* 2004).

It was concluded that: 1) more males vocalised during copula ejaculation than while being electro-ejaculated; 2) bio-acoustic analysis allows us to identify pain utterance vocalisations, which are characterised with higher frequencies than those from non-aversive emotions; and 3) at least with the anaesthetic combination and the responses studied, anaesthesia had no effect on the acoustic characteristics of the vocalisation emitted during EE in rabbits.

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