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Euthanasia using gaseous agents in laboratory rodents: how to refine and improve animal welfare and user safety

2024 Biotechnische Dagen
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A bit of etymology

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- The word ‘euthanasia’ is derived from the Greek words ‘eu’ meaning good and ‘thanatos’ meaning death
- A ‘good death’ would be one that occurs with minimal pain and distress
- In the case of animals, the word ‘euthanasia’ is often substituted by terms such as ‘humane death’ or ‘humane killing’
- For simplicity, the term ‘euthanasia’ will be used throughout this presentation

Why consider euthanasia for use in laboratory rodents?

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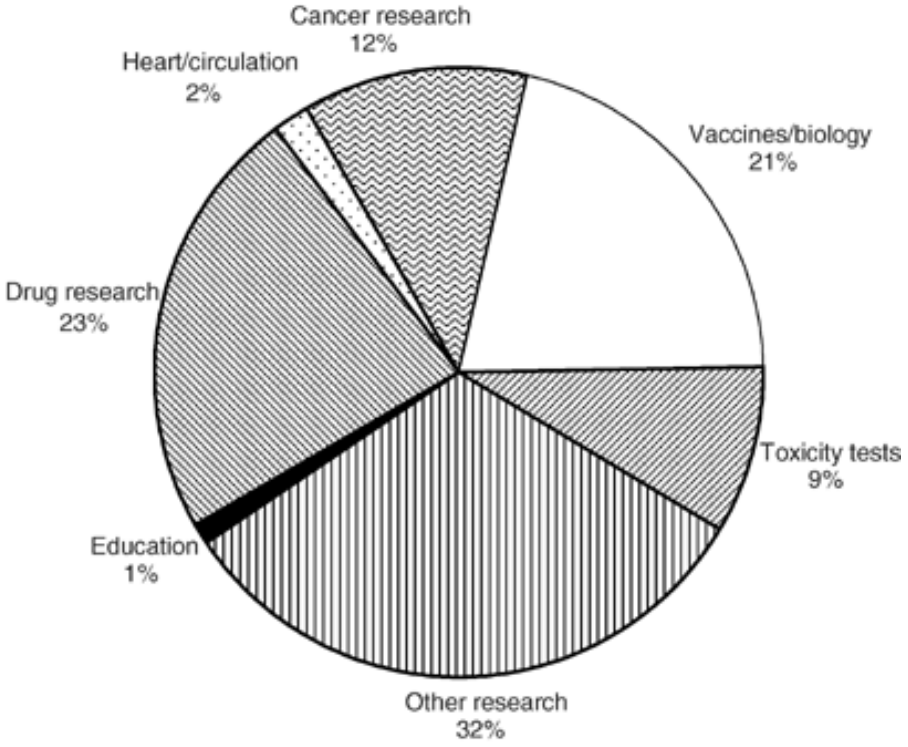
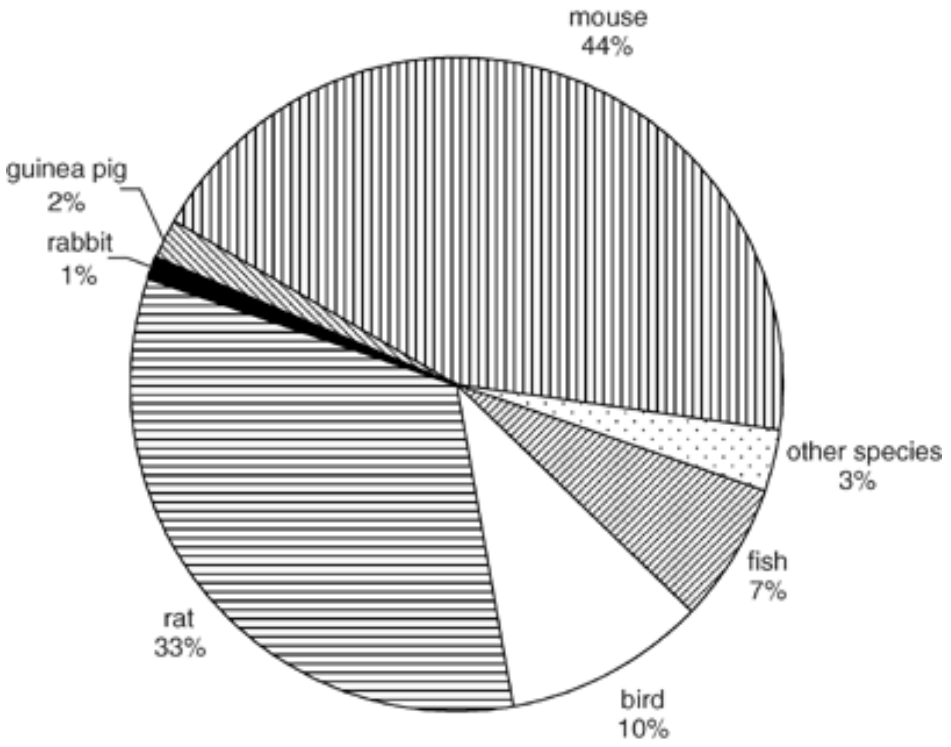
- Rodents remain the most widely used species for scientific research due to their small size, low cost, rapid sexual maturity and scope for genetic manipulation





Why consider euthanasia for use in laboratory rodents?

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- Despite significant interest in the replacement of animals for scientific purposes, the numbers involved are still growing

- Laboratory rodents are euthanized for various reasons

  To provide tissues for scientific purposes, at the end of an experiment

  When adverse effects (pain, distress, suffering, etc.) become excessive

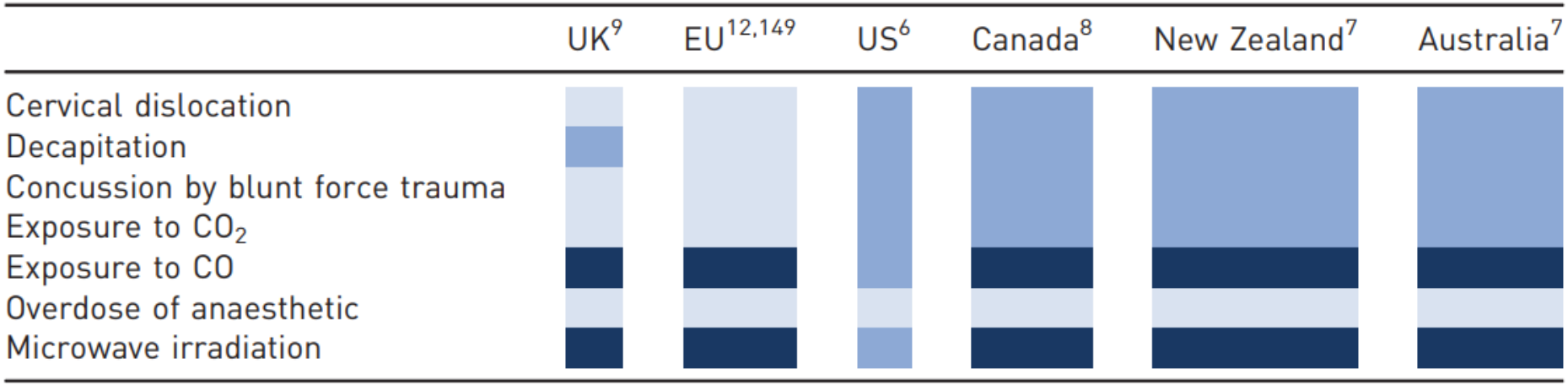
  When animals become unwanted stock

- Regulation requires animals to be euthanized upon completion of the work and when humane endpoints are reached

Identification and use of methods that offer a death with minimal suffering is a moral imperative, and crucial to support the justifiability of animal-based research based on utilitarian harm/benefit ethical reasoning

Overview of euthanasia methods

Overview of euthanasia methods from published euthanasia guidelines for adult laboratory rodents



CO, carbon monoxide, CO_2, carbon dioxide; EU, European Union; UK, United Kingdom; US, United States.

Methods permitted or recommended for use

Methods where additional permissions are required

Methods not permitted or recommended

Assessing animal welfare during euthanasia

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- Concern for animal welfare during killing stems from animals' ability to experience potentially negative sensations occurring during the conscious phase of the process

- To determine the welfare consequences of a euthanasia method

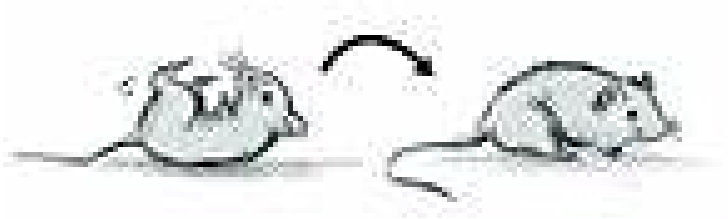
Time taken for the animal to be rendered unconsciousness inclusive of pre-handling time and euthanasia method application

Presence and character of potentially negative experiences during the conscious phase

Assessing animal welfare during euthanasia

By assessing the gradual loss of reflexes and goal-directed behaviours and ultimately, motor coordination

Typically measured in rodents by their inability to effectively right their posture, referred to as loss of righting reflex or loss of posture



Assessing animal welfare during euthanasia

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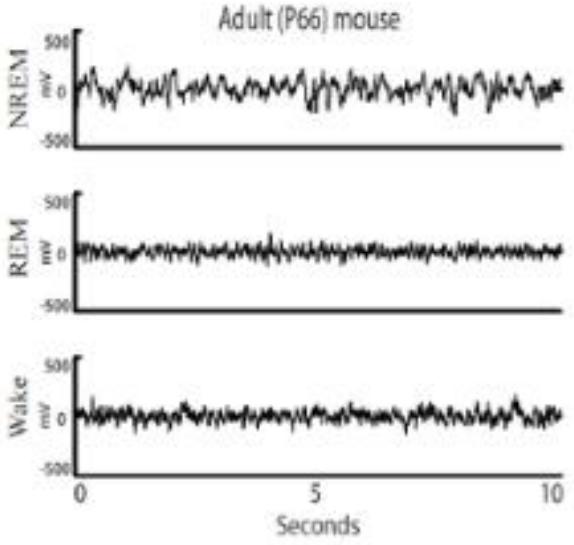
Changes in behavioural output can be measured through the transition from goal directed behaviour (motivated escape behaviours) to the presence of spontaneous ones such as jumping, gasping

Unconsciousness is also characterised by the presence of high amplitude, low frequency activity in the EEG signal



Assessing animal welfare during euthanasia

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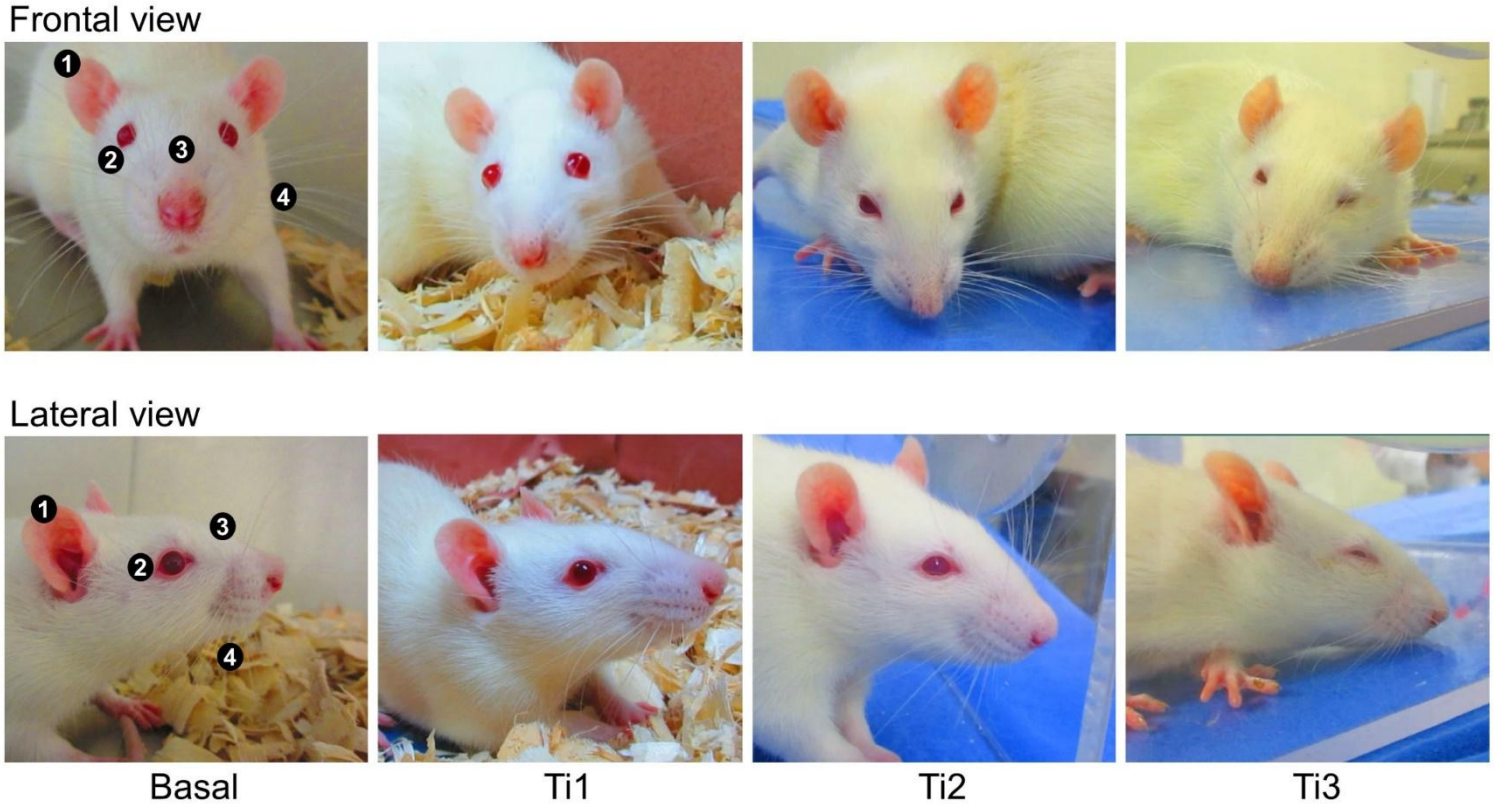
Presence and character of potentially negative experiences during the conscious phase



Example of the FAU and the assigned score. (1) ear change; (2) orbital tightening; (3) nose/cheek flattening; and (4) whisker change

Assessing user welfare during euthanasia

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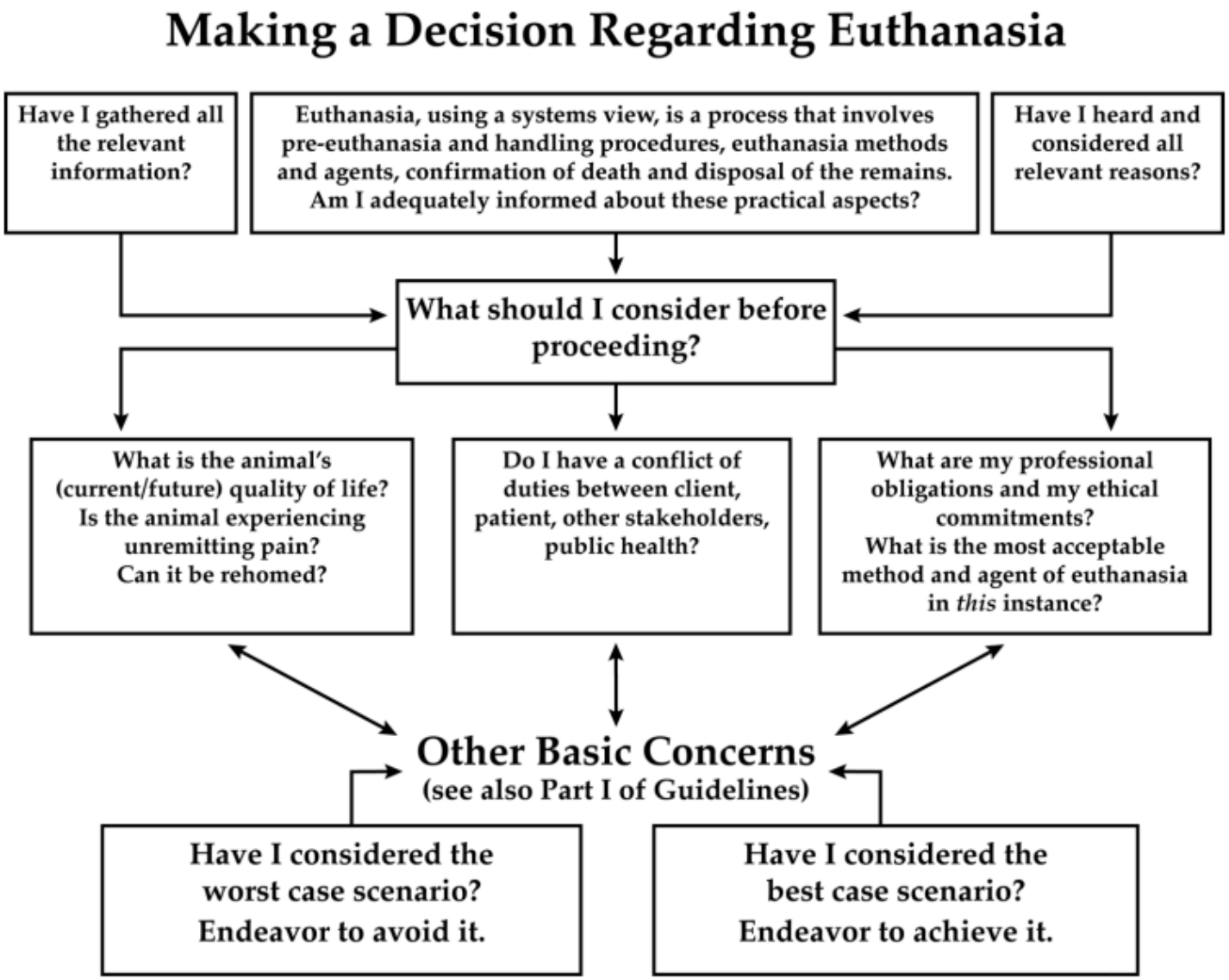

Assessing user welfare during euthanasia

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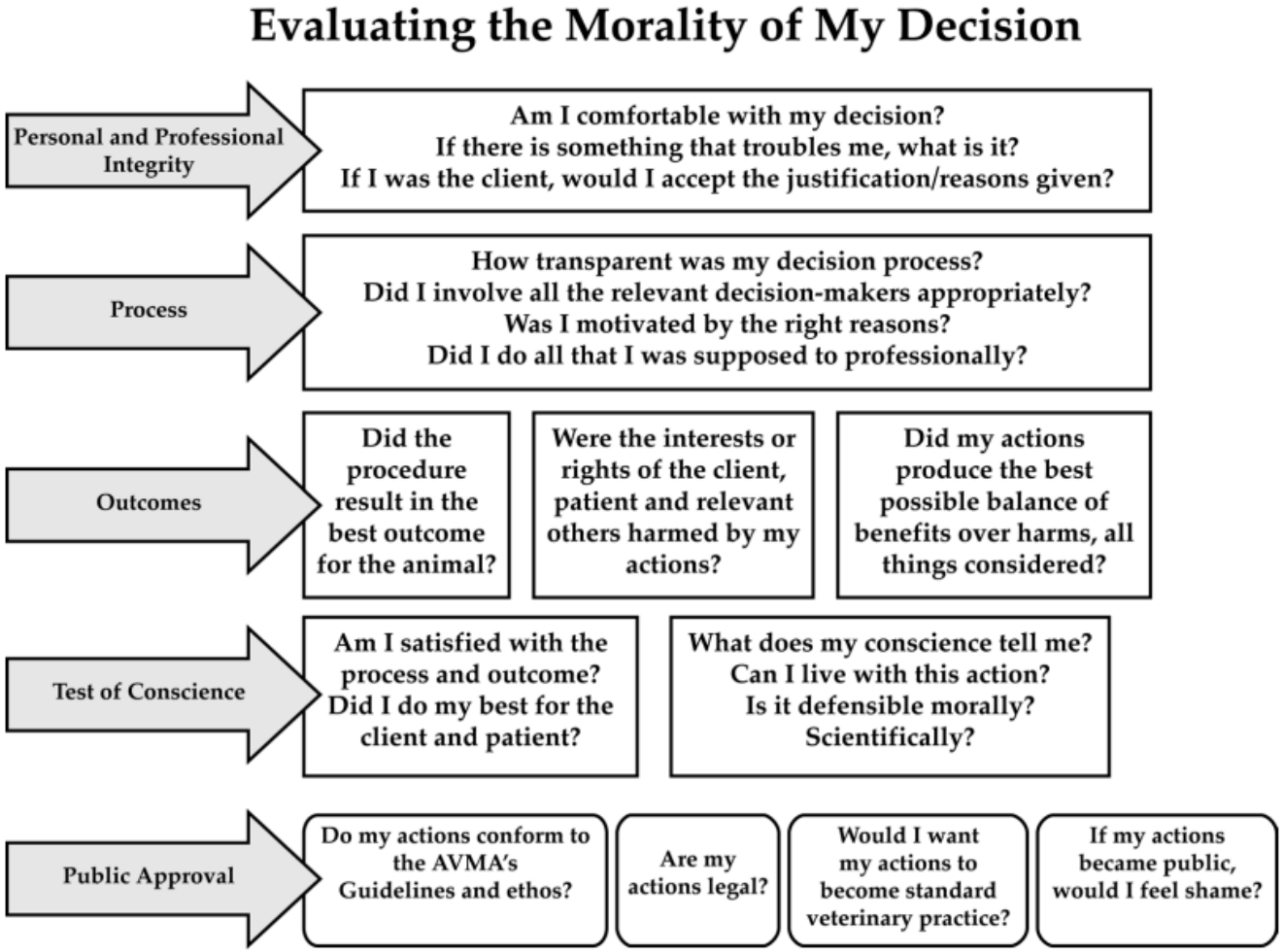

Euthanasia using various methods

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Agent or Method	2013 AVMA Guidelines[63]	2016 Society of Mammologists Guidelines[89]	2010 CCAC Euthanasia Guidelines[22]	2010 EU Directive[28]
Barbiturate	A[a]	A[b]	A[a]	A[c]
Dissociative Agent Combination	A	A[b]	—	A[c]
Ethanol	C[d]	—	—	—
T-6131	—	—	C[e,f]	A[c]
Carbon Dioxide	C[g,k]	—	C[g,h]	C[g,i]
Carbon Monoxide	C[l]	—	—	—
Cervical Dislocation	C[n,o]	C[p]	C[n,o,q,r]	C[s]
Decapitation	C[t]	—	C[t,u]	C[v]
Inhalant Anesthetic	C[w,x]	A[b]	A[w,y]	A[c]
Focused Beam Microwave Irradiation	C[a1]	—	—	—
Nitrogen, Argon	U	—	C[b1]	A
Nitrous oxide	U	—		
Exsanguination	U[m]	—	U[m]	U[m]
Thoracic Compression	U	C[c1]	—	—
Blunt Force Trauma to the Head	C[f,d1]	—	—	C[d1]

A = Acceptable; C = Acceptable with Conditions; U = Unacceptable when used as sole agent on conscious animals; - = not addressed.

Euthanasia using various methods

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a, IV preferred over IP; concentrated solutions may cause pain when given IP.
b, Drug use in field can present additional risks to investigators and stress to animals, risk of secondary toxicity if carcass left in field to be eaten.
c, Anesthetic overdose should, where appropriate, be used with prior sedation
d, 0.5 mL of 70% IP for mice; unacceptable for larger species
e, Only IV, slowly
f, Personnel must be well-trained
g, Gradual fill only, displacing 10% to 30% chamber volume per minute; source of gas should be compressed gas cylinder; euthanize in home cage or euthanasia chamber should be emptied and cleaned between uses; verify death has occurred
h, Must have written SOP, written records, regular postapproval monitoring, animals should be anesthetized prior to CO_2 delivery
i, Not to be used on fetuses/neonates
j, Prefilled chamber recommended for guinea pigs to minimize the experience of breathlessness
k, Prolonged exposure required for neonates
l, Requires properly maintained equipment; hazardous to personnel, acceptable only when conditions for safe use can be met
m, Acceptable under deep anesthesia
n, Personnel must be trained and their proficiency validated; availability of secondary method if initial attempt unsuccessful
o, For rodents < 200 g
p, Animals of small body size, performed by experienced personnel
q, Anesthetize or sedate first; scientific justification required for use on conscious animals
r, For rats > 200g use commercial dislocator
s, Mice, rats < 150 g
t, Properly maintained equipment: blades sharp, clean, in good condition; operator skilled in handling/restraint of animals; personnel should be trained on dead/anesthetized animals to demonstrate proficiency
u, Recommend anesthetizing first
v, Use only if other methods are not possible
w, Time to death may be prolonged, consider adjunctive method once animals are deeply anesthetized
x, Maintain compatible groups, clean and maintain induction/euthanasia chamber, adhere to recommended flow rates, it is important to verify death when inhalant method used
y, Not for use in species that breath-hold
z, Occupational health and safety issue for personnel exposed to waste anesthetic gas
a1, Purpose-built equipment, mouse and rat only
b1, When scientifically justified and approved by ACUC; Argon is aversive to rats; O_2 concentration must be <2%, only appropriate for use if O_2 concentration is known/measured; mixtures of argon and nitrogen should only be used if animal is already anesthetized
c1, Personnel skilled in technique and animal small enough to allow thoracic cavity to be collapsed and prevent inspiration
d1, Small laboratory rodents <1kg
e1, T-61 not available in the US

CO_2 as an euthanasia method

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- Carbon dioxide narcosis and asphyxiation have long been used for euthanasia of rodents and other laboratory species
- At present, the method is considered “acceptable with conditions” by the AVMA

Specified displacement rate

Prefilled chambers are unacceptable

Verify that an animal is dead after exposure to CO_2 as with other inhalant euthanasia techniques

- In the past, CO_2 was administered by putting animals in a prefilled chamber or delivering CO_2 at a very high rate (approximately 70%) of volume displacement

CO_2 as an euthanasia method

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- Cost-effective
- Relatively safe for users and the environment Suitable for euthanasia of multiple rodents at the same time
- Anesthetic at high concentrations (30% to 40% in rats)
- Renders animals unconscious before they die of respiratory arrest and hypoxia

- Reaction with the fluid in mucous membranes to form carbonic acid
- Produces a stinging sensation in the eyes and throat
- Produces anxiety responses in rodents at concentrations above 20%

➢ Controversies regarding its use, leading to find alternatives to CO_2 use

Isoflurane or sevoflurane as an alternative?

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- The use of CO_2 for euthanizing animals has been questioned as studies have shown that the exposure to CO_2 causes aversion in rats and mice, demonstrated by several behavioral tests
- Assessment and development of alternatives to CO_2 has become a focal point
- Recent studies recommend the use of volatile anesthetics, but it still needs to be clarified whether they cause less pain, distress, and suffering compared to CO_2

Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide?

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1 Institute of Pharmacology and Toxicology, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, **2** Institute of Veterinary Anatomy, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Isoflurane or sevoflurane as an alternative?

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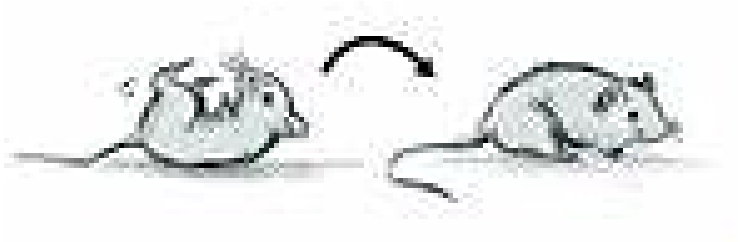
- The appropriate surrogate measure for unconsciousness in rodents is not finally defined
- The loss of righting reflex (LORR) has been described as a first indicator of insensibility and as a good correlate for the loss of consciousness in humans



C57Bl/6J	LORR [s]	
CO_2 20	108.0	[84.5/117.5]
CO_2 60	60.0	[57.0/63.75]
CO_2 100	50.0	[49.0/52.5]
Iso 2%	122.0	[108.0/136.0]*+
Iso 5%	67.0	[65.5/68.0]+#
Sevo 4.8%	122.0	[113.25/129.5]
Sevo 8%	82.0	[75.5/86.0]*+

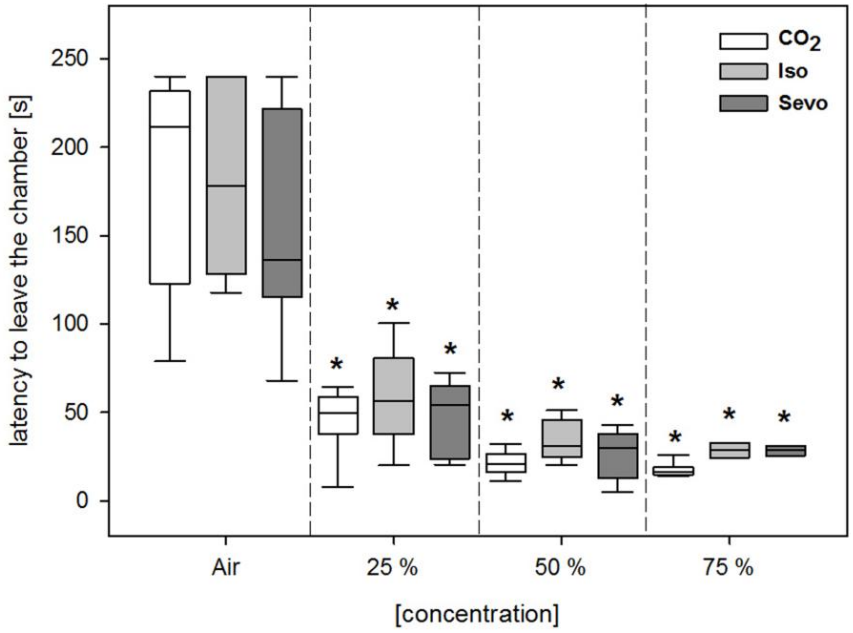
Isoflurane or sevoflurane as an alternative?

Set-up of the approach-avoidance test

Which gas is more aversive to animals?

- CO_2, isoflurane and sevoflurane were perceived similar aversive by the animals
- Indicated by shorter escape latencies compared to Air control

Difficult to weigh up the advantages and disadvantages of CO_2



Isoflurane or sevoflurane as an alternative?

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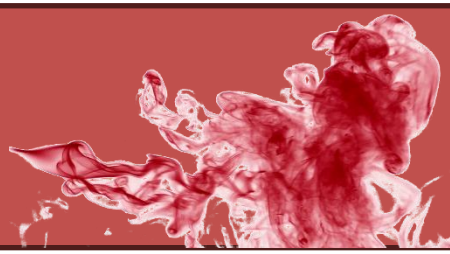
Data does not conclusively demonstrate the advantages of halogenated over CO_2

Price

User exposure to halogenated gases



Use of charcoal filters

- So why not try to improve the existing methods? Which will lead to improved animal welfare

How to increase animal welfare during CO_2 exposure

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➢ Know the basics : Gas source

- Carbon dioxide and CO_2 gas mixtures must be supplied in a precisely regulated and purified form without contaminants
  - ➢ Commercially supplied cylinder or tank

- Gas displacement rate is critical to the humane application of CO_2
  - ➢ Appropriate pressure-reducing regulator and flow meter with demonstrated capability for generating the recommended displacement rates for the size container being utilized

How to increase animal welfare during CO_2 exposure

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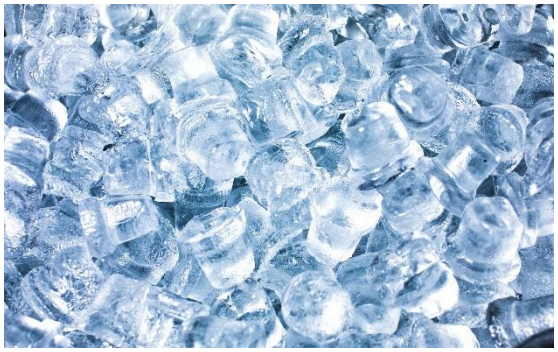
- Know the basics : Animal age is crucial

On the day of birth, rats and mice exposed to 100% CO_2 required exposure times of 35 and 50 minutes, respectively, to ensure death

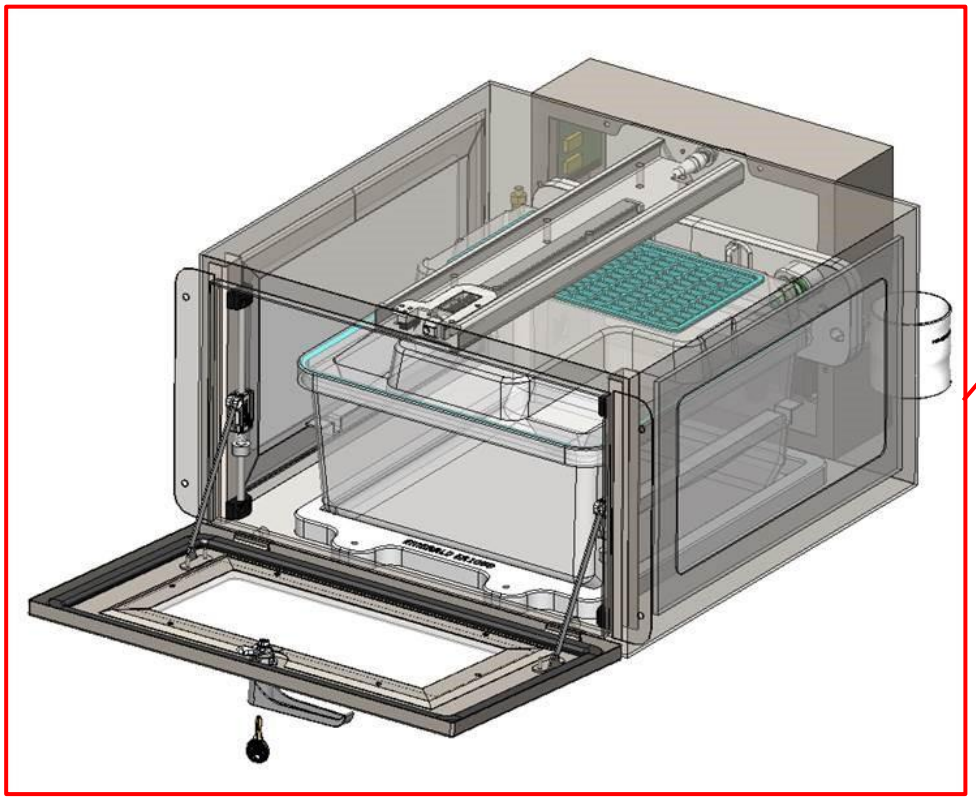
- If exposure time is not sufficient, pup will recover as shown by its pink coloration

Rat and mouse pups are born neurologically immature when compared with humans, and their afferent pain pathways are not well developed until after postnatal day 5 to 7



How to increase animal welfare during CO_2 exposure

- AVMA Guidelines on Euthanasia state that, to decrease potential distress of animals, the home cage should be used for the euthanasia of mice



How to increase animal welfare during CO_2 exposure

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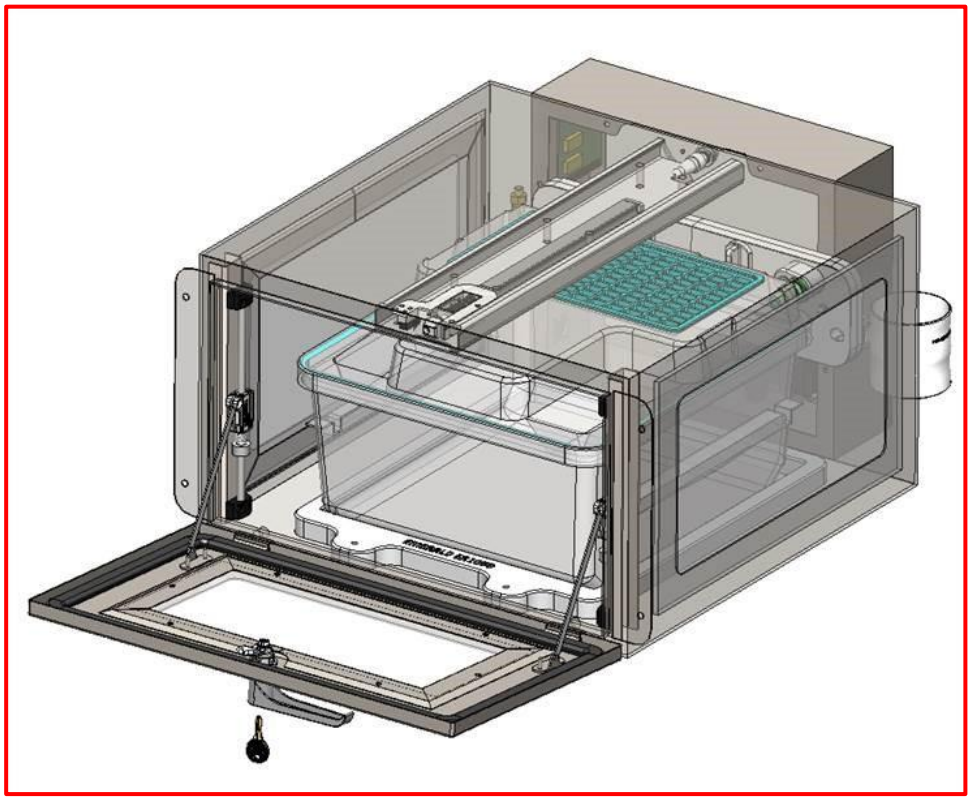
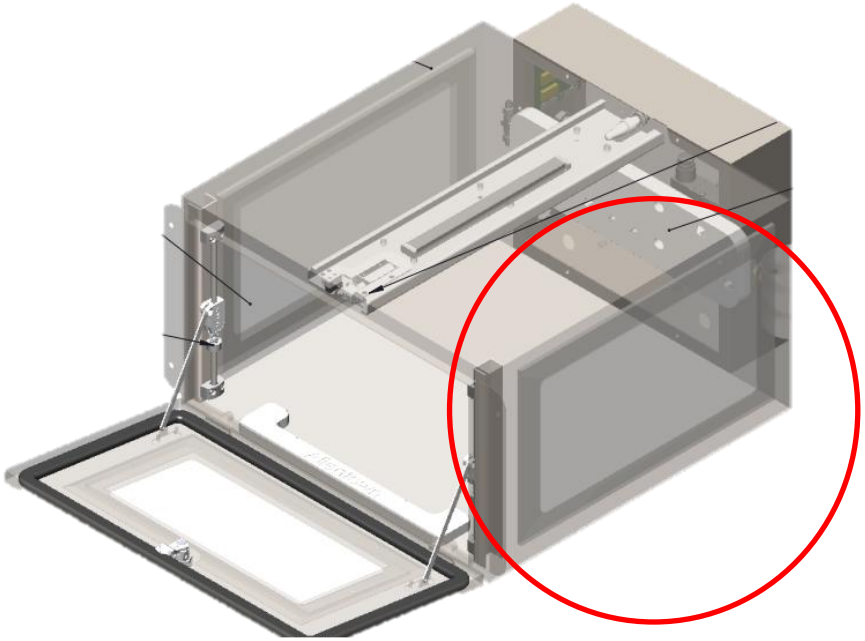


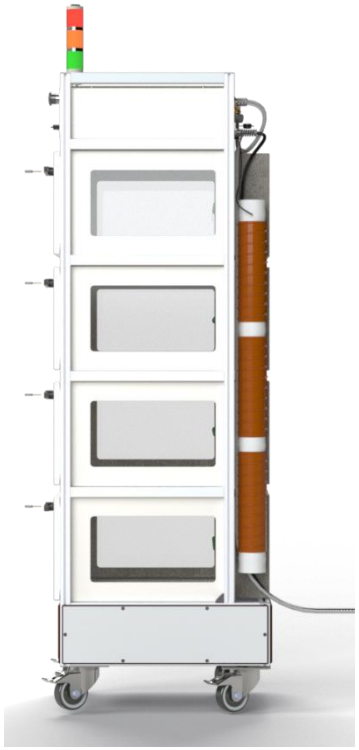
Offers several advantages over physical methods

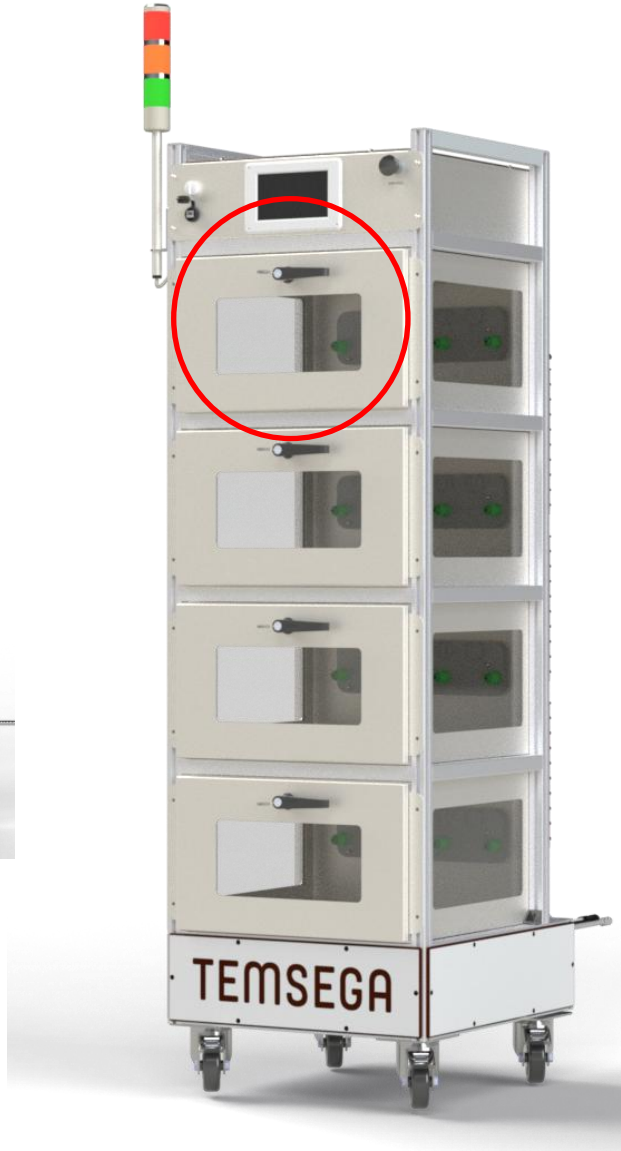
- Non-contact nature
- Elimination of stress associated with handling
- Isolation and restraint
- Minimising the impact of operator error

How to increase animal welfare during CO_2 exposure

- The euthanasia system should allow easy visibility of the animals







- Windows from each side of the system allowing optimal visibility
- Red tainted windows as an option

How to increase animal welfare during CO_2 exposure

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- Appropriate gradual displacement rate

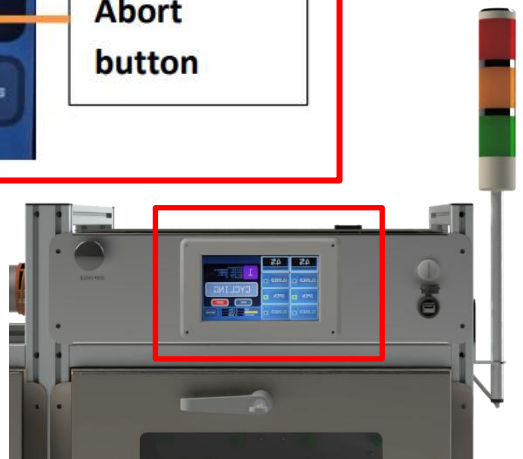
Displacement rate	Time to unconsciousness	CO_2 concentration
20% / min	106 s	30 %
10% / min	156 s	21 %
50% / min	< 80 s	45 %

➢ CO_2 exposure using a gradual-fill method is less likely to cause pain prior to unconsciousness due to nociceptor activation by carbonic acid

How to increase animal welfare during CO_2 exposure

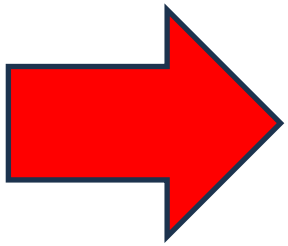
- AVMA Guidelines on Euthanasia recommends an optimal flow rate for CO_2 euthanasia systems that can displace 30% to 70% of the chamber or cage volume/min

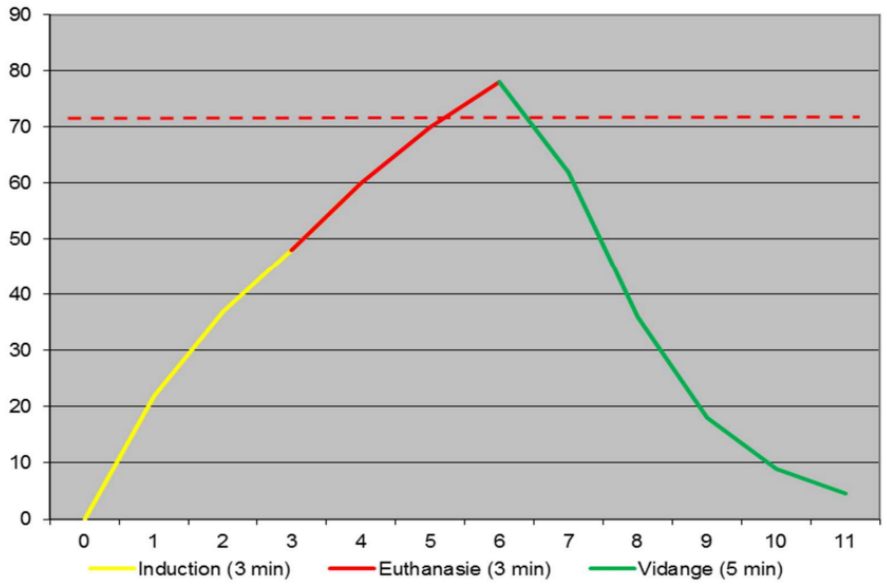
- Our system is equipped with independent modules
- Each module can be programmed with specifics flow rate
- Can evolve with AVMA and others guidelines



How to increase animal welfare during CO_2 exposure

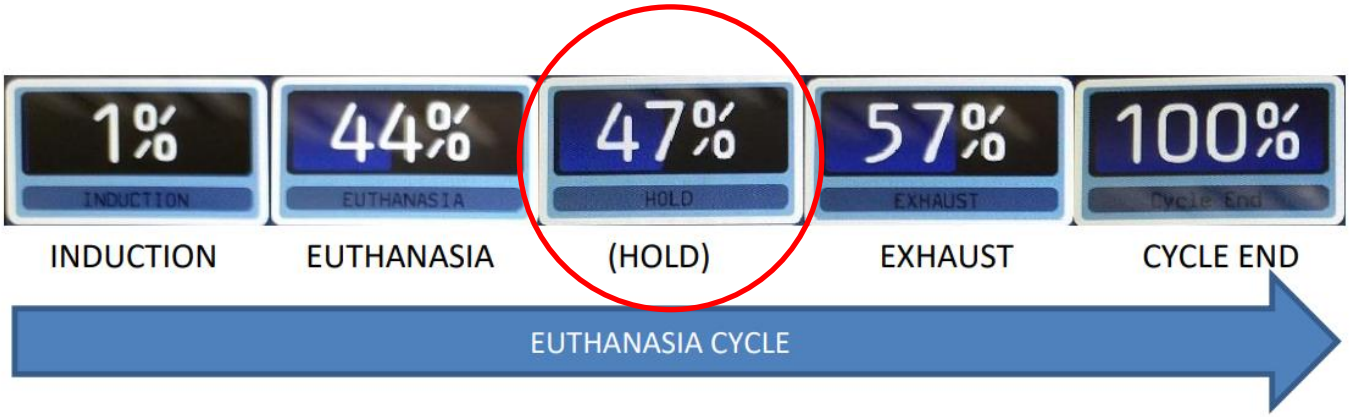
- Each program should include these specific steps :





How to increase animal welfare during CO_2 exposure

- Whenever gradual displacement methods are used, CO_2 flow should be maintained for at least 1 minute after respiratory arrest



- Our system allows the admin to increase or decrease the duration of each phase

How to increase animal welfare during CO_2 exposure

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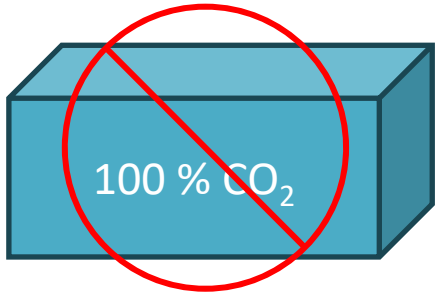
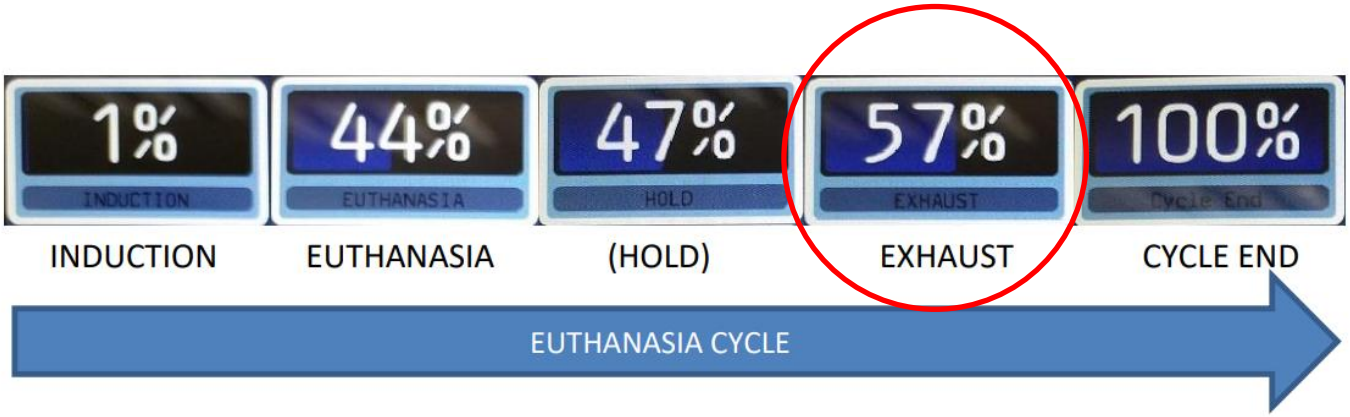
- What about prefilled chambers?

Prefilled chambers are not recommended due to the potential for significant pain upon inhalation of the gas



Our system is equipped with an exhaust phase allowing CO_2 to be flushed out of the cage (if the latter is to be reused)



➢ If euthanasia is not conducted in the home cage, induction chambers should be emptied and cleaned between uses

How to increase animal welfare during CO_2 exposure

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➢ More advices

- The practice of immersion, where conscious rodents are placed directly into a container prefilled with 100% CO_2, is unacceptable
  - A 2-step process, where animals are first rendered unconscious and then immersed, is preferred when gradual displacement methods cannot be used

- Consideration should be given to the benefits of using a darkened home cage, while also keeping in mind the need to have the animal under observation

- If animals need to be combined, they should be of the same species and, if needed, restrained so that they will not hurt themselves or others

- Addition of O_2 to CO_2 will prolong the time to death and may complicate determination of consciousness
  - There appears to be no advantage to combining O_2 with CO_2 for euthanasia

How to increase animal welfare during CO_2 exposure

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- More advices

Your euthanasia equipment has to be equipped with a CO_2 flow alarm

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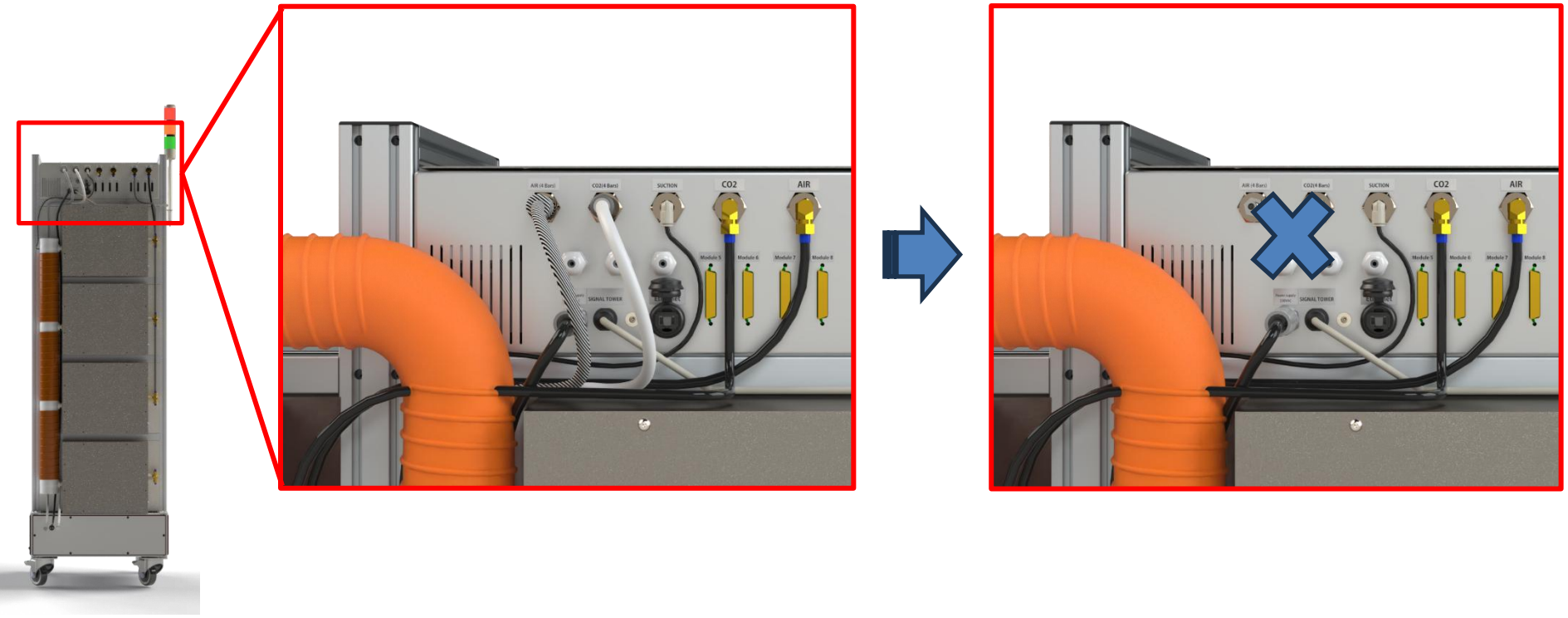
CO_2 room level alarm



How to increase animal welfare during CO_2 exposure

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- More advices

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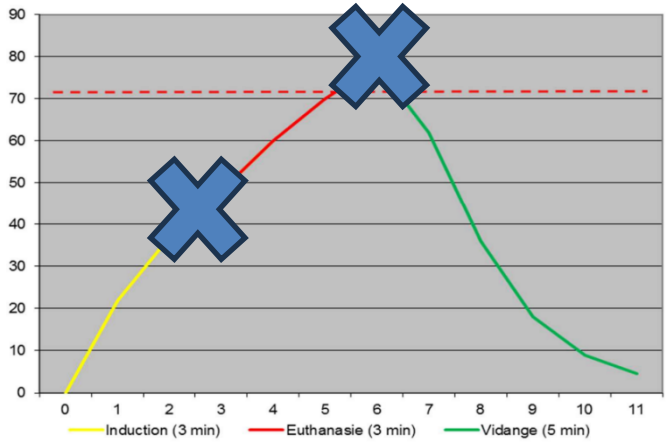

How to increase animal welfare during CO_2 exposure

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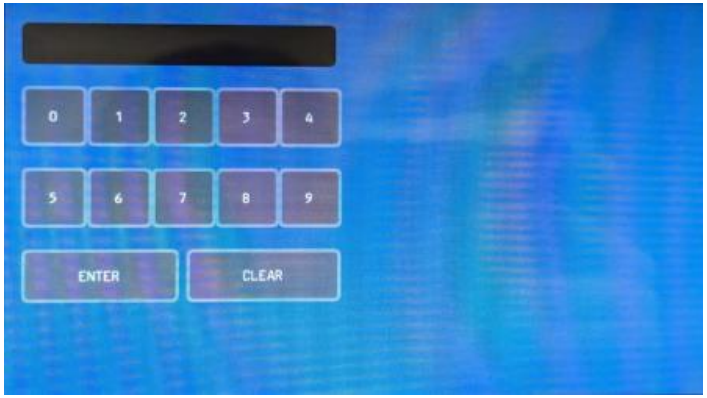
- What about user safety?

1

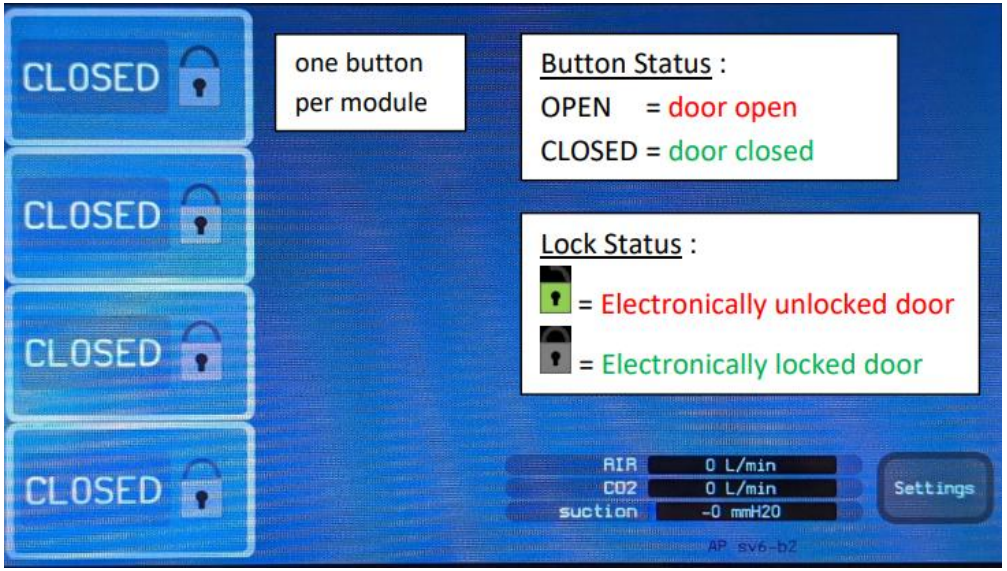


Password-equipped system for admin (can change cycle parameters) and users (can only use the system as set by the admin)

2

Each module will be locked automatically when closed to allow for the cycle launch

Modules can't be opened by user during cycle (a safety feature allows for emergency opening)



How to increase animal welfare during CO_2 exposure

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- What about user safety?

3

System equipped with a signal tower showing the cycle status:

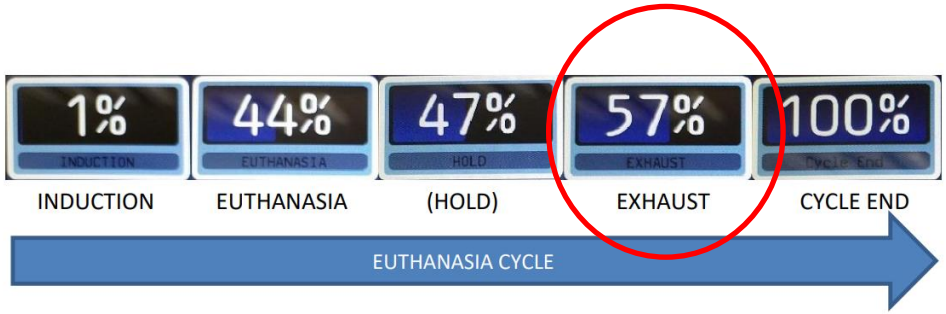
Green: system ready to be used

Orange: system in use, CO_2 is flowing to the cages

Red: system is broken and needs assistance

Signal tower can be seen from distance

4



System equipped with an exhaust cycle to filter CO_2

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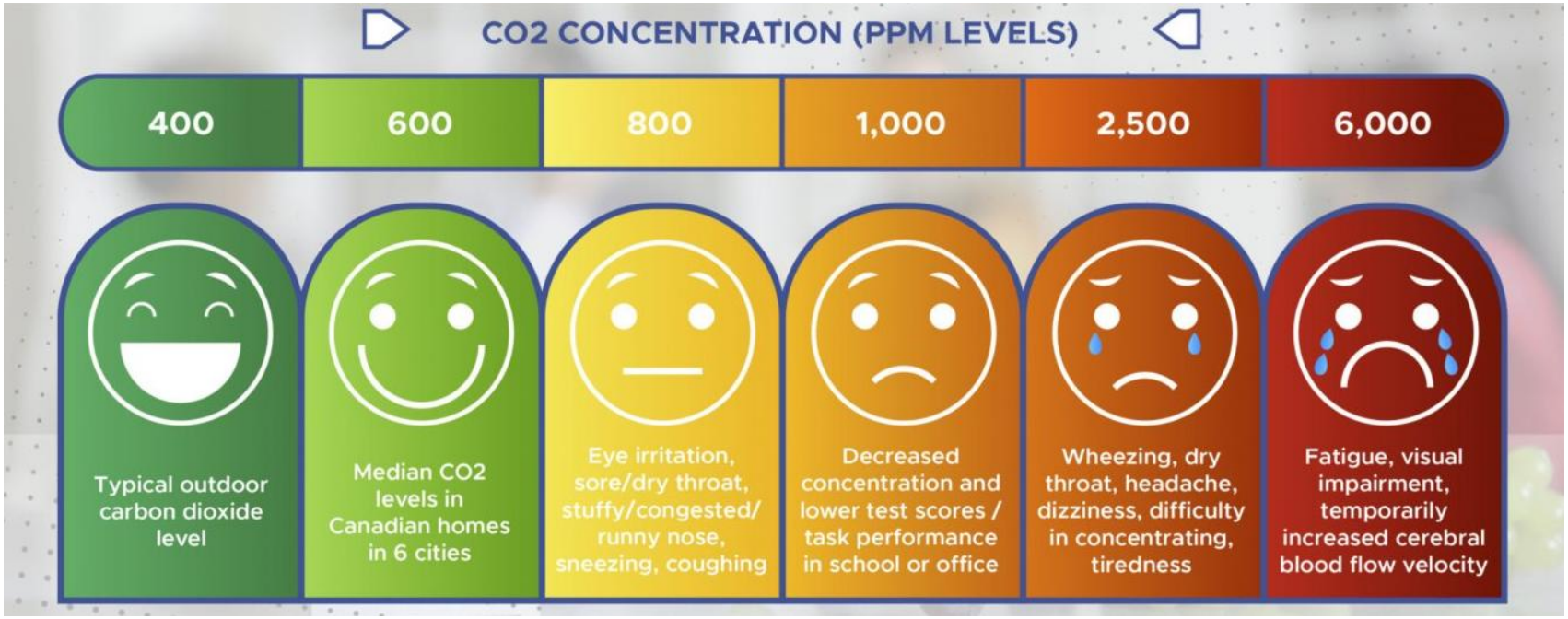
4

System equipped with an exhaust cycle to filter CO_2



5000 ppm (0.5%) is limit value of exposure (VLEP 8h)

How to increase animal welfare during CO_2 exposure

A CO_2 room alarm is required in the experimental area

Has to be set with a minimum value of 5000 ppm (0,5%) with sound alarm

Has to be set with a maximum value of 30.000 ppm (3%) with sound and light alarm

- If the sensor has to be installed in a place without ventilation, where CO_2 bottles are kept:

Alarm installed at a height of 30cm above the floor and very close to the potential leak source

How to increase animal welfare during CO_2 exposure

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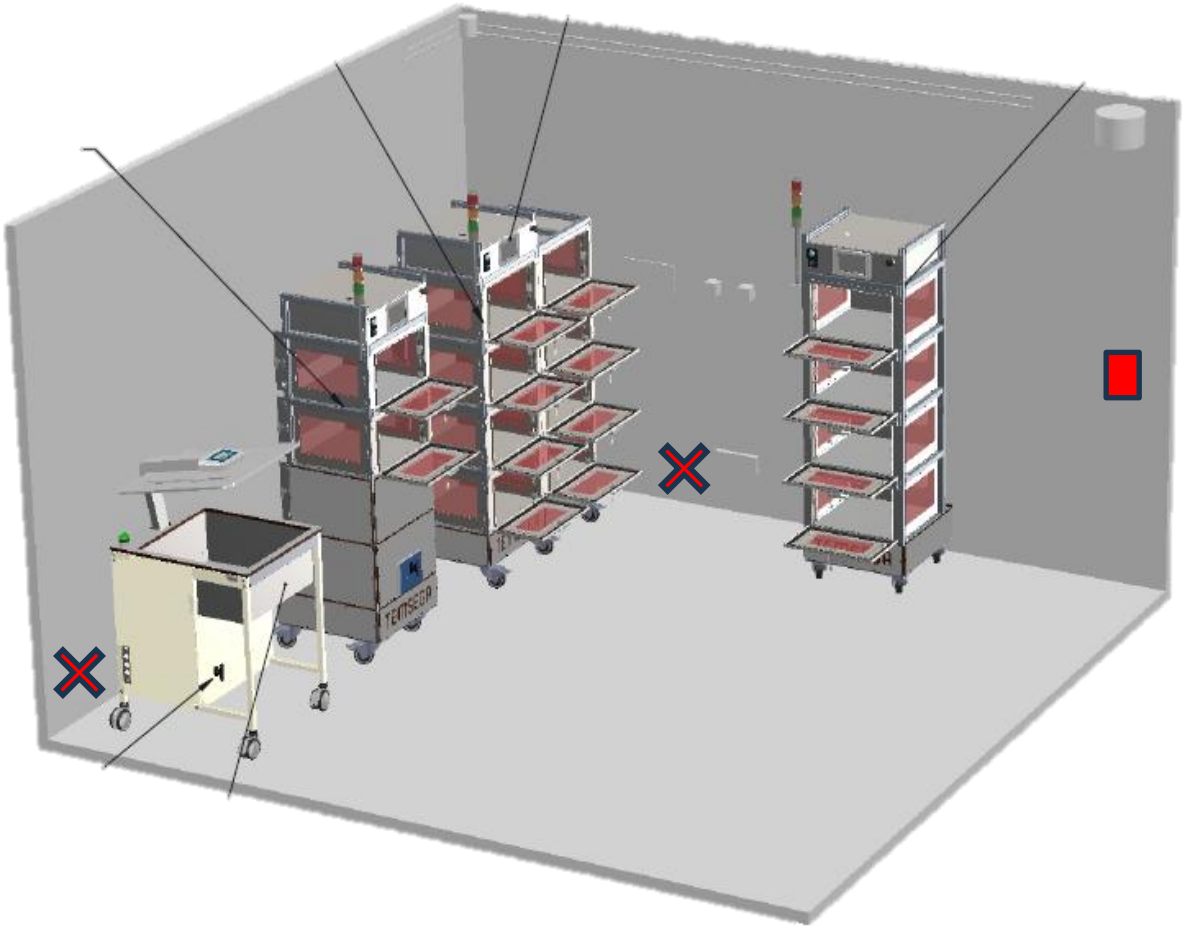
- CO_2 is 1.5x heavier than air
- Alarm has to be installed away from air vents, 1.5m above the floor and 1m from any system



How to increase animal welfare during CO_2 exposure

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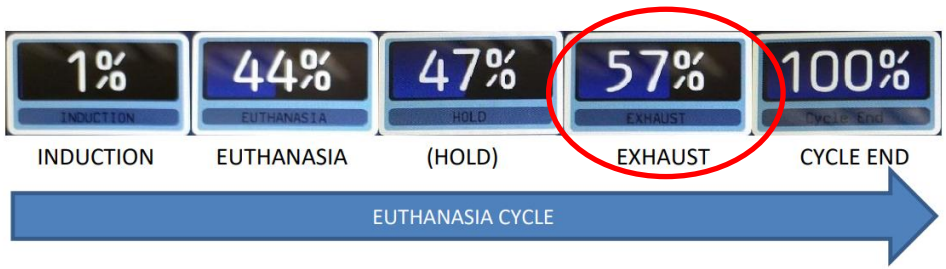
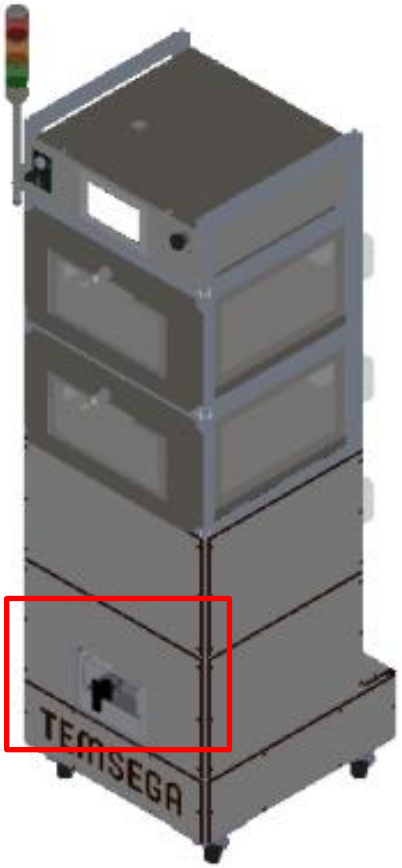
one touch device

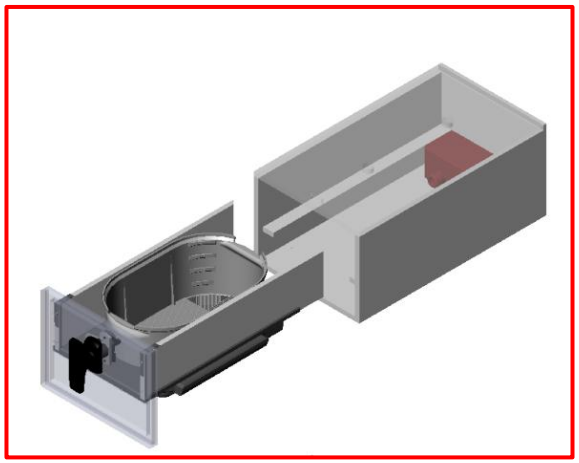
4



System equipped with an exhaust cycle to filter CO_2

Can be equipped with soda lime filter





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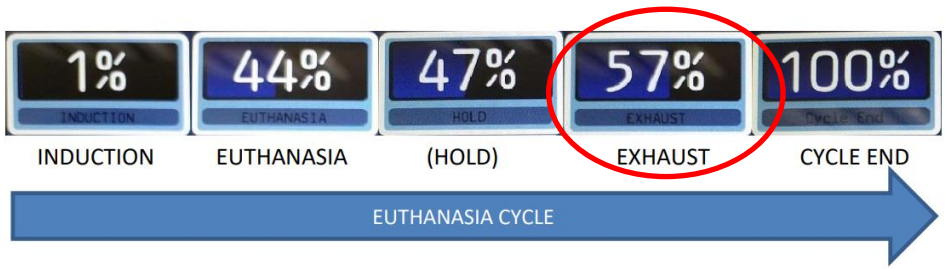
4



System equipped with an exhaust cycle to filter CO_2

Can be connected to building exhaust (active or passive)

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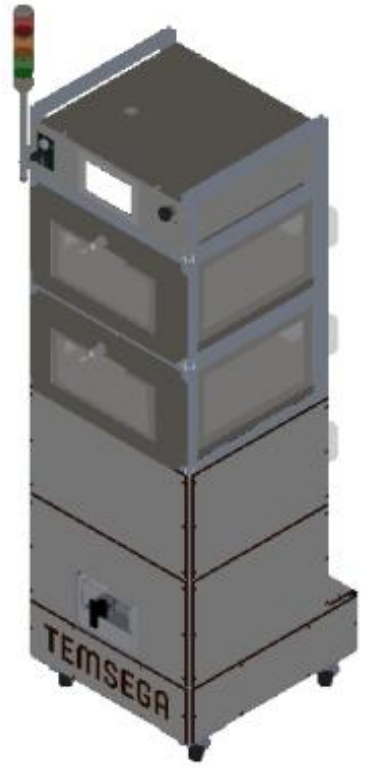
- Evolution capability



2 modules

4 modules

6 modules

How to increase animal welfare during CO_2 exposure

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- Euthanasia system compatible with several cage model from different manufacturers
- A specific adaptation can be made on request