Measurement of aversion to determine humane methods of anaesthesia and euthanasia

MC Leach*†, VA Bowell‡, TF Allan§ and DB Morton#

† Department of Clinical Veterinary Science, University of Bristol, Langford House, Langford, Bristol BS40 5DU, UK
‡ Department of Animal Behaviour Sciences, Scottish Agricultural College, Edinburgh EH9 3JG, UK
§ City University, Northampton Square, London EC1V 0HB, UK
# Biomedical Services Unit, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
* Contact for correspondence and requests for reprints: m.c.leach@bris.ac.uk

Abstract

The distress experienced by animals during the induction of unconsciousness remains one of the most important and yet overlooked aspects of effective methods of anaesthesia and euthanasia. Here we show that considerable differences exist in the aversive responses elicited by 12 common methods of inhalational anaesthesia and euthanasia in laboratory rats and mice. Carbon dioxide, either alone or in combination with oxygen or argon, was found to be highly aversive to both species. The least aversive agents were halothane in rats and enflurane in mice. Exposing these animals to carbon dioxide in any form, either for anaesthesia or for euthanasia, is likely to cause considerable pain and distress and is therefore unacceptable when efficient and more humane alternatives are readily available.

Keywords: anaesthesia, animal welfare, aversion, carbon dioxide, euthanasia, rodent

Introduction

The humane induction of unconsciousness is an important characteristic of any agent of anaesthesia or euthanasia, yet very little research has been conducted that considers the two key elements of humane induction: first, the animal's initial perception of the agent and, second, the potential pain and distress associated with the induction of unconsciousness. In the UK, approximately 40% of regulated laboratory procedures involve general anaesthesia, and since rodents comprise over 85% of research animals (Home Office 2002) this issue is of particular significance to laboratory rodents. Moreover, almost all research animals are eventually euthanased, along with those that are surplus, sick or injured, and the number of mice used in laboratory experiments stands to dramatically increase with the growth of transgenic research. The humane induction of anaesthesia or euthanasia is a moral responsibility of those who use animals or animal tissues in their research. In the UK, it is also a legal requirement to ensure the minimum amount of suffering necessary to attain a scientific objective (Animals [Scientific Procedures] Act 1986). After all, euthanasia means 'good death', which is accepted as being a method that causes the minimum of pain and distress (Merriam-Webster 2001), and the main purpose of anaesthetising animals is to prevent the pain and distress associated with surgical and other procedures.

A variety of agents is commonly used to anaesthetise animals (Green 1979; Flecknell 1996), including volatile liquid anaesthetics, which are considered to be both effective and non-irritant (Jones 1990; Atkinson et al 1993; Blackmore 1993). However, they vary in their pungency and this may affect an animal's willingness to breathe (Green 1979; Blackmore 1993; Flecknell 1995; Flecknell & Liles 1996; Flecknell et al 1996; Flecknell et al 1999; Hedenqvist et al 2000), and therefore might influence the distress it experiences during the induction of unconsciousness. Carbon dioxide (CO_2) is a relatively common method of euthanasia and has been used for short-term anaesthesia in laboratory rodents at concentrations above 40% (Fenwick & Blackshaw 1989; Blackmore 1993; Danneman et al 1997; Kohler et al 1999; van Luijtelaar & Coenen 1999; Hackbarth et al 2000), and in other laboratory species and farm animals at concentrations above 70% (Blackshaw et al 1988; Blackmore 1993; Coenen et al 1995; EU Working Party 1996; Danneman et al 1997; EU Working Party 1997; Kohler et al 1999; van Luijtelaar & Coenen 1999; Hackbarth et al 2000). Recently, concerns have been expressed over the humaneness of carbon dioxide induction because it is associated with breathlessness and hyperventilation (Hewett et al 1993; Raj & Gregory 1995; Lambooij et al 1999; Ludders et al 1999; van Luijtelaar & Coenen 1999; Raj & Whittington 2003) and with irritation of the nasal mucosa (Lucke 1979; Ewbank 1983; Iwarsson & Rehbinder 1993). As a result, modifications have been suggested to reduce the distress associated with carbon dioxide induction, including humidification (MacArthur 1978; Mouton et al 2001) and the addition of oxygen (O_2) (Iwarsson & Rehbinder 1993; Coenen et al 1995; EU

Table 1   Concentrations of anaesthetic and euthanasia agents used to assess aversion in Wistar rats and BALB/c mice. See text for further details.

Concentration of agent (%)	Rats			Mice		
	Low	Medium	High	Low	Medium	High
Halothane	1.8	3.9	7.4	2.0	3.5	8.5
Isoflurane	1.7	3.7	7.2	1.3	3.6	8.0
Enflurane	2.7	4.7	8.1	3.1	5.2	8.5
Sevoflurane	1.8	3.2	7.2	1.8	3.2	7.2
Desflurane	3.5	5.5	11.6	3.5	5.5	11.6
CO_2	25.5	34.9	50.8	28.0	36.0	53.0
Humidified CO_2	26.6	36.7	50.8	26.6	36.7	50.8
Argon	93.7	95.8	99.2	91.2	93.0	95.5
CO_2 + low O_2	15.5 + 5.0*	31.7 + 5.0*	59.2 + 5.0*	20.0 + 13.0*	20.0 + 7.9*	20.0 + 4.5*
CO_2 + low O_2	19.1 + 7.0*	32.1 + 7.0*	54.2 + 7.0*	30 + 14.0*	30 + 10.1*	30 + 5.3*
CO_2 + 20% O_2	29.3 + 20.0	37.2 + 20.0	60.7 + 20.0	29.3 + 20.0	37.2 + 20.0	60.7 + 20.0
CO_2 + 30% O_2	29.9 + 30.0	37.5 + 30.0	60.4 + 30.0	29.9 + 30.0	37.5 + 30.0	60.4 + 30.0

* Indicates the resultant oxygen concentrations in the carbon dioxide and argon mixtures. Oxygen levels are presented because argon is used to reduce the level of oxygen.

Working Party 1996; Danneman *et al* 1997; Smith & Harrap 1997; Kohler *et al* 1999).

Oxygen deprivation (ie hypoxia and anoxia) may offer a more humane method of euthanasia for many species (Freed 1982) and can be achieved using a variety of gases, such as argon and nitrogen, to displace the oxygen in air (Jones 1990; Blackmore 1993; Lambooij *et al* 1999). Argon has been the focus of research into humane farm animal euthanasia and appears to be both effective and non-aversive at concentrations above 90% (Raj & Gregory 1994; Lambooij *et al* 1999; Raj 1999; van Luijtelaar & Coenen 1999; Raj & Whittington 2003), although there has been no research into its value as a method for laboratory rodent euthanasia. The apparent lack of distress this agent causes may be associated with its odourless and inert properties, and its ability to induce a loss of consciousness without causing the breathlessness and painful irritation associated with carbon dioxide. Argon, however, takes longer to kill and therefore combinations of low carbon dioxide (30%) and high argon (above 60%) have been suggested as an alternative to argon alone; these mixtures kill within a similar timescale to carbon dioxide and are thought to be less aversive (Raj & Gregory 1994; Raj 1999; Raj & Whittington 2003).

The aim of the present study was to assess the initial responses of Wistar rats and BALB/c mice to 12 commonly used agents of anaesthesia and euthanasia at three different concentrations (low, medium and high), which represent a range of concentrations that could be used to induce unconsciousness for the anaesthetic agents, and unconsciousness and death for the euthanasia agents. The animals' initial responses to the agents were assessed in terms of locomotory and other behavioural responses that might indicate the level of aversion experienced. Aversion has been defined as "a tendency to extinguish a behaviour or to avoid a thing or situation and especially a usually pleasurable one because it is or has been associated with a noxious stimulus" (Merriam-Webster 2001). Such a situation is likely to cause psychological distress to an animal if it is unable to remove itself from the noxious stimulus.

Methods

Sixty female Wistar rats (190–220 g) from Charles River Laboratories (UK) and sixty female BALB/c mice (20–25 g) bred in-house (University of Birmingham) were used. The animals were nine weeks of age at the start of the study. Thirty animals of each species were allocated to the pilot study and the remaining animals were used in the main study. The animals were free from all common pathogens according to the Federation of European Laboratory Animal Science Associations (FELASA) health monitoring recommendations (FELASA 1994), and were housed in accordance with local ethical committee and Home Office guidelines, as outlined in Leach *et al* (2002a,b).

The six inhalational anaesthetics tested were carbon dioxide (BOC, Surrey MB28 2UT, UK) and five volatile liquid anaesthetics: halothane (Zeneca, Cheshire SK10 4TG, UK), isoflurane, enflurane, sevoflurane and desflurane (Abbot, Kent ME11 5EL, UK). The current recommended range of concentrations for effective induction to unconsciousness with these anaesthetics (Flecknell 1996; Hedenqvist *et al* 2000) includes the medium concentrations of the volatile liquid anaesthetics and the high concentration of carbon dioxide tested in this study. The seven euthanasia agents tested were carbon dioxide, humidified carbon dioxide, argon, two carbon dioxide–argon mixtures and two carbon dioxide–oxygen mixtures (BOC, Surrey MB28 2UT, UK). The current recommended range of concentrations for effective induction to unconsciousness and death with these euthanasia agents (Danneman *et al* 1997; Kohler *et al* 1999;

Hackbarth *et al* 2000) includes the high concentrations of all of the euthanasia agents tested in this study. The concentrations of the five volatile liquid anaesthetics were established by passing oxygen through calibrated vaporisers (fluotec, isotec, enflotec, sevotec and destec) at the desired concentration into the test chamber. The concentrations of carbon dioxide and the remaining euthanasia agents were established by flowing the gases into the test chamber until the desired concentration was reached according to a carbon dioxide monitor (carbon dioxide infrared gas analyser PA404, Servomex, Crowborough, UK) and an oxygen monitor (oxygen analyser 570A, Servomex, Crowborough, UK).

For both the pilot and main studies, animals of each species were numbered at random (between 1 and 30) and assigned to a randomly ordered gas treatment (ie order in which the different gases were to be tested) in a balanced incomplete block design (Cochran & Cox 1967, p 480 [Plan 1135]). Each concentration of each agent was treated as a separate treatment and all treatments were randomised. Each individual was exposed to seven treatments in total, either in the pilot or in the main study.

Pilot study

A pilot study was carried out to determine the three concentrations (low, medium and high) of each agent to be used in the main study (see Table 1). This was necessary to ensure that each of the three concentrations induced the same degree of ataxia (loss of muscular coordination [Blackshaw *et al* 1988]) irrespective of agent, within 30, 20, and 10 seconds in rats, and within 18, 12 and 6 seconds in mice. These times were chosen to allow accurate comparisons to be made between the agents. This also enabled the point at which animals became incapable of leaving the test chamber to be identified for each concentration, thus allowing differentiation between animals that chose not to leave and those that were unable to leave.

For each test treatment, a 12 litre transparent polycarbonate anaesthesia chamber was pre-filled with one of the agents at one of four concentrations that ranged from below to above those shown in Table 1, and an animal was placed into the chamber through a small door in the lid. The four concentrations were chosen to represent a range of levels from below that which would induce unconsciousness to a level well above the highest concentration recommended for induction with each agent. To maintain the concentration, the agent was flowed continuously into the chamber throughout the treatment. The time between the animal being placed into the chamber and it exhibiting initial signs of ataxia was recorded, and the animal was then removed and returned to its home cage. The concentrations of agents to be used in the main study were determined by plotting the observed mean time to ataxia against each of the four concentrations tested. These 'observed plots' were then compared with natural-logarithm, inverse, growth and exponential decay standard plots. The standard plot with the highest coefficient of determination (Montgomery & Peck 1992) was considered to be the closest match and was used to calculate the concentration of each agent that corresponded to

Figure 1
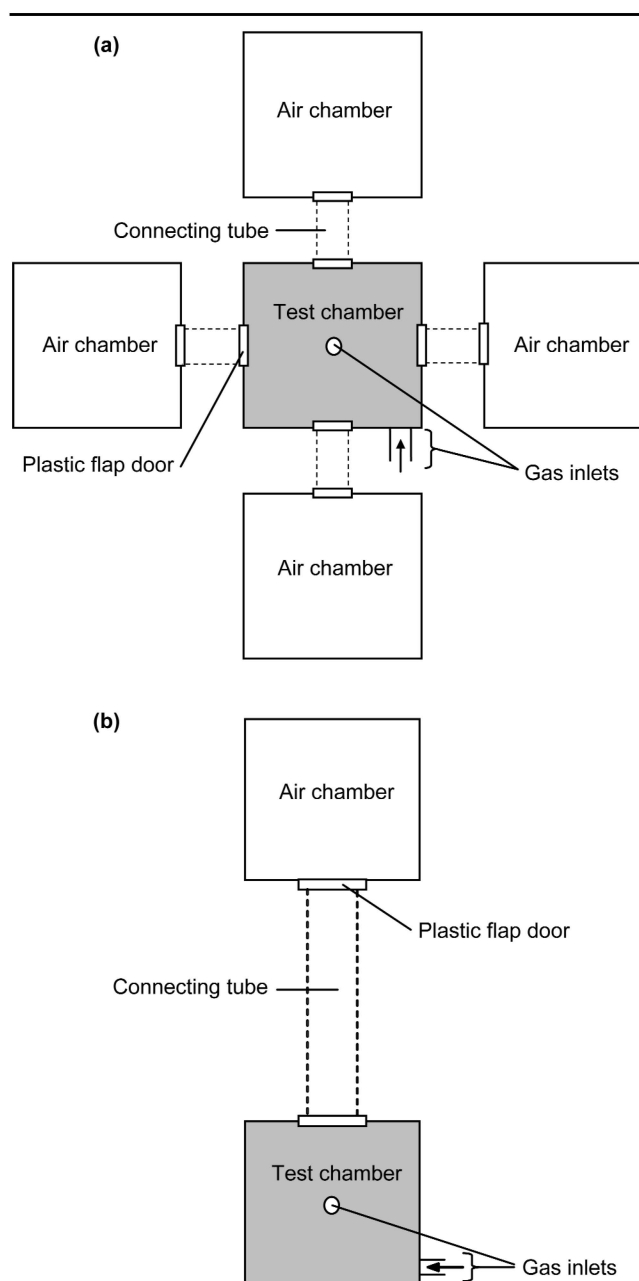
(a) Test apparatus for mice, consisting of a central test chamber surrounded by four identical air chambers (all chambers are 260 mm x 165 mm x 70 mm). **(b)** Test apparatus for rats, consisting of a test chamber connected to an identical air chamber (both chambers are 278 mm x 278 mm x 156 mm). Wire mesh tubes connect test and air chambers in both apparatus and each entry/exit point has a plastic door flap. The test chamber has two gas inlets, one in the centre of the chamber top and one at floor level.

low, medium and high, in terms of the time taken to induce ataxia (see Table 1).

Main study

Different apparatus were used to test rats and mice (Figure 1), but both comprised a main test chamber connected to one or more air chambers. The apparatus used to test mice (Figure 1a) was designed to have a greater number

Table 2 The coefficients of determination (adjusted R^2 values) for three locomotory and four behavioural measures of aversion to anaesthetic and euthanasia agents in female Wistar rats and BALB/c mice.

R^2 value for measure (%)	Anaesthesia		Euthanasia	
	Rats	Mice	Rats	Mice
Initial withdrawal time	27.7	41.7	70.6	60.6
Re-entry time	44.3	9.6	7.3	58.2
Total dwelling time	50.7	60.4	77.7	50.7
Grooming frequency	10.9	*	20.1	4.5
Rearing frequency	10.5	18.3	13.4	11.6
Sniffing frequency	4.3	5.6	15.1	5.9
Elimination frequency	4.4	2.6	7.8	3.2

* No grooming behaviour was observed in response to the anaesthetics in the BALB/c mice.

of entry/exit points than the rat apparatus (Figure 1b), because observations from preliminary trials suggested that, compared to rats, mice were less able to find entry/exit points in the presence of the agents.

The same experimental protocol was used for both rats and mice. First, each animal was placed into an acclimation apparatus (identical to the test apparatus) and left undisturbed for 30 mins. This ensured familiarity with the layout of the apparatus, therefore minimising the effects of neophobia (fear of novelty) and the possibility of carry over effects from previous treatments since the animals were always exposed to a period in which the system contained only air before being exposed to each treatment. Second, a 3 min control session was carried out in which the animal was placed into the test apparatus with all of the chambers containing only air. Finally, a 3 min test session was carried out in which the animal was placed into the test apparatus after the test chamber had been pre-filled with one of the agents at the specified concentration (see Table 1), while the other chamber(s) contained only air. In test and control sessions, the animals were introduced into the apparatus through a flap in one of the connecting tubes adjacent to the test chamber entrance. Each session began when the animal voluntarily entered the test chamber from this position, which was always within 5 s. After each treatment the animal was returned to its home cage. Animals were exposed to treatments in a random order, with no individual being exposed more than once per day. None of the animals tested remained in the test chamber for long enough for it to become unable to leave the test chamber.

During the control and test sessions, five locomotory measures were taken: 1) the time taken for an animal to withdraw from the test chamber after initially entering it ('initial withdrawal time', T_w); 2) the time taken for an animal to re-enter the test chamber after initially leaving it ('re-entry time',

T_r); 3) the total amount of time an animal spent in the test chamber ('total dwelling time', T_d); 4) the total number of test chamber entries; and 5) the total number of test chamber exits. In addition, frequencies of rearing ('up on hind legs with front feet off the floor'), grooming ('using front paws to groom face and snout'), sniffing entrance area to test chamber, and elimination (defecation and urination), were recorded. Changes in the frequency of these four behaviours have previously been associated with gas aversion (Wadham 1997; Ambrose 1999).

Data analysis

Statistical analyses were carried out using Minitab (Version 12, 1999) and SPSS (Version 9, 2000). The data were natural-logarithm transformed to meet the assumptions of parametric testing. An initial analysis compared the locomotory and behavioural responses of the animals between the control (air in all chambers) and test (agent in test chamber) treatments, using a paired Students *t*-test. The main analysis compared the animals' locomotory and behavioural responses between each of the test treatments using General Linear Modelling (GLM) with individual, species, agent type, and agent concentration as main factors, and with the frequency of test chamber entries and exits included as covariate factors. Significant differences were tested for, using Tukey post hoc multiple comparisons.

Results

The measures that best modelled aversive responses were identified as those that explained the greatest amount of variation between different agents, and were identified using the adjusted coefficient of determination ('adjusted R^2') values for each measure (generated as part of the GLM analysis, see Table 2). The adjusted R^2 refers to the amount of variation in a dataset that is explained by the variables being examined (Montgomery & Peck 1992), and the higher the adjusted R^2, the greater the variation accounted for. For clarity and brevity, only the aversion measures with the highest adjusted R^2 values for anaesthetic and euthanasia agents are reported here. For anaesthetic agents it was total dwelling time (T_d) both for rats and for mice, and for euthanasia agents it was total dwelling time (T_d) for rats and initial withdrawal time (T_w) for mice. The remaining locomotory response measures showed similar but less discriminatory patterns. Each of the suggested aversion-associated behaviours (ie rearing, grooming, sniffing entrance area and elimination) accounted for less than 20% of the total variation on average, and failed to show any clear trends.

When the animals' responses to each of the anaesthetic and euthanasia agents were compared with their responses to air (see Tables 3 and 4), all three concentrations of the anaesthetics carbon dioxide and desflurane induced significantly shorter T_d times both in rats and mice. In comparison with air, sevoflurane and halothane induced significantly shorter T_d times in mice at the medium and high concentrations, but, in rats, only at the high concentration. Medium and high concentrations of isoflurane and enflurane induced significantly shorter T_d times than air, in both rats and mice.
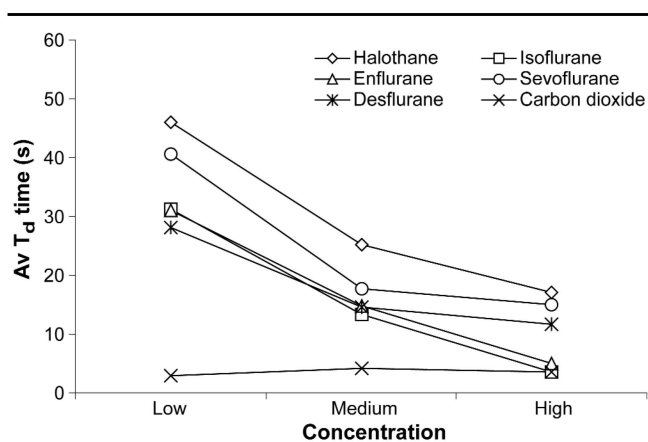
Table 3   Total dwelling times (mean ± SE) of female Wistar rats and BALB/c mice when the test chamber was filled with air (control), and with an anaesthetic agent (test). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

| Agent	Concentration (%)		Rat T_d times (s)		Mouse T_d times (s)	
			Control (air)	Test (agent)	Control (air)	Test (agent)
Halothane	Low	1.8	39.6 ± 6.2	42.7 ± 6.2	63.1 ± 11.4	52.5 ± 5.2
	Medium	3.9	39.2 ± 6.4	25.6 ± 2.8	54.6 ± 8.0	15.5 ± 5.9**
	High	7.4	55.4 ± 12.1	14.3 ± 6.3**	42.4 ± 5.9	14.2 ± 4.1***
Isoflurane	Low	1.7	37.5 ± 8.5	39.1± 5.6	47.0 ± 8.9	42.1 ± 14.3
	Medium	3.7	43.6 ± 4.1	12.9 ± 4.4**	61.6 ± 8.2	16.2 ± 5.5****
	High	7.2	60.4 ± 12.9	6.6 ± 3.2**	65.3 ± 13.8	10.1 ± 3.1*
Enflurane	Low	2.7	47.4± 5.5	35.1 ± 7.6	109.0 ± 11.5	68.9 ± 11.2**
	Medium	4.7	45.3 ± 7.1	18.6 ± 5.5**	54.2 ± 13.7	23.4 ± 8.7*
	High	8.1	33.6 ± 3.2	6.0 ± 1.8****	67.0 ± 12.9	24.8 ± 4.5*
Sevoflurane	Low	1.8	49.5 ± 9.9	39.0 ± 5.9	58.3 ± 8.9	39.7 ± 8.9
	Medium	3.2	25.4 ± 5.9	15.0 ± 6.2	81.3 ± 14.6	32.9 ± 9.3*
	High	7.2	44.8 ± 5.2	12.2 ± 3.1****	70.8 ± 10.5	31.5 ± 6.2**
Desflurane	Low	3.5	42.2 ± 4.7	25.5 ± 5.4**	62.2 ± 8.8	40.5 ± 6.1*
	Medium	5.5	43.6 ± 5.5	14.4 ± 5.2**	47.8 ± 5.8	22.0 ± 1.8**
	High	11.6	34.6 ± 6.4	10.1 ± 4.2**	86.6 ± 10.8	8.1 ± 1.2****
CO_2	Low	25.5	45.6 ± 7.9	2.1 ± 0.5****	48.0 ± 5.5	3.3 ± 0.8****
	Medium	34.9	51.3 ± 9.1	1.0 ± 0.1****	77.7 ± 14.6	2.3 ± 0.6***
	High	50.8	36.5 ± 6.6	0.7 ± 0.2****	38.4 ± 4.8	2.3 ± 0.6****

Table 4   Total dwelling times of female Wistar rats and initial withdrawal times of female BALB/c mice (mean ± SE) when the test chamber was filled with air (control), and with a euthanasia agent (test). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Agent	Concentration (%)		Rat T_d times (s)		Mouse T_w times (s)	
			Control (air)	Test (agent)	Control (air)	Test (agent)
CO_2	Low	25.5	45.6 ± 7.9	2.1 ± 0.5****	8.5 ± 1.8	1.3 ± 0.3**
	Medium	34.9	51.3 ± 9.1	1.0 ± 0.1****	13.7 ± 3.8	1.1 ± 0.1**
	High	50.8	36.5 ± 6.6	0.7 ± 0.2****	5.5 ± 0.7	0.7 ± 0.1****
Argon (Oxygen)	Low	93.7	61.9 ± 10.0	16.9 ± 1.5**	8.5 ± 3.4	20.3 ± 6.7*
	Medium	95.8	73.9 ± 13.9	13.5 ± 2.1**	6.2 ± 1.0	1.4 ± 0.3****
	High	99.2	41.4 ± 6.6	9.2 ± 1.8****	17.4 ± 4.4	2.6 ± 1.0**
Humidified CO_2	Low	26.6	47.7 ± 3.7	1.9 ± 0.6**	8.4 ± 1.0	2.1 ± 0.3****
	Medium	36.7	37.5 ± 9.2	0.8 ± 0.1**	5.7 ± 0.7	0.7 ± 0.1****
	High	50.8	45.5 ± 10.8	0.9 ± 0.2**	6.0 ± 0.9	0.7 ± 0.1****
CO_2 + Low O_2*	Low	15.5	53.9 ± 5.4	2.5 ± 0.6****	8.7 ± 1.6	3.3 ± 1.6**
	Medium	31.7	69.5 ± 13.9	1.4 ± 0.4***	5.0 ± 0.6	1.2 ± 0.3***
	High	59.2	56.1 ± 15.9	1.1 ± 0.3**	10.6 ± 1.3	1.2 ± 0.3***
CO_2 + Low O_2*	Low	19.1	61.2 ± 7.3	1.9 ± 0.2****	9.0 ± 2.3	1.1 ± 0.3***
	Medium	32.1	48.9 ± 8.9	1.1 ± 0.2****	7.2 ± 1.1	0.9 ± 0.2**
	High	54.2	39.0 ± 7.2	0.7 ± 0.2****	9.3 ± 1.9	0.6 ± 0.1*
CO_2 + 20% O_2	Low	29.3	57.5 ± 7.3	1.0 ± 0.2****	9.2 ± 3.6	1.3 ± 0.2*
	Medium	37.2	31.5 ± 8.7	1.0 ± 0.2**	4.9 ± 0.6	0.7 ± 0.1****
	High	60.7	56.7 ± 11.3	0.6 ± 0.1***	5.8 ± 0.4	0.7 ± 0.1****
CO_2 + 30% O_2	Low	29.9	42.3 ± 9.2	0.8 ± 0.3***	9.1 ± 1.6	1.0 ± 0.1****
	Medium	37.5	30.1 ± 6.6	0.6 ± 0.1***	5.0 ± 0.6	0.6 ± 0.1****
	High	60.4	28.7 ± 8.3	0.6 ± 0.1**	5.3 ± 0.5	0.6 ± 0.1****

*The low oxygen concentrations in the 'CO_2 + Low O_2' treatments were achieved using argon to reduce the level of oxygen.

Figure 2

The mean total dwelling times (T_d) of female Wistar rats in a test chamber pre-filled with one of six different anaesthetic agents at one of three different concentrations.

Figure 3
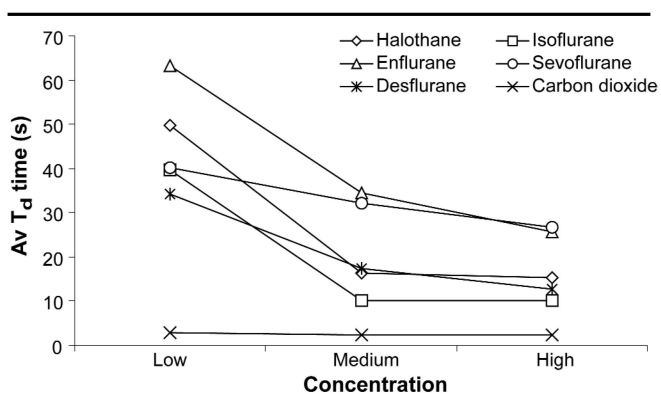
The mean total dwelling times (T_d) of female BALB/c mice in a test chamber pre-filled with one of six different anaesthetic agents at one of three different concentrations.

For euthanasia agents, all three concentrations of carbon dioxide, humidified carbon dioxide, carbon dioxide–argon mixtures and carbon dioxide–oxygen mixtures induced significantly shorter T_d times in rats, and significantly shorter T_w times in mice, compared to air. Low, medium and high concentrations of argon induced progressively shorter T_w times in mice and T_d times in rats, and these times were significantly different from one another.

The comparison between the anaesthetics and between the euthanasia agents showed that in both cases an animal's reaction was dependent upon an interaction between the type of agent and its concentration, since these were the only two factors to significantly affect T_d times in rats and both T_d and T_w times in mice ($P < 0.0001$ for all comparisons). Carbon dioxide resulted in significantly shorter T_d times in rats and mice than were observed for all concentrations of halothane ($P < 0.0001$ in rats, $P < 0.001$ in mice), isoflurane ($P < 0.05$ both in rats and mice), enflurane ($P < 0.01$ in rats, $P < 0.0001$ in mice), sevoflurane ($P < 0.0001$ in rats, $P < 0.001$ in mice), and desflurane ($P < 0.01$ in rats, $P < 0.05$ in mice) (see Figures 2 and 3). In

rats, halothane was associated with significantly longer T_d times than all other agents ($P < 0.05$ for all comparisons), with no significant difference being found between the other volatile liquid anaesthetics (see Figure 2). In mice, exposure to enflurane resulted in significantly longer T_d times than all other agents ($P < 0.05$ for all comparisons), with no significant differences being found between the other volatile liquid anaesthetics (see Figure 3). In terms of euthanasia agents, argon was associated with significantly longer T_d times in rats ($P < 0.0001$), and T_w times in mice ($P < 0.0001$), than all of the other agents tested (see Figures 4 and 5). There were no significant differences between the remaining euthanasia agents (ie carbon dioxide, humidified carbon dioxide, carbon dioxide–argon mixtures and carbon dioxide–oxygen mixtures) in terms of T_d times in rats or T_w times in mice.

Discussion

The measures used to model aversion in this study were chosen because they represent simple and objective measures of responses that are referred to in the definition of 'aversion' (see introduction). Similar measures have also been used successfully to assess aversion to gaseous agents of euthanasia in farm animal species (Raj & Gregory 1995). Although Raj and Gregory's study did not include rodents, it seems likely that different animals will respond to aversive situations in similar ways, for example, by trying to avoid or escape from a noxious stimulus. The current study and previous studies carried out by Leach *et al* (2002a,b) offer a method of assessing rodent aversion that is similar to that used in investigations of the effects of gaseous methods of euthanasia in farmed animals (Raj & Gregory 1995). Total dwelling times (T_d) and initial withdrawal times (T_w) can be considered the best measures of aversion since they accounted for the greatest amount of variation in the data (as demonstrated by their high adjusted R^2 values). In contrast, re-entry times (T_r) and the behaviours suggested to be associated with aversion (rearing, grooming, sniffing entrance area and elimination) had low adjusted R^2 values and showed few consistent trends in the analyses comparing test and control treatments and comparing different test treatments. This finding may help to explain contradictions in the literature surrounding the humaneness of carbon dioxide as a method of anaesthesia or euthanasia, since many studies have simply relied upon measurements of suggested aversion-associated behaviours as indicators of aversion (Danneman *et al* 1997).

A possible explanation for the differences between the three locomotory measures of aversion (T_d, T_w and T_r) is a difference in the underlying motivation of the animals. Initial withdrawal time may represent the first response of an animal to an agent and will therefore be dependent on the agent's aversiveness and novelty. Re-entry time may represent a more intermediate response in which an individual returns to re-assess an agent and so might depend more on aversiveness than novelty. Finally, total dwelling time may represent a more long-term 'considered' response that solely depends on aversiveness. However, these motivations will be affected

both by the individual's strategy for escaping from an aversive situation and by the aversiveness of the agent. Based upon frequencies of test chamber entries and exits, the animals tested could be said to fall into one of two categories: 'escapers', which exit the test chamber and never re-enter, and 'searchers', which continually move between the chambers looking for a way out, and so either intentionally or inadvertently enter the test chamber more than once. The severity of adverse effects experienced upon exposure to an agent is likely to affect an animal's motivation since aversion will decrease its willingness to explore the chambers. An animal's motivation may also be affected by its prior experience with the agent. It was to control for the effects of strategy and the severity of adverse effects that the frequencies of test chamber entries and exits were included as covariate factors in the analysis.

The effects of strategy and the severity of adverse effects may explain why the best model of aversion to the euthanasia agents in rats was dwelling time, whereas in mice it was initial withdrawal time. Mice were, in general, 'searchers' and showed considerable individual variation in the frequency with which they entered and exited the test chamber. This strategy would reduce the overall variation accounted for by total dwelling times, but would not affect withdrawal times since this latter measure represents an individual's initial response. In addition, withdrawal times may be more dependent on aversiveness than on novelty, because all of the euthanasia agents induced relatively high levels of aversion. Rats, however, were generally 'escapers', moving between the chambers less frequently than mice and exhibiting less individual variation in their responses. This strategy does not reduce the overall variation accounted for by dwelling times, and in this case total dwelling time is the best measure for explaining variation in the data.

The comparison of anaesthetic and air treatments (Table 3) suggests that in rats only halothane and sevoflurane can induce unconsciousness without causing distress, because these were the only agents in which aversion was first observed above the concentration recommended for the humane induction of anaesthesia. Using total dwelling times, the anaesthetic agents can be ranked according to their relative aversiveness. The ranking is across the three concentrations of each agent and is derived from the output of Tukey *post hoc* analysis of the General Linear Modelling used to compare between the test treatments. The ranking shows that carbon dioxide was by far the most aversive agent tested, both for rats and for mice. For rats, halothane was the least aversive agent, followed by sevoflurane, desflurane, enflurane and then isoflurane. However, in mice, enflurane was the least aversive agent, followed by sevoflurane, halothane, isoflurane and desflurane. The comparison of euthanasia agent and air treatments (Table 4) suggests that none was able to induce unconsciousness without potentially causing distress, since all of the agents induced aversion at concentrations well below those recommended for humane euthanasia. Using total dwelling times in rats and initial withdrawal times in mice, euthanasia agents can

Figure 4
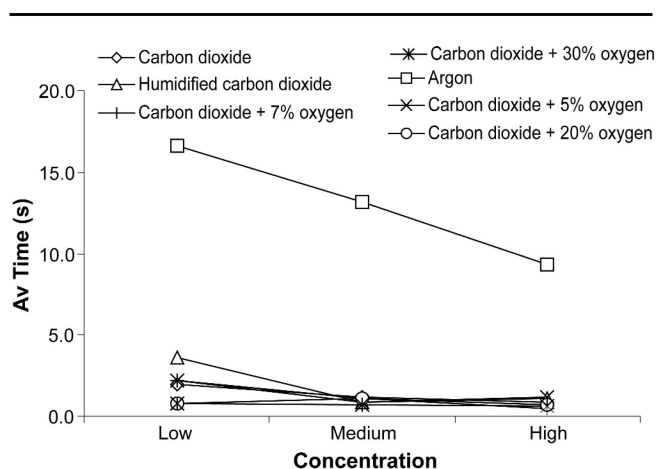
The mean total dwelling times (T_d) of female Wistar rats in a test chamber pre-filled with one of seven different euthanasia agents at one of three different concentrations. The carbon dioxide and low oxygen mixtures are represented by the 'carbon dioxide + 5% oxygen' and the 'carbon dioxide + 7% oxygen' lines.

Figure 5
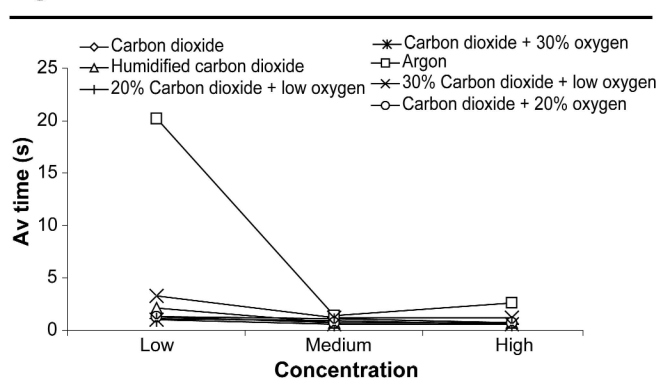
The mean initial withdrawal times (T_w) of female BALB/c mice from a test chamber pre-filled with one of seven different euthanasia agents at one of three different concentrations. The carbon dioxide and low oxygen mixtures are represented by the '30% carbon dioxide + low oxygen' and the '20% carbon dioxide + low oxygen' lines.

be ranked according to their relative aversiveness (based on Tukey *post hoc* output) and for both species argon was the least aversive agent tested. Carbon dioxide and its mixtures were all equally aversive.

The relative levels of aversion to the volatile liquid anaesthetics could be related to their differing characteristics. Both desflurane and isoflurane have been associated with adverse effects: desflurane induces coughing in humans (Hedenqvist *et al* 2000), and isoflurane produces a pungent vapour that some animals are reluctant to breathe (Green 1979; Blackmore 1993; Flecknell 1995; Flecknell & Liles 1996; Flecknell *et al* 1999). The aversive nature of desflurane observed in this study is in direct contrast to studies both in rabbits (Hedenqvist *et al* 2000) and in mice (Whelan unpublished data) that suggest desflurane to be less aversive than isoflurane. In these studies, aversion was assessed by

means of a subjective assessment of the level of struggling in restrained animals, and this measure may have been limited by high inter-observer variation. In addition, in the study on mice, aversion was assessed by measuring the frequencies of specific aversion-associated behaviours, which we have here found to be unreliable.

The lower level of aversion induced by argon compared to carbon dioxide is in agreement with the results of farm animal euthanasia studies using similar concentrations of these agents (Raj & Gregory 1994; Lambooij *et al* 1999; Raj 1999; van Luijtelaar & Coenen 1999; Raj & Whittington 2003), and may be due to argon's odourless, tasteless and inert properties. The reduced level of aversion shown upon exposure to argon, which acts by displacing oxygen in the air to induce hypoxia, suggests that oxygen deprivation is more humane than carbon dioxide, when administered either alone or in combination with other agents. However, appreciable aversion to argon was still observed and could be associated with feelings of hypoxia before unconsciousness. Another new finding is that the addition of argon to carbon dioxide failed to reduce the aversiveness of carbon dioxide. This result is in contrast to findings in chickens and turkeys, where such mixtures have been found to be less aversive than carbon dioxide alone (Raj & Gregory 1994; Raj & Whittington 2003). These species differences may reflect the higher ventilatory rate of rodents compared to chickens and turkeys (Olfert *et al* 1993, Appendix III), which could lead to the agent being detected more rapidly by rodents. The addition of oxygen also failed to reduce the level of aversion associated with carbon dioxide — a result that supports the findings of other studies (Hewett *et al* 1993; van Luijtelaar & Coenen 1999; AVMA 2000). The humidification of carbon dioxide also produced no reduction in aversion, contrary to a previous suggestion (MacArthur 1978). The inability of oxygen and the humidification process to reduce aversion to carbon dioxide, and the less aversive nature of argon, suggest that the aversiveness of carbon dioxide may be due to the rapid onset of mucosal membrane irritation and breathlessness, rather than to hypoxia.

Carbon dioxide alone, or in combination with oxygen or argon, at a concentration sufficient to induce loss of consciousness, is likely to cause considerable suffering before unconsciousness occurs. The highly aversive nature of carbon dioxide as an agent of anaesthesia (alone) or euthanasia (alone or in combination with other agents) is further illustrated by its clear aversiveness at concentrations below those deemed to produce humane induction of unconsciousness (Fenwick & Blackshaw 1989; Danneman *et al* 1997; Hackbarth *et al* 2000; Whelan unpublished data). It should be noted that in the current study, comparing the total dwelling times at the highest concentrations of agents, rats spent over 20 times longer in halothane than in carbon dioxide, and mice spent ten times longer in enflurane than in carbon dioxide. Furthermore, rats spent seven times longer in argon than in carbon dioxide, and mice exited the test chamber four times faster in carbon dioxide than in argon. Moreover,

the low levels of individual variation (as shown by standard error values) in the dwelling times and withdrawal times for carbon dioxide, compared to those for the volatile liquid anaesthetics and for argon, suggest a consistent and universal aversive response to this agent. The standard errors both for rats and for mice exposed to carbon dioxide alone or in combination were always within 1 s of the mean, suggesting that all animals found carbon dioxide similarly aversive, whilst for the least aversive anaesthetics, standard errors were between 3–14 s from the mean. The aversive nature of carbon dioxide should perhaps not be surprising since concentrations similar to those used for animal euthanasia have been used as noxious stimuli in human and animal pain research (Thurauf *et al* 1991; Anton *et al* 1992; Komai & Bryant 1993; Peppel & Anton 1993; Danneman *et al* 1997). Humans report exposure to carbon dioxide at concentrations of 40–50% (levels sufficient to cause unconsciousness in animals) to be "unpleasant and distressing", and at concentrations above 70% (levels sufficient to kill animals) to be "painful" (Paton 1983; Gregory *et al* 1990). The similarity between humans and other mammalian species in pain physiology (animals are considered good research models of human pain), and the level at which discomfort occurred in rodents in the current study with carbon dioxide (above 40%), strongly suggest that animals experience similar aversive sensations to humans when exposed to this agent.

Carbon dioxide is considered to be such a rapid, practical and economical method of euthanasia that further modifications have been proposed to make its use more humane, such as use at very high concentrations and the use of a rising rather than a static concentration. Some authors claim that at higher concentrations carbon dioxide produces such a rapid loss of consciousness that the animal suffers for only a short period of time, and that this is acceptable (Danneman *et al* 1997). It has been suggested that placing animals into a chamber and then introducing carbon dioxide might be potentially less distressing than placing them in a pre-filled chamber (Kohler *et al* 1999), since exposure to an increasing concentration of carbon dioxide might cause animals to lose consciousness before the higher concentrations that cause distress are reached. However, our results show that aversion to carbon dioxide occurs at very low concentrations, which are likely to be reached very rapidly even when a slow steady rising concentration is used (Fenwick & Blackshaw 1989; Kohler *et al* 1999). Alternative agents, such as argon, offer efficient killing, and these alternatives are associated with significantly less aversion and so can be considered more humane.

Conclusions and animal welfare implications

All of the anaesthetic and euthanasia agents tested in this study were associated with some degree of aversion depending on the type and concentration of the agent and on the species being tested. Based on our findings, we recommend that anaesthesia in rats should be induced with halothane at around 3–4%, and in mice with enflurane at about 5%, since these concentrations produce rapid and effective induction of anaesthesia with minimal distress.

Our results demonstrate that carbon dioxide, either alone or in combination with other agents at concentrations high enough to cause a loss of consciousness, is likely to be highly aversive. Regardless of whether these concentrations are experienced upon introduction to a pre-filled chamber or are reached by a gradual increase in concentration, we suggest that any conscious animal unable to escape from an environment containing these agents is very likely to experience considerable pain and distress before unconsciousness supervenes.

Therefore, carbon dioxide should not be used for the anaesthesia or euthanasia of laboratory rodents, and possibly not for other species, particularly since there are effective and more humane alternatives available. A single agent of euthanasia offers the simplest and most operator-friendly method; therefore we would recommend a high concentration of argon as a single agent of euthanasia both in rats and in mice as this represents a balance between producing rapid unconsciousness and death with minimum distress. However, to effectively and rapidly induce unconsciousness and death in the most humane manner, we would recommend using halothane or enflurane initially to cause a loss of consciousness followed by carbon dioxide or argon to produce death. Although, this method is not as simple or as user-friendly as the use of a single agent because the animals may require monitoring and the agents need to be flowed into the chamber at specific times, we consider this method to be the most humane.

Acknowledgements

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