The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK

Animal Welfare 2008, 17: 79-87
ISSN 0962-7286

Gas killing of rats: the effect of supplemental oxygen on aversion to carbon dioxide

RD Kirkden*, L Niel, SA Stewart and DM Weary

Animal Welfare Program, Faculty of Land and Food Systems, University of British Columbia, 2357 Main Mall, Vancouver, BC, V6T 1Z4, Canada
* Contact for correspondence and requests for reprints: rdk500@cs.york.ac.uk

Abstract

High concentrations of carbon dioxide (CO_2), used for killing laboratory rodents, are known to be more strongly aversive to rats than sweet food items are attractive. This study investigated whether the maintenance of a high oxygen (O_2) concentration, using a gas mixture of 70% CO_2 and 30% O_2, would reduce aversion to CO_2 during a gradual-fill procedure. Eight male Wistar rats, aged 10 months, were housed individually in an apparatus consisting of two cages, one higher than the other and joined by a tube. In a series of trials, subjects entered the lower cage for a reward of 20 sweet food items. The gas was turned on at the moment the rat started eating the reward items and flowed into the lower cage at a fixed rate. There were four treatments: 1) 100% CO_2 at 14.5% cage volume min^{-1}; 2) gas mixture at 14.5% min^{-1}; 3) gas mixture at 21.0% min^{-1}, which delivered CO_2 at approximately 14.5% min^{-1} and 4) air, with each subject tested with each treatment four times. Measures of willingness to stay and eat in the lower cage (latency to stop eating, latency to leave and the number of reward items eaten) were much lower in all three gas treatments than in air, indicating that the CO_2 and the CO_2 + O_2 mixture were both more strongly aversive than sweet food items were attractive. Comparing the gas mixture with 100% CO_2, the latency to leave and the number of reward items eaten were slightly higher in the CO_2 + O_2 mixture at 21% min^{-1} than in CO_2 at 14.5% min^{-1}, indicating that the addition of O_2 slightly reduced the aversiveness of CO_2 in the gradual-fill procedure. This reduction is not enough to warrant recommending the use of CO_2 + O_2 mixtures for killing rats.

Keywords: *animal welfare, aversion, carbon dioxide, euthanasia, oxygen, rat*

Introduction

Most laboratory animals are eventually killed, either for experimental purposes, or to dispose of unwanted genotypes, used subjects, or surplus stock. Carbon dioxide (CO_2) is the most widely-used agent for killing laboratory rodents. There are two methods. In the pre-fill procedure, animals are placed in a chamber containing a high concentration of CO_2; concentrations greater than 70% are typically used (eg Britt 1987; Blackshaw *et al* 1988; Hewett *et al* 1993; Iwarsson & Rehbinder 1993; Coenen *et al* 1995; Smith & Harrap 1997; Kohler *et al* 1999). In the gradual-fill procedure, the chamber initially contains air, and CO_2 is introduced at a fixed-flow rate to achieve a gradually increasing concentration. The flow rate varies widely between experimental studies, ranging from 3 to 125% of cage volume min^{-1} (eg Hornett & Haynes 1984; Britt 1987; Hewett *et al* 1993; Bowyer & Cubitt 1995; Coenen *et al* 1995; Smith & Harrap 1997; Hackbarth *et al* 2000; Niel & Weary 2007; Niel *et al* 2008), although a rate of 15–35% min^{-1} is common. Recommendations (eg 19.5%, Hornett & Haynes 1984; at least 20%, American Veterinary Medical Association 2000) are typically based on subjective assessments of distress. However, several recent studies using objective behavioural measurements have indicated that the flow rate should be no greater than 20%. Young (2006) reported that the incidence of immobility (interpreted as a fright response) at the onset of CO_2 exposure, and the frequency of rapid body movements (interpreted as excitement and agitation) occurring later in the procedure, were both lower when the flow rate was approximately 20% min^{-1} than when it was approximately 40 or 60%. Niel *et al* (2008), using a preference testing procedure, found that varying the flow rate from 3.4 to 26.7% min^{-1} made little difference to the CO_2 concentration that rats tolerated before leaving a chamber, but tolerance peaked at a flow rate of 13.5% min^{-1}.

There is evidence that the pre-fill procedure causes pain in laboratory rats, due to the formation of carbonic acid on mucous membranes (Yavari *et al* 1996; Golledge *et al* 2005; Hawkins *et al* 2006), whereas the gradual-fill procedure does not, provided that the flow rate is moderate (eg 17–20%: Golledge *et al* 2005; Hawkins *et al* 2006; Niel & Weary 2006). This is because consciousness is lost at a much lower CO_2 concentration in the gradual-fill method (between 30 and 40%: Smith & Harrap 1997; Hawkins *et al* 2006; Niel & Weary 2006). However, both methods can cause distress

https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

(defined as anxiety or pain: Oxford English Dictionary) and are perceived by rats as aversive (ie they are disliked: Oxford English Dictionary; Reber 2001). Signs of distress are not always observed (eg Blackshaw *et al* 1988; Hewett *et al* 1993; Bowyer & Cubitt 1995; Hackbarth *et al* 2000) and, as noted by Niel and Weary (2006), some behaviours are difficult to interpret. Behaviours that have been observed and interpreted as signs of distress include rapid breathing, laboured breathing or gasping (Hornett & Haynes 1984; Coenen *et al* 1995; Young 2006), elimination (Britt 1987; Young 2006), changes in activity level (Britt 1987; Young 2006), and escape attempts (Niel & Weary 2006). Aversion is easier to recognise and has been consistently observed at CO_2 concentrations of 3% and greater. Aversion is demonstrated by the fact that rats leave a chamber containing CO_2 more rapidly than one containing air and spend less time in it (Leach *et al* 2002a, b, 2004; Krohn *et al* 2003). However, simple preference tests of this kind do not assess the strength of aversion, so they cannot reveal whether the aversion is trivial or substantial (Dawkins 1990; Rushen 1990). More information is available from studies that have offered rats an incentive to remain in the gas chamber. Niel and Weary (2007) and Niel *et al* (2008) used sweet food items (Honey Nut Cheerios® [35% sugar], General Mills Inc, MN, USA) as an incentive and showed that rats chose to leave a chamber that was gradually filling with CO_2 when the concentration reached 13–18%, indicating that aversion to CO_2 at these concentrations was greater than their motivation to consume sweet food items. There is evidence that the strength of motivation for sweet foods in *ad libitum* fed rats is moderate, from which it follows that the strength of aversion to such CO_2 concentrations is probably at least moderate (Niel & Weary 2007). The most probable reason for aversion and distress during the gradual-fill procedure is a sensation of dyspnoea, or breathing discomfort (Niel & Weary 2006).

Some researchers have reported that the addition of oxygen (O_2) to CO_2 reduces distress during the pre-fill procedure, and also during the gradual-fill procedure when CO_2 flow rate is high. In the pre-fill method, the addition of 20% O_2 has been observed to reduce a subjective assessment of 'uneasiness' and the incidence of elimination (Iwarsson & Rehbinder 1993), although it is unclear whether this was caused by an increased O_2 or decreased CO_2 concentration. In the gradual-fill procedure, with a CO_2 flow rate of 125% of cage volume min^{-1}, the addition of 33% O_2 has been reported to eliminate 'agitation and excitation' and gasping (Coenen *et al* 1995). During the pre-fill procedure, animals are subjected to both hypercapnia (increased CO_2 partial pressure, pCO_2, in the blood and tissues) and hypoxia (decreased O_2 partial pressure, pO_2), and it has been suggested that the addition of O_2 reduces distress by preventing hypoxia (Iwarsson & Rehbinder 1993). Coenen *et al* (1995) suggested that this might also be true for gradual-fill CO_2 exposure with high flow rates. However, there is no evidence that the addition of O_2 to CO_2 reduces distress during the gradual-fill procedure when flow rate is moderate or low. Hewett *et al* (1993) reported that the addition of 20% O_2 made no difference to the behaviour of rats during CO_2 exposure when the flow rate was 20% of cage volume min^{-1}, although the only behavioural measurements they obtained in a systematic fashion were postural changes associated with the development of anaesthesia. At this flow rate, pure CO_2 produced unconsciousness without a significant decline in arterial pO_2, which may explain why the addition of O_2 appeared to have no effect. Young (2006) reported a reduced frequency of rapid body movements and a delayed onset of gasping when rats were exposed to a mixture of 80% CO_2 and 20% O_2 at a flow rate of approximately 20% min^{-1}, compared with 100% CO_2 at the same flow rate, but because the total flow rates of the two gas treatments were equalised, not the partial flow rates of CO_2, this finding could have been due to a slower inflow of CO_2 rather than a higher concentration of O_2. Despite the lack of evidence, O_2 supplementation is sometimes recommended for the gradual-fill procedure (Olfert *et al* 1993). It may be that a higher O_2 concentration, such as 30%, would be more effective, since hyperoxia is known to reduce the ventilatory and dyspnoea responses to hypercapnia (Nunn 1987, p 91; Banzett *et al* 1996; Masuda *et al* 2001).

Leach *et al* (2004) investigated the effect of supplemental O_2 upon aversion to CO_2 using preference testing. They reported that 20 or 30% O_2 did not reduce the amount of time rats spent in a chamber containing CO_2 at static concentrations ranging from 26 to 61%, and concluded that O_2 does not reduce aversion to CO_2. However, in this study subjects were given no incentive to remain in the chamber, with the consequence that time spent was always very low, so the ability of the study to detect differences between treatments was limited. Furthermore, the effect of supplemental O_2 upon aversion to CO_2 during a gradual-fill procedure has not been investigated.

The present study set out to investigate whether the addition of 30% O_2 would reduce the strength of aversion to CO_2 during a gradual-fill procedure, as shown by an increased willingness to remain in the chamber with a palatable food incentive. We employed a moderate flow rate of 14.5% of chamber volume min^{-1}, which was close to the optimal flow rate of around 13.5% min^{-1} identified by Niel *et al* (2008).

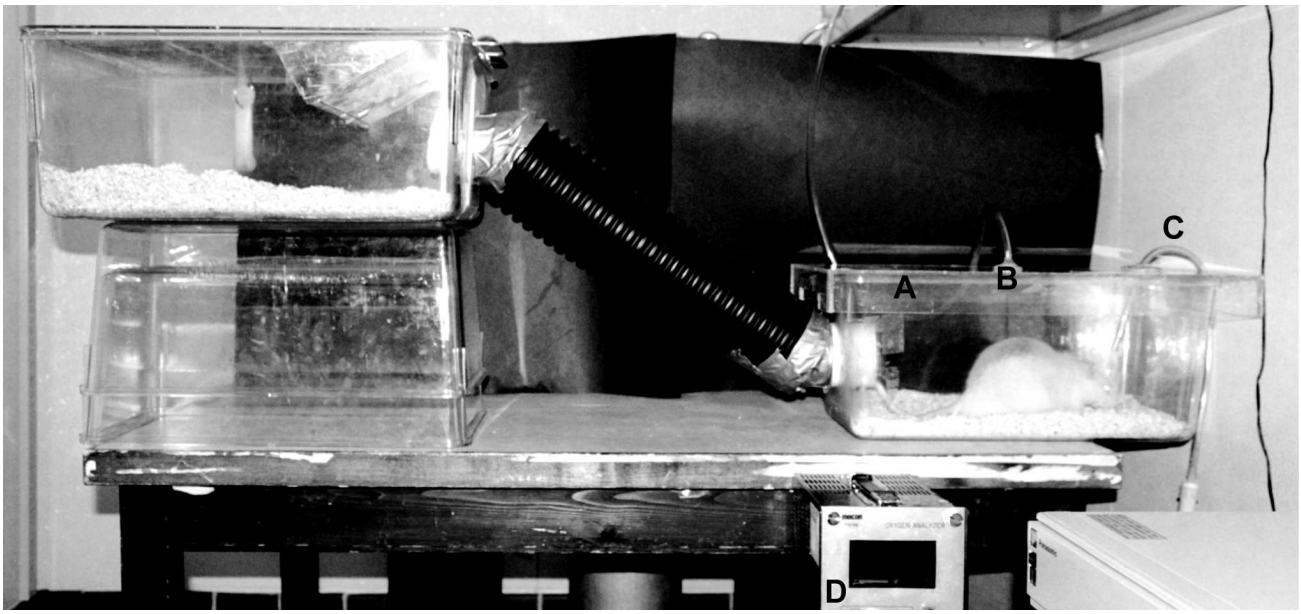
Materials and methods

Subjects and housing

The subjects were eight male Wistar rats, bred at the University of British Columbia Animal Care Centre in a line derived from Charles River Laboratories (Saint-Constant, QC, Canada). They were purchased as surplus stock, and would otherwise have been killed using CO_2 following this institution's standard operating procedure. Subjects were ten months of age at the start of the experiment. They had been used previously in similar experiments (Niel & Weary 2007; Niel *et al* 2008) and were familiar with all aspects of the experimental procedure except exposure to a $CO_2 + O_2$ mixture. All subjects were certified disease-free at the time of purchase and showed no clinical signs of respiratory disease during the study.

https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

Figure 1



Experimental apparatus. (A) Position of two gas outlet holes, (B) gas inlet tube, (C) sampling tube to O_2 analyser and (D) O_2 analyser.

The rats were housed individually in an apparatus consisting of two transparent polycarbonate cages with wire lids, one higher than the other and joined by a corrugated PVC tube (10 cm diameter and approximately 45 cm long) at an angle of about 45º. The upper cage was larger (approximately 46 × 37 × 20 cm; length × breadth × height) and contained wood shavings, a black Plexiglas nest box, a hard nylon dog chew, a water bottle and food in the cage lid hopper. The lower cage was smaller (approximately 45 × 23 × 20 cm) and contained wood shavings only. Food pellets (LabDiet 5001, PMI Nutrition International, USA) and tap water were available *ad libitum*. The rats were maintained on a LD 12:12 cycle, lights on at 0900h, and at a temperature of 23–24ºC. Humidity readings were not obtained.

Training procedure

The experiment was run twice with the same subjects. During a 1–2 week period prior to each run of the experiment, subjects were given four re-training sessions, to ensure that they were responding well. Training trials were conducted as follows, each subject receiving one trial on each training day. Subjects had previously been conditioned to ascend and descend the corrugated tube, in response to the noise of fingernails being dragged repeatedly along the tube, for a highly palatable reward item (Honey Nut Cheerios®). This technique was now used to move the subject to the upper cage, if they were not there already, for the start of the training procedure. The two cages were then carried separately through to the experimental room. The wire lid of the lower cage was replaced with a Perspex lid, fitted with a gas inlet tube and a sampling tube leading to an O_2 analyser (Mocon LF700D) (Figure 1).

Next the two cages were re-connected and a flow of air was initiated into the lower cage at a rate of 16.5% cage volume min^{-1}. (The volume of the lower cage plus the volume of the adjacent section of corrugated tube, up to the level of the gas outlets in the lower cage lid, was 24 l). The rat was allowed to explore the apparatus for 2 min. If the subject was in the lower cage at the end of this period, as was frequently the case, then they were induced to re-ascend the tube to the upper cage for a reward item. The top of the tube was then blocked off with a barrier, to confine the subject in the upper cage for a further 2 min. The air flow into the lower cage was turned off and 20 reward items were placed on the floor of the cage, directly below the O_2 sampling tube.

Finally, the barrier was removed and the subject descended the tube to consume the food reward. Descent of the tube was always rapid. At the moment the rat began eating, a flow of air was initiated into the lower chamber, at a rate of 16.5% cage volume min^{-1}. The trial ended either when the rat voluntarily re-entered the upper cage, or when 5 min had elapsed from the time at which the subject started eating. Any uneaten reward items were removed at this time, and if the subject was still in the lower cage they were induced to return to the upper cage for a final reward item.

Experimental procedure

Experimental trials proceeded in the same way as training trials, except that the type of gas delivered into the lower cage while reward items were present was varied from day-to-day, following a Latin square design in which treatment order was balanced across subjects (Jones & Kenward 2003). Experimental trials were conducted daily, each subject receiving one trial per day. There were four different gas treatments, each presented on two days to give a total of

https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

eight days for each run of the experiment. In total, each subject was exposed to each treatment on four occasions. The treatments were: 1) 100% CO_2 at 14.5% cage volume min^{-1}; 2) 70% CO_2 + 30% O_2 at 14.5% cage volume min^{-1}; 3) 70% CO_2 + 30% O_2 at 21.0% cage volume min^{-1}, delivering CO_2 at about 14.5% cage volume min^{-1} and 4) air at 16.5% cage volume min^{-1} (on one day), or 24.0% cage volume min^{-1} (on the other day). Air was used to control for the noise and humidity changes associated with gas flow, which might be aversive to rats (Hornett & Haynes 1984, but see Britt 1987; Bowyer & Cubitt 1995). Hence, it was necessary to ensure that air flow rate was at least as high as the flow rates of the gas treatments.

During experimental trials, the subject's behaviour was video recorded. Observations were made of the latency to stop eating, the latency to leave the lower cage and the number of reward items consumed in the lower cage. Behavioural observations were also carried out to look for the onset of ataxia, which was indicated by a sagging posture or loss of co-ordination.

Data analysis

Data were analysed using SAS, Version 9. Observations from repeat tests of the same subject on the same treatment were averaged to generate one value per subject per treatment. There were three dependent variables: latency to stop eating, latency to leave the lower cage and number of reward items eaten in the lower cage.

The two air controls were first compared, to ascertain whether flow rate *per se* affected rats' willingness to remain in the lower cage. Latency to leave the lower cage was not analysed, because in all air trials subjects remained in the lower cage for the entire 300 s testing period. The data could not be rendered normal, so Wilcoxon signed ranks tests were used to compare the effects of the two air flow rates (16.5 and 24.0%) upon the latency to stop eating and the number of reward items consumed in the lower cage.

The air controls were next compared with each of the three gas treatments. For this analysis, subjects in the air controls were assigned a latency to leave equal to the trial duration (300 s). Because the control data showed too little variability to be rendered normal, Wilcoxon signed ranks tests were used.

A mixed model with specified contrasts was used to compare the effects of the three gas treatments on all three dependent variables, including subject (df = 7) as a random effect. The dependent variables were square-root transformed to obtain normal distributions. Specified contrasts were CO_2 + O_2 at 14.5% cage volume min^{-1} versus CO_2 + O_2 at 21.0% cage volume min^{-1}, to confirm that the dependent variables declined with increasing CO_2 flow rate, and CO_2 at 14.5% cage volume min^{-1} versus CO_2 + O_2 at 21.0% cage volume min^{-1} to assess the effect of supplemental O_2 at a constant CO_2 flow rate.

In some trials, subjects showed clear signs of ataxia before leaving the lower cage, including sideways sliding of the legs while walking and sagging of the legs while standing. The number of trials in which each subject showed ataxia was scored for each treatment, and divided by the number of trials received (four) to give the proportion of trials in which ataxia was observed. These scores were compared among the three treatments and the air control within subjects, using a Friedman test. The same contrasts were carried out as described above, but in this case using Wilcoxon signed ranks tests.

Results

Comparison of the two air controls (16.5 vs 24.0% cage volume min^{-1}) showed that air-flow rate had no effect upon the latency to stop eating (271.5 vs 260.0 s, n = 8, $S = 10$, $P = 0.11$), or on the number of reward items eaten in the lower cage (20 vs 20, n = 8, $S = 0.5$, $P = 1.0$). The two air-flow rates were therefore combined for subsequent analyses.

Comparison of the gas treatments with the air controls (Figure 2) showed that all gas treatments reduced the latency to stop eating, the latency to leave the lower cage and the number of reward items eaten in the lower cage (in all cases: n = 8, $S = 18$, $P = 0.0078$).

Increasing the flow rate of the CO_2 + O_2 gas mixture from 14.5 to 21% cage volume min^{-1} (Figure 3) reduced the latency to stop eating (61.8 vs 42.0 s, $F_{1,14} = 27.1$, $P = 0.0001$), the latency to leave the lower cage (71.6 vs 51.0 s, $F_{1,14} = 26.1$, $P = 0.0002$) and the number of reward items eaten in the lower cage (4.8 vs 3.7, $F_{1,14} = 13.4$, $P = 0.0026$).

A comparison between CO_2 delivered at 14.5% cage volume min^{-1} and CO_2 + O_2 delivered at 21% cage volume min^{-1} (Figure 3) showed that O_2 supplementation at a constant CO_2 flow rate slightly increased the latency to leave the lower cage (43.3 vs 51.0 s, $F_{1,14} = 4.65$, $P = 0.049$) and the number of reward items eaten in the lower cage (3.1 vs 3.7, $F_{1,14} = 6.01$, $P = 0.028$), but not the latency to stop eating (35.2 vs 42.0 s, $F_{1,14} = 4.26$, $P = 0.058$).

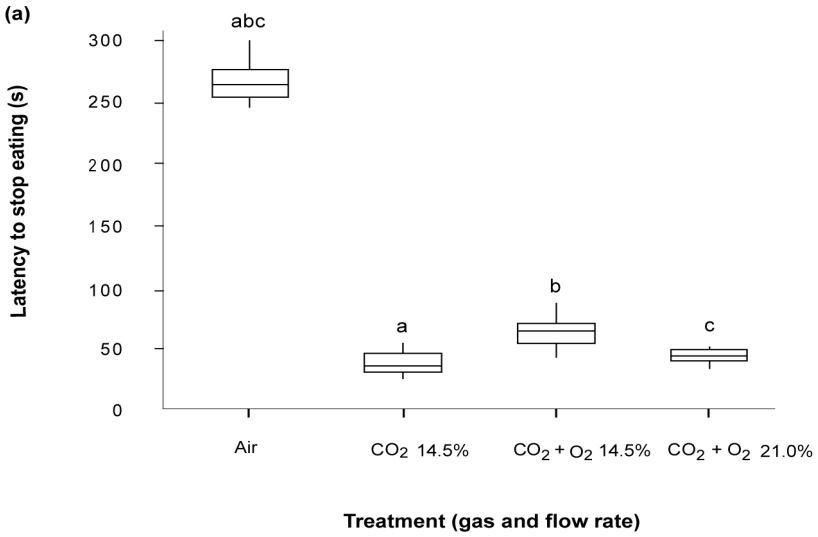
Although subjects always managed to leave the lower cage, on a number of occasions they were observed to show signs of ataxia before leaving. The proportion of trials in which each subject showed ataxia was scored for each treatment and the distributions of these scores are shown in Figure 4. It can be seen that ataxia was observed on some occasions during all three CO_2 treatments, but never during the air control. The median proportion of trials during which subjects showed ataxia was 0.25 with CO_2 at 14.5% cage volume min^{-1}, 0.5 with the CO_2 + O_2 mixture at 14.5% min^{-1}, and 0 with the gas mixture at 21.0% cage volume min^{-1}. A within-subjects comparison of the frequency of ataxia between treatments indicated that the proportion of trials during which ataxia occurred differed among the 3 treatments and the control (Friedman test: n = 8; $\chi^2 = 12.4$; $P = 0.0062$). However, the frequency of ataxia did not differ significantly between CO_2 + O_2 delivered at 14.5 versus 21.0% cage volume min^{-1} (Wilcoxon signed ranks test: n corrected for ties = 7, $S = 11.5$, $P = 0.078$), or between CO_2 delivered at 14.5% cage volume min^{-1} and CO_2 + O_2 at 21.0% cage volume min^{-1} (n corrected = 5, $S = 4.5$, $P = 0.38$).

Upon returning to the upper cage, subjects were occasionally observed to show deep breathing, visible as movements of the body wall, and inactivity for a number of seconds. This was only noticed when they had spent an unusually long time in the lower cage. Systematic observations of behaviour in the upper cage were not made.

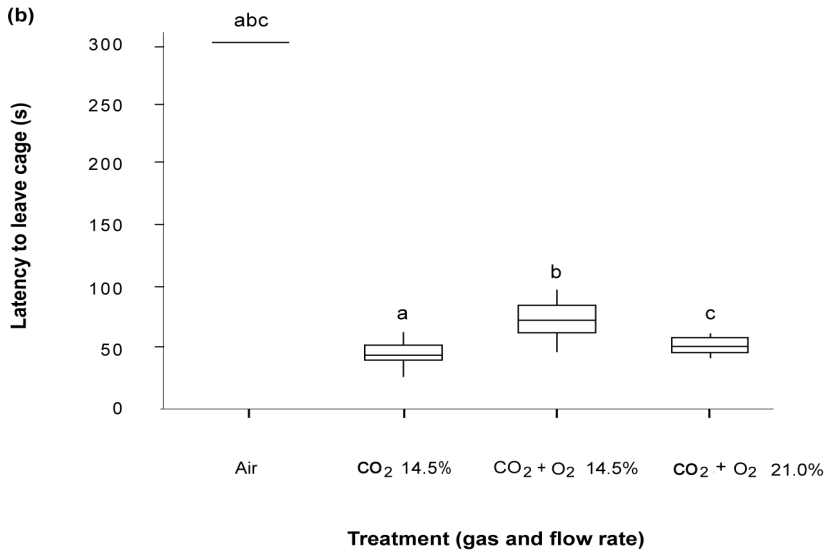
https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

Figure 2

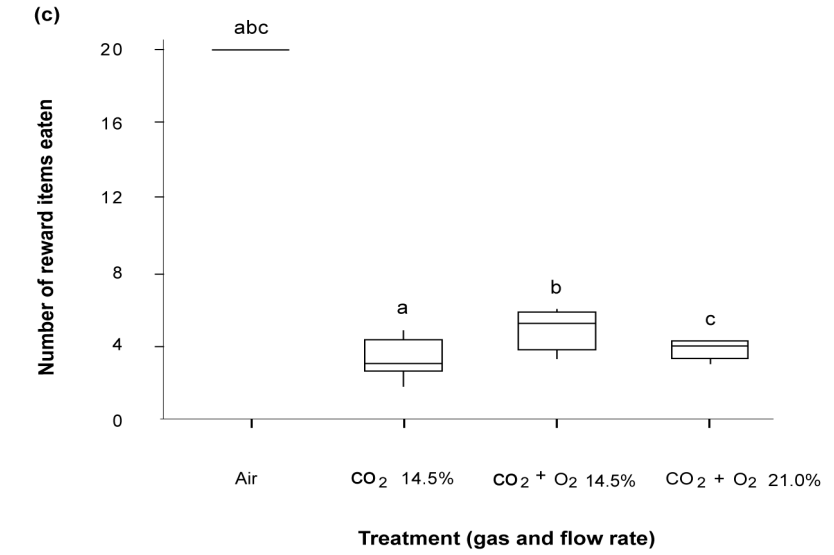
Comparison of gas treatments (CO_2 and $CO_2 + O_2$) with the air control, measuring latency to stop eating. Conditions with the same letters (a, b, c) are significantly different: $P < 0.01$. Note that in the air control, subjects did not leave the lower cage voluntarily, and these subjects have been assigned a latency equal to the trial duration (300 s). Gas flow rate was measured as a percentage of cage volume min^{-1}.



Comparison of gas treatments (CO_2 and $CO_2 + O_2$) with the air control, measuring latency to leave the lower cage.



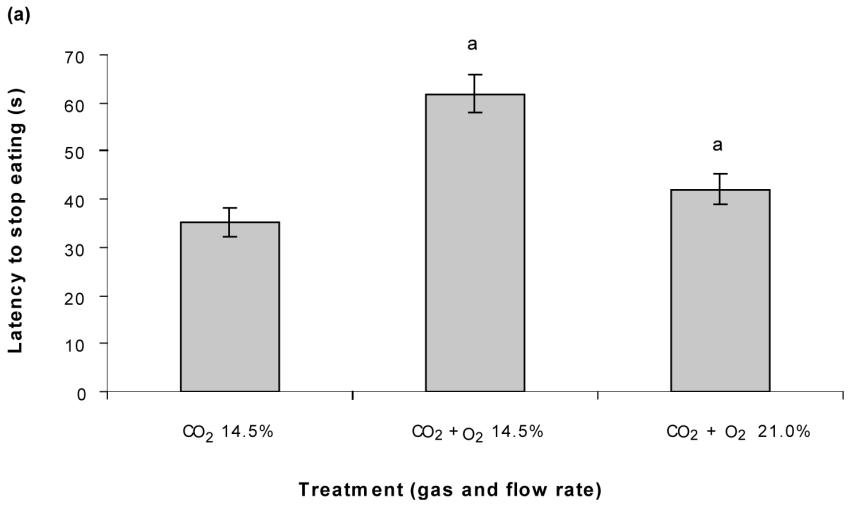
Comparison of gas treatments (CO_2 and $CO_2 + O_2$) with the air control, measuring number of reward items consumed.



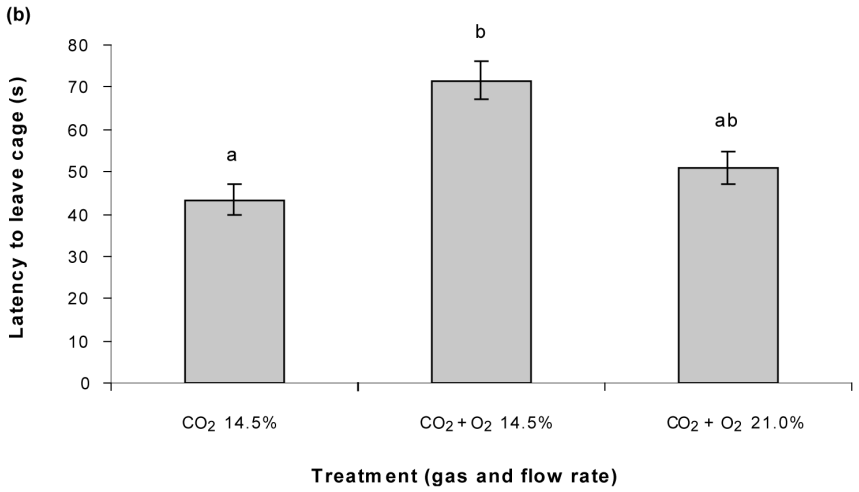
https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

Figure 3

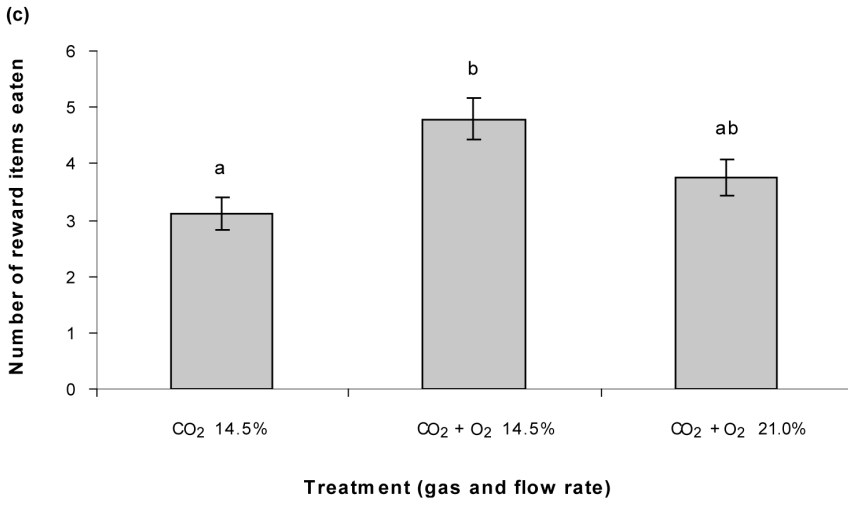
Two *a priori* comparisons between the gas treatments: CO_2 at a flow rate of 14.5% cage volume min^{-1} versus CO_2 + O_2 at 21.0% min^{-1} (showing the effect of adding O_2 at a constant CO_2 flow rate); and CO_2 + O_2 at 21.0% min^{-1} (showing the effect of increasing flow rate). The dependent variable is latency to stop eating (back-transformed mean ± SE). Treatments with the same letters (a, b) are significantly different: $P < 0.05$.



Dependent variable is latency to leave the lower cage (back-transformed mean ± SE).



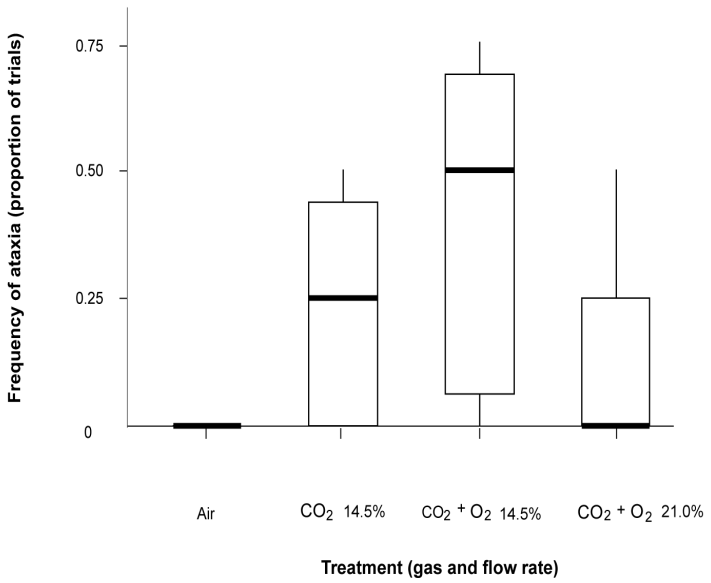
Dependent variable is number of reward items consumed (back-transformed mean ± SE).



https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

Figure 4

Effect of gas treatment upon the proportion of trials during which signs of ataxia were observed before leaving the lower cage. Proportions were scored for each subject and the distributions of these scores are shown. Gas flow rate was measured as a percentage of cage volume min^{-1}.



Discussion

Exposure to CO_2 in the lower cage substantially reduced the latency to stop eating, latency to leave and the number of reward items eaten compared with exposure to air, confirming previous findings (Niel & Weary 2007; Niel *et al* 2008) that CO_2 is more aversive to *ad libitum*-fed rats than sweet foods are attractive. When a mixture of 70% CO_2 and 30% O_2 was used, the rats' willingness to stay in the cage was still much reduced, indicating that in hyperoxic conditions the aversion to CO_2 remains stronger than the attraction to sweet food items. Because the motivation of *ad libitum*-fed rats to consume sweet foods appears to be moderate (Niel & Weary 2007), this finding suggests that the strength of aversion to the gas mixture, like the strength of aversion to pure CO_2, is at least moderate.

Oxygen supplementation reduced the strength of aversion to CO_2, shown by increases in the latency to leave the lower cage and the number of food items eaten compared with the delivery of pure CO_2. The magnitude of this effect was small, with subjects staying only 7.7 s longer on average. At first sight, this finding appears consistent with what is known about the effects of pO_2 on dyspnoea in humans: the dyspnoea experienced at a given pCO_2 is augmented by hypoxia and reduced by hyperoxia (Banzett *et al* 1996; Masuda *et al* 2001). However, these effects were documented under much more extreme levels of O_2 than those at which rats left the chamber in the present study. Although we were unable to obtain accurate measures of O_2 concentration, due to malfunction of the O_2 analyser, it is estimated that the O_2 concentration at the time of leaving would have been in the region of 23% in the CO_2 + O_2 mixture and approximately 18% in the 100% CO_2, based on a previous study using the same subjects and a similar flow rate (Niel *et al* 2008). Such small differences in O_2 concentration do not have a significant effect upon the ventilatory or dyspnoea response to CO_2 in humans (Dahan *et al* 1990; Duffin *et al* 2000; Masuda *et al* 2001). However, in rats, the ventilatory response to changes in O_2 concentration in the mildly hypoxic to hyperoxic range is greater than it is in humans (Hayashi *et al* 1983), and a reduction in dyspnoea seems the most likely explanation for the small but significant difference in the behaviour of our subjects.

Leach *et al* (2004) reported no difference in the amount of time rats spent in a chamber pre-filled with 25.5, 34.9, or 50.8% CO_2 versus similar concentrations of CO_2 with 20 or 30% O_2 added (the concentrations of CO_2 were, in fact, set slightly higher in the CO_2 + O_2 mixtures, in order to achieve similar times to ataxia). The authors concluded that the mixtures with and without O_2 were equally aversive. However, in all treatments the rats spent no more than 2.1 s in the chamber, much less than the period of 10–30 s required to produce ataxia. A probable reason for this very short occupancy, apart from the high concentrations of CO_2 used, is that the rats were given no incentive to remain in the chamber. Thus, the study only yielded information about the relative aversiveness of these gases during the first few moments of exposure to them. In the present study, rats were offered palatable food items as an incentive to remain in the chamber and, as a result, they stayed longer, approaching the onset of ataxia. The findings therefore reflect the relative aversiveness of the two gas treatments up to a later point in the time-course of the killing procedure. The results indicate that by the time rats are approaching ataxia in a gradual-fill procedure, a mixture of CO_2 and O_2 is slightly less aversive than CO_2 alone.

https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

It has been observed that the time to loss of consciousness is unaffected by the addition of O_2 during a gradual-fill CO_2 procedure with a moderate flow rate. Hewett *et al* (1993) compared 100% CO_2 with a mixture of 75% CO_2, 20% O_2 and 5% N_2, at a uniform CO_2 flow rate of 20% of cage volume min^{-1}, and found no difference in the time to ataxia or immobility, a slightly reduced time to loss of righting ability with the O_2 present, and no difference in time to loss of the pedal reflex. Ambrose *et al* (2000) had similar findings in BALB/c mice at a CO_2 flow rate of 30% min^{-1}. Young (2006) reported an increased time to loss of the pedal reflex in the presence of 20% O_2, but the addition of O_2 was confounded with a reduction in CO_2 flow rate. These negative findings are in contrast to the effect of supplemental O_2 at a high CO_2 flow rate of 125% of cage volume min^{-1}, where the onset of ataxia and loss of consciousness have been shown to be delayed (Coenen *et al* 1995). When flow rate is high, O_2 concentration declines rapidly in the pure CO_2 treatment and this may accelerate the development of anaesthesia. Another difference between the Coenen *et al* (1995) and Hewett *et al* (1993) studies is that Coenen *et al* used a higher O_2 concentration in their CO_2 + O_2 treatment (30 vs 20%), but this appears to make no difference to the time to onset of ataxia (Leach *et al* 2004). This means that when flow rate is moderate, a moment-by-moment reduction in the aversiveness of CO_2 could translate into an improvement in welfare during the procedure as a whole. However, it must be emphasised that the reduction in aversion that occurred prior to ataxia was slight.

It should also be noted that this study does not reveal whether a CO_2 + O_2 mixture is less aversive than pure CO_2 between the onset of ataxia and loss of consciousness. Indeed, it is not possible for an aversion test of this kind, in which the subject demonstrates aversion by escaping, to evaluate the period following the development of ataxia. A passive avoidance procedure, for example an assessment of reluctance to re-enter a chamber in which forced gas exposure has previously occurred, could in principle get beyond this point, but this procedure would probably be confounded by memory loss associated with CO_2 anaesthesia (Paolino *et al* 1966; Porter 1972; Leonard & Rigter 1975). The period between ataxia and loss of consciousness is also difficult to assess using behavioural measures of distress that involve locomotion, because the animal is increasingly incapacitated. Changes in breathing (variously described as rapid breathing, laboured breathing and gasping), which may be signs of dyspnoea, are often observed before and during this period (Hornett & Haynes 1984; Britt 1987; Iwarsson & Rehbinder 1993; Coenen *et al* 1995; Young 1996; Smith & Harrap 1997), and it has been reported that these signs are less prevalent when a high O_2 concentration is maintained (Coenen *et al* 1995), suggesting that the benefits of supplemental O_2 may persist until loss of consciousness. However, lung haemorrhage and oedema are more prevalent when a CO_2 + O_2 mixture is used (Iwarsson & Rehbinder 1993; Danneman *et al* 1997). Although the timing of these pathological changes is unclear, they could result in a sensation of drowning if they occurred in conscious animals (Ambrose *et al* 2000). Britt (1987) suggested that haemorrhages observed in rats following a gradual-fill procedure using pure CO_2 had occurred around the time of death; likewise in BALB/c mice, lung haemorrhages caused by CO_2 have been shown to occur after loss of consciousness (Ambrose *et al* 2000). However, Ambrose *et al* (2000) reported that haemorrhaging begins earlier in BALB/c mice when a CO_2 + O_2 mixture is used and starts before consciousness is lost.

Conclusions and animal welfare implications

We conclude that supplemental O_2 may slightly improve the gradual-fill CO_2 procedure for killing rats. However, a mixture of 70% CO_2 and 30% O_2 is almost as aversive as CO_2 alone and little is known about rats' experience of the CO_2 + O_2 mixture after the onset of aversion, including the occurrence of lung haemorrhage. Alternative killing methods are still urgently required.

Acknowledgements

Thanks to Jurgen Pehlke and Gilles Galzi for technical assistance, and to Marianne Pfaffinger and Mitja Sedlbauer for their help with running the experiment and with data collection. The Animal Welfare Program thanks the many supporters of our research, including NSERC, the BC SPCA, members of the BC VMA and many other donors listed on our web site, www.landfood.ubc.ca/animalwelfare.

References

Ambrose N, Wadham J and Morton D 2000 Refinement of euthanasia. In: Balls M, van Zeller A-M and Halder ME (eds) *Progress in the Reduction, Refinement and Replacement of Animal Experimentation* pp 1159-1170. Elsevier: Amsterdam, The Netherlands

American Veterinary Medical Association 2001 2000 Report of the AVMA Panel on Euthanasia. *Journal of the American Veterinary Medical Association 218*: 669-696

Banzett RB, Lansing RW, Evans KC and Shea SA 1996 Stimulus-response characteristics of CO_2-induced air hunger in normal subjects. *Respiration Physiology 103*: 19-31

Blackshaw JK, Fenwick DC, Beattie AW and Allan DJ 1988 The behaviour of chickens, mice and rats during euthanasia with chloroform, carbon dioxide and ether. *Laboratory Animals 22*: 67-75

Bowyer G and Cubitt S 1995 An improved method of carbon dioxide euthanasia for rodents. *Animal Technology 46*: 19-28

Britt DP 1987 The humaneness of carbon dioxide as an agent of euthanasia for laboratory rodents. In: *Euthanasia of Unwanted, Injured or Diseased Animals or for Educational or Scientific Purposes* pp 19-31. Universities Federation for Animal Welfare: Wheathampstead, Herts, UK

Coenen AM, Drinkenburg WH, Hoenderken R and van Luijtelaar GL 1995 Carbon dioxide euthanasia in rats: oxygen supplementation minimizes signs of agitation and asphyxia. *Laboratory Animals 29*: 262-268

Dahan A, DeGoede J, Berkenbosch A and Olievier IC 1990 The influence of oxygen on the ventilatory response to carbon dioxide in man. *Journal of Physiology 428*: 485-499

Danneman PJ, Stein S and Walshaw SO 1997 Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. *Laboratory Animal Science 47*: 376-385

https://doi.org/10.1017/S0962728600032012 Published online by Cambridge University Press

Dawkins MS 1990 From an animal's point of view: motivation, fitness, and animal welfare. *Behavioral and Brain Sciences 13*: 1-61

Duffin J, Mohan RM, Vasiliou P, Stephenson R and Mahamed S 2000 A model of the chemoreflex control of breathing in humans: model parameters measurement. *Respiration Physiology 120*: 13-26

Golledge H, Roughan J, Niel L, Richardson C, Wright-Williams S and Flecknell P 2005 Carbon dioxide euthanasia in rats - behavioural and autonomic system responses to exposure. *Proceedings of the SECAL-ESLAV 2005 International Congress* p 40. 5-7 October 2005, Elche, Spain

Hackbarth H, Kuppers N and Bohnet W 2000 Euthanasia of rats with carbon dioxide-animal welfare aspects. *Laboratory Animals 34*: 91-96

Hawkins P, Playle L, Golledge H, Leach M, Banzett R, Coenen A, Cooper J, Danneman P, Flecknell P, Kirkden R, Niel L and Raj M 2006 *Newcastle Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals*. 27-28 February 2006, University of Newcastle upon Tyne, UK. Published online by the NC3Rs (http://www.nc3rs.org.uk/ downloaddoc.asp?id=416&page=292&skin=0) and Laboratory Animals Ltd (http://www.lal.org.uk/pdffiles/ CO2%20Final%Report.pdf)

Hayashi F, Yoshida A, Fukuda Y and Honda Y 1983 The ventilatory response to hypoxia in the anesthetized rat. *Pflügers Archiv 396*: 121-127

Hewett TA, Kovacs MS, Artwohl JE and Bennett BT 1993 A comparison of euthanasia methods in rats, using carbon dioxide in pre-filled and fixed flow rate filled chambers. *Laboratory Animal Science 43*: 579-582

Hornett TD and Haynes AR 1984 Comparison of carbon dioxide/air mixture and nitrogen/air mixture for the euthanasia of rodents. Design of a system for inhalation euthanasia. *Animal Technology 35*: 93-99

Iwarsson K and Rehbinder C 1993 A study of different euthanasia techniques in Guinea pigs, rats, and mice. Animal response and post mortem findings. *Scandinavian Journal of Laboratory Animal Science 4*: 191-205

Jones B and Kenward MG 2003 *Design and Analysis of Cross-over Trials, second edition.* CRC/Chapman and Hall: Boca Raton, USA

Kohler I, Meier R, Busato A, Neiger-Aeschbacher G and Schatzmann U 1999 Is carbon dioxide (CO_2) a useful short acting anaesthetic for small laboratory animals? *Laboratory Animals 33*: 155-161

Krohn TC, Hansen AK and Dragsted N 2003 The impact of low levels of carbon dioxide on rats. *Laboratory Animals 37*: 94-99

Leach MC, Bowell VA, Allan TF and Morton DB 2002a Aversion to gaseous euthanasia agents in rats and mice. *Comparative Medicine 52*: 249-257

Leach MC, Bowell VA, Allan TF and Morton DB 2002b Degrees of aversion shown by rats and mice to different concentrations of inhalational anaesthetics. *Veterinary Record 150*: 808-815

Leach MC, Bowell VA, Allan TF and Morton DB 2004 Measurement of aversion to determine humane methods of anaesthesia and euthanasia. *Animal Welfare 13*: S77-S86

Leonard BE and Rigter H 1975 Changes in brain monoamine metabolism and carbon dioxide induced amnesia in the rat. *Pharmacology, Biochemistry and Behavior 3*: 775-780

Masuda A, Ohyabu Y, Kobayashi T, Yoshino C, Sakakibara Y, Komatsu T and Honda Y 2001 Lack of positive interaction between CO_2 and hypoxic stimulation for pCO_2-VAS response slope in humans. *Respiration Physiology 126*: 173-181

Niel L and Weary DM 2006 Behavioural responses of rats to gradual-fill carbon dioxide euthanasia and reduced oxygen concentrations. *Applied Animal Behaviour Science 100*: 295-308

Niel L and Weary DM 2007 Rats avoid exposure to carbon dioxide and argon. *Applied Animal Behaviour Science 107*: 100-109

Niel L, Stewart SA and Weary DM 2008 Effect of flow rate on aversion to gradual-fill carbon dioxide exposure in rats. *Applied Animal Behaviour Science 109*: 77-84

Nunn JF 1987 *Applied Respiratory Physiology, 3rd edition*. Butterworths: London, UK

Olfert ED, Cross BM and McWilliam AA 1993 *Guide to the Care and Use of Experimental Animals, Volume 1, 2nd edition*. Canadian Council on Animal Care: Ottawa, Canada

Paolino RM, Quartermain D and Miller NE 1966 Different temporal gradients of retrograde amnesia produced by carbon dioxide anesthesia and electroconvulsive shock. *Journal of Comparative and Physiological Psychology 62*: 270-274

Porter AL 1972 An analytical review of the effects of non-hydrogen-bonding anesthetics on memory processing. *Behavioral Biology 7*: 167-198

Reber AS 2001 *The Penguin Dictionary of Psychology, 3rd edition*. Penguin: London, UK

Rushen J 1990 Use of aversion-learning techniques to measure distress in sheep. *Applied Animal Behaviour Science 28*: 3-14

Smith W and Harrap SB 1997 Behavioural and cardiovascular responses of rats to euthanasia using carbon dioxide gas. *Laboratory Animals 31*: 337-346

Yavari P, McCulloch PF and Panneton WM 1996 Trigeminally-mediated alteration of cardiorespiratory rhythms during nasal application of carbon dioxide in the rat. *Journal of the Autonomic Nervous System 61*: 195-200

Young A 2006 Halothane induction results in differing behaviours compared with carbon dioxide mixed with oxygen when used as a rat euthanasia agent. *Animal Technology and Welfare 5*: 49-59