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Aversion of chickens to various lethal gas mixtures

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Abstract

In the event of a notifiable disease outbreak, poultry may need to be culled in situ. *This should be performed swiftly and humanely to prevent further spread of the pathogen while preserving the welfare of the animals prior to death. Here, we examined the aversion of broiler chicks (*Gallus domesticus*) to three lethal gas mixtures at various concentrations to determine the least aversive mix that could be used in whole-house gassing. For 1 h, individual chicks (n = 36) were allowed to place their heads inside three feeding and drinking stations (FDS) in order to access food and water. Each FDS was filled with a different gas mixture, and birds could access each FDS as much as they liked. Twelve chicks each were tested at low (50% carbon dioxide [CO_2] in air, 70% argon [Ar] in CO_2, 70% nitrogen [N_2] in CO_2), medium (55% CO_2 in air, 80% Ar in CO_2, 80% N_2 in CO_2) or high (60% CO_2 in air, 90% Ar in CO_2, 90% N_2 in CO_2) concentrations of gas mixtures. Aversion was assessed based on the time birds spent with head in each FDS (with more time indicating less aversive), and the frequency of head shakes relative to time spent with head in the FDS (with a lower proportion indicating less aversive). Data were analysed by ANOVA. On average, birds spent < 3 min with their head in any FDS. Mixtures containing 90% Ar or N_2 in CO_2 and 80% argon in CO_2 were least aversive and mixtures containing 70% N_2 in CO_2 and 60% CO_2 in air were most aversive, based on time spent with head in. Head shakes s^{-1} were more frequent with low concentration gas mixtures compared to high concentrations, and with all CO_2 in air mixtures, which suggests that the intensity of head shaking is related to the concentrations of CO_2. From these results, one concentration of each of the three gas mixtures (90% N_2 in CO_2, 80% Ar in CO_2, and 50% CO_2 in air) were chosen for assessment on a further 12 birds and the results showed that both inert gas mixtures were less aversive than 50% CO_2 in air based on time spent with head in. Frequency of head shakes s^{-1} did not differ between the three mixtures. Birds found all gases aversive, however it is concluded that inert gas in CO_2 mixtures were least aversive compared to CO_2 in air and these gases also caused less signs of respiratory discomfort.*

Keywords: *animal welfare, aversion, chickens, disease outbreak, lethal gas mixtures, preference testing*

Introduction

Worldwide, outbreaks of contagious diseases among farm animal species are becoming a familiar occurrence. Over the last decade, the UK and other parts of Europe have experienced foot and mouth disease, avian influenza, and blue tongue, all of which, in the EU, are notifiable diseases (Defra 2008b). It is essential that measures, such as culling, exist to reduce the risk of disease transmission to other susceptible animals and, if the disease is zoonotic, humans. Additionally, culling diseased animals can be necessary to eliminate pain and suffering, particularly where no cure is available. However, controlling and culling large numbers of animals is not a simple task, as was highlighted during the 2003 outbreaks of avian influenza in Europe and outbreaks in the UK (Defra 2008a). The most common methods of killing large flocks of birds in those cases was to expose birds to lethal concentrations of gases administered either directly into the house or removing birds to containers positioned outside the house into which gas was introduced. Whole-house gassing methods either used carbon dioxide (CO_2) or carbon monoxide (CO), while containerised gassing used either CO_2 in air or an inert gas (argon or nitrogen) and CO_2 mixture (Gerritzen & Lambooij 2004). The major advantages of whole-house gassing are that birds need not be handled prior to death, which is a significant bird welfare benefit, and that catching staff need not enter the house and come into direct contact with the infected environment, materials and birds. (For a full review, see Raj *et al* [2006]). In addition, gas mixtures have been used for stunning poultry, pigs (*Sus scrofa*) and lambs (*Ovis aries*) prior to slaughter (Hoen & Lankhaar 1999; Raj 1999; Machold *et al* 2003; Linares & Vergara 2009).

Regardless of the method used during gas culling or stunning, there is some concern that animal welfare could be compromised, because birds may suffer between the introduction of a gas or gas mixture and the onset of unconsciousness. Humans report feelings of breathlessness and a sense of suffocation and even pain when they inhale 50% CO_2 in

air (Manning & Schwartzstein 1995). Poultry are highly sensitive to CO_2 (Ludders 2001) and respond with depressed respiration, sneezing, gasping, and head shakes (Raj 1996; Lambooij *et al* 1999), which are thought to indicate respiratory distress. However, if loss of consciousness through exposure to gas mixtures is rapid, and birds do not regain consciousness before death, this may be considered an unavoidable but acceptable level of suffering compared to alternative culling methods, such as hyperthermia resulting from ventilation shutdown methods (HMSO 2006).

Previous work has shown that introducing broiler chickens (*Gallus domesticus*) to an environment of 90% argon in air (with 2% residual oxygen) resulted in loss of posture (a behavioural indicator of loss of consciousness [Raj & Gregory 1990; Gerritzen *et al* 2004, 2007]) after 13 s (average) and both broiler chickens and end-of-lay hens showed a profound suppression of spontaneous electrical activity in the brain recorded using electroencephalograms (EEGs) after 17 s (average) (Raj *et al* 1991; Raj 1997), suggesting that time between exposure and unconsciousness is brief. Mixtures of CO_2 in air or CO_2 alone take longer to induce unconsciousness in poultry compared to inert gas mixed with CO_2 or inert gas alone. For example, turkeys stunned with CO_2 and argon in a commercial stunning tunnel showed eyelid closure sooner (66 s) than turkeys stunned with CO_2 only (91 s) (Hänsch *et al* 2009). However, rapid induction of unconsciousness, as with anoxia induced by inert gases (eg < 2% residual oxygen) or hypercapnic anoxia induced by mixtures of inert gas and carbon dioxide (eg > 30% CO_2 and < 2% residual oxygen) produces more convulsions than mixtures that induce unconsciousness more slowly, such as mixtures containing higher concentrations of oxygen (for example, a mixture of 30% oxygen, 40% CO_2 and 30% nitrogen; Poole & Fletcher [1995]) or 30% carbon dioxide in air, which would contain 14% oxygen and 56% nitrogen; Raj & Gregory [1990]). This is immaterial if birds are unconscious during convulsions (as suggested by Raj & O'Callaghan 2001; Gerritzen *et al* 2004), however other work based on the use of correlation dimension as a determinant of the state of consciousness in poultry suggests that this may not always be the case (McKeegan *et al* 2007). Nevertheless, independent of the state of consciousness at the time of onset of convulsions, birds' preference or avoidance of a gas mixture may reveal their choice of gas mixtures that could be used to induce unconsciousness, and is easier to assess under well-controlled laboratory experiments. Preference testing is used to assess what animals want, or to determine what they want least, from a given set of choices. Preference testing has been used extensively in poultry studies (Gallagher 1976; de Jong *et al* 2007; Gunnarsson *et al* 2008; Struelens *et al* 2009) and across many other species of animals, such as rats (*Rattus norvegicus*) (Sørensen *et al* 2008), pigs (Baldwin & Meese 1977), dairy cows (*Bos taurus*) (Rioja-Lang *et al* 2009), and for various resources such as access to mates or companions (Akre *et al* 2009), food types (Bouvarel *et al* 2009), and environment (Nicol *et al* 2009). Preference testing has also been used previously to assess aversion to gas mixtures (Raj & Gregory 1991; Cooper *et al* 1998; Makowska *et al* 2008).

The aim of this study was to assess chickens' aversion to various lethal gas mixtures in a choice test, in order to determine which gas mixture might be least aversive to them for later use in whole-house culling.

Materials and methods

Animals and husbandry

In total, four batches of Ross 308 chicks were used. Chicks were housed at day-old in one climate-controlled room in batches of 20 (half male, half female) in litter-floor pens (2.5 m^2) with *ad libitum* access to food and water. Chick crumb (Target Feeds, Shropshire, UK), fed to 10 days of age, and pellets (Target Feeds, Shropshire, UK) thereafter were provided in food hoppers, and water was initially provided from chick drinkers and Spark nipple-and-cup drinkers (Roxell®, Belgium). Wooden feeder/drinker stations (FDS) 30 × 20 × 20 cm (length × width × height) were provided at day-old against each wall of the pen at floor level (Figure 1). The FDS, which chicks accessed through a 20 × 10 cm (length × height) aperture, contained Spark drinkers and a white feed cup which was filled with a mixture of the birds' normal feed and wholegrain wheat. Initially, chicks could climb into the FDS, but as they grew they could only place their head and neck through the opening. Once chick drinkers were removed at five days of age, Spark drinkers inside the FDS were the birds' only source of water, which they all successfully accessed. At 14 days of age, a curtain made from a clear plastic document holder cut into vertical strips 1 cm wide was pinned above the entrance to each FDS, but with the strips secured out of the way of the opening. The strips were hung across the FDS opening from 16 days of age. Every day, from 16 days of age onwards, the main food hopper was removed from the pen from 0830–1230h to encourage birds to access the feed cups in the FDS. Lights were initially on for 23 h a day from day-old, but, in order to facilitate use of the FDS in the testing phase, were gradually decreased to 9 h per day by 20 days of age. This lighting period was used to ensure that birds were more likely to be hungry when lights first came on, because although nocturnal feeding is known to take place in broilers, the rate is lower than that of diurnal feeding (Lewis *et al* 2009).

Apparatus and training

The aversion set-up consisted of two wooden chambers with a floor area of 1.49 m^2 (surrounded by a solid wall 61 cm high) housed in another climate-controlled room. One chamber was a true 'test' aversion set-up used for both acclimatisation and testing, the other a 'dummy' for acclimatisation only. Each chamber was raised 90 cm off the floor, mounted on top of another wooden box of the same dimensions. The top of the chamber was covered with a removable mesh lid. Directly above both chambers, approximately 2 m in distance from the chamber floor, was a tungsten bulb, illuminating the chamber to 30–35 lux. The floor of the chamber was covered in wood shavings, but around the inside lip of

Figure 1

An overhead view of a feeding/drinking station (FDS) used during rearing, acclimatisation to the test apparatus, and testing, showing the semi-circular feeder (right) and the nipple-and-cup drinker (left). A diffuser (centre, between the feeder and drinker) was added to the aversion set-up during acclimatisation and for choice tests. A gas sampling tube (far right) was used in the testing apparatus only. Arrow indicates the aperture through which a chick's head would enter the apparatus.

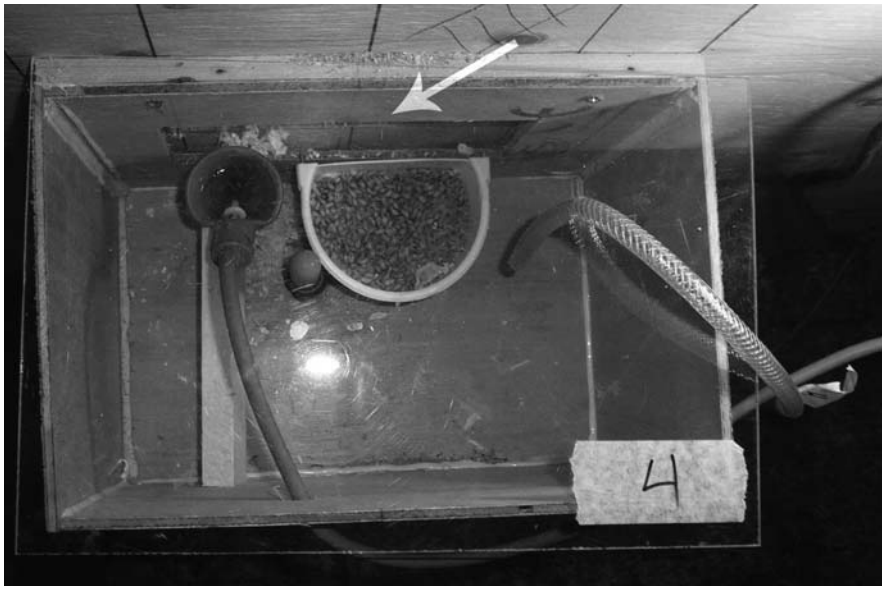
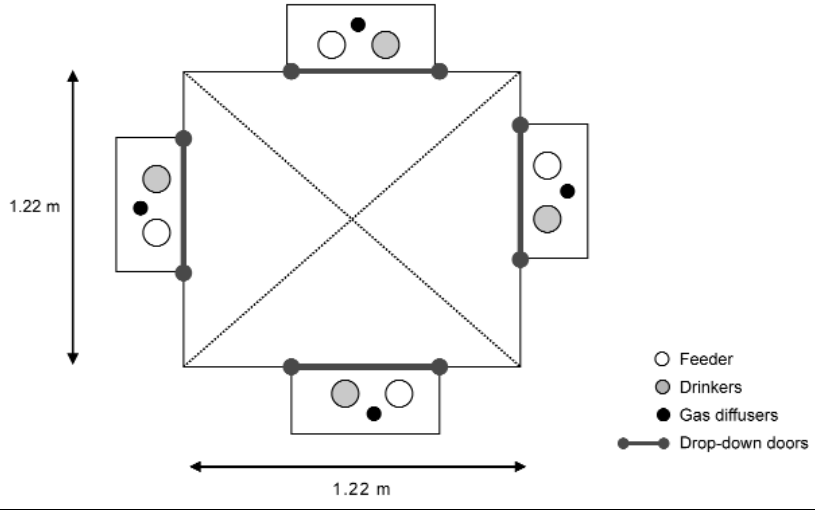


Figure 2

Overhead sketch of aversion set-up, with feeding/drinking stations (FDS). (Not to scale). The central chamber is shown split into quadrants with dashed lines for behaviour analysis of the video data.



the chamber was a 4 cm wide gap covered in fine mesh. Below the test aversion set-up only, in the bottom box, was a tube connected to a vacuum pump, which gently extracted air away from the central chamber. This ensured the safe removal of denser than air gas mixtures that might spill out of the FDS (see below) and into the central chamber.

One FDS as described previously was mounted to each outer wall of the two chamber set-ups. Birds could access the FDS aperture via a 10 × 8 cm hole in the side wall of the chambers. A drop-down door was mounted above each FDS access hole. As well as the drinker and feed cup, a diffuser (56 mm high, 18.5 mm in diameter; pneumatic plastic silencer, RS Components, Northamptonshire, UK) was mounted in the centre of the floor of each FDS. Each diffuser was connected to a tube (10 mm internal diameter) that ran through the wall into the neighbouring control room, where they could be connected to an air compressor or to gas mixing panels. The dummy aversion set-up was only ever connected to the air compressor. The lid of each FDS was made of clear acrylic and rested on top of the wooden FDS walls for ease of access to the feeder and drinker, and a light was placed next to this lid to illuminate the resources within (range: 72–80 lux). In the test aversion set-up, a gas sampling tube was inserted through a hole in the lid of each FDS to be approximately level with a chick's head and within 12 cm horizontal distance and 4 cm vertical distance of the diffuser (Figure 2).

From 21–25 days of age, all 20 birds were acclimatised once a day to the aversion set-up, initially without the plastic curtain in front of the FDS. On the first day, groups

Table 1 Predicted and determined mean (± SD) percentage levels of gases used during aversion tests on chickens. Each gas mixture/concentration combination was tested on 12 chicks, apart from those in bold which were tested on a further 12 chicks in the final test. The overall determined means for the gas concentrations (columns) or gas mixtures (rows) are also given.

	Gas concentration									
	Low			Medium			High			
	CO_2	Inert gas	Air	CO^2	Inert gas	O_2 Air	CO_2	Inert gas	O_2 Air	Overall mean
CO_2 in air	**50**		**50**	55		45	60		40	CO_2 53.2 (± 4.6)
Determined	49.5 (± 1.26)			57.2 (± 1.98)			60.1 (± 0.6)			
N_2 in CO_2	30	70 ≤ 2		20	80 ≤ 2		**10**	**90** ≤ 2		
Determined	29.0 (± 2.6)	3.7 (± 3.1)		19.7 (± 0.5)	2.5 (± 2.4)		10 (± 0.4)	2.4 (± 1.68)		CO_2 17.2 (± 8.1) O_2 2.8 (± 2.3)
Ar in CO_2	30	70 ≤ 2		**20**	**80** ≤ 2		10	90 ≤ 2		
Determined	30.3 (± 0.7)	2.1 (± 1.3)		19.9 (± 0.86)	2.6 (± 2.7)		10 (± 0.5)	2.8 (± 1.77)		CO_2 20.0 (± 7.3) O_2 2.5 (± 2.2)
Overall mean	39.6 (± 10.2)	2.9 (± 2.5)		28.3 (± 14.9)	2.6 (± 2.6)		22.5 (± 21.9)	2.5 (± 1.7)		

of five birds were placed into the two chambers for 3 h, then for two days pairs of birds were placed in the chambers for 1 h (the plastic curtain was installed in front of the FDS openings on the second day), then for two days individual birds were acclimatised for 0.5 h. During the acclimatisation periods, compressed air was pumped into each FDS through the diffuser, to accustom birds to the sound and flow of gas. The only feed and water available to birds during this time was from the four FDS. Once testing began, individual re-acclimatisation of two chicks not yet tested was carried out the day before their test was conducted, for 0.5 h. Only 12 birds per batch were used for testing.

Choice tests

Thirty-six chicks from the first three batches of birds were used for the initial choice tests. Four target gases (carbon dioxide [CO_2], carbon monoxide [CO], argon [Ar] and nitrogen, [N_2]) at three concentrations were selected for testing. The gases were obtained from a commercial supplier (Air Liquide UK Ltd, UK). When supplying gas at different concentrations it is commercial practice to balance the mixture with air or CO_2. To simplify the nomenclature, concentrations were denoted as 'low', 'medium', or 'high' based on the relative level of the target gas in that gas mix. The gas mixtures and various concentrations were selected based on previous research that showed that they induce rapid unconsciousness and death (Raj *et al* 2006) and were likely to be suitable for whole-house gassing in later studies. Similar concentrations of CO_2 in air, CO in air, and mixtures of N_2 or Ar in up to 30% by volume of CO_2 have been used previously for stunning and/or killing of chickens and turkeys in laboratory studies (Raj & Gregory 1993; Poole & Fletcher 1995; Raj & Tserveni-Gousi 2000) and to cull poultry during avian influenza outbreaks in 2003 (Gerritzen *et al* 2006). Furthermore, European regulation 1099/2009 (due to be enforced in Europe from January 2013), stipulates that during depopulation or killing of poultry in houses, a minimum of 40% by volume of carbon dioxide in air or up to 40% by volume of carbon dioxide in inert gases should be used (European Commission 2009). It was not feasible to expose each chick to all 12 gas mixture × gas concentration combinations. So, each bird was exposed to either all low, all medium, or all high gas concentrations of the four gas mixtures (12 birds per concentration over three batches). The position of gas mixture presentation in the FDS was balanced within low, medium, or high gas concentrations, across all three batches of birds. These sequences were then randomly allocated to the three batches. However, the CO in air mixtures (2, 4 and 6% CO) were abandoned after two tests due to small amounts of CO (ie > 30 ppm) leaking into the central chamber and room, triggering personal gas monitor alarms and presenting a potential risk to the health and safety of personnel when they re-entered the room after testing. As a consequence, in all remaining tests, only three gas mixtures were examined and one FDS drop-down door remained closed (Table 1).

Each of the remaining three gas mixtures × three concentrations was combined using a mixing panel in the control room adjacent to that containing the test and dummy aversion set-ups and pumped into the designated FDS of the test set-up in the adjoining room. Every 20 s, a sample of gas was extracted in turn from each FDS and assessed for CO_2 concentration (infra red sensor, Servomex, UK) for both CO_2 in air mixtures and inert gas mixtures, and O_2 concentrations (magnetic sensor, Servomex, UK) for inert gas mixtures. With inert gas mixtures, the amount of Ar or N_2 was calcu-

lated by subtracting the measured CO_2 value from 100% which left a balance of Ar or N_2 and residual air. By measuring the O_2 level (aim: $O_2 < 2\%$), then the remaining calculated balance was Ar or N_2 (plus N_2 from residual air).

The fourth batch of 12 chicks tested (birds 37–48) was exposed to the three gas mixtures whose concentrations were selected based on what was deemed the least aversive with the first three batches of birds (shown in bold, Table 1). The sequences of gas presentation were randomly allocated per bird.

Prior to testing each batch of birds, leakage of gases from the FDS into the central arena within 10 cm of the curtain was determined, and found to be very low (< 1% CO_2 and a reduction in O_2 of less than 0.05%). Gas analysers were calibrated three times over the course of the trial: calibration gases of known concentration (50% CO_2 in air, 70.5% Ar in CO_2 and 70.8% N in CO_2; Air Liquide Deutschland GMBH, Krefeld, Germany) were sampled and the CO_2 monitor was accurate to within 0.12 (± 0.93)%, (n = 9) and O_2 monitor was accurate to 0.01 (± 0.18)%, (n = 6).

For all batches of birds, testing was carried out over six days between 26–32 days of age. The evening before testing began, one chick was placed into the central pen of each aversion set-up, with the drop-down doors lowered to prevent feeding. The two birds could not see each other, but had auditory contact. A small, hand-filled drinker was placed in the middle of each pen. In the morning, after approximately 16 h without access to food, the chick and drinker in the test set-up were removed from the pen while the drop-down doors were raised, curtains positioned, and the appropriate gases administered into each FDS. Once the desired gas concentrations were reached, the first chick was placed in the test aversion chamber for 1 h. The second bird remained in the identical dummy aversion set-up a few metres away. After the 1-h test, the test FDS were purged, the chicks were swapped, and the second chick tested. Each chick in the pair was tested at a different concentration, with all possible permutations (ie low-medium, medium-high, high-low) tested eight times over all 24 pairs. At the end of the second test, the chicks were returned to the home pen and two new chicks placed in the chambers. At the end of all tests, the birds were culled.

A video camera was mounted above the aversion set-up so that behaviour could be observed and recorded remotely. All behaviours performed by the chicks, ie standing, sitting, walking, head in an FDS, head shaking, head scratching, gasping and staggering were continuously recorded throughout the 1-h test onto a palmtop computer, using Keybehaviour (Deag 1995). Data were processed using Keytime (Deag 1993). Standing, sitting, and walking behaviours were ignored as incidental to the study. Frequency and duration of 'head in' were calculated per bird. Only frequency could be calculated for all other behaviours. Head scratching, gasping and staggering occurred at frequencies too low to analyse. From the data, bout length of head in and the frequency of head shakes s^{-1} of time spent head in with each gas mixture/FDS were calculated. In order for head shakes to be attributed to a particular gas mixture, they had to be performed within the quadrant (area = 0.37 m^2) for that particular FDS, as dictated by the dotted lines overlaid on the video screen, as shown in Figure 2.

Statistical analysis

Data were square-root transformed to provide normality and then analysed by ANOVA using Genstat 11th Edition (2008). For the first 36 birds, the model was a two-way analysis of treatment: gas × level, block: bird batch. For birds 37–48, the model was a one-way analysis of treatment: gas, and no blocking. Pair-wise comparison of significant variables was carried out using least significant differences of means (LSDs). *F*-test and *P*-values are given.

Ethical considerations

This work was carried out under the Home Office (Scientific Procedures) Act 1986 and Project Licence number PPL 60/3508. This experiment passed an assessment by SAC's Animal Experiments Committee (consisting of animal scientists, lay person, statistician, Named Veterinary Officer and Named Animal Care & Welfare Officer), which considers the ethical use of animals (including numbers of animals, for what purpose, severity of the procedures conducted, and likely benefits). The study was discussed with a statistician to ensure that the appropriate sample size was used. Any bird found to be suffering from pain, injury or lasting distress during the trial was to be treated or culled (however this was not necessary). At the end of the study, all birds were culled using a Schedule 1 method.

Results

All birds learned to use the FDS during rearing and acclimatisation. During testing, gas levels achieved in the FDS were close to target levels (Table 1), although overall oxygen levels in N_2 in CO_2 mixtures were more difficult to achieve than in Ar in CO_2 mixtures. During testing of birds 1–36, four birds never entered one of the three FDS available to them: two birds presented with low gas mixtures did not use the 70% N_2 in CO_2 mixture FDS, one bird presented with medium gas mixtures did not use the 80% Ar in CO_2 mixture FDS, and one bird on high gas mixtures did not use the 60% CO_2 in air FDS. The mean time spent with 'head in' according to gas concentration × mixture combinations ranged from approximately half a minute to over two minutes (Table 2). The number of visits ranged from 10–20, with mean bout length of visits ranging from 3.1–7.6 s. Mean number of head shakes associated with any gas concentration × mixture ranged from 3.1–7.5, while the mean number of head shakes s^{-1} of head in was < 1. With time spent with head in, all three concentrations of Ar in CO_2 accounted for three out of the four least aversive gas mixtures (Table 3). Birds spent more time with their head in FDS containing 90% Ar in CO_2 or 90% N_2 in CO_2 mixtures when compared to 80% N_2 in CO_2, 70% N_2 in CO_2, and all concentrations of CO_2 in air ($F_{4,97} = 2.89$, $P = 0.026$). Time spent with head in FDS filled with 80% Ar in CO_2 did not differ significantly from mixtures of 90% Ar in CO_2 or 90%

Table 2 Mean (± SD) of various behaviours when birds 1–36 accessed feeder/drinker stations (FDS) infiltrated with various gas mixtures of different concentrations from a central chamber in a 1-h test (n = 12).

Relative concentration of target gas	Gas concentration	Mean time head in (s)	Mean number of visits	Mean bout length (s per visit)	Mean number of head shakes	Mean head shakes s^{-1} head in
High	90% Ar in CO_2	132.3 (± 68.4)	20 (± 10)	7.6 (± 3.6)	6.8 (± 5.1)	0.07 (± 0.08)
	90% N_2 in CO_2	126.4 (± 140.3)	17 (± 12)	6.5 (± 3.8)	3.4 (± 3.4)	0.04 (± 0.04)
	60% CO_2 in air	34.6 (± 31.7)	11 (± 8)	3.1 (± 1.4)	5.3 (± 4.6)	0.17 (± 0.14)
Medium	80% Ar in CO_2	98.0 (± 121.0)	19 (± 17)	5.1 (± 3.1)	5.3 (± 4.0)	0.09 (± 0.05)
	80% N_2 in CO_2	46.8 (± 44.1)	11 (± 7)	3.9 (± 2.0)	3.1 (± 3.6)	0.10 (± 0.17)
	55% CO_2 in air	37.3 (± 20.6)	11 (± 6)	4.0 (± 2.5)	6.4 (± 4.1)	0.24 (± 0.27)
Low	70% Ar in CO_2	59.9 (± 54.5)	15 (± 14)	4.5 (± 3.1)	7.5 (± 5.8)	0.17 (± 0.19)
	70% N_2 in CO_2	38.7 (± 38.6)	10 (± 9)	4.9 (± 5.3)	4.8 (± 5.0)	0.15 (± 0.16)
	50% CO_2 in air	57.6 (± 73.5)	13 (± 6)	3.8 (± 3.4)	7.3 (± 4.4)	0.26 (± 0.22)

Table 3 Mean (± SED) time (s, [square-root transformed]) spent with 'head in' feeding/drinking stations (FDS) filled with various gas concentrations, mixtures, and their interaction, for birds 1–36.

Factor	Descriptor	Mean time head in (s, square-root transformed)	SED	*P*-value
Relative concentration of target gas (C)	High	8.8[a]	0.826	0.011
	Medium	6.8[b]		
	Low	6.3[b]		
Gas mixture (M)	Ar in CO_2	8.9[a]	0.826	0.002
	N_2 in CO_2	7.1[a]		
	CO_2 in air	5.9[b]		
C × M	90% Ar in CO_2	11.2[a]	1.431	0.026
	90% N_2 in CO_2	9.9[ab]		
	80% Ar in CO_2	8.4[abc]		
	70% Ar in CO_2	7.1[bcd]		
	50% CO_2 in air	6.7[cd]		
	80% N_2 in CO_2	6.2[cd]		
	55% CO_2 in air	5.9[cd]		
	70% N_2 in CO_2	5.3[d]		
	60% CO_2 in air	5.2[d]		

Where superscripts within a factor differ, values are significantly different at the level shown. Least significant differences (LSDs) are provided: gas concentration (5% level) = 1.64; gas mixture (1% level) = 2.17; C × M (5% level) = 2.84.

N_2 in CO_2. Birds spent less time with head in FDS filled with CO_2 in air with increasing concentrations of CO_2, although these were not significantly different from one another. Overall, mean time with head in was greater in FDS filled with high concentrations ($F_{2,97} = 4.78$, $P = 0.011$) of gases, and lower with CO_2 in air mixtures ($F_{2,97} = 6.6$, $P = 0.002$). Over the course of the 1-h tests, two birds spent more than seven minutes with their heads in the 80% Ar in CO_2 FDS (bird 8, 447 s over 59 bouts), and 90% N_2 in CO_2 FDS (bird 18, 508 s over 34 bouts). Gas monitoring of these birds showed that mean O_2 and CO_2 concentrations were 5.5 and 20.3%, respectively for bird 8, and 4.0 and 9.1% for bird 18. When data from these two birds were ignored, the order of preferences shown in Table 3 for gas concentration ($F_{2,97} = 4.19$, $P = 0.018$) and gas mixture ($F_{2,97} = 5.78$, $P = 0.004$) did not alter, but in the C × M interaction, 90% N_2 in CO_2 was now more aversive than 90% Ar in CO_2 based on time spent with head in ($F_{4,97} = 3.23$, $P = 0.016$), rather than being statistically similar. The effect of ignoring these two birds on head shakes s^{-1} was insignificant.

Table 4 Mean (± SED) number of head shakes s^{-1} of time (square-root transformed) spent with head in feeding/drinking stations (FDS) filled with various gas concentrations, mixtures, and their interaction for birds 1–36.

Factor	Descriptor	Mean time head in (s, square-root transformed)	SED	*P*-value
Relative concentration of target gas (C)	High	0.252[b]	0.0425	0.004
	Medium	0.314[ab]		
	Low	0.397[a]		
Gas mixture (M)	Ar + CO_2	0.292[b]	0.0425	< 0.001
	N_2 + CO_2	0.249[b]		
	CO_2 in air	0.423[a]		
C × M	50% CO_2 in air	0.468	0.0737	0.868
	55% CO_2 in air	0.428		
	60% CO_2 in air	0.371		
	70% Ar in CO_2	0.363		
	70% N_2 in CO_2	0.359		
	80% Ar in CO_2	0.281		
	80% N_2 in CO_2	0.235		
	90% Ar in CO_2	0.231		
	90% N_2 in CO_2	0.154		

Where superscripts within a factor differ, values are significantly different at the level shown. Least significant differences (LSDs) are provided: gas concentration (1% level) = 0.112; gas mixture (0.1% level) = 0.145.

Table 5 Mean (± SD) of various behaviours when birds 37–48 accessed feeder/drinker stations (FDS) from a central chamber. (One bird's video data were irrecoverable, so data shown is n = 11 where full information was available).

Gas mixture	Mean time head in (s)	Mean number of visits	Mean bout length (s per visit)	Mean number of head shakes	Mean head shakes s^{-1} head in
80% Ar in CO_2	200.7 (± 182.0)	17 (± 12)	11.2 (± 4.6)	10.5 (± 13.3)	0.05 (± 0.04)
90% N_2 in CO_2	178.9 (± 140.5)	16 (± 15)	15.1 (± 11.4)	4.6 (± 4.2)	0.03 (± 0.03)
50% CO_2 in air	46.3 (± 37.8)	9 (± 6)	5.3 (± 4.4)	4.3 (± 4.8)	0.10 (± 0.08)

Table 6 Mean time (s) that birds 37–48 (n = 11, one bird's data irrecoverable) spent with head in feeding and drinking stations filled with various gas mixtures, and mean number of head shakes s^{-1} of time spent head in (both square-root transformed).

Factor	Gas mixture	Square root	SED	*P*-value
Head in (s)	80% Ar in CO_2	13.1[a]	2.09	0.005
	90% N_2 in CO_2	12.3[a]		
	50% CO_2 in air	6.2[b]		
Head shakes s^{-1}	80% Ar in CO_2	0.19	0.058	0.148
	90% N_2 in CO_2	0.14		
	50% CO_2 in air	0.25		

Where superscripts within a factor differ, values are significantly different at the level shown. Least significant difference (LSD) is provided: head in (1% level) = 5.8.

A significantly greater proportion of head shakes s^{-1} were performed with CO_2 in air mixtures ($F_{2,93} = 9.02$, $P < 0.001$) and where target gas concentrations were low compared to high concentrations ($F_{2,93} = 5.83$, $P = 0.004$), whereas medium concentrations did not differ (Table 4). There was no interaction effect between gas concentration and mixture on head shakes s^{-1}.

From these results, we selected one of each gas mixture, at the most 'preferred' concentration, based on what appeared to be least aversive to birds 1–36. A fourth batch of 12 birds (birds 37–48) was tested on these selected mixtures: 90% N_2 in CO_2, 80% Ar in CO_2, and 50% CO_2 in air. Data on one bird were irrecoverable, thus data are

given for 11 birds (Table 5). Birds spent up to four times longer with their head in inert gas mixtures than 50% CO_2 in air, with almost twice as many visits. Overall bout length of visits were one third to half as short with 50% CO_2 in air than with inert gas mixtures. Mean number of head shakes were twice as high with 80% Ar in CO_2, but when calculated as relative to time spent with head in, head shakes s^{-1} were greatest with 50% CO_2 in air. In the analysis of transformed data, there was no significant difference in the proportion of head shakes s^{-1} ($F_{2,30} = 2.04$, $P = 0.148$), but birds spent significantly less time with head in with 50% CO_2 in air, compared to either inert gas mixtures ($F_{2,30} = 6.46$, $P = 0.005$) (Table 6).

Discussion

By food-depriving chicks overnight, during a longer than typical dark period (ie 15 h), we ensured that chicks were highly motivated to feed at the start of the test approximately 1 h later. Despite this, birds spent on average as little as 35 s with their head inside feeding/drinking stations filled with 60% CO_2 in air, and at most just over 2 min with a 90% Ar in CO_2 mixture, in a 1-h test. The short time spent by most birds with their heads in any gas atmosphere used here is unsurprising because these gas mixtures inhibit biological functions and, if birds were to remain with their heads in any station, would eventually cause unconsciousness and death. It could be argued that a control station filled with normal air would have been a useful comparison for measurements presented here. However, the risk of birds using this station exclusively once they discovered it would have produced limited or no results on the relative adverse effects of the gas mixtures, which was the primary objective of this study. This assumption is corroborated by results from a previous study by Raj and Gregory (1991), in which they showed that hens learned to recognise the presence of non-lethal concentrations of CO_2 or Ar over a period of time due to repeated exposure and, as a result, occupied a chamber filled with normal air compared to a chamber where CO_2 concentrations were raised above 5% or the residual O_2 in argon was reduced to 10% or less. Nevertheless, birds used in this study spent relatively more time in the central arena containing atmospheric air, which could be considered as 'control', albeit without food and water, than FDS containing gas mixtures. The inference could therefore be that all the gas mixtures and concentrations tested in this study were to a certain extent aversive and that birds would prefer to remain in air.

Relative to one another, high concentrations of inert gas mixtures (ie 90% inert gas and 10% CO_2) were less aversive (in terms of time spent with head in an FDS) than 50% or more of CO_2 in air, although both 80% Ar and 80% N_2 mixtures (containing 20% CO_2) were not preferred to 50 or 55% CO_2 in air. Mixtures of 80% N_2, 70% Ar and 70% N_2 in 20–30% CO_2 were not preferred to 60% CO_2 in air. Previous work has indicated that hens and broilers can detect CO_2 at concentrations as low as 10%, based on behaviour and respiratory responses (McKeegan *et al* 2005, 2006). In a study by Webster and Fletcher (2004), in which hens could access a lower chamber filled with various gas mixtures to feed, birds tested on 30, 45 and 60% CO_2 in air stopped on their descent to the chamber and retreated as much as when they were tested with a mixture of 70% argon and 30% CO_2. However, in this study, when birds 37–48 were able to access three gas mixtures, birds spent approximately four times longer with their head in FDSs filled with inert gas mixtures containing 10 or 20% CO_2 compared to the 50% CO_2 in air. It appears that the different concentrations of CO_2 presented to birds in these choice tests are critical to assessing what is 'preferred'. An alternative argument is that aversion to lethal gases is based on gas potency: gas mixtures that induce loss of posture and/or unconsciousness more quickly may be likely to be more aversive. However, this is not necessarily the case: Lambooij *et al* (1999) found that 90% Ar in air caused gasping and head shaking — signs of aversion — later than 40% CO_2 + 30% O_2, but caused loss of posture sooner.

Two birds spent between 7.5 and 8.5 min feeding in FDS filled with inert gas mixtures over several visits: they seemingly had a strategy to feed, exit for fresh air, and then re-enter. The long time spent with head in the FDS to access the feeders and drinkers will inevitably have allowed some of the gas mixture to escape, but a constant flow rate of gas through the diffuser, positioned directly between the feeder and drinker, meant that birds were continuously exposed to the gas mixtures. It is notable that O_2 levels were harder to maintain at < 2% with N_2 mixtures: this is most likely because N_2 is lighter than air and thus harder to control within the FDS, whose acrylic lid was not sealed shut.

Head shakes are thought to be an alerting response to novel or disturbing stimuli (Hughes 1983) and have been used previously as an indicator of aversion (Lambooij *et al* 1999; Webster & Fletcher 2001) and respiratory distress (Raj 1996; Webster & Fletcher 2001; McKeegan *et al* 2006). In Webster and Fletcher's (2001) study, no difference was found in the number of head shakes performed by broiler chickens exposed to varying concentrations of CO_2 in air, 70% Ar in CO_2 or 100% Ar. However, in Raj's (1996) work on turkeys, birds showed less severe head shaking with 60% Ar + 30% CO_2 in air than with 72% CO_2 in air. In the present study, with birds 1–36, the proportion of head shakes s^{-1} was significantly greater with low, compared to high, concentrations of the target gases, and with all CO_2 in air mixtures compared to inert gas mixtures. But, because the inert gas mixtures were balanced with CO_2, when the target gas was an inert gas at 'low' concentration, the birds were exposed to relatively high concentrations of CO_2 (eg 70% of inert gas mixed with 30% CO_2) compared to 'high' concentration (eg 90% of inert gas mixed with 10% CO_2). So, it is likely that the increased head shaking associated with low concentrations of gas was the result of the relatively high levels of CO_2 in the inert gas mixtures. McKeegan *et al* (2006) noted that the proportion of chickens performing head shakes increased as the concentration of CO_2 increased from

10–70% in air, but that birds exposed to inert gas mixtures (without any CO_2) showed considerably less head shaking. Lambooij *et al* (1999) found that chickens tended to perform more head shakes where CO_2 concentrations were higher (40% CO_2 in 30% O_2 versus 60% argon in 30% CO_2), although these were not statistically significant. The fourth batch of birds in our study showed no difference in the proportion of head shakes s^{-1} despite gas mixtures varying in CO_2 from 10 to 50%, however mean head shakes s^{-1} were very low compared to birds 1–36.

Animal welfare implications and conclusion

Based on birds' least aversive responses, chicks found inert gas mixtures, such as nitrogen or argon containing low concentrations of CO_2, to be preferable to CO_2 in air. However, data suggest that all gas mixtures, at all concentrations, were aversive to a degree and that their use for culling poultry has to be balanced against alternative methods of culling and the relative duration of suffering any one of them would incur. Although gases are aversive, if they kill relatively quickly, this may be considered acceptable in order to prevent the spread of highly pathogenic and/or zoonotic disease.

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References

Akre AK, Bakken M and Hovland AL 2009 Social preferences in farmed silver fox females (*Vulpes vulpes*): Does it change with age? *Applied Animal Behaviour Science 120*: 186-191

Baldwin BA and Meese GB 1977 Sensory reinforcement and illumination preference in domesticated pig. *Animal Behaviour 25*: 497-507

Bouvarel I, Chagneau AM, Lecuelle S, Lescoat P, Ferreira G, Duvaux-Ponter C and Leterrier C 2009 Feed composition and hardness interact in preference and intake in chickens. *Applied Animal Behaviour Science 118*: 62-68

Deag J 1993 *Keytime: A program system for recording and analysing behavioural data. v3.4d.* Penicuik, Midlothian.

Deag J 1995 *Keybehaviour: A programme for recording behavioural data on the DIP pocket PC and Atari Portfolio computer. v3.5a.* Penicuik, Midlothian

Defra 2008a *Disease Factsheet: Avian Influenza, History and Spread of the Disease.* http://www.defra.gov.uk/animalh/diseases/notifiable/ai/index.htm#history

Defra 2008b *Notifiable Diseases.* http://www.defra.gov.uk/animalh/diseases/notifiable/index.htm

De Jong IC, Wolthuis-Fillerup M and van Reenen CG 2007 Strength of preference for dustbathing and foraging substrates in laying hens. *Applied Animal Behaviour Science 104*: 24-36

European Commission 2009 *Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing* pp 1-30. http://ec.europa.eu/food/animal/welfare/slaughter/regulation_1099_2009_en.pdf

Gallagher J *1976* Sexual imprinting: effects of various regimens of social experience on mate preference in Japanese quail, *Coturnix coturnix japonica. Behaviour 57*: 91-115

Gerritzen M and Lambooij E 2004 *Killing poultry for disease control. Report ID-Lelystad 04/101242* pp 1-55. Wageningen University and Researchcenter Publications: Wageningen, The Netherlands. http://en.scientificcommons/org/14167211

Gerritzen M, Lambooij B, Reimert H, Stegeman A and Spruijt B 2004 On-farm euthanasia of broiler chickens: effects of different gas mixtures on behavior and brain activity. *Poultry Science 83*: 1294-1301

Gerritzen M, Lambooij B, Reimert H, Stegeman A and Spruijt B 2007 A note on behaviour of poultry exposed to increasing carbon dioxide concentrations. *Applied Animal Behaviour Science 108*: 179-185

Gerritzen M, Lambooij B, Stegeman A and Spruijt B 2006 Slaughter of poultry during the epidemic of avian influenza in the Netherlands in 2003. *Veterinary Record 159*: 39-42

Gunnarsson S, Heikkila M, Hultgren J and Valros A 2008 A note on light preference in layer pullets reared in incandescent or natural light. *Applied Animal Behaviour Science 112*: 395-399

Hansch F, Nowak B and Hartung J 2009 Evaluation of a gas stunning equipment used for turkeys under slaughterhouse conditions. *Livestock Science 124*: 248-254

HMSO 2006 *The Welfare of Animals (Slaughter or Killing) (Amendment) (England) Regulations 2006. Statutory Instrument 2006 No 1200* pp 1-2. http://faolex.fao.org/docs/pdf/uk75865.pdf

Hoen T and Lankhaar J 1999 Controlled atmosphere stunning of poultry. *Poultry Science 78*: 287-289

Hughes BO 1983 Headshaking in fowls: the effect of environmental stimuli. *Applied Animal Ethology 11*: 45-53

Lambooij E, Gerritzen MA, Engel B, Hillebrand SJW, Lankhaar J and Pieterse C 1999 Behavioural responses during exposure of broiler chickens to different gas mixtures. *Applied Animal Behaviour Science 62*: 255-265

Lewis PD, Danisman R and Gous RM 2009 Photoperiodic responses of broilers I. Growth, feeding behaviour, breast meat yield, and testicular growth. *British Poultry Science 50*: 657-666

Linares MB and Vergara H 2009 Light lamb meat quality packed under modified atmospheres: effect of stunning systems (electrically v gas). *Animal 3*: 1763-1771

Ludders JW 2001 Inhaled anaesthesia for birds. In: Gleed RD and Ludders JW (eds) *Recent Advances in Veterinary Anesthesia and Analgesia: Companion Animals.* International Veterinary Information Service: Ithaca, USA

Machold U, Troeger K and Moje M 2003 Gas stunning of pigs: a comparison of carbon dioxide, argon, a nitrogen-argon-mixture and argon/carbon dioxide, (2 steps-system) under animal welfare aspects. *Fleischwirtschaft 83*: 109-114

Makowska IJ, Niel L, Kirkden RD and Weary DM 2008 Rats show aversion to argon-induced hypoxia. *Applied Animal Behaviour Science 114*: 572-581

Manning HL and Schwartzstein RM 1995 Pathophysiology of dyspnoea. *New England Journal of Medicine 333*: 1547-1553

McKeegan DEF, Smith FS, Demmers TGM, Wathes CM and Jones RB 2005 Behavioral correlates of olfactory and trigeminal gaseous stimulation in chickens, *Gallus domesticus*. *Physiology & Behavior 84*: 761-768

McKeegan DEF, McIntyre J, Demmers TGM, Wathes CM and Jones RB 2006 Behavioural responses of broiler chickens during acute exposure to gaseous stimulation. *Applied Animal Behaviour Science 99*: 271-286

McKeegan DEF, McIntyre JA, Demmers TGM, Lowe JC, Wathes CM, Van den Broek PLC, Coenen A and Gentle M 2007 Physiological and behavioural responses of broilers to controlled atmosphere stunning: implications for welfare. *Animal Welfare 16*: 409-426

Nicol CJ, Caplen G, Edgar J and Browne WJ 2009 Associations between welfare indicators and environmental choice in laying hens. *Animal Behaviour 78*: 413-424

Poole GH and Fletcher DL 1995 A comparison of argon, carbon dioxide, and nitrogen in a broiler killing system. *Poultry Science 74*: 1218-1223

Raj ABM 1996 Aversive reactions of turkeys to argon, carbon dioxide and a mixture of carbon dioxide and argon. *Veterinary Record 138*: 592-593

Raj ABM 1997 Novel on-farm killing system. *Poultry International August*: 48-49

Raj ABM 1999 Behaviour of pigs exposed to mixtures of gases and the time required to stun and kill them: welfare implications. *Veterinary Record 144*: 165-168

Raj ABM and Gregory NG 1990 Effect of rate of induction of carbon dioxide anaesthesia on the time to onset of unconsciousness and convulsions. *Research in Veterinary Science 49*: 360-363

Raj ABM and Gregory NG 1991 Preferential feeding-behaviour of hens in different gaseous atmospheres. *British Poultry Science 32*: 57-65

Raj ABM and Gregory NG 1993 Time to loss of somatosensory-evoked potentials and onset of changes in the spontaneous electroencephalogram of turkeys during gas stunning. *Veterinary Record 133*: 318-320

Raj ABM and O'Callaghan M 2001 Evaluation of a pneumatically operated captive bolt for stunning/killing broiler chickens. *British Poultry Science 42*: 295-299

Raj ABM and Tserveni-Gousi A 2000 Stunning methods for poultry. *World's Poultry Science Journal 56*: 291-304

Raj ABM, Gregory NG and Wotton SB 1991 Changes in the somatosensory evoked potentials and spontaneous electroencephalogram of hens during stunning in argon-induced hypoxia. *British Veterinary Journal 147*: 322-330

Raj ABM, Sandilands V and Sparks NHC 2006 Review of gaseous methods of killing poultry on-farm for disease control purposes. *Veterinary Record 159*: 229-235

Rioja-Lang FC, Roberts DJ, Healy SD, Lawrence AB and Haskell MJ 2009 Dairy cows trade-off feed quality with proximity to a dominant individual in Y-maze choice tests. *Applied Animal Behaviour Science 117*: 159-164

Sørensen DB, Mortensen K, Bertelsen T and Vognbjer K 2008 Enriching the metabolic cage: effects on rat physiology and behaviour. *Animal Welfare 17*: 395-403

Struelens E, Tuyttens FAM, Ampe B, Odberg F, Sonck B and Duchateau L 2009 Perch width preferences of laying hens. *British Poultry Science 50*: 418-423

Webster AB and Fletcher DL 2001 Reactions of laying hens and broilers to different gases used for stunning poultry. *Poultry Science 80*: 1371-1377

Webster AB and Fletcher DJ 2004 Assessment of the aversion of hens do different gas atmospheres using an approach-avoidance test. *Applied Animal Behaviour Science 88*: 275-287