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Animal Welfare 2012, 21(S2): 123-129
doi: 10.7120/096272812X13353700593923
ISSN 0962-7286

Aversion to the inhalation of nitrogen and carbon dioxide mixtures compared to high concentrations of carbon dioxide for stunning rabbits

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Abstract

*Stunning by inhalation of nitrogen (N_2) and carbon dioxide (CO_2) mixtures reduces aversion compared to high concentrations of CO_2 in pigs and poultry. The objective of the study was to assess the aversion to 90% of CO_2 (90C) and an alternative gas mixture of 80% N_2 and 20% CO_2 (80N20C) in commercial rabbits (*Oryctolagus cuniculus*). Sixty animals, divided into two groups, were used. During the first day, the rabbits of both groups were lowered in pairs into the pit with atmospheric air and their behaviour was recorded as control. During the second day, one group was exposed, again in pairs, to 90C and the other to 80N20C for 1 min. Exploratory behaviour and general activity were assessed 2 min before the exposure, during the exposure and for 2 min subsequently. During the exposure, signs of respiratory distress, loss of balance, muscle twitching and recovery of balance were also assessed. In the control sessions (atmospheric air), animals did not show respiratory distress or muscle twitching and were less active while the crate was descending than when gas treatments were applied. The percentage of animals with respiratory distress was higher in 90C (97%) than 80N20C (40%). Muscle twitching occurred earlier in 80N20C (97%; 23.9 s) than in 90C (17%; 37.4 s). A second phase of muscle twitching occurred only in 90C at 93.0 s. Mean latency of lost of balance and recovery were lower in 80N20C (24.2 and 98.6 s, respectively) than in 90C (28.2 and 110.2 s, respectively). It is concluded that rabbits showed less signs of respiratory distress to inhalation of 80N20C than 90C but more signs of aversion than when they were exposed to atmospheric air.*

Keywords: *animal welfare, aversion, carbon dioxide, nitrogen, rabbits, stunning*

Introduction

Domestic rabbits (*Oryctolagus cuniculus*) kept for meat production represent 1.2% of the meat produced in Europe. However, they are in fact the second species, after poultry, in terms of the number of animals slaughtered per year in the European Union, with 321,334 × 1,000 rabbits in 2009 (FAOSTAT). Due probably to the local consumption of this meat (mainly restricted to the Mediterranean countries), this is by far the least studied meat production species in terms of animal welfare (EFSA 2005), especially when compared to pigs, poultry, cattle, sheep and goats. Eurogroup for Animals stated that further research is needed on rabbits to improve handling during transport and slaughter (Eurogroup for Animals 2010). Stunning before slaughter is a statutary requirement in Europe (Council Regulation [EC] No 1099/2009) and is performed to induce unconsciousness and insensibility in animals in order that the slaughter can be performed without causing the animals any avoidable anxiety, pain, suffering or distress. Under commercial conditions, two main methods are used to stun rabbits for meat production, electrical and mechanical stunning. In both systems animals have to be handled and restrained before stunning. According to the EFSA (2005), rabbits should be lifted by grasping the loose skin at the back of the neck and supported by placing the hand beneath the hindquarters. However, due to possible fur damage, most abattoir personnel avoid catching the animals by the skin on the neck and use the legs or ears instead. This latter method is painful and is not allowed by the Regulation 1099/2009 (EFSA 2005). Alternatively, carbon dioxide stunning is used in pigs and birds, and allows the exposure of animals in groups, and avoids the need for shackling of live animals. This reduces human contact during handling and decreases pre-slaughter stress (Velarde *et al* 2000). In this stunning system, animals contained in cages, cradles, crates or conveyor belts, are exposed to high concentration of CO_2 or a predetermined gas mixture contained within a well or tunnel. However, carbon dioxide is not allowed for the stunning of rabbits for meat consumption (Regulation 1099/2009) because of its aversiveness at high concentrations (Conlee *et al* 2005). Furthermore, loss of consciousness is not immediate and other species, such as pigs, mice or rats, also react aversely on its exposure (Smith & Harrap 1997; Leach *et al* 2002) especially when the concentration of CO_2 is above 30% (Raj & Gregory 1995; Velarde *et al* 2007; Dalmau *et al* 2010b). Raj and Gregory (1995) reported that pigs tried to escape from an atmosphere of 90% CO_2 in less than 5 s. Prior to loss of balance, pigs show vigorous head shaking (EFSA 2004), a big gasp through the wide open mouth, which is indicative of the onset of breathlessness, and escape attempts (Raj & Gregory 1996). In comparison to high concentrations of CO_2, hypoxia induced by the inhalation of inert gases such as argon (Ar) or nitrogen (N_2), causes less aversion in pigs (Raj & Gregory 1995; Raj 1999; Dalmau *et al* 2010b; Llonch *et al* 2011). The relative density of nitrogen (0.97) is slightly lower than air (1.00) and therefore its stability, defined as the capability of the gas to be sustained within the pit without being displaced by oxygen, is low (Dalmau *et al* 2010a). Nevertheless, this stability is improved combining nitrogen with CO_2. Dalmau *et al* (2010a) reported very good results with the mixture 80% N_2 and 20% CO_2 in a commercial gas system. Furthermore, Dalmau *et al* (2010b) concluded that pigs exposed to 80% N_2 and 20% CO_2 showed less aversion than those exposed to 90% CO_2. The objective of the present study was to assess the aversion in rabbits for meat production to 90% of CO_2 (90C), and an alternative gas mixture of 80% N_2 and 20% CO_2 (80N20C) in a commercial gas-stunning system.

Materials and methods

Study animals

Sixty commercial rabbits (crossbred) weighing between 1.8 and 2.0 kg were used. Six days prior to the study, the rabbits were transported from their farm of origin to the IRTA facilities and housed in 15 wire cages (4 animals per cage) measuring 80 × 50 × 30 cm (length × width × height). The cages were adjacent to the experimental facilities and water and food were available *ad libitum*.

Experimental procedure

The experiment was carried out in IRTA, Spain with a Dip Lift XL ESPECIAL stunning system (Butina Aps, Copenhagen, Denmark). This system contained a crate (299 × 138 × 100 cm) that descended to the base of a pit 290 cm deep. The floor of the crate (perforated to facilitate gas distribution within) was marked with lines 50 cm apart to assess displacements of animals. When 90% CO_2 was used, the pit was automatically pre-filled and the concentration controlled by a sensor placed 1.5 m below the top of the pit (the position of the crate after 8 s of descent) and connected to a pump into the stunning system. The gas mixture 80N20C was administered manually by two flow meters and conducted by tubes to the bottom of the pit. The concentrations of both gases inside the pit were controlled by a mixed O_2/CO_2 analyser (MAP Check Combi, PBI Dansensor, Denmark) placed 1.5 m below the top of the pit. The gas mixture was considered adequate when the CO_2 concentration was 20% and the O_2 below 2%. The experiment was carried out during two consecutive days. The rabbits were divided in two groups of 30 animals each. The first day, all animals were placed in pairs in the crate and lowered for 1 min to the pit with atmospheric air. A total of 30 runs were carried out. Before, during and after exposure behaviour was recorded and used as control. The following day, the same pairs of animals were placed in the crate. One group was exposed to 90C and the other to 80N20C. Therefore, 15 runs for each gas treatment were carried out. In both cases, animals were subjected to the gases during one minute (15 s descending, 30 s stationary and 15 s ascending).

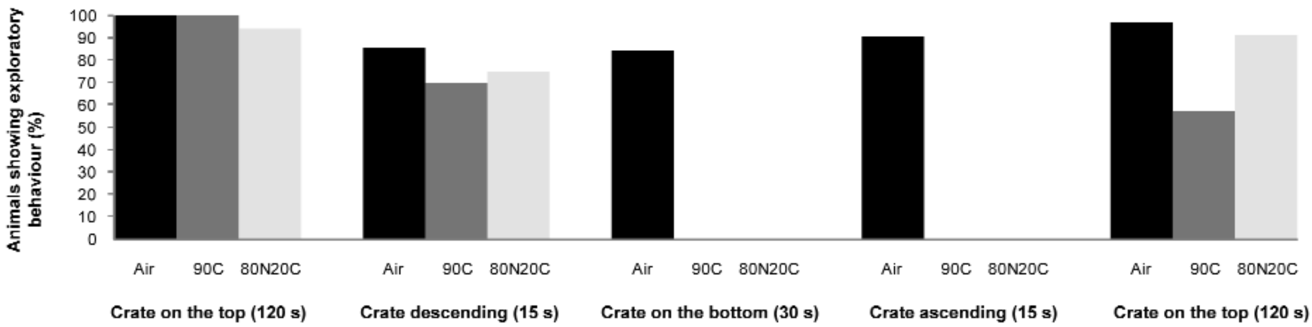
Behaviour and physiological measures

The first and second day an open-field trial was carried out on both pairs of animals during 2 min prior to the beginning of the descent, during the minute of descent and ascent and during 2 min after ascent. Animals were positioned one in front of the other in the two sides of the crate, with a distance of 150 cm between them (three floor marked lines). During this period the onset and latency of the explorative behaviour, defined as chewing or licking crate elements, sniffing the environment, gnawing or marking with the chain (Prinz *et al* 2008; Shepers *et al* 2009) and general activity (counted as number of moves, ie number of times that rabbits crossed one of the lines painted on the floor [Dalmau *et al* 2009]) were assessed. When an animal went out of the crate (this was only possible at the top position) this was also recorded. In addition, during the descent into and ascent from the pit, signs of respiratory distress similar to gasps observed in pigs (Dalmau *et al* 2010b) were recorded as indicators of breathlessness (Velarde *et al* 2007). The onset and duration of muscle twitches, defined as muscle contractions similar to spams or convulsions (Forslid 1987; Raj & Gregory 1996) in the whole body or part of it, were recorded, as was the time to loss of balance (considered the first indicator of onset of unconsciousness [Raj & Gregory 1996]), and of the recovery of balance were also recorded. Defaecation, described in rodents as signs of distress during exposure to gas (Smith & Harrap 1997; Ambrose *et al* 2000), was assessed. All recording times were synchronised with the time the rabbits started to descend into the pit.

Statistical analysis

Analyses were carried out with the Statistical Analysis System (SAS; Software SAS Institute Inc, Cary, NC, USA; 2002–2008). In all cases, the fixed effect included in the models was gas treatment and cage of origin was considered as a random effect. General linear models (PROC MIXED) were used to analyse the dependent variables of the onset of respiratory distress, loss of balance, muscle twitches and recovering, but also the duration of muscle twitching. When the analysis of variance showed significant differences ($P < 0.05$), the least square means of the fixed effect (LSMEANS) was used for comparisons. General models (PROC GENMOD) by means of a binomial distribution were used to analyse the percentage of animals showing respiratory distress, loss of balance and muscle twitching. General models (PROC GENMOD) by means of a binomial distribution and with animal as a repeated measure were used to analyse the percentage of exploratory behaviour when the crate was on the top of the pit, descending, stationary at the bottom of the pit, ascending and again at top of the pit. The models were subjected to three covariance structures: compound symmetric, autoregressive order

Figure 1



Percentage of animals showing exploratory behaviour in relation to the different positions of the crate and gas treatment (atmospheric air [Air], 90C and 80N20C).

one, and unstructured covariance. The compound symmetric structure was finally used as it yielded the smallest Schwarz's Bayesian criterion and closest to Likelihood values. The residual maximum likelihood was used as a method of estimation. We used the least square means of fixed effects (LSMEANS) when analysis of variance indicated differences at $P < 0.05$. General models (PROC GENMOD) by means of a multinomial distribution with a cumulative logit as link function were used to analyse general activity when the crate was on the top of the pit, descending, stationary at the bottom of the pit, ascending and again at the top of the pit. The estimate statements were used to analyse the gas mixtures comparisons. Significance was fixed at $P < 0.05$.

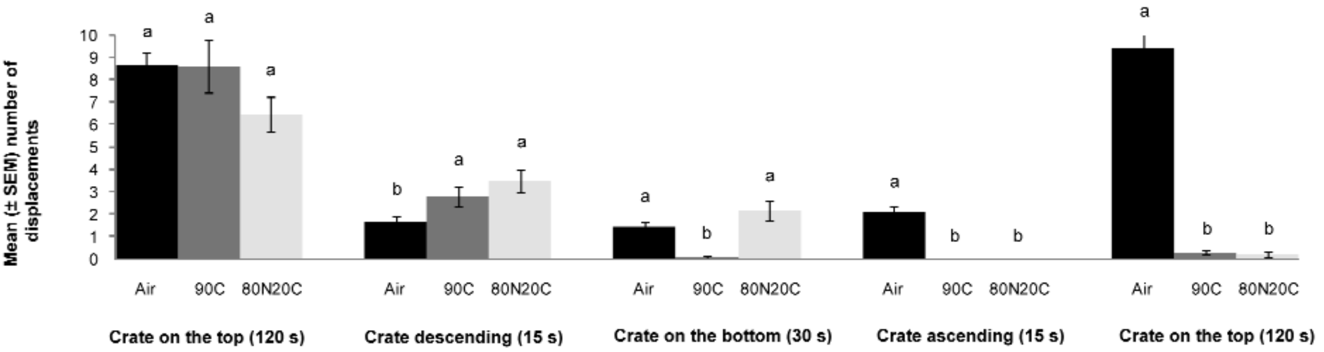
The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

Results

Prior to the descent of the crate, the percentage of animals exploring and the number of moves (general activity; Figures 1 and 2) did not differ significantly between air and gas treatment days. During the descent of the crate, the percentage of animals showing exploratory behaviour was also similar (Figure 1). However, an effect was found for general activity (C2 = 11.84; $P = 0.0027$), the number of moves being higher during 90C and 80N20C than during atmospheric air ($P < 0.05$ in both cases; Figure 2). When the crate was stationary at the bottom of the pit and ascending, 84 and 90% of the rabbits, respectively, performed exploratory behaviour the first day, significantly higher than during the gas treatment day (C2 = 113.88; $P < 0.0001$ and C2 = 127.15; $P < 0.0001$, respectively), when none performed this behaviour (Figure 1). When the crate was at the bottom of the pit, an effect was found for general activity (C2 = 34.79; $P < 0.0001$), the number of moves (Figure 2) being higher when the pit contained atmospheric air (1.44 [± 1.939]) and 80N20C (2.23 [± 1.991]) than when it contained 90C (0.07 [± 0.365]; $P < 0.0001$ in both cases). When the crate was ascending in the presence of air, animals showed 2.10 (± 2.006) moves, with a significant effect of day (C2 = 106.30; $P < 0.0001$), and no moves were observed in the case of 90C and 80N (Figure 2). During the ascent, animals performed moves only when exposed to atmospheric air (2.10 [± 2.006]; C2 = 106.30; $P < 0.0001$; Figure 2). After the exposure cycle, an effect was also found (C2 = 13.26; $P = 0.0013$), but in this case the percentage of animals performing exploratory behaviour (Figure 1) was higher in atmospheric air (97%) and 80N20C (91%) than in 90C (57%; $P < 0.01$ in both cases). In the presence of atmospheric air, rabbits showed 9.37 (± 5.366) moves during the last phase (2 min after ascent), significantly more (C2 = 137.47; $P < 0.0001$) than with 90C (0.27 [± 0.450]) or 80N20C (0.19 [± 0.535]; Figure 2). Eleven percent of animals tried to go out of the crate when it was at the top position during the moves. Forty-three percent of these events occurred during the first 2 min in the atmospheric air treatment, 33% just after ascending during the atmospheric air treatment and 24% during the first 2 min before descent when the pit contained 80N20C (14%) or 90C (10%).

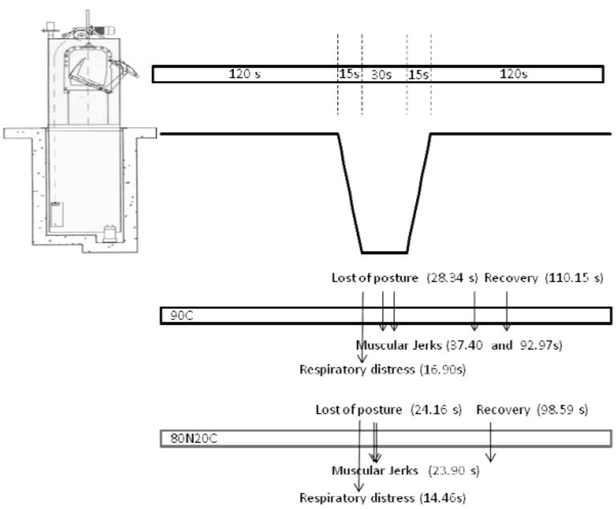
Animals exposed to atmospheric air did not show signs of respiratory distress. On the other hand, the percentage of animals with respiratory distress was significantly higher (C2 = 12.31; $P = 0.0005$) when exposed to 90C than to 80N20C (97 vs 42%). In addition, respiratory distress appeared later ($F = 5.17$; $P = 0.0284$) in 90C than in 80N20C (Figure 3). During the gas treatment day, all animals, with the exception of one exposed to 90C, lost balance. Animals exposed to 80N20C lost balance earlier ($F = 9.70$; $P = 0.0028$) than those exposed to 90C (Figure 3). During the gas-treatment day, two phases of muscle twitching were observed in 90C, one during exposure to the gas treatments and another subsequently, when the crate was at the top of the pit, but only the first was observed in 80N20C. In this last case, more animals showed muscle twitches in 80N20C than in 90C (C2 = 39.65; $P < 0.0001$) and these muscle twitches also appeared earlier ($F = 41.41$; $P < 0.001$) and for a longer duration ($F = 6.48$; $P = 0.0156$) in 80N20C than in 90C (Figure 3).

Figure 2



Mean (± SEM) number of moves (see text) in relation to the different positions of the crate and gas treatment (atmospheric air [Air], 90C and 80N20C). Different superscripts mean significant differences at P < 0.01 within the position.

Figure 3



Mean times of appearance of respiratory distress, lost of balance, muscle twitches and recovery of balance in 90C and 80N20C treatments in relation to the position of the crate into the pit.

The mean duration of muscle twitching during the second phase in 90C was 17.03 (± 5.372) s. Finally, all the animals exposed to 80N20C and 90C recovered balance. However, this recovery was earlier (F = 12.81; P = 0.0007) in 80N20C than in 90C (Figure 3).

Discussion

Carbon dioxide (CO_2) has been commonly used for euthanasia of laboratory animals, especially when a large number of individuals must be euthanised in a short period of time (Conlee *et al* 2005). In the meat industry, the use of high concentrations of CO_2 for stunning pigs has increased in recent years, especially in the larger plants, with the development of new equipment such as was used in this project. Velarde *et al* (2007) stated that high CO_2 concentrations (70 and 90%) increase aversiveness in pigs due to early detection of the gas at the entrance of the crate and the stimulation of more nociceptors. Llonch *et al* (2011) suggested that pigs exposed to gas mixtures with 15 to 30% of CO_2 do not react adversely before entering the crate. In rabbits, the lack of difference in exploratory behaviour and general activity between treatments (air, 90C and 80N20C) when the crate was at the top position, confirms that there is no aversion to the presence of gas in the pit at this point. The fact that animals recovered their balance shortly after the end of the exposure cycle also suggests that the presence of gas with 90C and 80N20C on the top position is very low, as described by Dalmau *et al* (2010a). During the descent of the crate rabbits increased their activity on the second day, when exposed to the gas treatments. The lower general activity of the first day might be the consequence of an increase in the fear response of immobility (freezing reaction) provoked by the novel situation of being lowered into the pit (Whishaw *et al* 1978; Ewell *et al* 1981). The fear response also includes alertness in rabbits (EFSA 2005), indicated by an increase in exploratory behaviour. On the other hand, the increased activity during the second day, might be a consequence of the aversiveness to the 90C and 80N20C exposure, described in pigs as escape attempts (Raj & Gregory 1996; Dalmau *et al* 2010b), in mice as increased locomotion (Ambrose *et al* 2000), and in rats as abnormal activity, excitation and agitation (Coenen *et al* 1995). Although rabbits exposed to 80N20C lost balance at a mean time of 24 s, that is, 9 s after arriving at the bottom of the pit, the number of times they crossed lines at this position was similar to that by animals in atmospheric air that had 30 s to do it (21 s more). In contrast, although animals exposed to 90C lost balance later than when in 80N20C, only one showed moves. Therefore, according to the previous description of the behaviours, animals exposed to 80N20C were more excited and animals with 90C showed more immobility than when atmospheric air was used, but we cannot conclude which is more aversive to rabbits. However, the most interesting points are the greater occur-

https://doi.org/10.7120/096272812X13353700593923 Published online by Cambridge University Press

rence of respiratory distress in animals exposed to 90C (97%) compared with animals exposed to 80N20C (42%) and the fact that animals in 80N20C lost balance earlier than in 90C. Respiratory distress is considered a physiological reaction associated with breathlessness during the inhalation of CO_2 (Raj & Gregory 1996). Dalmau *et al* (2010b) found a higher percentage of pigs showing gasping in 70N30C than AR or 85N15C and higher in 85N15C than AR. Llonch *et al* (2011) also found in pigs a higher percentage of animals showing gasping in 70N30C than in 80N20C and 85N15C, confirming the effect of the CO_2 concentration on respiratory distress. Gerritzen *et al* (2007) described the occurrence of gasping behaviour in poultry with concentrations below 20% of CO_2 in atmospheric air and Raj (1998) stated that, although gasping is present in poultry stunned with mixtures of CO_2 up to 30%, the response is less severe than when using higher CO_2 concentrations. Coenen *et al* (1995) described a higher percentage of gasping with mouth open and head turned up and backward in rats with higher CO_2 concentration. In fact, an increase in CO_2 concentration increases pCO_2 in the blood, which is subsequently detected by the respiratory centre in the medulla oblongata producing an increased rate of breathing (Guyton & Hall 2000), so higher CO_2 concentrations can produce deeper breathing due to higher CO_2 pressure in the blood. On the other hand, gasping and loss of balance appear earlier in pigs (Raj *et al* 1997; Velarde *et al* 2007) and broilers (Raj *et al* 1992) when CO_2 concentration increases. Britt (1986), Danneman *et al* (1997), Ambrose *et al* (2000) and Leach *et al* (2002) described in rodents also that as the inhaled CO_2 concentration increases, the time to unconsciousness decreases. The opposite has been observed in the present study for rabbits. In our opinion, the effect found in 80N20C compared to 90C in the present study could be due to a better distribution of the gas mixture 80N20C along the whole pit, as described Dalmau *et al* (2010a) when ten different mixtures, including the two in the present study, were explored. However, other explanations, such as a high sensibility of rabbits to anoxia or, rather, tolerance to CO_2, as described Hertrampf and von Mickwitz (1979) cannot be ruled out and they are, in fact, not mutually exclusive. Other behaviours, such as defaecation, described in rodents as sign of distress during exposure to gas (Smith & Harrap 1997; Ambrose *et al* 2000), were not observed in rabbits during the present study.

According to Raj and Gregory (1996), muscle twitches before or during the loss of balance are associated with escape attempts in conscious animals. On the other hand, muscle twitches after loss of balance are associated with involuntary convulsions in unconscious animals (Forslid 1987, 1992), although Rodríguez *et al* (2008) stated that animals could be conscious during these movements. Coenen *et al* (1995) described four phases during the exposure to high CO_2 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); and disappearance of muscle tone and head sinking (phase IV) — but it is not clear at what point the onset of unconsciousness occurs. In the present study, the state of consciousness of the rabbits was not measured directly, but it was observed as muscle twitches (compatible with phase III) appeared in the moment the animals lost balance when 80N20C was applied and a mean time of 9 s later and 18 s prior to recovery when 90C was applied. Again, two very different patterns can be observed in both gas treatments. Raj (1999) described muscle twitches as lasting longer when pigs were exposed to either argon or to a mixture of argon and CO_2 than when they were exposed to a high concentration of CO_2. Lawson *et al* (2003) concluded for mice that CO_2 at high concentrations is preferable for euthanasia due to the rapidity of effects and lack of muscle rigidity and spasms in comparison to argon and nitrogen. However, the mean total times of muscle twitches for rabbits in the present study were 10.7 s in 80N20C and 6.4 (+ 17.0 s later) in 90C. Therefore, it is harder to draw this conclusion from the results we obtained, although further studies are needed to ascertain in which moment the animals are unconscious by assessing brain activity and to know if the different muscle twitches periods observed correspond to the same state in the animal. Finally, animals exposed to 80N20C recovered balance earlier than animals exposed to 90C. This is in accordance with other studies (Dalmau *et al* 2010b; Llonch *et al* 2011) that found that the lower the CO_2 concentration, the quicker the recovery of pigs. Therefore, further studies are needed to assess the exposure time required to guarantee unconsciousness until brain death via exsanguination. In this case, it will be also necessary to study the effect on meat quality in relation to other stunning systems.

Animal welfare implications

Exposure to gas mixtures should be considered as an alternative to mechanical or electrical stunning in rabbits, as it reduces the stressful procedures of restraining and shackling. However, CO_2 is not authorised for use in stunning rabbits for meat production because it is aversive to animals in high concentrations. Other gas mixtures, such as 80N20C, could be a good alternative, as the adverse effects of the CO_2 are reduced. Therefore, from an animal welfare point of view, of the gas mixtures tested here, 80N20C is recommended.

Conclusion

According to the results, rabbits did not react adversely to 90C or 80N20C when they were at the top of the crate. However, during crate descent, animals immersed in 90C or 80N20C showed an increase in general activity in comparison to atmospheric air. Furthermore, animals showed respiratory distress in both treatments, but in a higher percentage of animals when exposed to 90C as opposed to 80N20C. In 1 min of exposure time, rabbits lost balance before 30 s and recovered it after 30–40 s of exposure, but none died. Muscle twitches were observed in both treatments, but appeared earlier in 80N20C than in 90C. Therefore, it is concluded that in rabbits, exposure to 80N20C is more aversive than atmospheric air, but that it causes less respiratory distress than 90C.

Acknowledgements

Thanks are due to Oriol Rafel, Miriam Piles and Josep Ramón for their collaboration.

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doi: 10.7120/096272812X13353700593923