

USE OF HIGH CONCENTRATIONS OF CARBON DIOXIDE FOR STUNNING RABBITS REARED FOR MEAT PRODUCTION

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Abstract: An investigation was performed to determine whether high concentrations of carbon dioxide (CO₂) at 70-98% in atmospheric air are a suitable alternative for stunning rabbits compared to conventional approaches such as electronarcosis. Aversion to the gas and efficacy in causing prolonged unconsciousness and death were studied in a total of 480 rabbits by means of behavioural parameters, physiological indicators (presence of rhythmic breathing and corneal reflex) and electroencephalography (EEG, brain function). The use of any of the 4 studied concentrations of the gas caused more nasal discomfort and vocalisations than the use of atmospheric air ($P<0.001$). EEG activity confirmed that loss of posture is a good indicator of the onset of unconsciousness in rabbits exposed to CO₂, occurring earlier ($P<0.05$) at 90 and 98% than at 70 and 80%. Rabbits showed signs of aversion for 15 s before the onset of unconsciousness, which occurred around 30 s after the beginning of the exposure to the gas, similar to species such as swine in which high concentrations of CO₂ are also used for stunning. CO₂ at 80 to 98% is suggested as a reasonable concentration range to induce a long state of unconsciousness and death in rabbits, while 70% CO₂ is not recommended because it requires too long duration of exposure (more than 360 s) to ensure effectiveness. Despite the advantages in terms of pre-stun handling and irreversibility, CO₂ is not free of animal welfare concerns. In consequence, a debate is necessary to ascertain if CO₂ can be considered a suitable alternative to stun rabbits, considering the advantages and drawbacks cited, quantified in the present study as 15 s of aversion (nasal discomfort and vocalisations) before losing posture.

Key Words: animal welfare, aversion, behaviour, CO₂, rabbits, stunning.

INTRODUCTION

Stunning animals before slaughter is a statutory requirement in Europe (Council Regulation (EC) N° 1099/2009). Under commercial conditions, 2 main methods are used to stun rabbits for meat production, namely electrical (the most used) and mechanical stunning. Both systems need to handle and restrain the animals before stunning. According to the European Food Safety Authority (EFSA, 2005), rabbits should be lifted by grasping the loose skin at the back of the neck and supported by placing the hand under the hindquarters. However, due to possible damage to the fur (in most cases it is a valuable sub-product of rabbits reared for meat production), abattoirs avoid grabbing the animals by the skin on the neck and they use legs or the ears. This latter procedure is considered painful by EFSA (2005) and is not allowed by Regulation 1009/2009. However, the faster the slaughter-line, the higher the risk could be for the animals handled this way. In addition, the animals waiting in the cages to be taken, in close contact to humans in the hoisting area, may also be subjected to stress at this time.

Alternatively, carbon dioxide stunning is used in pigs and birds, and allows the exposure of animals in groups, eliminating the shackling of conscious animals. Thus, as in other species, reducing human contact during handling

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could decrease pre-slaughter stress (Velarde *et al.*, 2000). In this stunning system, animals kept in the cages used for transport, cradles, crates or conveyors are exposed to high concentrations of CO₂ or a predetermined gas mixture contained within a well or tunnel. In comparison to electronarcosis, which provides a short period of unconsciousness (from 15 s to 30 s) before the animals begins to recover consciousness (EFSA, 2004), CO₂ offers the possibility of providing irreversible stunning by killing the animal, thus avoiding any kind of recovery by rabbits in the event of a stop in the slaughter-line or an incorrect bleeding. Furthermore, a significant improvement in the quality of the carcass (lower presence of hematomas) and meat (presence of PSE) has been found in other species when moving from electronarcosis to gas stunning (Velarde *et al.*, 2000).

However, the use of carbon dioxide to stun rabbits for meat consumption is not allowed in the EU (Regulation 1009/2009) because of its aversion in other species, such as mice, rats or swine (Raj & Gregory 1995; Smith and Harrap, 1997; Leach *et al.*, 2002; Conlee *et al.*, 2005; Velarde *et al.*, 2007; Dalmau *et al.*, 2010a). Inhalation of CO₂ at high concentrations causes irritation of the nasal mucosal membranes and lungs in rats (Peppel & Anton, 1993), where the presence of chemoceptors acutely sensitive to this gas has been described (Manning & Schwartzstein, 1995). In humans, inhalation of high concentrations of CO₂ causes irritation of the respiratory tract and a sensation of breathlessness, and in pigs CO₂ induces severe respiratory distress causing hyperventilation and breathlessness during the induction phase prior to loss of consciousness (Gregory *et al.*, 1990). Before loss of posture, pigs show vigorous head shaking (EFSA, 2004), a very deep breath through the wide-open mouth, which is indicative of the onset of breathlessness, and escape attempts (Raj and Gregory, 1996), all of them considered to be signs of aversion to the gas. In rabbits, Llonch *et al.* (2012a) indicated that general activity, nasal movements and head shaking could also be indicative of aversion in this species.

In comparison to high concentrations of CO₂, hypoxia induced by the inhalation of inert gases such as argon (Ar) or nitrogen (N₂), or combinations of N₂ with CO₂ up to 30% in atmospheric air, have been found to cause less aversion in pigs (Raj and Gregory, 1995; Raj, 1999; Dalmau *et al.*, 2010a; Llonch *et al.*, 2012b). However, Llonch *et al.* (2012a) concluded that mixtures of N₂ and CO₂ were still aversive to rabbits, and the duration of signs of unconsciousness, such as loss of posture, were lower than when high concentrations of CO₂ were used.

In 2005, the EFSA report published on stunning of rabbits concluded: "Electrical stunning is the method of choice in commercial slaughter plants. However, due to insufficient data, no recommendations can be given with regard to the magnitude, type and duration of the current required to ensure that all rabbits are adequately stunned and remain unconscious until death from bleeding supervenes. Gaseous stunning would remove the stress of handling the animals while they are still conscious and would therefore be the method of choice. However, it has not been scientifically investigated whether the induction phase might cause distress and even pain for the animal and in addition the gas concentrations and exposure needed to achieve a reliable stun are unknown. Therefore, the method cannot be recommended at this time".

The purpose of the present study is to provide this information to the public and the scientific community. As mentioned above, the 2 advantages of CO₂ stunning are a reduction in handling prior to slaughter and the possibility of ensuring that 100% of the animals after the onset of unconsciousness will never recover consciousness by killing them with the gas, something impossible to do with electronarcosis (EFSA, 2004). In addition, even in the event of finding some signs of aversion to the gas (which could be indicative of suffering and distress), as electronarcosis is not problem-free from an animal welfare standpoint, the duration of this aversion to CO₂ may be another key point to study for future debates. On the other hand, combinations of concentrations and exposure times ensuring that animals do not recover consciousness must be ensured. For instance, if the stun-to-stick (exsanguination by neck-cutting) interval is presumed to be 2 min for the last animal in a cage, the duration of unconsciousness must be at least two and a half minutes (2 min+30 s for bleeding to be effective in impeding the animal's recovery). Moreover, the capacity of the system to kill rabbits per se also needs to be assessed to ascertain the times needed to induce irreversible stunning in a high percentage of rabbits. Consequently, the possible use of CO₂ as an alternative to the existing methods for stunning rabbits must contain studies of aversion (quantifying its duration), irreversibility of the system (animals killed by the system) and effectiveness when combined with sticking at different times (as what will occur in commercial conditions).

The scientific objectives of the present study are thus to assess aversion in rabbits during exposure to 70, 80, 90 and 98% CO₂ in atmospheric air and determine the duration of signs of aversion before the onset of unconsciousness, to ascertain if these times are indeed comparable with the time rabbits are subjected to stress with other stunning systems. In addition, to ascertain the combinations of exposure times and concentrations to achieve a long duration of unconsciousness, including irreversible stunning, and finally to ascertain the concentrations and exposure times to produce an effective stunning with a stun-to-stick interval of 70 and 120 s. To make the study more representative in terms of the typical size of rabbit slaughtered in Spain, we decided to work in all cases with 2 weight categories: heavy rabbits (around 2.4 kg) and light rabbits (around 1.8 kg) in a ratio of 70 and 30%.

MATERIALS AND METHODS

The experiment was approved by the Institutional Animal Care and Use Committee of IRTA (DAAM=7318).

Facilities and treatments

Rabbits, from the New Zealand breed, were transported from commercial farms in cages in groups of 6 individuals per cage (0.8×0.5×0.33 m). Once in the IRTA experimental facilities, the animals were housed in a pen 2.2 m wide×8 m long. The pen was provided with straw bedding and food (commercial pellets for rabbits from the farm of origin) and water *ad libitum*.

The IRTA experimental slaughterhouse in Monells is equipped with a Dip Special XL gas stunning system lift (Butina Aps, Denmark), which consists of a 350 cm deep pit, down which a crate 299 cm long, 138 cm wide and 100 cm high is lowered. Gas was supplied by Carburos Metálicos (Barcelona, Spain) in packs with liquid carbon dioxide which was heated to 13-15°C before being delivered into the pit, which contained only atmospheric air prior to the CO₂ delivery. In the present study, the system worked with a density from 0.9 to 1.2 kg of rabbit/m². The cage floor was labelled by including 50 cm spaced lines forming a grid that was used to estimate the rabbits' activity. Before lowering the crate, oxygen and CO₂ concentrations at different levels inside the pit were measured up to a depth of 1 m. Although the system had a sensor located at 1 m depth in the pit to automatically monitor the carbon dioxide concentration, a continuous gas flow analyser (Check Mate II, PBI Dansensor; Spain) was also used to assess the oxygen and carbon dioxide concentrations at different levels within the pit. With this extra gas-flow analyser, assessments were carried out at different heights in the pit during the study, such as at +10 cm (rabbit's head position when the cage was in the initial position) and to 0, -10; -20; -30; -40; -50; -60; -70; -80 and -90 cm in depth. Additionally, to control the environmental temperature around the rabbits, continuous recordings of environmental temperature and relative humidity inside the crate were also made using 2 sensors (Hobo U10-003, Onset Computer Corporation; Massachusetts) placed in the crate at the height of the animal's head.

During the study, 4 different gas concentrations were tested: 70% carbon dioxide in atmospheric air (70%), 80% carbon dioxide in atmospheric air (80%), 90% carbon dioxide in atmospheric air (90%) and 98% carbon dioxide in atmospheric air (98%). The study was divided into 3 different phases, in which the 4 treatments were assessed (except in the case of 70%, which was not assessed in Phase 3): Phase 1 assessed aversion to the gas, Phase 2 measured stunning effectiveness without sticking and Phase 3 treated stunning effectiveness with sticking.

Phase 1. To study aversion to 4 carbon dioxide (CO₂) concentrations: 70, 80, 90 and 98%

For the aversion assessment, a study with 20 rabbits per treatment (70, 80, 90 and 98%, in total 80; Table 1) was carried out. Fifty-six rabbits had an average weight of 2.45±0.20 kg and 24 had an average weight of 1.89±0.13 kg. A total of 14 heavy animals and 6 light animals were used in each treatment. Animals were placed in the crate in pairs, facing each other and with freedom of movement. The animals began to descend into the pit 30 s after being placed in the crate. Then, the crate was lowered to 1 m depth (in 9 s), remained at the bottom position for 45 s and then returned to the initial position (in 9 s). After ascending to the starting point, the animals were returned to their pens after 30 s of behavioural observation. This process was repeated twice for each pair of rabbits, the first time with atmospheric air in the pit and the second with one of the 4 treatments assigned previously and in a random order. In all cases, the total cycle lasted 63 s and animals were exposed to the fixed (maximum) gas concentration

Table 1: Number of animals in relation to the gas treatments, times of gas exposure and in the case of Phase 3 stun-to-stick interval.

	CO ₂ concentrations								
	70%	70%	70%	80%	80%	90%	90%	90%	98%
Phase 1									
Exposure (s)	45			45		45			45
Animals	20			20		20			20
Phase 2									
Exposure (s)	80	270	360	90	170	50	80	90	60
Animals	10	30	40	40	40	40	8	32	40
Phase 3									
Stun-to-stick interval (s)				120		120	120	70	120
Exposure (s)				200		130	150	110	110
Animals				30		30	30	22	30

for 45 s. Within each treatment, the heavy animals were tested first, followed by the animals of 1.8 kg (light rabbits). Therefore, a total of 40 cycles were performed. In all cases, animals were individually identified using a collar with a number and the same pairs were used in the repeated tests with atmospheric air and gas. After the tests, animals were euthanised by prolonged exposure to CO₂.

Behavioural observations were made directly from outside the crate by 2 observers (one per rabbit) and by using a videotape camera (Ex-view Sony; Barcelona Spain) located on the roof of the crate (used only in the event of doubts in any of the parameters assessed). The observations were made from 30 s before the beginning of the descent to the pit until the end of the ascent. During exposure to the atmospheric air and the gas treatments, the following variables were measured: 1. Activity, considering the number of lines crossed by animals (defined as a line crossed when the 2 forelimbs completely exceeded one of the marked lines on the floor of the crate); 2. Vocalisations (when the animal screamed); 3. Nasal discomfort (when animals began to touch the nose with the forelimbs and shook the head from side to side), and 4. Loss of posture (when the animal touched the floor of the crate with the abdomen or side with outstretched limbs). This last measure has been used in other species as an indicator of the onset of unconsciousness, to be able to anticipate the end of the aversion period (Raj and Gregory, 1996). Another parameter assessed was the presence of muscular jerks, defined as repeated muscular movement of the whole body, where it is not clear whether it occurs in conscious or unconscious animals (Rodríguez *et al.*, 2008). For each of these behaviours, the presence and time taken to occur in relation to the start of the descent was recorded.

Phase 2. Stunning effectiveness without sticking

The objective of the second phase was to ascertain times for the different exposure concentrations (shown in Table 1) to produce an irreversible stunning (killed by the gas) in most of the rabbits, so sticking was not performed to avoid confounding factors. To assess the effectiveness to induce unconsciousness (capacity to induce an unconscious state in the animals and duration of this state), a total of 280 rabbits were used (Table 1). Eighty animals were tested with 70, 80 and 90% treatments, respectively and only 40 with 98%. The times were decided according to experience in previous studies with rabbits and the results achieved in Phase 1 of the present study. In all cases, the exposure time is only considered to be the time the animals remained in the bottom position, not taking into account the 9 s of the crate ascending or descending.

Before exposure to the gas, 20 animals (14 of 2.5 kg and 6 of 1.8 kg) per treatment and exposure time were selected for monitoring brain activity. To do this, each animal was shaved in the frontal area of the head, where three 27-gauge-needle stainless steel electrodes (Ambu® Neuroline Subdermal, AMBU; Spain) were placed. The reference electrode was positioned on the midline of the skull between the base of the ears and lateral commissure of the eyes. The other 2 measuring electrodes were placed in line with it, approximately 1 cm on either side of the midline for a transhemispherical electroencephalography (EEG) recording. To ensure a good grip on the electrodes, rabbits were restrained-wrapped with a piece of cloth. Subsequently, the 3 electrodes were connected to a computer by means

of a 150 cm coaxial cable (IoC® view monitor; Morpheus Medical; Barcelona) to record brain activity using EEG as described in EFSA (2013) and Llonch *et al.* (2015). The electrodes were connected to the computer to register brain activity 5 min before the descent, but the baseline EEG activity of the animals was recorded for 2 min. Afterwards, the animals were placed into the crate and exposed to the gas treatment. These animals were always stunned in pairs, 1 connected to the EEG equipment and 1 with freedom of movement and not connected. The EEG was recorded during the exposure to the gas treatment until 5 min after the end of the exposure or until the animal showed signs of death, such as glassy eyes (EFSA, 2004). During this time, physiological reflexes, such as rhythmic breathing (in a continuous way) and corneal reflex (every 10 s) were also assessed. Although a large percentage of the animals died before these 5 min were up, the animals showing signs of recovery of consciousness, after being reported, were exposed again to the gas for longer times (around 10 min) to ensure their death.

The EEG signal before being analysed was filtered to remove noise, such as very low frequency components and electrical signals of 50 Hz. To this end, a Butterworth stop filter (Butterworth, 1930) between frequencies 49 Hz and 51 Hz was used. The signal was divided into 1-s intervals to analyse the EEG parameters.

The parameters used to assess the EEG are described in EFSA (2013) and Llonch *et al.* (2015), consisting of:

- a. Time of appearance of slow waves (high amplitude and low frequency). Determining the time at which low-frequency signals appeared after stunning was performed using the median frequency of the power of the signal. For this calculation, the signal was transformed to the frequency domain by applying Fourier transform, and from the Fourier transform the power spectrum was calculated (EGG power), which means the distribution of the signal's power for each frequency. This process was performed for the EEG signal in windows of 1 s, as previously mentioned. For each time interval, the frequency which accumulated 50% of the EEG power, beginning from 0 Hz, was determined. To determine if the signal was really of low frequency for the time interval (1 s) under scrutiny, the value of the median frequency of the power signal should be 4 Hz or lower (EFSA, 2013).
- b. The moment a significant change in the EEG power appears. To determine the occurrence of a significant change in the EEG signal, the baseline EEG power was compared with the EEG power of the signal during the stunning of each animal. As in the previous case, these comparisons were made at 1-s intervals. The onset of a significant change occurred when the EEG power during stunning fell below the baseline level.
- c. Appearance of profoundly suppressed or quiescent EEGs, indicative of a complete loss of spontaneous brain activity or a reduction of EEG total power content to less than 10% of the pre-stun EEG power content. As in the previous case, a comparison between the power of the signal after the beginning of the stunning procedure with the pre-stun EEG values was carried out. In this case, the criterion applied was that the signal should have an EEG power not exceeding 10% of the power of the baseline signal (EFSA, 2013).
- d. Appearance of a continuous reduction of the EEG total power content to less than 10% of the pre-stun EEG power content. This case is almost identical to case c, but the criterion was that the signal power could be equal to or less than 10% of the baseline over an extended time interval. For this reason, in this case the condition set was that the signal should remain within the cited premise for over 80% of the periods considered in the following 50 periods (s).
- e. EEG recovery. The signal was considered recovered when, after having an EEG power below 10% for the first time, the signal reached and maintained at least 30% of the pre-stun EEG power until the end of the recorded EEG signal.

Consciousness after exposure to the gas treatment was also assessed using the following physiological and behavioural indicators: 1. Presence of rhythmic breathing, defined as the presence of at least 2 respiratory movements assessed by means of movements of the mouth and/or flanks. 2. Presence of corneal reflex, defined as the rabbit closing its eye when the cornea was touched with a pen. It was assessed at intervals of 10 s. 3. Presence of vocalisations, defined as rabbits screaming. 4. Presence of righting reflex, defined as rabbits recovering their posture. 5. Presence of a state of awareness, defined as rabbits being aware of the surroundings (reacting to stimulation) when touched or when hands were moved in front of them. Another parameter assessed was the presence of muscular jerks although, as mentioned previously, it is not clear whether this occurs in conscious or unconscious animals (Rodriguez *et al.*, 2008). The assessment was monitored by 2 observers (1 per rabbit) from outside the crate, and videotape was used as a support.

Phase 3. Stunning effectiveness with sticking

The stun-to-stick (neck cutting with sectioning of both jugular veins and carotid arteries) interval was established at 70 s or 120 s. The exposure times for each gas treatment were set up according to the results of Phase 2, and were established at: 200 s in 80%, 130 s and 150 s in 90% and 110 s in 98%, when the stun-to-stick interval was 120 s and 110 s of exposure with bleeding at 70 s in 90%. The 70% treatment, due to the results observed in Phase 2, was not included in this phase. Thirty animals were used per treatment, except for 110 s exposure in 90%, in which 22 animals were used. In total, 142 rabbits were assessed (102 of 2.56 ± 0.22 kg and 40 of 1.96 ± 0.19 kg). Before being exposed to the gas, in 15 animals of each treatment and 11 for 90% for 110 s (71 in total), the brain activity was monitored by EEG until death.

During the induction phase and up to 2 min after bleeding, the unconsciousness of animals was also assessed continuously by means of absence of rhythmic breathing, vocalisations and righting reflex, and every 10 s by means of absence of corneal reflex. In addition to indicators of unconsciousness, signs of death, such as the presence of glassy eyes and lack of muscle tone, were also assessed.

Statistical analysis

Analyses were carried out with the Statistical Analysis System (SAS software, SAS Institute Inc.; Cary, NC, USA; 1999-2001). Count data not normally distributed, such as number of lines crossed or time to show nasal discomfort, vocalisations, loss of posture, muscular jerks, rhythmic breathing and corneal reflex, and EEG activity, such as appearance of slow waves in EEG activity, and reduction of EEG total power content to less than 10% of the pre-stun EEG power content, were analysed with general models (Proc Genmod) and repeated measures. Poisson or Negative Binomial distribution was applied in relation to the deviance and according to Cameron and Trivedi (1998). The residual maximum likelihood was used as a method of estimation. The least square means of fixed effects were used when the analysis of variance indicated differences. The fixed effect was gas treatment (including atmospheric air). The effect of the animals' weight was also tested initially (small animals [1.8 kg] vs. big animals [2.5 kg]), but no effect was found in any case, so it was not considered in the final analysis. On the other hand, when presence or absence of nasal discomfort, vocalisations, loss of posture and muscular contractions were analysed, the Proc Genmod procedure with binomial distribution was used. The fixed effect was again gas treatment. As temperature and humidity inside the crate showed a normal distribution, they were analysed with the Proc mixed procedure. The fixed effects were gas treatment and time into each gas treatment. In all cases, significance was fixed at $P < 0.05$ and models took into account the pair of animals which descended into the pit each time as a factor.

RESULTS AND DISCUSSION

Gas treatment concentration and conditions

Table 2 shows the mean CO₂ concentrations at different positions from +10 cm to -90 cm in relation to the floor for each gas treatment. According to the results, the concentration of CO₂ at +10 cm from the floor showed similar concentrations to those found in atmospheric air (from 0.8 to 1.2% at 70 and 90%). When the crate was descending into the pit it took 9 s from the initial point to the deepest, -90 cm in relation to the floor, at a speed of 1 s per 10 cm. The higher the final concentration of each treatment (98 vs. 70%), the higher was the concentration of CO₂ at different heights in the pit, as the concentration followed a gradient similar to those found in Dalmau *et al.*, (2010b). Therefore, although in the whole study the exposure times did not take into account the time taken

Table 2: Carbon dioxide concentration at different positions into the stunning system for each treatment (70, 80, 90 and 98%).

Distance to the floor (cm)	70%	80%	90%	98%
+10	1.2	0.6	0.8	1.9
0	2.2	4.7	3.5	2.3
-10	15.9	32.4	41.8	40.0
-20	26.0	47.8	55.8	65.0
-30	36.6	51.1	70.0	83.0
-40	45.3	53.6	76.3	97.0
-50	54.4	66.1	80.0	97.4
-60	59.2	72.2	83.1	97.4
-70	63.2	74.5	85.2	97.8
-80	66.9	77.7	87.2	97.5
-90	70.3	80.0	89.9	97.8

to raise and lower the crate, it should be considered that animals were always exposed to high concentrations of CO₂ some seconds earlier (4-5 s) at the highest concentrations due to this effect (Table 2). The environmental temperature ranged between 12 and 23°C and the relative humidity from 30 to 90% in the IRTA facilities (crate) during the study days. The temperature and relative humidity in the crate did not show differences ($P>0.05$) throughout the experimental trials and no significant differences ($P>0.05$) between gas treatments were found when temperature and humidity were taken into account. These combinations of temperatures and humidity can be considered within a reasonable range for these species and clearly outside the conditions deemed potentially harmful by EFSA (2004) when using CO₂, due to the freezing conditions of the gas if used directly after vaporisation without a previous heating process.

Phase 1. Aversion study

For the assessment of aversion to the gas, the behaviour of the animals during the first exposure to atmospheric air was compared with the second exposure to one of the gas treatments. For the activity analysis, the exposure period of each animal was separated into 2 parts. The first part was the initial 39 s (30 s prior to descent and the 9 s of descent) and the second was the other 54 s (45 s at the bottom position and 9 s of ascent). The results show few significant differences between the 2 parts (Figure 1). Only at 90% did the number of lines crossed while the crate was at the bottom position or ascending decrease during the gas exposure in comparison with the air treatment ($P=0.0003$); this was not the case for 70%, 80% or even 98%. Within each period, the animals from the 98% treatment were less ($P<0.05$) active in the first part compared to the other treatments when both air and gas was used. Surprisingly, in the second part, especially when atmospheric air was used, the opposite pattern was found, rabbits in the 98% treatment being the most active ($P<0.001$). Therefore, although Lonch *et al.* (2012a) found increased activity when rabbits were subjected to 90% and even when exposed to a mixture of 80% Nitrogen and 20% CO₂ in comparison to the exposure to atmospheric air, the results of the behavioural indicators of activity used in the present study for rabbits are too inconsistent to associate them with the magnitude of the aversion. One difference between the study of Lonch *et al.* (2012a) and the present study is that in the former the animals were subjected to air and gas on different days, whereas in the present study both exposures (air and gas) were tested the same day.

Figure 2 shows the percentage of animals with nasal discomfort and vocalisations, considered to be 2 signs of aversion (EFSA, 2004), and loss of posture (considered the first sign of onset of unconsciousness; Raj and Gregory, 1996). None of these signs was observed when rabbits were subjected to atmospheric air. Nasal discomfort is related to the irritation CO₂ causes in the nasal mucosal membranes (Peppel and Anton 1993), where the presence of chemoreceptors acutely sensitive to this gas has been described (Manning and Schwartzstein, 1995), and can thus be considered a sign of pain. In Lonch *et al.* (2012a) signs of nasal discomfort

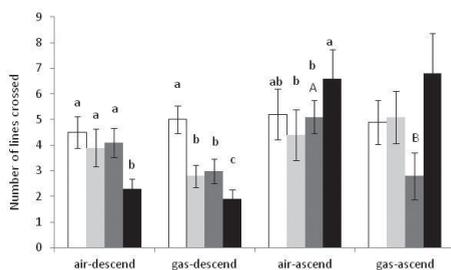


Figure 1: Number of lines crossed by the rabbits exposed to atmospheric air 30 s before descending the crate and while it was going down (air-descent), the crate stationary at the bottom position for 45 s and going up (air-ascent) and when subjected to a gas treatment of 70 (□), 80 (▒), 90 (■) and 98% (■) CO₂ (gas-descent and gas-ascent). Lowercase indicates differences between gas concentrations at each stage and uppercase differences between air-ascent and gas-ascent in 90% treatment at $P<0.05$.

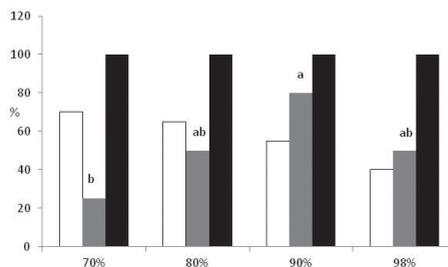


Figure 2: Percentage of animals showing nasal discomfort (□), vocalisations (▒) and loss of posture (■) in 70, 80, 90 and 98% CO₂. Different letters within a variable mean significant differences between treatments at $P<0.05$.

were considered as part of a general pattern of respiratory distress and recorded as indicators of breathlessness. This sense of breathlessness has been well described in pigs exposed to CO₂ (Gregory *et al.*, 1990; Velarde *et al.*, 1997), but is difficult to assess in rabbits, where the most clear sign to observe was when the animal began to touch the nose with the forelimbs and shook the head from side to side, defined here as nasal discomfort. However, the sense of breathlessness must not be ruled out in rabbits, at least during the phase in which the animal presents head shaking.

Although rabbits tend to show very low frequency vocalisations when compared to other species such as swine, loud, piercing screams, described by Bradley *et al.* (2006) as signs of pain and fear, as when the rabbit is caught by a predator, were found in the present study. Nodari *et al.* (2009) also found vocalisations in conscious animals after electrical stunning and slaughter at a commercial abattoir. The percentage of animals showing vocalisation and loss of posture did not differ among treatments, but a higher ($P<0.05$) percentage of animals showed nasal discomfort at 90 than at 70%. On the other hand, loss of posture appeared earlier when exposed to 90 and 98 than to 70 and 80% ($P<0.05$; Figure 3). In addition, vocalisations appeared later ($P<0.05$) at 90% than in the other treatments. Therefore, the time between the appearance of the first vocalisation until loss of posture was shortest at 90% when compared to the other treatments (Figure 3).

Finally, the percentage of animals showing muscular jerks increased at higher CO₂ concentrations, being 15 at 70%, 40 at 80%, 70 at 90% and 90 at 98%. In this case, 98% had significantly higher ($P<0.05$) values than did 80 and 70%, and 90% significantly higher ($P<0.05$) values than did 70%. The muscular jerks started at a mean time of 26.2 ± 5.41 s at 98%, 32.2 ± 10.01 s at 90%, 29.8 ± 5.15 s at 80% and 32.7 ± 6.42 s at 70%, but without significant differences among gas treatments ($P>0.05$). Llonch *et al.* (2012a) described that muscular jerks appeared after the loss of posture when 90% was used and before the loss of posture when 80% N₂ and 20% CO₂ was used. According to Raj and Gregory (1996), muscle jerks before or during the loss of balance are associated with escape attempts in conscious animals. On the other hand, muscle jerks after loss of balance are associated with involuntary convulsions in unconscious animals (Forsslid, 1987, 1992), although Rodríguez *et al.* (2008) stated that animals could be conscious during these movements. Coenen *et al.* (1995) described 4 phases during exposure to high CO₂ concentrations in rats: normal behaviour (Phase I); continuous abnormal activity, excitation and agitation at a higher rate than normal (Phase II); sagging of hind legs and loss of body control (Phase III); disappearance of muscle tone and head sinking (Phase IV), but it is not clear where the onset of unconsciousness occurs. In the present study, muscle jerks (compatible with phase III) appeared at 26.2; 32.2; 29.8 and 32.7 s at concentrations of 98, 90, 80 and 70%, respectively, so after loss of posture (see Figure 3). Therefore, they occurred after the onset of unconsciousness according to Raj and Gregory (1996).

In all cases, before loss of posture, animals showed aversion to the gas, (vocalisations and nasal discomfort), behaviours not seen when the animals were exposed to atmospheric air. These behaviours are comparable to those studied in pigs as signs of aversion, such as vocalisations, retreat or escape attempts and gasping (Dodman, 1977;

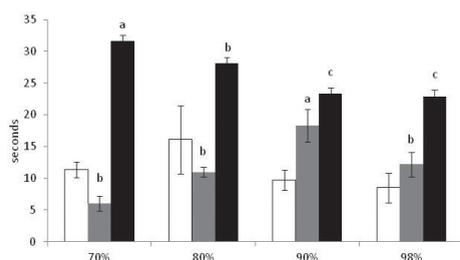


Figure 3: Time to show nasal discomfort (□), vocalisations (■) and loss of posture (■) after the start of the exposure to 70, 80, 90 and 98% CO₂ treatments. Different letters within a variable mean significant differences among treatments at $P<0.05$.

Raj and Gregory, 1996; Velarde *et al.*, 2007; Rodríguez *et al.*, 2008; Dalmau *et al.*, 2010a; Llonch *et al.*, 2012b), where high CO₂ concentrations are also used for stunning. Moreover, the time until loss of posture appears in pigs is also similar to that in rabbits, around 30s (Raj and Gregory, 1996; Llonch *et al.*, 2012b). The presence of animals vocalising was higher at 90% compared to the other treatments, but it occurred later at 90% than in the other treatments, closer to the onset of unconsciousness (loss of posture), with no differences found between treatments in the number of vocalisations per animal. Therefore, after Phase 1 all the gas treatments were considered suitable (few differences among them and with similar results to those observed in other species in terms of signs of aversion) for study in the next phase.

Phase 2. Stunning effectiveness without sticking

In the second phase of the study, in addition to the loss of posture, the EEG signal and physiological reflexes were used to ascertain the moment of the onset of unconsciousness. In relation to the physiological reflexes, such as presence of corneal reflex or respiratory rhythm (EFSA, 2013), no animals in any treatment showed any sign at the end of the exposure, confirming that animals were well stunned at this point. In relation to the EEG, from the 4 parameters assessed, the mean time in low-frequency and high-amplitude waves appearing was the first sign to be significantly different from the basic EEG signal, being 27.5 ± 14.67 s at 70%, 24.8 ± 14.94 s at 80%, 22.2 ± 16.27 s at 90% and 12.7 ± 8.19 s in the case of 98% (Figure 4). Immediately after that, a significant effect was found for the time taken for a change in the EEG power to appear, being 31.6 ± 30.52 s at 70%, 30.7 ± 21.92 s at 80%, 31.2 ± 17.66 s at 90% and 33.9 ± 23.84 s at 98% (Figure 4). In all cases, this change in the EEG power coincided with the lapse of time in which the animals lost their posture (Figure 4), as mentioned previously, which is considered to be the first sign of onset of unconsciousness (Raj and Gregory, 1996). Therefore, the combination of the 3 signs confirms that onset of unconsciousness occurs about 30 s after the beginning of gas exposure. In fact, the appearance of low frequencies in the EEG and changes in EEG power were already considered good indicators of a change in brain activity in previous studies when animals were stunned with gas (EFSA, 2013) or even with other methods, such as electricity (Lonch *et al.*, 2015) or the use of anaesthetics. For instance, Lonch *et al.* (2011) found a change in brain activity 7 s after the loss of balance in pigs anaesthetised with propofol. Other parameters considered in the study of the EEG, and observed later (Figure 5) were the moment in the first wave with a power equal to or lower than the 10% of the EEG basal value (from 70 s to 130 s) appearing, and the moment at which a continuous EEG with values under this 10% was observed (from 105 s to 300 s). This latter sign is a symptom of deep brain disruption (EFSA, 2013). In fact, it is clear that the waves with a power equal to or lower than 10% of the basal EEG can be useful to assess deep states of unconsciousness but not for the onset of unconsciousness, when other measures, such as the appearance in the EEG of low-frequency signals or the appearance of statistically significant changes in the EEG power are preferred.

Animals stunned without sticking will die (in the case of an irreversible stunning) or will recover consciousness (in the case of a reversible stunning). From the 142 animals assessed with EEG (130 with complete registers) in this second phase of the study, 93 died and 29 recovered consciousness (12 at 70%, 15 at 80%, 17 at 90% and 5 at 98%). Recovery of the basal EEG in these animals was found at about 119 s from the start of exposure when 70% was used and an exposure time of up to 80 s was used (awareness was observed at 119 ± 16.6 s), and 350 s when the exposure time was 270 s (awareness was observed at 348 ± 47.8 s). In the case of 80%, the EEG recovery took place in a mean time of 187 s with an exposure time of 160 s (although awareness was detected at 326 ± 64.5 s). In the

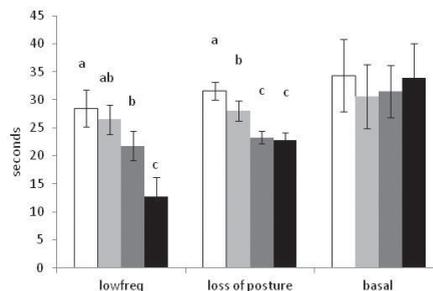


Figure 4: Mean times in the appearance of low frequencies in the electroencephalography (lowfreq) and electroencephalography different from the basal values (basal) in addition to time in loss of posture. Different letters means significant differences at $P < 0.05$. 70% (□), 80% (▤), 90% (▥) and 98% (■).

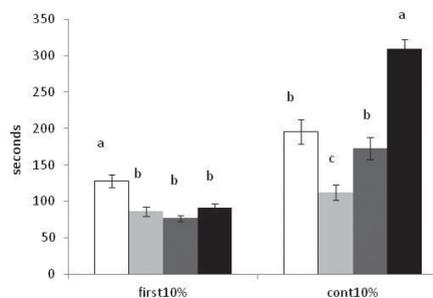


Figure 5: The mean time for the signal to have a power equal to or lower than 10% of the basal value of the electroencephalography (first10%) and when they appeared continuously (cont10%) for the different treatments. Different letters means significant differences at $P < 0.05$. 70% (□), 80% (▤), 90% (▥) and 98% (■).

case of 90%, recovery of the EEG was within a mean time of 221 s with 50 s of exposure (awareness was detected at 192 ± 73.4 s) and, finally, in 98%, the mean time of EEG recovery was at 301 s with an exposure time of 60 s, while awareness was detected at 258 ± 50.3 s. Therefore, although a relationship is found between the recovery of a basal level in the EEG and a state of awareness in rabbits, the latter is probably a better indicator of complete recovery of consciousness than the first (based on statistical analysis). Righting reflex and signs of awareness coincided in all cases in the time, so both can be considered to assess the same in rabbits stunned with CO₂.

Other measures that could indicate that the animals were recovering consciousness were rhythmic breathing and corneal reflex, which are described as appearing before the righting reflex and a complete state of awareness (EFSA, 2004). In the present study, we observed that during the recovery phase rhythmic breathing was the first reflex to reappear, in comparison to corneal reflex or righting reflex (Figure 6) and, in consequence, as well before the recovery of a basal EEG signal. This outcome agrees with Llonch *et al.* (2013), who found animals with rhythmic breathing and corneal reflex with brain activity below the basal. However, as Anil and McKinstry (1991) suggested, some symptoms commonly considered to be relevant for the assessment of consciousness are indicative of brain stem activity only and do not relate to cortical function. In fact, the EEG assesses cortical activity rather than brain stem activity, and recovery of the brain stem reflexes precedes consciousness.

On the other hand, the fact that the righting reflex appears after rhythmic breathing and corneal reflex is not surprising (EFSA, 2004), but this is not the case for corneal reflex, which has been described as being the first reflex to reappear during recovery in pigs (Rodríguez *et al.*, 2008). Consequently, in contrast to studies carried out in other species, we found a higher percentage of animals with rhythmic breathing than with corneal reflex (Figure 7). In fact, some animals that recovered rhythmic breathing subsequently died before recovering any other reflex (1 at 80%, 1 at 90% and 2 at 98%). This is an interesting detail to consider in studies carried out in rabbits in commercial conditions. A possible explanation is that some of the respiratory movements were confounded with involuntary gagging movements. Gagging has been defined as low-frequency inhalations with the neck towards the front legs and occasional emitting of sounds similar to snoring in pigs (Rodríguez *et al.*, 2008) and it is considered an indicator of deep unconsciousness (EFSA, 2004). According to Llonch *et al.* (2013), gagging is a rudimentary brain stem response occurring in association with the process of exhalation in pigs that takes place when general anaesthesia is achieved in gas exposures. However, it is possible than in rabbits movements and expression are more similar to and difficult to distinguish from a normal respiratory movement.

In any case, the time between the end of the exposure to the gas and the recovery of rhythmic breathing, the first sign of recovery, was lower ($P < 0.0001$) at 70% than in all the other gas treatments. In addition, the time for the corneal reflex to appear was also lower ($P < 0.0001$) at 70% than in the other three treatments and higher ($P < 0.0001$) at 98

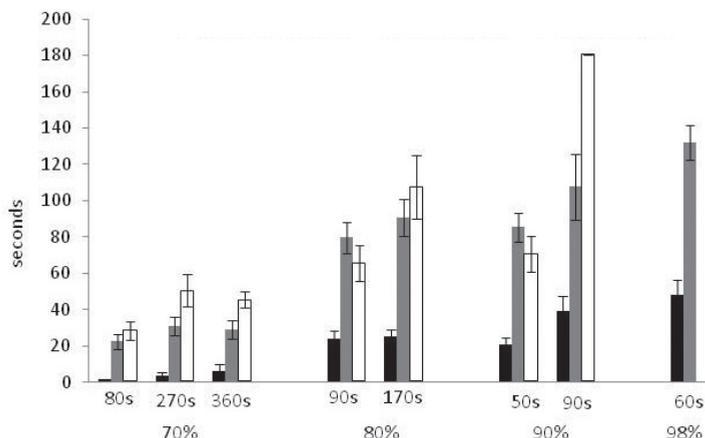


Figure 6: Times in the appearance of rhythmic breathing (■), corneal reflex (■) and showing a phase of muscular jerks (□) in treatments at 70, 80, 90 and 98% CO₂ with different exposure times.

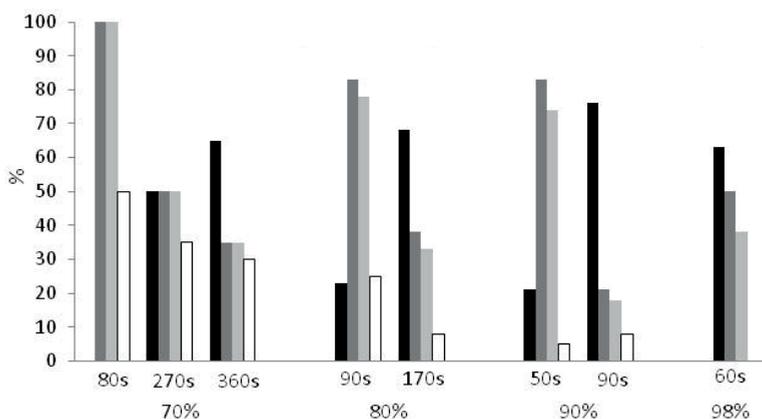


Figure 7: Percentage of animal deaths (■) after the exposure, animals recovering rhythmic breathing (▒), corneal reflex (□) and showing a phase of muscular jerks (◻) in treatments at 70, 80, 90 and 98% CO₂ with different exposure times.

compared to 80 and 90%. Therefore, the duration of consciousness is related with the CO₂ concentration, as has previously been described (Raj *et al.*, 1999; LLonch *et al.*, 2013).

On the other hand, as observed in Phase 1, here again muscular jerks appeared (mean time of 43.4±20.58 s at 70%, 73.6±35.65 s at 80% and 106.7±64.29 s at 90%) after the mean times in the appearance of the loss of posture (33.2±3.80 s at 70%, 31.9±4.12 s at 80% and 25.0±3.67 s at 90%), so it should be considered a sign appearing after the onset of unconsciousness in most of the animals, as stated by Rodríguez *et al.* (2008). Moreover, as can be observed in Figures 6 and 7, this occurred earlier and in a higher proportion at the lowest CO₂ concentration.

From our experience (not in laboratory tests, but in commercial conditions), it takes around 30 s for rabbits to lose physiological reflexes such as rhythmic breathing and corneal reflex when only exsanguination is applied (data not published). Therefore, a good stunning system should last at least 30 s after the sticking. According to the results of Phase 2, to ensure a state of unconsciousness with a stun-stick interval from 1 min up to 2 min, none of the treatments carried out was effective enough, as in no case was the death of 100% of the animals achieved, and those recovering consciousness did so in less than the two-and-a-half minutes needed (2 min of stun-stick interval+30 s of an effective bleeding). In the case of 70%, with 360 s of exposure, 60% of the animals died, but the others recovered signs of consciousness, such as rhythmic breathing and corneal reflex at 5 s and 22 s respectively, the EEG recovering basal values around 30 s after the end of exposure and a state of awareness being detected in these animals at 87.5 s. With these times, it was not possible to fulfil a time of unconsciousness of two and a half minutes (150 s). Moreover, as longer exposure times (more than 6 min) are difficult to apply in commercial conditions, the 70% treatment was deemed unsuitable in commercial abattoirs and, accordingly, not tested in Phase 3 of the study. Additionally, as the exposure at 80% for 170 s induced the death of only 68% of the animals (Figure 6), 90% for 90 s induced the death of 75%, and 98% for 60 s the death of 63% of the animals, the exposure times of these 3 treatments were also modified for Phase 3 of the study.

Phase 3. Stunning effectiveness with sticking

All animals exposed to 80% for 200 s died without any sign of recovery of consciousness (no rhythmic breathing, no corneal reflex, etc.) during a stun-stick interval of 2 min. In the case of 90%, 2 exposure times were assessed with a stun-stick interval of 2 min, 130 s and 150 s. With 130 s of exposure, 93% of the animals died without signs of recovery of consciousness at any moment, while 2 out of 30 (7%) showed rhythmic breathing at 60 and 140 s after the exposure, and 1 out of 30 (3%) presented corneal reflex at 180 s after the exposure and before death. In the case of 150 s of exposure, all of the animals died before exsanguination and not one of them recovered any sign of

consciousness at any point. Finally, for the 98% treatment an exposure time of 110 s was set, resulting in death of 100% of the animals, without any showing signs of recovery at any point.

Another test with 90% was also carried out, but this time with a shorter stun-to-stick interval of 70s. Two animals showed from 2 to 4 respiration movements, the first being at 60 s and 90 s, respectively, after the end of the exposure. However, no other signs of recovery were seen in these animals before death due to exsanguination.

Therefore, according to the results, with a stun-stick interval of up to 2 min, 200 s of exposure at 80%, 150 s at 90% and 110 s at 98% are recommended in the case of a stun-to-stick interval of 2 min. In the case of a stun-stick interval up to 70 s, the exposure time with 90% could be reduced to 110 s, but, as in other cases where the stunning is reversible, the quality of the sticking must be checked regularly.

In conclusion, exposure to CO₂ at high concentrations can produce irreversible stunning in rabbits. This is a clear advantage in comparison to the main system used nowadays for stunning rabbits, electronarcosis, a reversible system in which animals recover consciousness after 15 to 30 s if bleeding is not performed. However, as in other species, aversion to the gas was observed, lasting 15 s in rabbits before the animal lost its posture. Loss of posture occurred, as in swine, within the first 30 s after the beginning of the exposure to the gas. Therefore, despite the advantages that the system provides in terms of pre-stunning handling and irreversibility, this is not free of animal welfare problems. In consequence, a debate is necessary to ascertain if CO₂ can be an alternative to stunning rabbits, taking into account the advantages and the disadvantages mentioned, quantified in the present study as 15 s of aversion (nasal discomfort and vocalisations) before losing posture.

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