Question to EURCAW-Pigs
10 July 2020

Question
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EURCAW-Pigs received the following issue and questions from a Ministry:

- What is the current scientific knowledge on the use of nitrogen for stunning and killing of poultry and piglets?
- Is there scientific knowledge on additional benefits and risks if nitrogen-filled foam is used for this purpose?

Answer
Several EURCAW experts contributed to the response below. The EURCAW secretariat did the final editing, and may be contacted for queries: info.pigs@eurcaw.eu.

In short, the answers are:

- Due to the physical properties of nitrogen, there is a lack of studies on stunning and killing animals with pure nitrogen. Studies on the effect of pure nitrogen can only be carried out in closed containers/boxes, in mixtures with CO_2 or by using a high expansion foam. Like nitrogen, argon is an inert gas, but it is heavier than air and therefore easier to concentrate in stunning systems. To describe the effect of inert gases, thus, reference is made not only to the few studies with nitrogen, but also to investigations with argon and with mixtures of these both inert gases with carbon dioxide.
- The addition of CO_2 to argon to produce hypercapnic anoxia seems more promising for stunning and killing poultry than inert gases such as nitrogen alone: hypercapnic anoxia accelerates the time to death and reduces the degree of wing flapping and convulsions. Particularly during these vigorous movements, loss of consciousness cannot be assured. Therefore, stronger respiratory symptoms during hypercapnic anoxia seem to be justifiable, when compared to the increased risk of injury during pronounced convulsions of conspecifics in the absence of additional CO_2.
- Study results on anoxia (argon) and hypercapnic anoxia (mixtures of argon and carbon dioxide) in suckling piglets are contradictory. Hypercapnic anoxia does not seem to be as beneficial for stunning and killing piglets as in poultry.
- The residual oxygen content is decisive for the effectiveness of stunning and killing by anoxia. Research on the selection and use of inert gases for stunning and killing, in particular of moribund animals, e.g. those with impaired lung function, should be extended. In addition, basic research should be conducted on the regulation of respiration, whether it is regulated not only by pCO_2, as previously assumed, but also by pO_2.
- Gas-filled foam enables the use of pure nitrogen in an open system. The process is not only promising for use on farm level, but also as an alternative to whole-house-gassing in case of a disease outbreak. Further research work should be carried out on the emergency killing of piglets, which, with parallel optimisation and adaptation of the technology, should include investigations into the assured killing effect of the process and the use of gas-filled foam on groups of animals. Risks arise from the lack of visibility of the animal due to the opaque foam

and the possible stress to the animal during submersion in foam. The size of the bubbles must be large enough, depending on the animals to be killed, to prevent foam from obstructing the airways. A sufficient supply of foam during stunning and killing of animals particularly by anoxia should reduce the risk that the foam is destroyed too quickly by movements so that the animals have access to oxygen-rich ambient air (with the risk of prolonged induction of unconsciousness or reawakening).

- Possible key parameters for the process of stunning and killing by a gas-filled foam are bubble size (to prevent trachea displacement), residual oxygen, expansion rate, sufficient foam production rate (especially with inert gases), gas source and gas purity, foam concentration, gas and water temperature.

Further information can be found in the background section below.

Scope

The current knowledge on stunning and killing of poultry and pigs with argon and nitrogen and mixtures of these inert gases with carbon dioxide is described below. With regard to the questions, single-phase controlled atmosphere stunning (CAS,anoxia and hypercapnic anoxia) procedures are described, especially for poultry. Two-phase approaches (hypercapnic hyperoxygenation; McKeegan et al., 2007, Abeyesinghe et al., 2007) will not be discussed further despite possible advantages for animal welfare, as they are intended for use at farm level for emergency killing.

Background

- *What is the current scientific knowledge on the use of nitrogen for stunning and killing of poultry and piglets?*

Gas stunning and killing procedures do not immediately render animals unconscious (Raj et al., 1998a, EFSA, 2004). The induction of unconsciousness with gases and gas mixtures is a gradual process (EFSA, 2004). To ensure that the introduction phase is not stressful for the animals, the gas mixture itself should not be aversive and the time to loss of consciousness during gas exposure should be as rapid as possible (Raj et al., 1998a; Gerritzen et al., 2000; Raj and Tserveni-Gousi, 2000; EFSA, 2004; EFSA, 2019a). The rate of induction, the depth and the duration of unconsciousness, and death depend on exposure times and gas concentrations. Both parameters can be used to control gas stunning and killing (EFSA, 2019a).

Inert gases and mixtures of those with carbon dioxide

Poultry

Inert gases such as argon and nitrogen used at normobaric conditions and in high concentrations with a residual oxygen content of less than 2% induces anaesthesia by anoxia (Barton Gade et al., 2001; Raj and Tserveni-Gousi, 2000; McKeegan et al., 2007) based on the displacement of oxygen from the inhaled atmospheric air (Poole and Fletcher, 1995; Hoen and Lankhaar, 1999; EFSA, 2004; Webster and Fletcher, 2004). The residual oxygen content is a decisive factor for the stunning effectiveness of anoxia processes including the time to induction of unconsciousness. According to the investigations of Raj et al. (1990), it should even be lower than 1% if, during stunning and killing of several animals (e.g. in a cage with floor), an increased amount of oxygen-rich ambient air enters the prepared gas atmosphere via air pockets between the animals, so that the effective inert gas concentration is reduced.

Since the molecular weights of nitrogen and atmospheric air are only slightly different, it is difficult to maintain the required high nitrogen concentrations for stunning (Poole and Fletcher, 1995; Raj and Tserveni-Gousi, 2000; Sandilands et al., 2011; Llonch et al., 2012). The stability of nitrogen (=maintaining a high gas concentration without being displaced by oxygen) is increased in mixtures with CO_2 as a gas heavier than air (Troeger et al., 2004b; Dalmau et al., 2010a, 2010b). Since argon is heavier than air and therefore easier to concentrate in stunning systems, studies on the effects of inert gases are primarily available with argon. The investigation of nitrogen is limited to mainly closed stunning systems (e.g. boxes with an active gas inlet) or to the use of nitrogen in combination with carbon dioxide (hypercapnic anoxia). Since nitrogen, like argon, is an inert gas, the following description refers mainly to studies with argon due to the current lack of studies on the effects of pure nitrogen. With exposure times less than 3 min, anoxia as well as hypercapnic anoxia are considered as simple stunning methods (EFSA, 2019a).

Behavioural tests showed that inert gases such as argon and nitrogen are less aversive to the animal than carbon dioxide in high concentrations. In the study of Webster and Fletcher (2004), using approach-avoidance-tests, hens showed the least stops and retreats on the way to the argon atmosphere compared to CO_2 containing mixtures. The percentage of hens that lost their posture in the gas-filled chamber and not on the way towards it was highest for argon and for 30% CO_2 in air. Hesitating to enter the chamber filled with 60% CO_2, most hens were stunned already in the corridor leading to the chamber. Intermediate percentages of hens entered the chamber before losing posture in the presence of 45% CO_2 in air or 30% CO_2 in Argon. The study of Raj (1996) has produced similar results: 50% of turkeys refused an atmosphere containing 72% CO_2 in air whereas the majority of them entered the feeding chamber containing either 90% argon in air or 30% CO_2/60% argon in air (92% and 83% of the tested turkeys, respectively).
In the search for alternative gases, inert gases and mixtures of inert gases with a limited amount of carbon dioxide (e.g. up to 40%) were used for stunning and killing of poultry.

Studies to stun hens, broiler chickens and turkeys have shown that inert gases can lead to a rapid loss of consciousness and onset of death (Raj et al., 1991, 1992, 1998a; Raj and Gregory, 1994). Loss of somatosensory evoked potentials (SEP) serving as unequivocal indicator of the loss of consciousness was reached in hens, broiler chickens and turkeys 29 s, 32 s and 44 s, respectively, after exposure to 90 % argon in air (2% residual oxygen; Raj et al., 1991, 1992, 1998a; Raj and Gregory, 1994). The time to unconsciousness in an atmosphere of nitrogen (< 2% residual oxygen) was 48 s in broilers based on averaging the values of time to an isoelectric EEG and to CD (correlation dimension) values below 60% (Coenen et al., 2009). The time to death of the animals in the nitrogen atmosphere was 194.3 s based on an isoelectric EEG and a reduction of the heart rate to less than 180 bpm (Coenen et al., 2000, 2009). In the study of McKeegan et al. (2007), assuming an isoelectric EEG and a respiratory arrest as the definition of the onset of death, this was already achieved after an average of 94.25 s in nitrogen and after a mean of 107.5 s in argon. Only based on respiratory arrest, death of broiler chickens occurred in argon after 79 s and in nitrogen after 75 s (Poole and Fletcher, 1995). The addition of CO_2 to the inert gases and thus the induction of hypercapnic anoxia significantly shortens the time to unconsciousness and death. Thus, the loss of consciousness in N_2/CO_2 occurred after an average of 33 s (instead of 48 s, Coenen et al., 2009). Taking into account the differing definitions, death in N_2 /CO_2 occurred in the study by Coenen et al. (2009) already after 124.1 s and according to McKeegan et al. (2007) only after 80.71 s.

Anoxia by the use of pure argon and nitrogen is associated with the occurrence of more vigorous convulsions in form of wing flapping compared to CO_2 and CO_2 containing gas mixtures including especially those with oxygen (hypercapnic hyperoxygenation; Woolley and Gentle, 1988; Poole and Fletcher, 1995; Barton Gade et al., 2001; Raj and Tserveni-Gousi, 2000; Abeyesinghe et al., 2007; McKeegan et al., 2007, Coenen et al., 2009). Patterns of wing flapping appear to be less pronounced during hypercapnic anoxia induced by gas mixtures containing 30% CO_2/60% argon/nitrogen in air (McKeegan et al., 2007; Coenen et al., 2009). The wing flapping bout duration was significantly increased in anoxia compared to hypercapnic anoxia (McKeegan et al., 2007). While argon and nitrogen provoked longer bouts, hypercapnic anoxia seemed to induce more bouts of shorter length (McKeegan et al., 2007). Coenen et al. (2009) could not detect this difference in the length of the bouts per se, but in this study the N_2 anoxia led to the highest total duration of wing flapping. During the investigations of Mc Keegan et al (2007), the wing flapping lasted the longest during Argon anoxia, followed by nitrogen and the two mixtures to produce hypercapnic anoxia. However, differences in wing flapping duration between N_2 anoxia and hypercapnic anoxia were not significant (McKeegan et al., 2007).
Whether the animals are still conscious during these convulsions is a matter of debate (Raj et al., 1991; Raj and Tserveni-Gousi, 2000; McKeegan et al., 2007). On the one hand, it is assumed that convulsions result from the abolition of the inhibitory effect of higher (and already stunned) centres on subcortical centres (Ernsting, 1965). The occurrence of convulsions per se should therefore be taken as an indicator of loss of consciousness (Raj et al., 1998a; Raj and Tserveni-Gousi, 2000). On the other hand, McKeegan et al. (2007) concluded -also with regard to the results of Raj et al. (1991, 1998a)- that these anoxic convulsions occur at a time when unconsciousness is not guaranteed. According to McKeegan et al. (2007), this is the case if e.g. SEPs are not completely lost in the EEG (the suppression of SEP is not sufficient) or if the EEG was not substantially suppressed or isoelectric in temporal proximity to wing flapping. Also in hypercapnic anoxia, although it leads to a faster loss of consciousness probably due to the narcotic effect of carbon dioxide (Martoft et al., 2003; McKeegan et al., 2007), the conscious experience of convulsions by the animal cannot be safely excluded. While the onset of convulsions in hypercapnic anoxia in hens and broilers in studies of Raj et al. (1992, 1998a) occurred temporally after the loss of SEP, this occurred in the study by Coenen et al. (2009) only at a stage when consciousness was still a possibility.

Birds have intrapulmonary chemoreceptors (IPC) in their lungs which are sensitive to carbon dioxide but do not react to anoxia (Ludders, 2001). Anoxia procedures therefore have no direct effect on respiration like CO_2 containing gas mixtures. Parameters of respiratory disruption like open bill gaping and cessation of breathing (apnoe) were thus only visible in later phases of euthanasia due to anoxia (McKeegan et al., 2007). Respiratory disruption was more frequent during hypercapnic anoxia compared to anoxia alone (McKeegan et al., 2007; Abeyesinghe et al., 2007). Hypercapnic anoxia led to strong respiratory 'deep breathing' responses like an increased inhalation depth and duration (McKeegan et al., 2007). However, it should be noted that vigorous behavioural movements as described above might mask a potential hyperventilation at an earlier stage of stunning with inert gases (McKeegan et al., 2007; Coenen et al., 2009).

Pigs and piglets

When anoxia is induced with argon, no signs of aversion (Machold et al., 2003a, 2003b; Raj and Gregory, 1995) and no respiratory distress symptoms before loss of consciousness (Raj and Gregory, 1995) are detectable in slaughter pigs. Due to the lack of stimulation of the respiratory centre, the time until the onset of loss of consciousness and insensibility is not associated with stress for the animals (Llonch et al., 2012). Hypercapnic anoxia using 30% CO_2 in argon led to a rapid loss of SEPs in the EEG within 11-20 seconds (Raj et al., 1997), comparable with CO_2 in high concentrations. In contrast to CO_2 in high concentrations, the majority of pigs (75%) did not show aversive reactions when CO_2 and argon were combined (Raj and Gregory, 1995).

These results for slaughter pigs cannot be directly transferred to piglets. The anaesthesia and killing of suckling piglets with argon compared to 100% carbon dioxide (both in the prefill procedure and independent of the disease status) was associated with a delayed onset of unconsciousness (loss of posture) and a prolonged and increased occurrence of ethological stress indicators. These included an increased duration of open-mouth breathing, ataxia, more righting responses of increased intensity, increased escape attempts and a prolonged time to respiratory arrest (Sadler et al, 2013b). A reduction of the stress in neonatal piglets based on these stress-related behaviours could not be achieved by applying a 50 % carbon dioxide / 50 % argon mixture (Sadler et al., 2013a). This contradicts the results of the approach-avoidance tests of Rault et al. (2013), in which a lower aversion to a gas mixture of 30% CO_2/60% Ar compared to 90% CO_2 was found for piglets. Kells et al. (2018) also failed to demonstrate fundamental advantages of hypercapnic anoxia in the stunning and killing of piglets. Even though in this study a loss of consciousness in the argon atmosphere compared to CO_2 and CO_2/Argon was still delayed as in Sadler et al. (2013b), now under anoxic conditions animals were less distressed (f.ex. based on escape attempts, deep breathing) and the number and total duration of convulsions was increased similar to poultry. The authors attributed vocalizations (grunting) before loss of posture and not during escape attempts in argon and Ar/CO_2 to exploration behaviour under non-aversive environmental conditions. A higher number of convulsive bouts as well as more frequent and longer (unconscious) vocalizations under anoxic conditions (100% argon) were confirmed in the study by Sutherland (2011) compared to 100% CO_2. Contrary to the authors cited above and although no significant differences were found between argon and argon/CO_2 mixtures, based on the calculation of a welfare index, Sutherland (2011) recommends the use of gases to induce hypercapnic anoxia over pure argon due to significantly more rapid loss of consciousness, faster onset of isoelectric EEG and respiratory arrest.

According to Sadler (2013b), the general condition of the piglets (severely depressed piglets vs. other piglets) is not a decisive factor in the effectiveness of gas stunning and killing with carbon dioxide (regardless of the way the gas is provided), but it does have a negative effect on the anoxia process using argon. The time to loss of consciousness as well as the duration of open-mouth breathing is significantly prolonged ($p<0.05$) in moribund piglets with decreased respiration rate and tidal volume in argon compared to other piglets, while the time of last limb movements is faster and the duration of ataxia and righting attempts is shortened. Sadler et al (2013b) attribute these differences in effectiveness to the different effect mechanisms of the two gases. CO_2 causes hypercapnia and affects multiple organ systems by lowering the pH value (even in the blood and interstitial fluid), so that an euthanasia process is equally possible regardless of

the disease status of the animal. The acidotic status of the moribund animal (Straw et al., 2009, cited by Sadler et al., 2013b) is not an inhibiting factor. Argon, on the other hand, produces a hypoxic state, which can additionally complicate euthanasia in animals with impaired lung function. The insertion and especially the necessary removal of the animals into and out of the gas atmosphere was, independent of the disease status, associated with a critical increase of the residual oxygen content in the prepared argon atmosphere (< 7 %) according to Raj et al. (1990). Target values of less than 3 % were only regained within 45 s with ongoing gas supply.
The widespread opinion that euthanasia is more difficult to achieve in neonatal compared to older weaned piglets could be rejected by the studies of Sadler et al (2013b). In neonatal piglets, with the exception of the duration of ataxia, an earlier loss of posture and earlier time of last movements during gas killing could be demonstrated while stress-indicating behaviour is less pronounced.

- *Is there scientific knowledge on additional benefits and risks if nitrogen-filled foam is used for this purpose?*

By incorporating nitrogen in highly expansive foam (expansion rate > 1:300, 10-20 mm bubble size), it is now possible to use this gas in high concentrations next to the animal in an open system without necessary sealings (Raj et al., 2008b; Gerritzen and Sparrey, 2008; Gerritzen et al., 2010) or CO_2 proportions. According to EFSA (2019b), gas-filled foam as stunning and killing method is more designed as large-scale killing method than for individual killing. The effectiveness of this method has already been proven for poultry (Raj et al., 2008; Gerritzen et al., 2010; McKeegan et al., 2013). The opaque foam limited behavioural observations (Gerritzen et al., 2010; McKeegan et al., 2013). Bubble size was 10-20 mm in order to minimize risk of occluding the trachea (Gerritzen et al., 2010; McKeegan et al., 2013).

Due to the low concentration of residual oxygen in the foam below 1%, the average time until loss of consciousness through nitrogen anoxia based on a suppressed EEG was 30 s for hens (Mc Keegan et al., 2013) and 18 s (McKeegan et al., 2013) resp. 13 s for broilers (Gerritzen et al., 2010). A loss of consciousness using CO_2 filled foam was evident in broilers after 16 s (Gerritzen et al., 2010; McKeegan et al., 2013). With anoxic foam (nitrogen), transitional EEG states were achieved significantly ($p=0.024$) later after complete submergence of the animal. Using CO_2 in the foam, however, transitional EEG states were already detectable prior to submergence (Gerritzen et al., 2010; McKeegan et al., 2013). This positive accelerating effect on the reduction of consciousness is due to the narcotic properties of the CO_2 released from the foam. In addition to gasping, CO_2 indeed leads to more frequent and earlier headshaking in the animals, but the released gas can serve as a safeguard in case of destruction of the foam structure by wing flapping or convulsive spasms (Gerritzen et al., 2010, McKeegan et al., 2013). If movements destroy the foam, the surrounding CO_2 does not allow the animals to reach oxygen-rich ambient air directly, thus reducing the associated risk of re-awakening (Gerritzen et al., 2010, McKeegan et al., 2013). The onset of convulsions and vigorous wing flapping was comparable between nitrogen and carbon dioxide (Gerritzen et al., 2010) or occurred in the transitional phase after ataxia and loss of posture (McKeegan et al., 2013). Particularly in anoxia processes, a sufficiently high capacity of the foam generators must therefore be ensured so that the destruction of the foam by these vigorous movements does not outweigh the subsequent supply of foam (Gerritzen et al., 2010, McKeegan et al., 2013). The height of the foam layer above the animals can thus be used as a key parameter to control the process (McKeegan et al., 2013). Time to cessation of movements as a sign of death was reached in broilers 51 s and in hens 65 s after start of N_2 foaming (McKeegan et al., 2013). By using the nitrogen-filled high expansion foam, the time to cessation of movements was approximately half the time to death compared to air- or CO_2-filled foams of lower expansion rates (Alphin et al., 2010, McKeegan et al., 2013).

A rapid onset of death by nitrogen anoxia could not be demonstrated in the investigations of Balzer (2017) on the killing of moribund, 3-day-old sucking piglets. The residual oxygen content was less than 1%. The target bubble size of 15 mm successfully prevented obstruction of the respiratory tract. However, it could be shown that animal reactions (vocalizations, latency to last limb movement, catecholamines) were not reduced compared to data from the literature on CO_2. Compared to CO_2, the duration of vocalizations was longer with 14.02±11.84 s (Sadler et al., 2013). Transcutaneous blood gas measurements indicated a rapid reduction of pO_2 as well as a constant increase of pCO_2 during foam-induced N_2 anoxia potentially because of CO_2 rebreathing of the animal. The onset of apnea, characterized by a basal value increased by more than 20 mm Hg (Nagakawa et al., 2011), occurred after 4.1±0.99 minutes. Death was determined by electrocardiogram, phonendoscopic auscultation and reflex tests (e.g. corneal and pupillary reflexes, pain-associated motor reflexes). Although, after extension of the exposure time to 12 minutes, there was no longer any evidence of auscultation of the heartbeat in any of the animals, a cessation of the electrical activity of the heart was still not achieved. A confirmed cardiac arrest in case of N_2-anoxia for the determination of death (AVMA, 2020) was not evident, but the areflexia present in each animal after 10 or 12 minutes of exposure suggested the induction of brain death. The hypoxia-related changes in ECG were associated with an increase in catecholamine concentrations and occurred later in animals with increased duration of movements (continuous and sporadic movements). The latency period until the last movement was 296.8±155.33 s.

This contrasts with the results from the recently completed "Conture" project at the Swedish University of Agricultural Sciences: in a technically advanced facility compared to the FENDA project (Balzer, 2017), the use of nitrogen-filled foam for stunning pigs was investigated in comparison to air-filled foam and application without foam (Wallenbeck et al., 2020). The animals heavier than those in the FENDA project (Balzer, 2017) (27.8 ± 3.4 kg, per treatment and control group n= 20) showed no aversive behaviour when exposed to the air-filled and nitrogen-filled foam. However, they avoided dipping the head and the mouth area when the foam level increased and showed increased escape attempts with the filling of the box. Expected increases in heart and breathing rate with reduction of oxygen content were observed. The loss of posture occurred after an average of 57.9 s, followed by violent convulsions, which changed into less strong and irregular movements. After an average of 131.2 s, no convulsions were detected. At the stunning control, the animals were unconscious or dead within five minutes after the start of foam production (Wallenbeck et al., 2020).

The use of foam in closed systems (with lid) with modification of the foam supply (from below) and the foaming agent showed promising results under practical conditions during stunning and killing of piglets in groups. A comparable animal size should be used to reduce the risk of injury during euthanasia (Van der Aa et al., Anoxia BV, 2020).

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