UNDERSTANDING INDIVIDUAL VARIATION IN RAT RESPONSES TO CARBON DIOXIDE

by

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Abstract

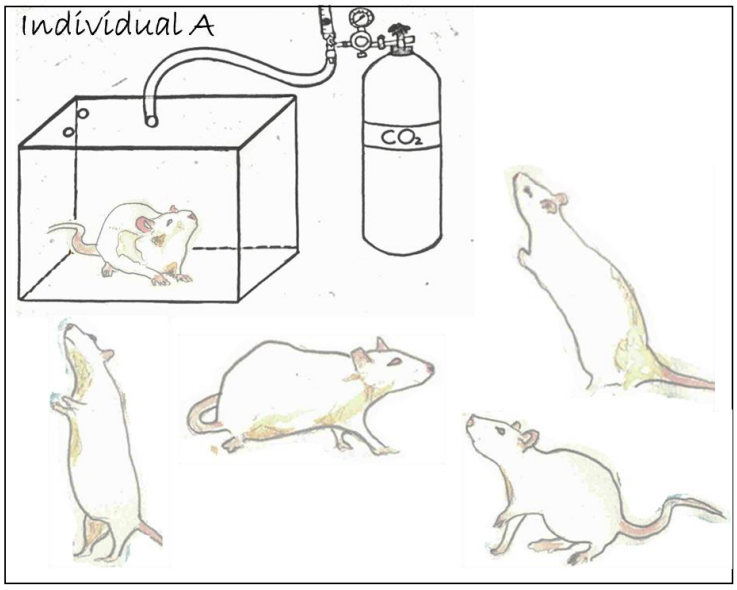
Carbon dioxide (CO_2) is commonly used to kill laboratory rats, but the humanness of this method remains controversial. Cumulative evidence indicates that CO_2 elicits negative emotions in rats. However studies using inescapable exposure (forced exposure) have shown contrasting results. Understanding individual differences could allow for stronger inferences regarding rat experiences when exposed to CO_2. The main aim of this thesis was to determine if CO_2 sensitivity is variable between rats. In Chapter 2, I described rat active and passive behaviours during CO_2 forced exposure and assessed consistency of individual differences in rat response to CO_2. Results from Chapter 2 confirmed that rats do not express passive behaviours when exposed to gradually increasing concentrations of CO_2, showed that the individual rat is an important source of variation in the behavioural responses to CO_2, but this variation was not related to individual differences in coping strategies. In Chapter 3, I investigated consistency and stability of rat individual thresholds of aversion to CO_2 across repeated exposures, and I assessed whether other situational-dependent personality traits could account for the variation in response to CO_2. My results suggest that individual differences in rat thresholds of aversion are not related to other personality traits but to sensitivity to CO_2. In Chapter 4, I assessed the effects of an anxiolytic on the individual thresholds of aversion to CO_2. I found that rats experience anxiety when exposed to lower CO_2 concentrations and variation in rat CO_2 sensitivity is driven by individual differences in the onset of these feelings. Collectively these studies suggest that the emotional experience of rats exposed to CO_2 varies among individuals, likely due to differences in the onset of CO_2-induced anxiety. In these studies using aversion tests, all rats avoided CO_2 before losing consciousness, even less sensitive rats when treated with an anxiolytic. Indicating

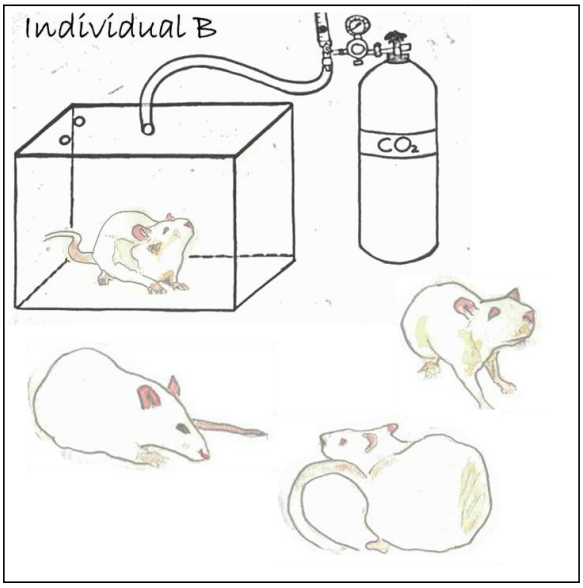
that CO_2 concentrations required to render rats unconscious elicit negative affective states. Further research is necessary to determine what type of emotions, in addition to feelings of anxiety, are experienced by rats at higher concentrations (e.g. intense air hunger or panic), and whether these experiences also vary between individuals.

Lay Summary

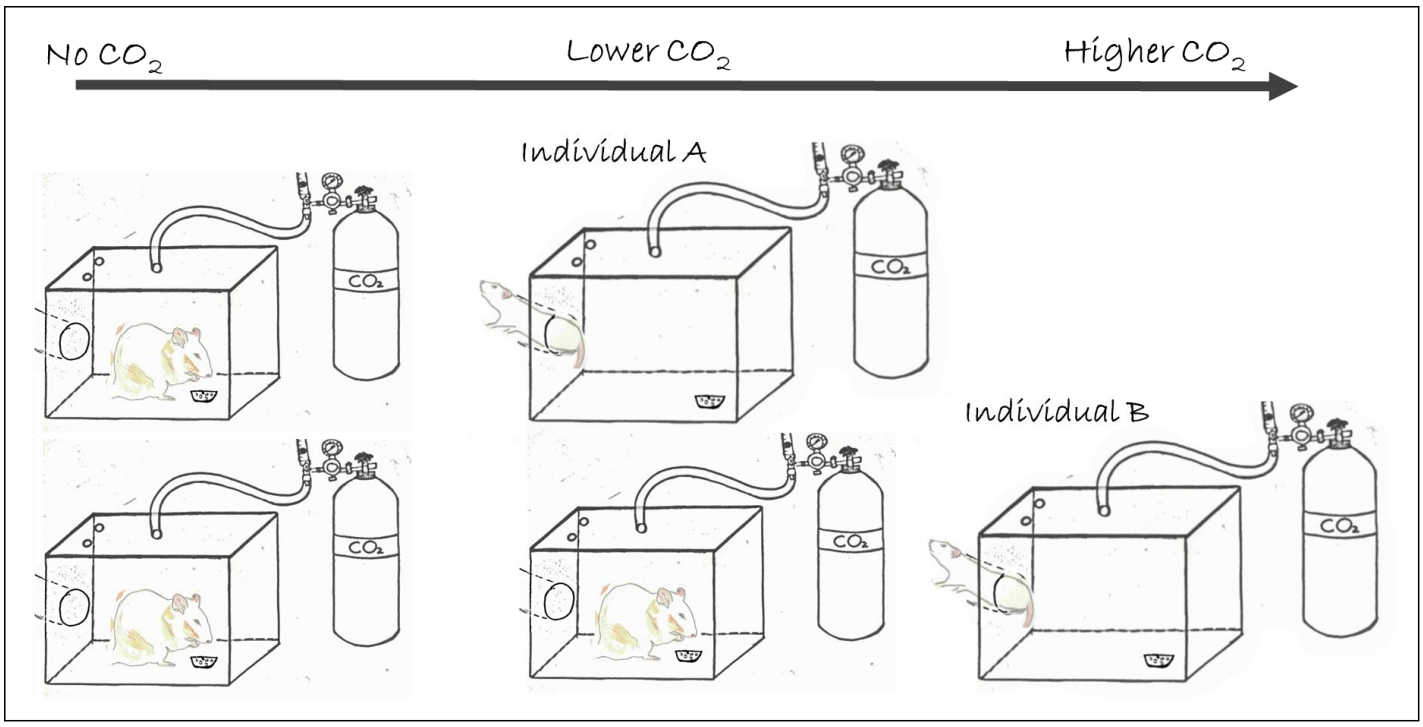
The majority of rats used for science are likely killed with carbon dioxide (CO_2), but the use of this agent remains controversial. Research has shown that rats experience negative emotions when killed with CO_2. However, several studies report great variability in behavioural responses to CO_2, making inferences regarding rat emotional states difficult. In my PhD thesis I found that the variation in behavioural responses to CO_2 is related to individual differences in CO_2 sensitivity. That is, some rats consistently find inhalation of CO_2 more aversive than others. Moreover, my results showed that rats experience anxiety when inhaling low CO_2 concentrations, and the onset of these feelings varies among individuals. I found that CO_2 induces negative emotions in rats and, although the emotional experience varies among individuals, CO_2 compromises the welfare even of least sensitive rats.

214,000 lab rats were used in Canada last year
Over 50% of them were likely killed with CO_2

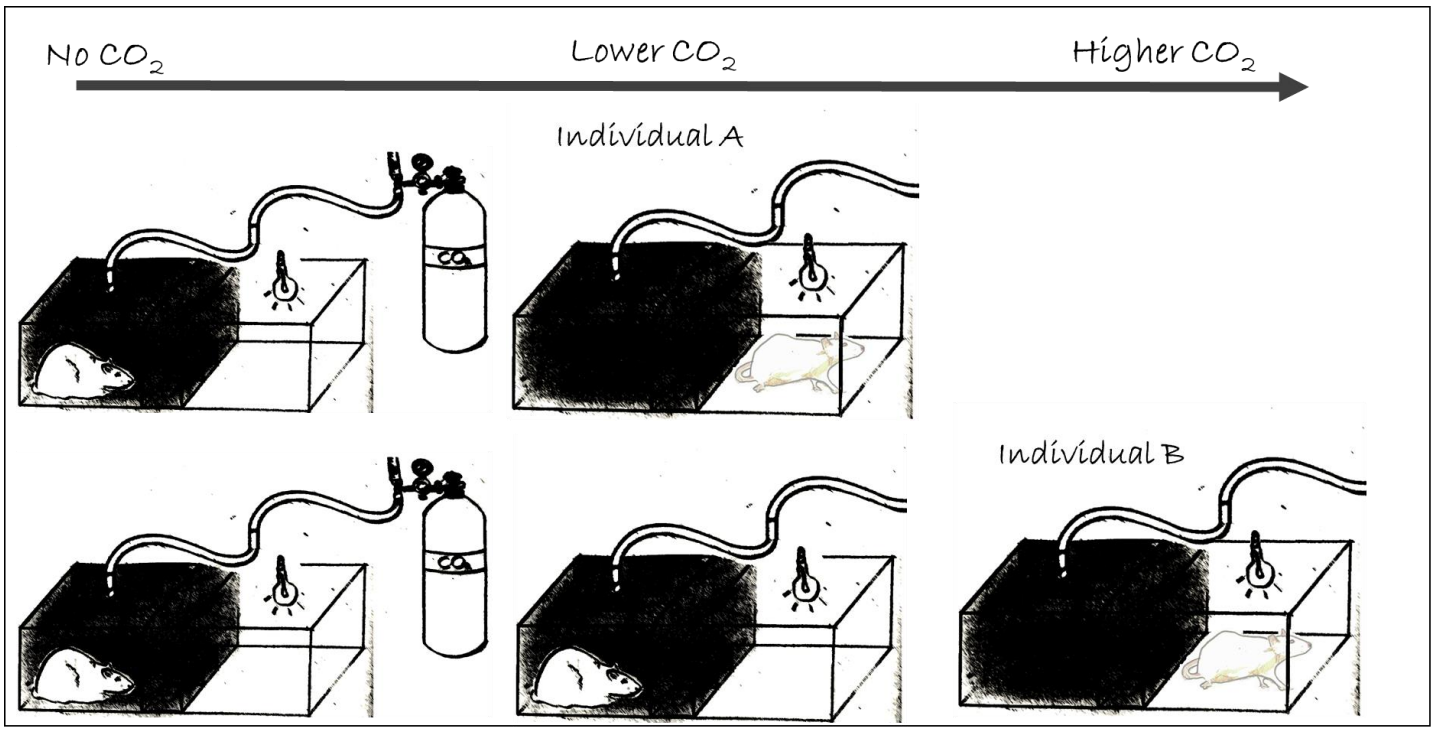




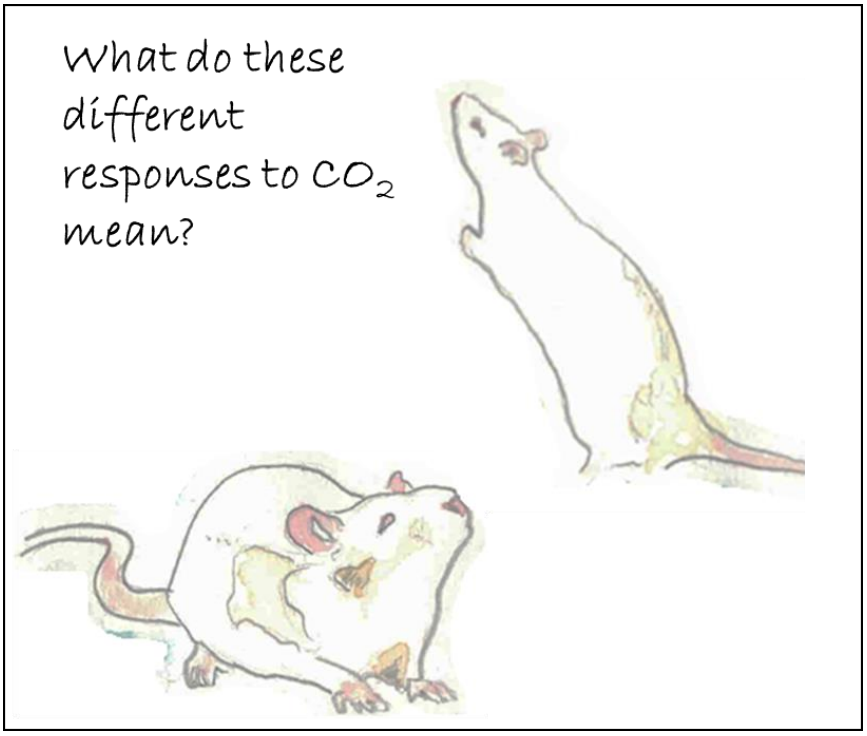
When rats are killed with CO_2 not all individuals show the same behavioural responses.
Differences between individuals are maintained every time that rats encounter CO_2

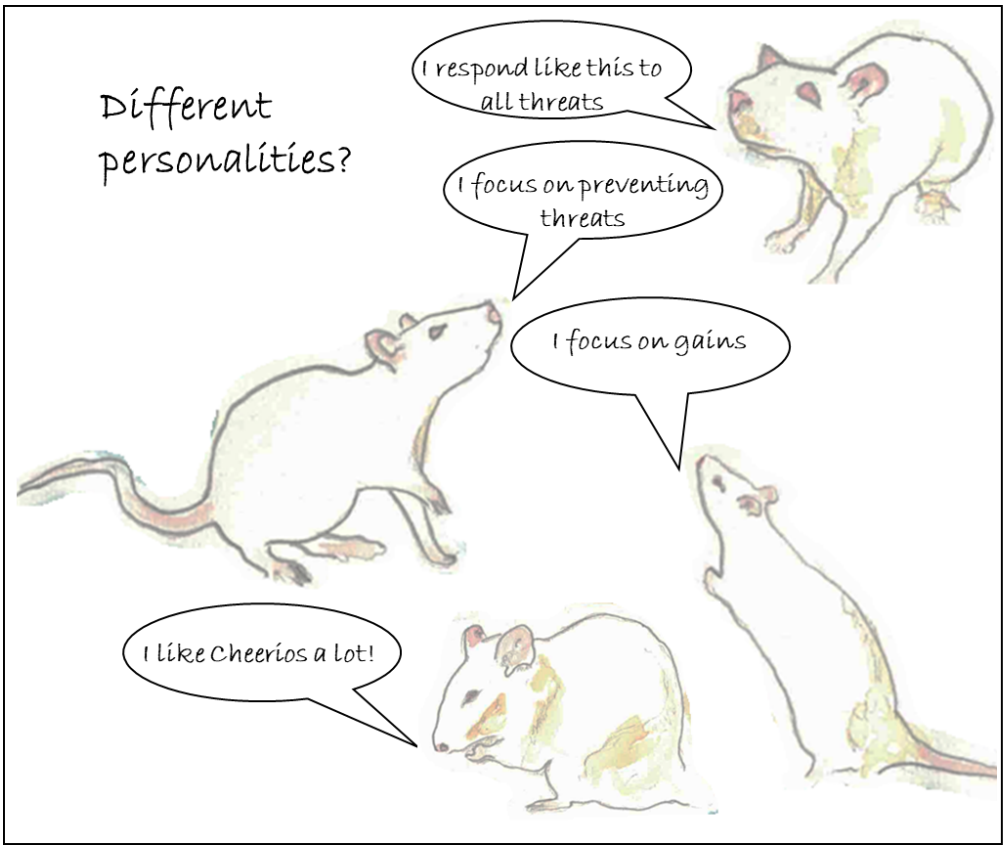


Some rats consistently tolerate more CO_2 than others, to gain sweet Cheerios…

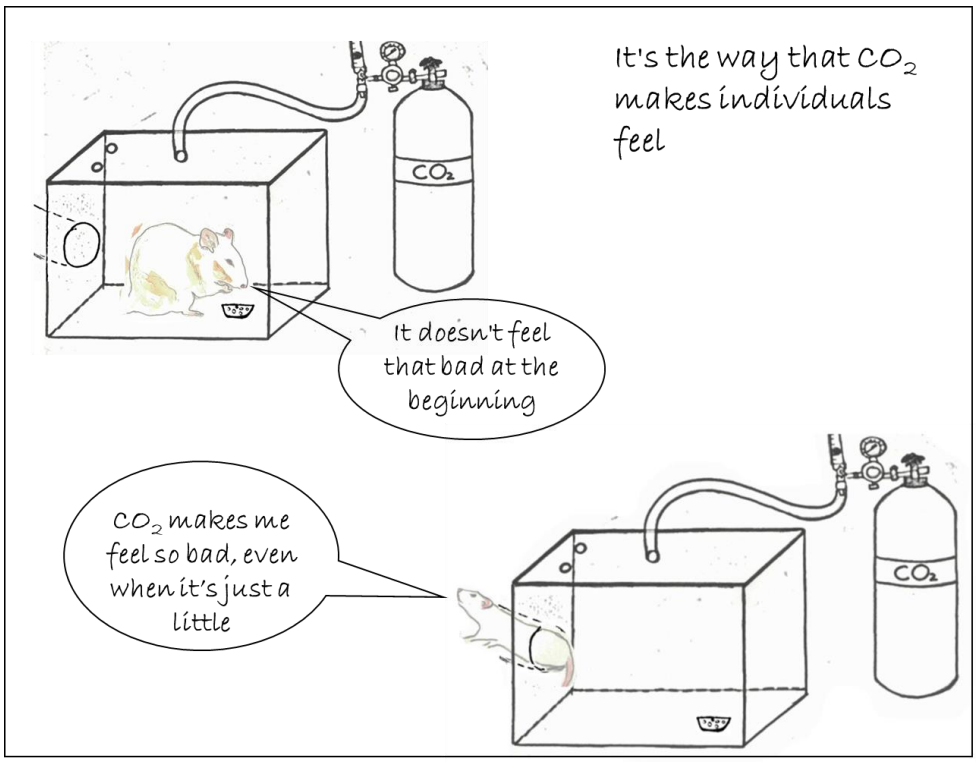


Some rats consistently tolerate more CO_2 than others, to avoid a bright light

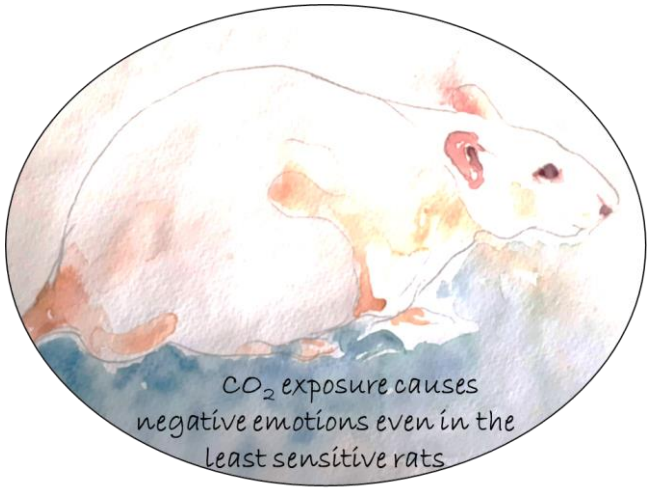




Rats have different personalities, but…
That is not why rats behave differently when exposed to CO_2



At lower CO_2 concentrations rats feel anxiety…
More sensitive individuals experience an early onset of these feelings



Killing rats with CO_2 is incompatible with the definition of euthanasia

Preface

A version of Chapter 1 has been submitted for publication. Améndola, L. and Weary, D.M. Understanding rat responses to CO_2. Améndola developed the main ideas and wrote the manuscript, Weary supervised, reviewed and edited the manuscript. This chapter did not require ethics approval.

A version of Chapter 2 has been published. Améndola, L. and Weary. 2019 Evidence for consistent individual differences in rat sensitivity to carbon dioxide. *PLoS ONE,* e.0215808. Améndola and Weary conceptualized the project. Améndola designed and performed the experiments, collected and analyzed data, and wrote the original draft. Weary helped design the study, and supervised, provided input, reviewed and edited the manuscript. This project received UBC Animal Care Committee approval (protocol A15-0071).

A version of Chapter 3 is in preparation for publication. Améndola, L. Ratuski, A., and Weary, D.M. Individual differences in rat sensitivity to CO_2. Améndola and Weary conceptualized the project. Améndola designed and performed the experiments, collected and analyzed data, and wrote the original draft. Ratuski helped designing and performing experiments and provided input on the manuscript. Weary helped design the study, and supervised, provided input, reviewed and edited the manuscript. This project received UBC Animal Care Committee approval (protocol A15-0071).

A version of Chapter 4 has been published. Améndola, L., Ratuski and Weary. 2019. Variation in the onset of CO_2-induced anxiety. *Scientific reports,* 9, 19007. Améndola and Weary conceptualized the project. Améndola designed and performed the experiments, collected and analyzed data, and wrote the original draft. Ratuski helped designing and performing experiments and provided input on the manuscript. Weary helped design the study, and

supervised, provided input, reviewed and edited the manuscript. This project received UBC Animal Care Committee approval (protocol A15-0071).

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
Ar	Argon
ASCIs	Acid sensing ion channels
bpm	Breaths per minute
BNST	Bed nucleus of the stria terminalis
BLA	Basolateral amygdala
CFR	Corticotropin releasing factor
CO_2	Carbon dioxide
DLPAG	Dorsolateral periaqueductal gray
DMH	Dorsomedial region of hypothalamus
DMPAG	Dorsomedial periaqueductal gray
EPM	Elevate plus maze
GABA	γ- aminobutyric acid
HPA	Hypothalamic-pituitary-adrenocortical
LC	Locus coeruleus
LORR	Loss of righting reflex
NA	Noradrenaline
N_2	Nitrogen
O_2	Oxygen
OF	Open field
P_{CO_2}	Carbon dioxide partial pressure

Po_2	Oxygen partial pressure
PAG	Periaqueductal gray
PSL	Panic symptom list
PVN	Paraventricular nucleus
SNS	Sympathetic nervous system
SRI	Serotonin reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
STAI	State trait inventory
VASA	Visual analogous scale for affect
VAS	Visual analogous scale
VLPAG	Ventrolateral periaqueductal gray

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Dedication

To all lab rats. A day may come when euthanasia in the lab is not your only fate.

Chapter 1: Introduction

Approximately 214,000 rats were used in research in Canada during 2017 (CCAC, 2018). Most rats used for scientific purposes are killed with carbon dioxide (CO_2) when the study is finished or when humane end-points are reached. In a recent international meeting on laboratory animal euthanasia, 80% of the participants reported using CO_2 to kill rats (Hawkins et al., 2016). Although CO_2 killing for rodents is controversial (e.g. National Research Council, 2011), the practice likely persists because it is inexpensive, requires little human effort, and poses little risk to the people who perform it (Leary et al., 2013).

The humaneness of CO_2 as a killing method for rats is typically assessed through behavioural, physiological and neurological signs of negative experiences. In the first sections of this Chapter, I review the research available on rat experiences when exposed to CO_2. In addition, I briefly review research on human experiences when inhaling CO_2. In the final sections of this Chapter, I present possible explanations for individual variability and argue that the study of individual differences in rat responses to CO_2 can help explain some of the inconsistencies within and between studies found in the literature.

1.1 Rat welfare during killing

1.1.1 Guides and regulations on CO_2 as a killing method for rats

The interest in ensuring humane treatment of research animals is reflected in guidelines, regulations, and laws that define euthanasia as gentle or humane killing of animals (e.g. Leary et al., 2013). For a method to be considered humane, it is generally agreed that pain, distress, fear, and anxiety should be absent or minimal. Additionally, preferred methods cause a rapid loss of

consciousness, have high reliability, are non-reversible, are compatible with research objectives, and safe for the people performing the procedure (CCAC, 2010; European Union, 2010; Leary et al., 2013; National Research Council, 2011; USDA, 2017).

Most countries recommend or accept CO_2 as a killing method for rats. The Canadian Council on Animal Care (CCAC) sets standards for animal care in Canada and has published guidelines on euthanasia of animals used in science. In this document, the use of CO_2 to kill rats as a sole method is listed as conditionally acceptable, this is to say, it is only to be used if other methods are impractical or inadequate for the research purposes (CCAC, 2010). In the United States of America, the care of animals used in science is regulated by the Animal Welfare Act (AWA), the federal law that establishes minimum requirements (USDA, 2017), and the Public Health Service Policy (PHS Policy), through the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The PHS Policy follows the recommendations of the American Veterinary Medical Association (AVMA) panel on euthanasia; CO_2 for killing rats is acceptable at a flow of 10% to 30% CO_2 of chamber vol. min^{-1} (Leary et al., 2013). The European Union and its member states adhere to the Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes, to establish at a national level, the minimum welfare standards for animals used in science; this document describes CO_2 gradual fill as an "appropriate method" for killing rats (European Union, 2010).

The ability of rats to feel emotions is presumed possible by most of the guidelines, regulations and laws. In the following section, I will present definitions of emotions available in the literature that will be useful to assess these experiences during CO_2 killing.

1.1.2 Negative affective states as welfare concern during rat killing

Animal welfare can be viewed as encompassing three aspects: the animals' biological functioning, ability to perform natural behaviours, and affective states (Fraser et al., 1997). The latter, considered to reflect the overall welfare status of an animal (Mellor, 2016), is most relevant in the assessment of euthanasia methods. Pain, distress, and anxiety are considered high-arousal, negatively valenced affective states (following Mendl et al., 2010).

Attempts to define emotions, feelings and affect have been subjected to much debate (Kagan, 2009). As stated by Fehr and Russell (1984), "Everyone knows what an emotion is, until asked to give a definition. Then, it seems, no one knows". Here I provide a functional approach that identifies emotional responses as objectively observable, and feelings as the conscious awareness of emotions (Adolphs, 2010, 2013, 2017; Damasio, 2004; Panksepp, 2008). Emotions are 'central states' that link situational-dependent stimuli with sets of behavioural, physiological and brain responses (Adolphs, 2013). Not all stimuli elicit an emotional response; the competence of the stimuli will depend on the individual's evolutionary history (innate response), personal experience (developmental plasticity and learning), discriminative properties of the stimuli (intensity and type of stimuli), and the current situation (e.g. controllability). Induction regions in the brain are responsible for the emotional cascade (chemical and neural reaction) that leads to the execution of appropriate behavioural, physiological and brain responses (Damasio, 2004).

Feelings of emotions are the conscious awareness of affective states experienced as positive or negative (e.g. Adolphs, 2010; Damasio, 2004; Panksepp, 2008). These can be described using an arousal axis (high or low) and a valence (positive or negative) axis (Mendl et al., 2010; Russell, 2003), and comprise different patterns of neural, behavioural and

physiological responses (for a review, see Adolphs, 2013). In the scientific community, there is little consensus regarding what constitutes the feelings of emotions, and how and where these feelings are evoked in the brain. For example, some argue that this requires an internal self-representation of the body changes (i.e. interoception; homeostatic state, state of preparedness to cope, and motivational state) that accompany emotions, a process thought to occur in cortical areas of the brain (e.g. insula and cingulate cortex). Within this view, species that possess interoception could feel emotions (Adolphs, 2010, 2013; Craig, 2008; Damasio, 2004). Other authors emphasize the role of neocortical working memory (i.e. temporary hold and manipulation of information while doing mental work) as a requirement for feeling emotions, an idea that may exclude many non-human animals (LeDoux, 2016). Panksepp (2004, 2008) argued that basic neurobiological subcortical areas present within all mammals are responsible for both emotion and feelings.

Whether animals are experiencing feelings of emotions remains a major question within the literature (Panksepp, 2008), but felt emotions are at the core of our concerns about animal welfare (Mellor, 2016; Weary et al., 2017). Stronger inferences about felt experiences can be made through a combination of evidence from 1) central state emotions (i.e. regional and local brain arousal and behavioural and physiological changes when a competent situation-dependent stimuli is present; Adolphs, 2013; Damasio, 2004; discussed by Mellor, 2016), and 2) indications of awareness (i.e. behavioural plasticity, direction and maintenance of attention, and agency; Weary et al., 2017), 3) functional homology in which human felt experiences and their associated physiological, neural and behavioural responses can be compared to that of animal responses to the same stimuli and used as a proxy to the animal's felt emotions (Panksepp, 2008;

e.g. Beausoleil and Mellor, 2015), and 4) drug treatments that target specific feelings of emotions in humans to infer specific feelings in animals (discussed by Weary et al., 2017).

It is non-trivial to identify negative affective states during euthanasia. Feelings of pain emerge from receptors responding to tissue damage or the perception of such damage (VandenBos, 2015). Feelings of fear and anxiety serve as a signal of danger and are dependent upon threat proximity and context (see Adolphs, 2013). Fear is considered a short-lived negative state (VandenBos, 2015) related to present or imminent danger (Fanselow and Lester, 1988; McNaughton and Gray 2000) that gives rise to appropriately oriented responses to avoid the threat. Anxiety is related to uncertainty and lack of control, as an anticipation or assessment of future danger (Barlow, 2002; LeDoux and Pine, 2016; VandenBos, 2015), or distant threat (Fanselow and Lester, 1988; McNaughton and Gray 2000). Some authors support the idea that different neural structures are responsible for emotional states of fear and anxiety (e.g. Davis et al., 2010), while others state that given the close proximity and interconnectivity between structures, dissociation is challenging (Adolphs, 2013). Panic (attack) can be defined as feelings of intense fear or terror (APA, 2013; see Panksepp, 1998 for a disctintion between the fear and panic systems in relation to separation distress) and has been stated to be a response to more proximal threat imminence (a continuum of threat imminence, with anxiety at the distal end and panic at the proximal end, with fear in the middle; Fanselow and Lester, 1988; McNaughton and Gray 2000). The negative emotional experience related to respiration is known as breathlessness (defined as dyspnea in humans; for a discussion, see Beausoleil and Mellor, 2015). Breathlessness comprises the sensory experience, feelings of unpleasantness and the emotions evoked from experiencing the latter (see Beausoleil and Mellor, 2015), this experience depending on intensity, can evoke for example mild fear to terror (Mongeluzi et al., 2003). Air

hunger is consider a qualitative distinctive sensation of breathlessness, that humans describe as "not getting enough air", "shortness of breath", "fighting for air" or "suffocation", among others (see Beausoleil and Mellor, 2015; Banzett et al., 1996).

1.2 Biological responses to carbon dioxide

1.2.1 Mechanisms of causing death by CO_2

When rats are killed with CO_2, the time required to achieve unconsciousness, often measured as recumbency or as the loss of righting reflex (LORR), varies depending on the concentration (for prefill methods) and flow rate (for gradual fill methods). For example, at a flow rate of ~17% CO_2 chamber vol. min-1 rats became recumbent after 106 s corresponding to approximately 33% CO_2 (Niel and Weary, 2006). At a flow rate of 20% CO_2 chamber vol. min-1, rats became unconscious after 115 ± 31 s measured as recumbency, or after 136 ± 53 s when measured as LORR (Chisholm and Pang, 2016).

To sustain life, rats rely on: 1) the inspiration of air from the environment, usually containing 20.95% oxygen (O_2), 78.08% nitrogen (N_2), <1% Argon (Ar), and <1% CO_2, among other gases at lower concentrations, 2) gas exchange on the alveoli (i.e. diffusion of O_2 across the alveoli into pulmonary capillaries and diffusion in the opposite direction of CO_2 by-product of cellular respiration) and 3) the exhalation of metabolic waste CO_2 (Prange, 1996).

Gas diffusion across the alveoli varies with the partial pressures of O_2 (P_{O_2}) and CO_2 (P_{CO_2}). When using CO_2 to kill rats, room/atmospheric air is gradually displaced by CO_2 within the chamber, and rats inhale high concentrations of CO_2 causing the P_{CO_2} in the alveoli to increase. This impedes diffusion, and consequently metabolic waste CO_2 cannot be properly removed from the organism. High arterial P_{CO_2} disturbs pH homeostasis. Most of the metabolic

waste CO_2 is processed by the bicarbonate buffering system that maintains physiological pH balance: CO_2 reacts with water, a reaction catalyzed by the enzyme carbonic anhydrase, to form carbonic acid (H_2CO_3), which dissociates into ion bicarbonate (HCO_3^-) and hydrogen ions (H^+; $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H+ + HCO_3^-$). The pH homeostatic balance is maintained by a 20:1 ratio of HCO_3^-:H_2CO_3. Therefore when P_{CO_2} increases (hypercapnia) this results in an increase in H_2CO_3 which forces more proton production and decreases blood pH (Prange, 1996).

Hypercapnia and acidification of the blood results in other serious disturbances. It reduces hemoglobin affinity for O_2 due to conformational changes of its globin portions (Bohr shift; Prange, 1996; Rigs, 1988). Additionally, CO_2 diffuses through the blood-brain barrier, causing dilatation of the cerebral retinal vessels, and increasing the volume of blood within the skull (Westlake, 1958). Other effects of hypercapnia are life threatening including a decrease of pH in the heart that reduces muscle contractibility that can led to arrhythmias and heart arrest (Fenn and Cobb, 1934; Westlake, 1958). Acidification also has detrimental effects on cell structural and functional protein synthesis and degradation (Langenbuch, 2006).

CO_2 is an effective killing method; cellular functions that sustain the life can prevail only under small variations of pH (Guyenet and Bayliss, 2015). Since acid-base and PCO_2 balance are highly relevant for survival, animals possess complex mechanisms capable of detecting changes in pH and CO_2 and respond to these threats to homeostasis.

1.2.2 Ventilatory response to hypercapnia

In mammals, one of the primary homeostatic mechanisms for the removal of excessive CO_2 is ventilation (Griez et al., 1987; Guyenet and Bayliss, 2015; Prange, 1996). Changes in ventilation occur through modulation of breathing depth (tidal volume) and frequency, whose

product gives a measure referred to as minute ventilation (Straus et al., 1998). Exposure of rats to CO_2 increases breathing frequency, tidal volume, and consequently, minute ventilation. For example, when exposed to the 20% CO_2 challenge (rapidly increasing concentration stabilizing at 20% CO_2 after 5 min), rats showed increased breathing frequency from 100-130 breaths per minute (bpm) within the first min, to 130-165 bpm after 2 min of exposure (Hickman et al., 2016).

A rise in arterial P_{CO_2} and decrease in pH (measured as H^+) are detected by peripheral and central chemoreceptor cells (e.g. acid sensing ion channels; ASCIs). A synergic output from the integration of peripheral and central chemoreceptor inputs (Blain et al., 2010), adjusts the ventilatory response according to the blood gas stimuli (Prange, 1996). Peripheral chemoreceptors are located in the aortic and carotid bodies (Straus et al., 1998); the carotid is more sensitive and functionally important to changes in pH and P_{CO_2} than the aortic bodies (for a review, see Kumar, 2009). Central chemoreceptors are located principally in ventral medulla (for reviews, see Guyenet and Bayliss, 2015; Nattie and Li, 2012).

By modulating ventilation, mammals can cope with slight increases in CO_2, but when CO_2 is used as a killing method, the control of ventilation is not sufficient to alleviate the excess of CO_2. When natural responses to maintain homeostasis are no longer effective, the animal may be experiencing negative emotions, for example air hunger may be intense (see Beausoleil and Mellor, 2015). Next I will present behavioural, physiological, and brain activation as evidence of negative emotional states elicited by hypercapnia in rodents, and where relevant refer to parallel research in humans.

1.2.3 Behavioural responses to CO_2

Defence behaviours (often called fear or anti-predator behaviours) are elicited from detected competent negative stimuli or potential threats (Dugatkin, 2009). The rat's defence behaviours include active (flight) and passive responses (freeze), as well as responses associated with fight (for a review, see Blanchard et al., 1990; de Boer and Koolhaas, 2003).

The expression of defence behaviours is plastic and sensitive to situational contingencies. If escape is available rats may flee, while rats may freeze if escape is restricted (e.g. Blanchard et al, 1976; McGregor et al., 2002; Vernet-Maury et al., 1992; Wallace and Rosen, 2000). When rats are provided the opportunity to explore a closed cage before exposure to a cat, rats froze more than rats exposed to the cat but without the opportunity to explore the closed cage first, suggesting that inescapability of the cage was perceived prior to exposure (Blanchard et al., 1976). Reponses also vary with threat proximity (Blanchard et al., 1986) and intensity of the stimuli. For example, rats avoid predator scent when provided the opportunity to escape (McGregor et al., 2002; Vernet-Maury et al., 1992) but rats freeze during inescapable exposure, this response varies in intensity depending on scent concentration (Wallace and Rosen, 2000).

Since defence behaviours are situation dependent, I provide the behavioural evidence in support of negative emotional states in three sections: 1) studies where animals cannot escape exposure to CO_2, 2) studies where animals can avoid exposure to CO_2 and 3) other situations where behaviour was used to infer negative emotional states during or after exposure to CO_2.

1.2.3.1 Inescapable exposure to CO_2

To assess emotions evoked by CO_2, one common approach is exposing rats in confined inescapable environments (forced exposure; e.g. Blackshaw et al., 1988; Britt, 1987; Niel and

Weary, 2006). Considering behavioural plasticity in defence behaviours, the working premises are that the frequency, duration and intensity of these responses reflect the intensity of the rat's negative emotional experience to the procedure. I describe below some examples of rat behavioural responses and discuss areas where there is a lack of agreement between studies.

Several studies that report baseline periods and detailed descriptions of the behaviours have found behavioural evidence of negative states in rats exposed to CO_2. Niel and colleagues (2006, 2008), using medium flow rates (~17% CO_2 chamber vol. min^{-1}), found that rats showed increased frequencies and intensities of several behaviours associated with distress. The onset of rearing and increased locomotion occurred at around 5% CO_2 and peaked at around 20% CO_2. Escape behaviours (i.e. pushing and scratching at the lid) were observed at between 20% and 28% CO_2. These results are consistent with those obtained by Makowska and Weary (2012), using a slightly higher flow rate (23% CO_2 chamber vol. min^{-1}). In contrast, Burkholder et al. (2010) found that locomotion and rearing did not increase relative to baseline levels when rats were exposed to lower concentrations (10 % CO_2 chamber vol. min^{-1}). Rats vocalized in the range of 6 to 103 kHz when exposed to CO_2 flow rates between 17 and 30% chamber vol. min^{-1} (Chisholm et al., 2013; Niel and Weary, 2006). Vocalizations within the range of 30 to 70 kHz are associated to emotional arousal not exclusive to negative states (Knutson et al., 2002). One other study reported that rats exposed to 10 % CO_2 chamber vol. min^{-1} did not vocalize (Burkholder et al., 2010).

Rats exposed to the 20% CO_2 challenge also show variable results. Some studies have reported increases in locomotion but not freezing (e.g. Hickman et al., 2016) and others have found the reverse (e.g. Johnson et al., 2005). These results suggest that the type of defence behaviour varies (between active and passive), but that some response is usually present.

Different delivery methods may account for some differences, but even when comparing similar methods results differ. When exposed to high concentrations of static CO_2 (CO_2> 97%), several studies have found that rats are less active and do not show struggling, vocalizations (e.g. Blackshaw et al. 1988), or other signs of distress (e.g. Smith and Harrap, 1997), but other studies have found that rats show signs of asphyxia and behavioural excitation (e.g. Coenen et al., 1995). In these studies, behaviours were recorded without baseline or acclimation periods (e.g. Blackshaw et al. 1988). Also, behaviours were not clearly defined, for example head rising was described as "inquisitive or agitated movements of head", vocalizations as "squealing and other noises", and escape as "attempts to get out of the box" (Smith and Harrap, 1997), or responses were simply mentioned without description (Coenen et al., 1995). Without control animals, baseline period, and a clear description of behaviours, interpretation of these results is challenging.

Strain differences in behaviour have been reported (for a review, see Cavigelli et al., 2013). For example, in response to predator scent, Sprague Dawley rats show low freezing levels (Farook et al., 2001) and less avoidance than Wistar rats (Staples and McGregor, 2006). Strain differences may also explain the lack of agreement between studies, although these differences have been seldom assessed in the euthanasia literature. Winter and colleagues (2017) showed that exposure to 10% static CO_2 elicits freezing behaviour in Long Evans and Wistar Kyoto strains, but not in Sprague Dawley and Wistar strains (Winter et al., 2017). Sprague Dawley rats often respond to CO_2 exposure by increased active defence behavioural responses (e.g. Britt, 1987; Chisholm et al., 2013; Hickman et al., 2016; Makowska and Weary, 2012; Niel and Weary, 2006), but the absence of responses has been also reported for this strain (Burkholder et al., 2010; Smith and Harrap, 1997). In contrast to Sprague Dawley rats, Lister Hooded rats

decrease activity during CO_2 exposure (Britt, 1987). Blackshaw et al. (1988) reported a decrease in activity by Wistar rats during exposure to CO_2, a result that differs from those of Niel et al. (2008 b). Fisher rats - a strain selected for lower activity - showed no behavioural signs of distress when exposed CO_2 gradual fill (Hackbarth et al., 2000). These results suggest that strain differences may limit comparability among studies.

Sex differences also have been often reported. For example, female rats show higher locomotion (Gray and Lalljee, 1974; Perrot-Sinal, 2004), rearing (Gray and Lalljee, 1974) and exploration of open areas (Domonkos et al., 2017; Johnston and File, 1991). However, it seems unlikely that sex differences can account for disagreements between studies since most CO_2 exposure studies have used male rats, and results are still inconsistent. Male rats responded with increased active defence in some studies using forced exposure to CO_2 (Coenen et al., 1995; Hickman et al., 2016; Johnson et al., 2012; Makowska and Weary, 2012; Niel et al., 2008 b; Niel and Weary, 2006), but other studies report no changes in behaviour (e.g. Burkholder et al., 2010; Hackbarth et al., 2000; Smith and Harrap, 1997). Female rats vocalized during CO_2 forced exposure in one study (Chisholm et al., 2013), but in a second study showed no increase in active defence behaviours in response to CO_2 (Blackshaw et al., 1998).

In summary, there is considerable variation in responses of rats to forced CO_2 exposure. Strain and sex differences may account for some of this lack of agreement, but contrasting results within the same sex and strain still exist. Differences in methodology, including the use of baselines, controls, and induction method (e.g. static versus gradual fill, variable concentrations and flows rates), and the lack of well-defined behavioural categories and interpretation of behaviours limit comparability between studies. However, considering both passive and active responses, and the behaviour's adaptive function during hypercapnia (i.e. avoidance of death),

there is some indication that when escape is prevented CO_2 elicits negative emotional states in rats. A summary of these results is presented in Table 1.1. The table specifies delivery methods, concentration or flow rate used, strain and sex, whether the study contained baseline and well defined behavioural categories, and a summary of results.

Table 1.1 Summary of forced exposure studies.

Strain	Delivery method	Concentration/ flow rate	Sex	Baseline	Defined behaviours	Results	Reference
W	Gf	~17%	M	✓	✓	↑ line crossing (locomotor activity), ↑ rearing, ↑ nose to lid, ↑ escape behaviours and ↑ ultrasonic vocalizations (mean range 22 ± 19 kHz)	Niel and Weary, 2006
W	Gf	17%	M	✓	✓	↑ line crossing (locomotor activity; 50% of the rats), ↑ rearing, ↑ nose to lid, ↑ escape behaviours (60% of the rats)	Niel et al., 2008
W	Pf	10%	×	✓	✓	↔ in freezing/immobility, ↔ rearing	Winter et al., 2017
W	Pf	> 97%	M/F	×	Vague	↓ wall touching, ↑ wall climbing (rearing), ↔ vocalizations *	Blackshaw et al., 1988
W	Pf	100%	M	×	×	↓ normal behaviour, ↑ behavioural agitation and excitation, ↓ immobility/freezing, ↑ signs of asphyxia	Coenen et al., 1995
SD	Gf	10%	M	✓	✓	↔ line crossing (locomotor activity), ↔ rearing, ↔ escape behaviours	Burkholder et al., 2010

Strain	Delivery method	Concentration/ flow rate	Sex	Baseline	Defined behaviours	Results	Reference
SD	Gf_a	20%	M	✓	Telemetric recordings	↑ locomotor activity, ↔ freezing/immobility	Hickman et al., 2016
SD	Gf_a	20%	M	✓	×	↑ freezing /immobility	Johnson et al., 2005
SD	Gf	23%	M	✓	✓	↑ line crossing, ↑ rearing	Makowska and Weary, 2012
SD	Gf	30%	F	✓	✓	↑ ultrasonic vocalizations (median range 51 kHz)	Chisholm et al., 2013
SD	Gf	High but undefined	M	✓	✓	↑ wall climbing (rearing), ↑ activity, ↑ shaking (undefined), ↔ ultrasonic vocalizations	Brit, 1987
SD	Pf	10%	×	✓	✓	↔ freezing/immobility and ↓ rearing	Winter et al., 2017
SD	Pf	~75%	M	×	Vague	↔ signs of distress, ↔ vocalization, ↔ escape behaviours, ↔ tail lashing	Smith and Harrap, 1997
LE	Gf	High but undefined	M	✓	✓	↔ wall climbing (rearing), ↓ activity, ↑ shaking (undefined), ↔ ultrasonic vocalizations	Brit, 1987

Strain	Delivery method	Concentration/ flow rate	Sex	Baseline	Defined behaviours	Results	Reference
LE	Pf	10%	×	✓	✓	↑ freezing/immobility and ↓ rearing	Winter et al., 2017
F	Gf	35%**	M	×	×	↑ interest and curiosity, ↔ vocalizations, ↔ signs of pain	Hackbarth et al., 2000
WK	Pf	10%	×	✓	✓	↑ freezing/immobility, ↔ rearing	Winter et al., 2017

Strain: W = Wistar, SD = Sprague Dawley, LE = Long Evans, WK = Wistar Kyoto, F = Fisher; Delivery method: Pf = pre-fill, Gl = gradual fill, GF_a = 20% CO_2 challenge; Concentration or flow rate: static concentration (%) or flow rate (% CO_2 chamber vol. min^{-1}); Sex: M = male, F = female, × = unspecified; Baseline: ✓ = present, × = absent; Defined behaviours: ✓ = if a clear ethogram, Vague = if an unclear ethogram, × = absent; Results: ↑ = increase, ↓ = decrease, ↔ no change in behaviour or absent.

*Within the same study, two different results were found depending upon age: for young rats no change in activity but increase in stationary episodes, while old rats decreased both activity and stationary episodes. These authors concluded arrived to the same conclusion for both young and old rats.

**Flow rate was given as 6 l/min^{-1} and cage size unspecified, calculations were made based on the brand and type of the cage (Makrolon type III = ~17.25 l).

1.2.3.2 Escapable exposure to CO_2

Here I describe the behavioural responses of rats when exposure to CO_2 can be avoided. One approach to assess emotions during CO_2 exposure is through choice and motivational tests. This approach is based upon the "hedonic principle"; i.e. that animals are motivated to avoid undesired end-states (e.g., potential harms, pain, etc.) and approach desired ones (see Fraser and Duncan, 1998).

Choice tests involve giving animals two or more alternative conditions (e.g. different agents or the same agent at different concentrations), and measuring the amount of time spent in each alternative as an expression of preference (e.g. Leach et al., 2002). Studies have shown that rats prefer lower concentrations of CO_2. Using choice tests, consisting of chambers prefilled with very low (<1%), low (25.5%), medium (34.9%), and high (50.8%) CO_2 concentrations, total time in the chamber and latency to leave the chamber varied with concentration (Leach et al., 2002, 2004). For example, total time in the chamber was 2.1 s and 0.7 s for the low and high concentrations respectively. In contrast, rats spent between 36 and 51 s in the chamber with very low CO_2 (Leach et al., 2004).

The strength of aversion can be measured by giving animals the ability to avoid agent exposure (e.g. at different concentrations or delivery method), with an added cost to their decision-making (Kirkden and Pajor, 2006). For euthanasia agents, strength of aversion has been investigated through aversion- and approach-avoidance tests. In the aversion-avoidance test, the cost of avoiding the agent in a preferred dark compartment is the exposure to an aversive brightly lit compartment (Bertolus et al., 2015; Wong et al., 2013). Using the aversion-avoidance test with a flow rate of 24% chamber vol. min^{-1}, all rats left the dark chamber filling with CO_2, escaping to the previously avoided bright chamber (Wong et al., 2013).

In the approach-avoidance test, the cost of avoiding the agent by escaping to an agent free cage is the loss of a sweet reward (Bertolus et al., 2015; Makowska and Weary, 2009; Niel et al., 2008 b; Niel and Weary, 2007). Rats are highly motivated to eat the sweet rewards even when fed their regular diet *at libitum* (Kirkden et al., 2008). When tested with different static concentrations of CO_2 in the approach-avoidance apparatus, rats tolerated concentrations ranging from <1% to 10% CO_2, entering the test chamber, eating the sweet rewards and staying in the gas chamber for ~300 s. Latency to leave the gas chamber decreased to 46 s at 15% CO_2, and to 5 s with 20% CO_2. Other studies using flow rates between 14 and 20% CO_2 chamber vol. min^{-1}, have shown that aversion is variable; rats leave the chamber at between 14% and 18% CO_2 depending on the study (Kirkden et al., 2008; Niel et al., 2008 b; Niel et al., 2008 a; Niel and Weary, 2007). However, all aversion studies report that rats avoid CO_2 before becoming ataxic or recumbent, even when they were fasted for 24 h (Kirkden et al., 2008).

These results suggest that onset of negative emotional states occurs between 10 to 18% CO_2. These concentrations are consistent with the onset and peak of active behaviours during forced exposure (see Niel and Weary, 2006), and freezing behaviours reported using the 20% CO_2 challenge (Johnson et al., 2005). This evidence shows that CO_2 concentrations required to achieve loss of consciousness (~33% CO_2; Niel and Weary, 2006) are aversive to rats (e.g. Kirkden et al., 2008; Niel et al., 2008 b; Niel et al., 2008 a; Niel and Weary, 2007).

1.2.3.3 Exposure to CO_2 in other situations

CO_2 is often used as an unconditioned stimulus in studies designed to induce negative emotional states in rodents. These studies support the conclusion that CO_2 exposure is anxiogenic. In the Vogel test two opposing motivations - gaining a reward versus avoiding a

punishment - are used to determine anxiolytic and anxiogenic effects of drugs. Food or water deprived rats can choose to receive a reward (water or food) at the cost of receiving punishments (shocks). Punishments suppress the reward consumption. Anxiogenic drugs suppress reward consumption and anxiolytics increase reward consumption (for a review, see Millan and Brocco, 2003). Using the Vogel test, rats previously exposed for 60 s to CO_2:O_2 (35:65%) chamber vol. min^{-1}, suppressed water licking by 40% relative to control rats (Cuccheddu et al., 1995). Thus CO_2 could be considered anxiogenic.

In the open field test, rats are placed in the centre of an open arena. The tendency to avoid the central area and display thigmotaxis (locomotion close to the walls of the apparatus) is enhanced by anxiogenic drugs, while anxiolytics increase locomotion in the central areas of the arena (for a review, see Prut and Belzung, 2003). In the social interaction test, rats are introduced in an open field arena to an unfamiliar conspecific; anxiolytic drugs increase the frequency of social interactions (e.g. sniffing, following, grooming) while anxiogenics have the opposite effect (for a review, see File and Seth, 2003). After exposure to the 20% CO_2 challenge, rats showed a 15% increase in thigmotaxis (Johnson et al., 2012) and a 50% decline in social interactions compared to rats exposed to air (Hickman et al., 2016).

Conditioning tests are also used to assess the aversiveness of a stimulus, and different variants of this test can be found. Potentially aversive stimuli can be paired with a neutral stimulus (Pavlovian conditioning), or with a specific neutral environment (place conditioning). Rats are then exposed to the potentially aversive stimulus, and conditioning is assessed later with the paired stimulus or environment, in absence of the aversive stimulus. Avoidance of the environment or the paired stimulus is an indicator of aversion; when avoidance is restricted immobility is used as a measure of aversion (Carlezon, 2003). Rats exposed to vanilla scent

before 30 s of forced inhalation of different concentrations of CO_2 (<1%, 5%, 35% or 100%), showed a conditioned response to vanilla 24 h later. Rats that inhaled <1% CO_2 froze less than rats that inhaled higher concentrations. Rats exposed to 100% CO_2 froze more and this conditioning resisted extinction relative to rats exposed to 5% CO_2. These results indicate that CO_2 can be used to condition rats, and that the degree of behavioural response (freezing) and extinction reflect the severity of the experience (Mongeluzi et al., 2003).

In summary, there is evidence that rats express defence behaviours when exposed to inescapable CO_2. This response is plastic, depending on the situation and stimulus' intensity, with some studies reporting freezing (passive response) and others active responses. If escape is possible all rats avoid CO_2, but when motivated rats will tolerate medium concentrations (<18% CO_2). CO_2 has an anxiogenic effect, which is sustained after exposure, and acts as an unconditioned stimulus especially at higher concentrations. Overall, these studies indicate that CO_2 elicits behavioural responses reflective of negative emotional states in rats, with an onset at between 10 and 18% CO_2. In addition to defence behavioural responses, negative emotional states can be inferred from physiological responses to exposure. These responses are reviewed in the following section.

1.2.4 Physiological responses to CO_2

Physiological changes help confront threats by preparing the animal to respond actively (i.e. fighting and fleeing) or passively (i.e. freeze). The physiological changes shared between high intensity emotional states include activation of the sympathetic nervous system (SNS; originally proposed by Cannon, 1915), and the neuroendocrine response known as hypothalamic–pituitary–adrenocortical axis activation (HPA axis; for reviews, see Armario et al.,

2012; Kemeny and Shestyuk, 2008; Ulrich-Lai and Herman, 2009). Sympathetic activation is linked to noradrenergic activity, and can be measured through heart rate, blood pressure, and plasma catecholamines (for a review, see Kvetnansky et al., 2009). Activation of the HPA axis results in the release of corticotropin releasing factor (CFR) by the hypothalamus, stimulating the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH induces glucocorticoid release (corticosterone in rats) from the adrenal cortex (Ulrich-Lai and Herman, 2009), which stimulates gluconeogenesis and inhibits glucose uptake by adipocytes, among other functions (Munck et al., 1984). Changes in the HPA axis can be measured through changes in CRF, ACTH, and corticosterone (for review see Armario et al., 2012).

The sympathetic response in rats exposed to CO_2 are increased blood pressure (e.g. Smith and Harrap, 1997) and bradycardia before LORR (e.g. Chisholm and Pang, 2016). Rat arterial blood pressure increases during the 20% CO_2 challenge (Hickman et al., 2016). Bradycardia has been reported for rats exposed to flow rates between 10% and 20% CO_2 chamber vol. min^{-1} (Burkholder et al., 2010; Chisholm and Pang, 2016), and to the 20% CO_2 challenge (Hickman et al., 2016). The cardiovascular response also correlates with changes in P_{CO_2} and pH (Johnson et al., 2012). Altholtz et al. (2006) found that rats anesthetized with 70% CO_2 showed increased plasma corticosterone levels after 30 min; this result is consistent when rats were exposed to 35% CO_2 (Barbaccia et al., 1996).

The sympathetic and neuroendocrine responses to CO_2 exposure indicate arousal and are least reflective of valence. However, taken together with the associated defence behaviours, these responses indicate negative emotional states in rats. In the following section I will provide evidence of different neural activation during hypercapnia.

1.2.5 Neurobiological responses to CO_2

Many brain regions, and nuclei within regions, are involved in the emotional states of fear and anxiety. In the following section, I briefly review the literature on induction and execution regions related to predator scent and hypercapnia; unfortunately, the neural circuits and interactions that support these emotional states are complex and, to some extent unknown (Adolphs, 2013).

To study the link between stimulus-response and the activation of brain regions, different *in vivo* and *in vitro* techniques can be used. *In vivo* techniques include focal microinjections (e.g. Coates et al., 1993), damage to specific brain areas (e.g. Khalsa et al., 2016), and creating genetically (e.g. Ziemann et al., 2009) or optogenetically modified animals (modifications that confer loss or gain of function and light detection capability to specific cell groups; Deisseroth, 2011), and real-time functional magnetic resonance imaging (fMRI) that detect changes in cerebral blood flow due to local neural activation (Buxton, 2009). *In vitro* studies provide information regarding local activation by specific stimuli, through measures of neuron firing rate, membrane action potential (e.g. Caradini and Fester, 2002), and c-Fos expression - an indirect marker of neuronal activation (Herdegen and Leah, 1998).

The amygdala is one of the regions implicated in emotional responses to sensory inputs, and in generating the behavioural and physiological adaptive responses (Davis and Whalen, 2010). In rats, exposure to predator scent increases c-Fos expression in the amygdala (Day et al., 2004; Dielenberg et al., 2001), and lesions in this area reduce freezing responses to predator scent (see Takahashi et al., 2005).

Johnson et al. (2011) found that rats exposed to the 20% CO_2 challenge tended to increase c-Fos expression in the amygdala, and this increase was related to increased fecal boli

production (indicative of fear and anxiety) and thigmotaxis in the open field tests. In a study by Ziemann and colleagues (2009), wild mice[1] (with intact acid sensing ion channels ASIC1a+/+ in the amygdala and in other brain regions) and mice without intact ASIC1a (ASIC1a-/- mice), were exposed to CO_2 in a series of experiments. The authors found that wild mice ASIC1a+/+ froze when exposed to 10 % static CO_2, and that exposure to 20% CO_2 in an open field test increased thigmotaxis. These responses were attenuated in the ASIC1a-/- mice. In a choice test, wild mice ASIC1a+/+ preferred an air chamber to a chamber prefilled with 20% CO_2, while the ASIC1a-/- mice spent similar amounts of time in both chambers. Both, wild ASIC1a+/+ and ASIC1a-/- mice showed similar minute ventilation increase due to hypercapnia. In vitro amygdala neurons of wild ASIC1a+/+, but not ASIC1a-/- mice, responded to a reduction in pH. Overall, these results suggest that the amygdala can act as a chemoreceptor for changes in PCO2/H+, and its activation is involved in the behavioural (but not ventilatory) response to CO_2.

The bed nucleus of the stria terminalis (BNST), frequently referred as the extended amygdala, is associated with modulation of the behavioural responses to threatening stimuli (Davis and Shi, 1999). Exposure to predator scent increases c-Fos expression in the BNST (Day et al., 2004; Dielenberg et al., 2001). Loss of function of the BNST leads to a reduction in the freezing responses due to predator scent exposure (Fend et al., 2003). Furthermore,

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[1] This review focuses on rats. Research has shown that ASIC1a in the rat amygdala respond to a reduction on pH, which is related to emotional states of fear and anxiety (e.g. Pidoplichko et al., 2014). Nonetheless, to my knowledge the role of ASIC1a during hypercapnia has not yet been tested in rats.

noradrenergic neurons that are activated during stressful events, project to the BNST (Moore and Card, 1984). During predator scent exposure, noradrenaline (NA) release in the rats' BNST increases; by locally blocking NA release in the BNST, the freezing response to predator scent is eliminated (Fendt et al., 2005). Taugher and colleagues (2014) have shown that the BNST is a chemoreceptor region that is also involved in the behavioural response to hypercapnia. These authors found that lesions in the BNST decreased freezing responses of mice during 10% CO_2 exposure without altering changes in minute ventilation due to hypercapnia.

Activation of the hypothalamus is related to the execution of different behavioural and physiological stimuli-responses; the most commonly identified is the activation of the HPA axis and modulation of autonomic responses. During the HPA axis cascade, the paraventricular nucleus (PVN) in the hypothalamus, which receives incoming inputs from the noradrenergic system, secretes CRF (Leibowitz et al., 1989; Swanson and Sawchenko, 1983). High density of c-Fos expression has been found in the rat's hypothalamic PVN nucleus when exposed to predator scent (Dielenberg et al., 2001). When rats are exposed to CO_2 concentrations between 5 and 20%, the PVN shows a high density of positive c-Fos expression; suggesting that the PVN is activated during hypercapnia (Haxhiu et al., 1996; Johnson et al., 2011; Kc et al., 2002).

The dorsomedial region of hypothalamus (DMH) is involved in the execution of cardiovascular (DiMicco and Monroe, 1998) and behaviour responses. For example, this region modulates 'escape' behaviours (locomotion, rearing, jumping out) in inescapable environments (Shekhar and DiMicco, 1987), and behaviours in an elevated plus maze test (Shekhar, 1993). The elevated plus maze is a cross shaped apparatus with two closed arms and two open arms; anxiogenic drugs decrease open arm entries, and anxiolytics have the opposite effect (for a review, see Walf and Frye, 2007). Lesions of the DMH increase open arm entries in the elevated

plus maze and interactions in the social test (Inglefield et al., 1994). When rats are exposed to predator scent, the DMH shows high density of c-Fos expression (Dielenberg et al., 2001). In rats exposed to the 20% CO_2 challenge, the DMH shows high c-Fos expression (Johnson et al., 2005), particularly in orexin neurons (Johnson et al., 2012) found only in the DMH and perifornical nucleus of the hypothalamus (Williams et al., 2007). Thigmotaxis in the open field was attenuated in rats treated with an orexin receptor antagonist before the 20% CO_2 challenge (Johnson et al., 2012, 2015). Orexin neurons are chemosensitive; firing rate of *in vitro* orexin neurons increases with fluctuations in CO_2 and pH (Williams et al., 2007). These results show that the DMH and the perifornical nucleus of the hypothalamus are not only involved in the behavioural response to hypercapnia, but also act as chemoreceptors.

The locus coeruleus (LC) is entirely composed by noradrenergic neurons; around 43% of the rats' noradrenergic neurons are situated in the LC, which presents a high density of innervations in the PVN, DMH, BLA, periaqueductal gray, medullary raphe, and spinal cord, among other regions (for a review, see Moore and Bloom, 1979). The LC is involved in the execution of several defence behaviours and physiological responses, including regulation of cardiovascular (Singewald and Philippu, 1996) and ventilatory (Biancardi et al., 2008) responses, cognitive flexibility, sensory processing (Sara and Bouret, 2012), and arousal related to emotional states of fear and anxiety (Southwick et al., 1999; Zitnic, 2015). High density of c-Fos positive cells has been found in rats exposed to predator scent (Dielenberg et al., 2001). The firing rate of *in vitro* LC neurons increased by 93% when stimulated with 15% CO_2 (Filosa et al., 2002; Li and Putnam, 2013). These results indicate that LC acts as a chemoreceptor and its activation may be related to arousal during CO_2 exposure.

Another region relevant in the modulation of behavioural responses to threatening stimuli is the periaqueductal gray (PAG). The ventrolateral PAG (VLPAG) is involved in the execution of passive behavioural responses (i.e. freezing; Bago and Dean, 2001; Bandler and Shipley 1994), while the dorsal PAG (dorsolateral DLPAG and dorsomedial DMPAG) is related to flight behaviours (active defence responses; Bandler and Shipley 1994; Beckett and Marsden 1997). In the brain of rats exposed to predator scent (Dielenberg et al., 2001), and to the 20% CO_2 challenge (Johnson et al., 2011), VL, DL and DMPAG show increased c-Fos expression. In rats, exposure to CO_2 concentrations between 8% and 13% increases the expression of immobility and active behaviours due to PAG electrical stimulation (Schimitel et al., 2012). Lesions in the DL and DMPAG of rats exposed to low concentrations of CO_2 (7% CO_2) decreased the ventilatory response compared to controls, without altering the cardiovascular response (Lopes et al., 2012).

A key central chemoreceptor brainstem region is the medullary raphe; local acidification of the medullary raphe produces an increase in the ventilatory response (Nattie and Li, 2001; for a review, see Richerson, 2004). Serotonin (5HT) is originated in the medullary raphe by tryptophan hydroxylation. Serotonin is implicated in mediating emotional states, perception, cognition, and sympathetic arousal (for a review, see Lesch et al., 2012). During predator scent exposure, rats pre-treated with a serotonin agonist showed less freezing, and lower plasma corticosterone levels (Shields and King, 2008). Administration of serotonin reuptake inhibitors (SRI), which increase synaptic serotonin, is known to decrease anxiety in humans and reduce respiratory rate of rats exposed to 6% CO_2 (Olsson et al., 2004).

The role of the γ- aminobutyric acid (GABA) inhibitory neurotransmitter has been highlighted in the response to hypercapnia. In rats, stressful events like acute handling (Andrews,

Zharkovsky and File, 1992), chronic restraint (Gruen et al., 1995), and social isolation (Serra et al., 2000) decrease $GABA_A$ receptor function. Similarly, exposure to 35% CO_2 (Cuccheddu et al., 1995; Sanna et al., 1992), the 20% CO_2 challenge (Johnson et al., 2015), and 35% CO_2 paired with footshock (Concas et al., 1993) decrease $GABA_A$ function. The administration of benzodiazepines (like lorazepam and alprazolam) before exposure to CO_2 increases $GABA_A$ receptor functioning (Sanna et al., 1992), increases reward consumption in a Vogel conflict test (Cuccheddu et al., 1995), and enhance social interactions in the social test (Johnson et al., 2015). These results indicate that CO_2 acts as negative stimulus on GABA functioning, and that following drug treatment with agents known to serve as anxiolytics the behavioural effect of CO_2 is diminished.

In summary, brain areas and neurotransmitters involved in fear and anxiety due to threatening stimuli, such as predator scent, are also activated by hypercapnia. These regions include the amygdala, BNST, hypothalamus, PAG, LC, and medullary raphe. This large body of evidence shows that rats likely experience negative emotions when inhaling CO_2. Since these responses can, to some extent, be counteracted by the administration of anxiolytics and SRIs, these results suggest that rats experience anxiety and fear when exposed to CO_2. As I will review in the following section, this inference is consistent with human responses when inhaling CO_2.

1.2.6 Human responses to CO_2 exposure

In this section I summarize evidence of healthy humans' physiological responses and felt experiences during CO_2 inhalation. Exposure to CO_2 has been extensively used in humans, because of its high potential to cause symptoms of anxiety and panic attacks (PAs). Panic is characterized by feelings of pounding of the heart, shortness of breath (dyspnea), sweating,

trembling or shaking, choking, chest pain, nausea, dizziness, light-headedness, depersonalization, derealization, fear of losing control, fear of dying, chills and hot flushes (APA, 2013). PAs can be assessed through, for example, the panic symptoms list (PSL; Schruers et al., 2000), and acute panic inventory (API; Goetz et al., 2001). Anxiety is often accompanied by feelings of tension, apprehension, nervousness and worry, and is often measured through the state trait anxiety inventory-S (STAI-S; Spielberger and Reheiser, 2004), the visual analogous scale (VAS), and the visual analogous scale for affect (VAAS), among others (e.g. Griez et al., 2007; Leibold et al., 2013).

Similar physiological responses as those reported for rats have been found in humans. When subjected to inhalation of 5% CO_2 and 7% CO_2, healthy humans increase both tidal volume and respiratory frequency as a compensatory mechanism to remove excess of CO_2 (Gorman et al., 2001). People inhaling CO_2 concentrations between 7 and 14% for 10-20 min show an increase in minute ventilation, blood pressure, heart rate, plasma noradrenaline and cortisol (Sechzer et al., 1960). A single inhalation of 35% CO_2 activates the HPA axis, and cortisol increases for approximately 30 min after exposure. In addition, blood pressure increases and heart rate decreases with exposure (Argyropoulos et al., 2002; Kaye et al., 2004).

When healthy humans are exposed to CO_2 they typically become anxious and sometimes panic. The emotional experience to CO_2 is dose dependent, with a greater response at higher CO_2 concentrations (Griez et al., 2007, Leibold et al., 2016; Schruers et al., 2011). Healthy humans exposed to a double inhalation of different CO_2 concentrations (0, 9, 17.5, 35% CO_2), reported that symptoms increased with CO_2 concentration. With increasing the dose (e.g. double inhalation of 35% CO_2), CO_2 is an effective method to induce PAs in healthy people (Leibold et al., 2013). In addition to feeling fear and anxiety, in healthy humans a single inhalation of 35%

CO_2 increases the feeling of wanting to leave the room and feeling paralyzed (Argyropoulos et al., 2002). Similarly, Schmidt et al. (2008) found that when exposed to 20% CO_2 inhalation during 20 s, people reported an increase in feelings of immobility (freezing) and desire to flee.

Physiological and subjective experiences when exposed to 7.5% and 35% CO_2 are often correlated. A positive correlation was found between plasma cortisol and blood pressure, feelings of fear and anxiety were also correlated with an increase of blood pressure (Bailey et al., 2003). Repeated inhalation of 5% CO_2, produces dyspnea in healthy people (Wan et al., 2008). During 8% CO_2 inhalation, the experience of dyspnea in healthy humans is correlated with activation of the amygdala, PAG, hypothalamus and anterior insula (Liotti et al., 2001). Although the role of the amygdala in the subjective experience of air hunger due to hypercapnia has been noted in the study above, patients with bilateral amygdala lesions still experience fear and panic when inhaling 35% CO_2 (Feinstein et al., 2013), but not when exposed to external life-threatening stimuli (Feinstein et al., 2011). These studies have led to the suggestion that fear to external and internal stimuli can be dissociated (Adolphs, 2017).

Treatment with serotonin antagonists, or tryptophan depletion before one or two inhalations of 35% CO_2, enhances the subjective experience of anxiety, fear, dyspnea and panic (Ben-Zion et al., 1999; Klaassen et al., 1998; Schruers et al., 2000). Treatment with serotonin precursors and selective serotonin reuptake inhibitors (SSRIs) has the opposite effect (Perna et al., 2002; Schruers et al., 2002). Similarly, pre-treatment with alprazolam diminishes the experience of panic elicited by one inhalation of 35% CO_2 (Bailey, et al., 2009).

In this section I have reviewed evidence that CO_2 exposure elicits physiological and subjective experiences of fear, anxiety, dyspnea, and panic in humans. The felt emotions reported by healthy humans, suggest that at lower CO_2 concentrations anxiety is experienced,

and fear and panic are felt at higher concentrations. Physiological responses are similar to those reported for rats, and some reported feelings resemble defence behaviours. Behavioural responses are often restricted in human research, but "feeling like leaving the room" and "desire to flee" may be seen as an active response, or "feeling paralyzed" and "feelings of immobility" as freezing responses. Several brain regions activated during CO_2 inhalation in rats are also activated in humans. In addition, similarly to behavioural changes in rats, benzodiazepines, serotonin precursors and SSRIs ameliorate the feelings of anxiety, fear, distress, dyspnea and panic due to hypercapnia in humans.

1.2.7 Summary of rat emotions during CO_2 exposure

In this section, I will summarize evidence of negative emotional states in response to CO_2. I will argue that rats likely feel emotions of anxiety, fear, distress and air hunger, rather than pain.

In mammals, inhalation of above atmospheric concentrations of CO_2 is biologically relevant; CO_2 is produced as metabolic waste, forms part of the normal gas exchange during respiration, and when the rate of production exceeds that of removal, can be life threatening. The importance of the appropriate removal of CO_2 metabolic waste is evident from the complex biological mechanisms that detect and respond to changes in P_{CO_2}. From forced exposure studies it is evident that rats respond with active and passive defence behaviours to concentrations that surpass 10% CO_2. Motivation tests show that CO_2 is aversive to rats, with aversion increasing as CO_2 concentrations rise. The behavioural responses to CO_2 are accompanied with neuroendocrine and sympathetic activation. The behavioural responses to hypercapnia are similar to those elicited by other well-studied threatening stimuli (e.g. predator scent).

Furthermore, similar brain-region activation and executed responses (both behavioural and physiological) are shared between CO_2 and other threatening stimuli. This evidence indicates that CO_2 is a dose-dependent competent stimulus that evokes negative emotional states in rats, although the specific emotions are not clear.

The core issue regarding the humaneness of CO_2 relies on the felt experience of negative emotions. It may be possible to draw stronger inferences regarding the felt experiences associated with CO_2 by examining responses after exposure when CO_2 is no longer present. The behavioural responses in the open field and social tests show that negative emotions were sustained when CO_2 was no longer present. Furthermore, after acute exposure to CO_2 rats showed conditioned responses. The level of conditioning and extinction resistance depend upon CO_2 concentration, implying that the magnitude of the emotional response increased with the intensity of the stimuli.

Human reports of felt experiences of fear, anxiety, dyspnea, distress and panic during CO_2 inhalation suggest that rats feel similar emotions. It seems less likely that rats feel pain during CO_2 gradual fill (e.g. Hawkins et al., 2016). Electroencephalogram arousal of anesthetized rats exposed to 15% CO_2 has been suggested to be associated with a noxious respiratory sensation rather than pain (Kells et al., 2018). Loss of consciousness during gradual fill frequently occurs before CO_2 concentrations reach 40% of the chamber vol., but human reports of pain begin at concentrations above 47% CO_2 (Anton, Euchner and Handwerke, 1992) and most response thresholds for rats and human nociceptors in the nasal mucosa range from 37% to 50% CO_2 (Peppel and Anton, 1993).

Caution is required when functional homology is used to draw inferences regarding felt experiences (see Weary et al., 2017). However, human feelings of anxiety, fear, dyspnea, and

panic in response to hypercapnia can be attenuated by benzodiazepines and SSRIs. That these drugs also diminish the defence behavioural responses to CO_2 allows for stronger inferences regarding similar emotions in rats.

In summary, concentrations below 10% CO_2 are tolerated, do not elicit intense behavioural responses, and cause mild conditioning in rats. At 15% CO_2, rats respond with freezing behaviours, but if motivated some will still tolerate exposure. Rats do not tolerate concentrations that surpass 18% CO_2, which correspond with the peak in active defence behavioural responses. Negative emotions elicited by 20% CO_2 forced exposure, which resemble those of anxiogenic drugs, are sustained even after exposure. Exposure to concentrations over 35% - required for loss of consciousness in rats - have anxiogenic effects and produce strong conditioning. During the course of CO_2 killing, rats likely feel negative emotional states that worsen as CO_2 increases. During gradual fill, these feelings most likely correspond to fear, anxiety, air hunger, or distress, but not pain. However, rats vary in their behavioural responses.

Variation in behavioural responses could indicate differences in the magnitude of the emotional experience. That is, when exposed to the same delivery method, CO_2 concentration or flow rate, some rats may experience a higher degree of negative emotion than others (i.e. variation in sensitivity). In the next sections I will explore this idea and discuss proximal explanations for this individual variability. Firstly, I will discuss different individual personality traits that may account for variability in responses among individuals on a situation-dependent basis. Secondly, I will present human evidence on variable sensitivity to CO_2.

1.3 Variable responses to CO_2

1.3.1 Rat variability: personality and CO_2 sensitivity

In the previous sections I reviewed studies that considered within-individual variation in behaviour (i.e. behavioural plasticity) as an indicator of the animal's experience during CO_2 exposure. I have explored variability between studies as a result of human error, situation (e.g. escapable vs. inescapable), intensity (CO_2 concentrations), strain, and sex differences. Interest has been growing on identifying and understanding individual differences (Dingemanse et al., 2010). In this section, I focus on the individual as a relevant source of variation that could account for differences between studies.

I will first describe individual variability in strategies under forced exposure, and then discuss motivational differences in choice and aversion tests. In all cases, I will make inferences regarding the emotional experiences of rats when exposed to CO_2, linking personality traits and sensitivity.

1.3.1.1 Inescapable exposure: strategies and styles

Individual differences in the rat responses to CO_2 are often mentioned, but not explicitly evaluated. Using forced exposure to CO_2, one study reported that only 20% of rats climbed the cage and 20% circled (i.e. moving around the perimeter of the cage; Smith and Harrap, 1997); other studies reported that some rats expressed little and others numerous escape behaviours (Niel et al., 2008 b; Niel and Weary, 2006), and that only half of the rats tested increased locomotion (Niel et al., 2008 b).

Individual rats exposed to CO_2 may be adopting different behavioural strategies. Within the same sex, strain, situation and stimuli intensity, rats vary between individuals in the

behavioural strategy (i.e. passive vs. active responses) used to cope with an aversive or threatening context. For example, when exposed to a prod that delivered shocks in confined cages, wild-type rats were highly variable in the strategy used to avoid the shock; some individuals spent almost 80% of the test burying the prod and less than 10% of the test immobile away from the prod, while others spent less than 5% burying and around 60% immobile (de Boer and Koolhaas, 2003). These different behavioural strategies could represent personality traits, if the responses are consistent within individuals across time and contexts (Réale et al., 2007). Note that different approaches and conceptual frameworks can be found in the animal personality literature (e.g. Coppens et al., 2010; Gosling, 2001; Koolhaas et al., 1999), and personality does not imply absolute consistency within the individual (see Dingemanse et al., 2010).

Behavioural differences during forced exposure to CO_2 may reflect different strategies rather than variation in sensitivity to CO_2 among rats, which implies that the different types of response may not be related to the intensity of the emotional experience. In the following section, I will examine choice, approach- and aversion-avoidance tests more thoroughly, with particular focus on individual differences.

1.3.1.2 Motivational differences in choice and aversion tests

Some evidence for variability in rat sensitivity to CO_2 can be found in choice and aversion tests. Leach and colleagues (2002, 2004), using choice tests, reported high inter-individual variability in responses. These authors described two strategies: 'escapers' that left the test chamber and never came back, and 'searchers' that constantly moved between chambers, returning to the test chamber repeatedly. In the aversion-avoidance test, rat aversion to CO_2 was

variable. For example, latency to avoid CO_2 ranged from 7 s to 48 s between individual rats in the study by Wong et al. (2013). In the approach-avoidance, the mean concentration avoided varied among individuals (Kirkden et al., 2008; Niel et al., 2008 a) ranging from 11% to 18.6% CO_2 (Niel et al., 2008 a). These results indicate that the individual rat may be a relevant source of variation in behavioural responses to CO_2. Moreover, if consistent within and between situations (e.g. between forced exposure and between aversion tests), variation in responses would be a strong indicator of CO_2 sensitivity.

In choice and aversion tests, the underlying assumption is that rats are motivated to approach and avoid stimuli that elicit positive and negative end-states, respectively. In approach-avoidance tests, CO_2 exposure is paired with a positive emotion-eliciting stimulus (i.e. sweet reward), which animals seek to approach. Aversion-avoidance tests pair the rats' motivation to avoid CO_2 exposure in a dark 'safe chamber' against a known negative emotion-eliciting stimulus (i.e. a brightly lit chamber), that animals normally seek to avoid. Is assumed that motivation to approach or avoid a stimulus is stronger as the value of the stimulus increases. Thus, in these between-motivations tests, a known strong motivation to approach or avoid a stimulus is compared to the strength of motivation to avoid CO_2. Rat motivation is strong when approaching sweet rewards, even without food deprivation (Kirkden et al., 2008), and when avoiding light exposure (1650 lux), even at the cost of losing a food reward when food deprived (Barker et al., 2010).

When variation among individuals is found within these tests, one first step could be to determine if variability arises as a response to the assessed (i.e. CO_2) or the paired (i.e. sweet reward or bright light) stimuli. The question would be: is the value of the paired stimuli the same for all rats? In the aversion-avoidance test, variation in CO_2 aversion could reflect differences in

the strength of motivation to avoid a bright light. In one study, rats were housed for 14 days in a two-chamber apparatus (one brightly lit and one dark), and individuals consistently differed in the time spent in the bright chamber (Whishaw, 1974). Similarly, in the approach-avoidance test, the strength of motivation to approach a sweet reward could vary among rats. Several studies have shown that motivation for sucrose varies between individuals and is repeatable; rats that consume higher freely available sucrose also make a higher effort to earn sucrose (Brennan et al., 2001; de Sousa et al., 1998; Tõnissaar et al., 2006). If strength of motivation to avoid a brightly lit chamber, or to approach sweet rewards is variable, then variation between individuals in aversion tests may simply reflect these motivational differences rather than variability in CO_2 sensitivity.

Treating the hedonic principle as unitary (i.e. approach positive end-states and avoid negative ones) makes predictions difficult (see Cornwell et al., 2014). For instance, in approach-avoidance tests by avoiding CO_2 rats are also approaching safety making it difficult to conclude which motivation is driving the behaviour. Predictions can be more easily made by examining motivation using regulatory focus theory (Higgins, 1997). Regulatory focus theory distinguishes between two different types of approach motivations: 1) motivation to approach gains, growth, or advancement (i.e. promotion), and 2) motivation to approach safety and non-losses (i.e. prevention). To attain a desired goal, two strategies can be used; individuals can approach matches to end-states or avoid mismatches to end-states. These two strategies are situational dependent, approaching matches is more likely to occur when incentives are related to promotion goals, versus avoiding mismatches to prevention ones. These approach motivations are related to different types of positive and negative emotional experiences. Consistent with a dimensional view of emotions (i.e. arousal and valence axis), when achieved, promotion goals correspond to

experiences of high arousal positive states, and when failed, to low arousal negative states. In contrast prevention goals, when successful, correspond to low arousal positive states, and if failed, to high arousal negative end-states (Figure 1.1).

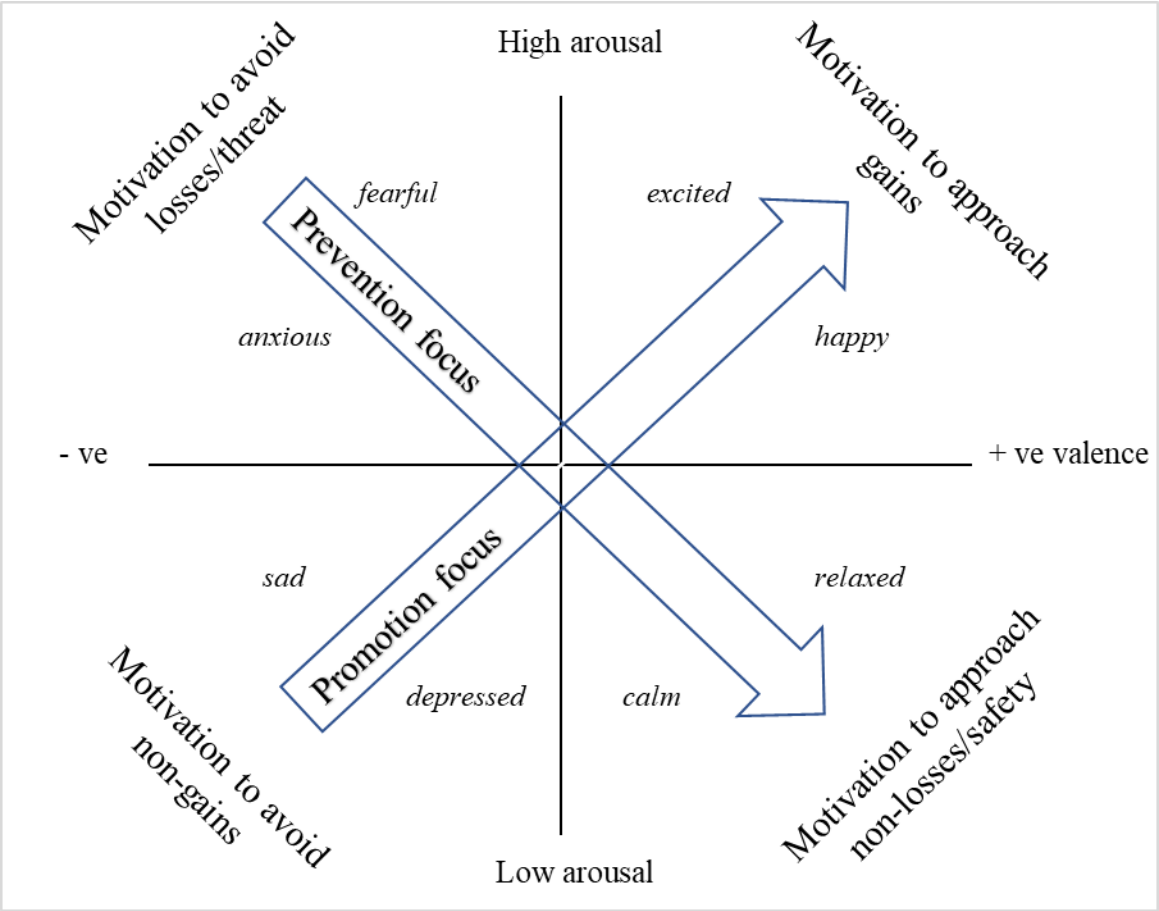


Figure 1.1 Illustration of approach-avoidance distinctions between promotion and prevention motivations and its relationship with a bi-dimensional representation of affect.

Adapted from Mendl et al. (2010), Molden et al. (2008), Panksepp et al. (2002), and Russell and Barrett (1999).

Individual differences in the strength of promotion and prevention motivations have been reported in the human literature. Individuals highly focused on promotion are more motivated to approach gains, and are more sensitive to their presence or absence. Whereas individuals highly

focused on prevention are more motivated and sensitive to safety related incentives (e.g. Shah and Higgins, 1997). Promotion and prevention motivations are independent; hence individuals can be high or low on promotion or prevention, or both (e.g. Franks et al., 2014). These motivational foci have been also identified in rats. Franks and colleges (2012, 2014) tested rats in a modified open field arena containing a treat location and a dark location (if the rat approached it, lights would go off); rats could approach gains, safety, or engage in other activities (e.g. exploration). Authors found consistent individual differences in promotion (i.e. approaching treats) and prevention (i.e. approaching darkness) motivations. This pursuit of treats or darkness varied between individuals, and was consistent over time ($r = 0.81$; Franks et al., 2012).

By taking a regulatory focus approach, we can distinguish approach- and aversion-avoidance tests in terms of situational frames. Approach-avoidance tests involve gain and safety incentives. Therefore, both promotion and prevention strategies are likely to be elicited, and individual differences in promotion and prevention motivations could account for variation in CO_2 thresholds of aversion. One prediction is that promotion focused individuals tolerate risky CO_2 concentrations to maximize gains, and prevention focused individuals maintain safety by avoiding non-threatening CO_2 concentrations. The aversion-avoidance test involves only prevention incentives, so the prediction is that prevention focused rats would tolerate risky CO_2 concentrations to maintain safety.

In summary, variation in CO_2 concentrations tolerated by rats could indicate that some rats find CO_2 more aversive than others. To determine if rats vary in CO_2 sensitivity in aversion tests, I first proposed to assess whether the paired stimulus (i.e. sweet reward and bright lit chamber) is equally valued among rats. Then I propose using regulatory focus theory to predict individual differences in CO_2 aversion in a situational-dependent way. By identifying the sources

of among individuals' variation in aversion tests, more accurate inferences can be drawn regarding the rats' experience during CO_2 exposure. In the next section I will present human evidence of between individual variation in felt emotions during CO_2 inhalation.

1.3.2 Variability in humans' felt experience during CO_2 inhalation

Human sensitivity to CO_2 lies in a continuum with healthy people at the lower end, and panic disorder (PD) patients on the other end (Colasanti et al., 2008, 2012). False suffocation alarm theory posits that high CO_2 sensitivity arises from inappropriate activation of systems that monitor suffocation (Klein, 1993). Several candidates have been proposed to mediate the response and sensitivity to hypercapnia, including the noradrenergic (for a review, see Bailey et al., 2003), serotoninergic (for a review, see Leibold et al., 2015), and GABAergic systems (for a review, see Bailey and Nutt, 2008). Not all healthy humans experience fear, anxiety and panic when inhaling CO_2, but when individuals respond, the experience is repeatable. Furthermore, vulnerability increases in first-degree relatives (parent, sibling or child) of PD patients, by a diagnosis of PD, or by a positive screen on trait anxiety. Inhalation of 7% CO_2 during 20 min, consistently (between two exposures) induces anxiety in healthy responders (60% of the volunteers; Poma et al., 2005). In healthy humans, increases in fear and anxiety and panic were reported in only 50% of the participants after a double inhalation of CO_2 concentrations between 9 and 35% (Griez et al., 2007). When healthy humans inhale 20% CO_2 during 20 s, 13% and 20% of the individuals experience modest to greater feelings of immobility and desire to flee, respectively (Schmidt et al., 2008). Between 47 and 68% of healthy humans experience PAs with a double inhalation of 35% CO_2 (Leibold et al., 2013). Inhalation of 35% CO_2, elicits anxiety responses in 48% of healthy first-degree relatives of PD patients (Perna et al., 1999); this

response being greater than control humans, and was highly correlated between two exposures (Coryell and Arndt, 1999). With single inhalation of 35% CO_2, 44% healthy humans with a first-degree relative with PD, experience panic symptoms, in contrast to 12% of control humans (van Beek and Griez, 2000). In PD patients, a single inhalation of 35% CO_2 reliably induces PAs ($r = 0.7$), and anxiety ($r = 0.4$; Verburg et al., 1998), in between 43 and 94% of the individuals, depending on the subtype of PD (for a review, see Colasanti et al., 2012). Trait anxiety (i.e. consistent individual differences in anxiety proneness; Spielberger and Reheiser, 2004) is a predictor of the affective response to CO_2 (Schmidt et al., 2008). Individuals positive for trait anxiety experience greater feelings of fear and anxiety when inhaling 7.5% CO_2 for 20 min, than did those screened negative for this trait (Fluharty et al., 2016).

In summary, human experiences of fear, anxiety and panic due to CO_2 inhalation vary between individuals. Human variation in the emotional experience due to inhalation of low CO_2 concentrations corresponds to feelings of anxiety, whereas inhalation of higher CO_2 concentrations elicits panic attacks. Intrinsic individual characteristics have been shown to be predictive of sensitivity (e.g. a diagnosis of, or a first-degree relative diagnosed with, PD). Thus like humans, it is possible that rats vary in CO_2 sensitivity which is linked to the emotional experience during inhalation. Considering that rat aversion to CO_2 has been shown to vary between individuals at concentrations below 18.6% CO_2, variation in the emotional experience may be related to feelings of anxiety.

1.4 Thesis aims

The literature reviewed above indicates that rats feel negative emotional states when exposed to CO_2. This research also suggests that identifying sources of variation in the responses

of individual rats during exposure to CO_2 would allow for stronger inferences regarding their experiences. The general aim for my thesis was to assess whether rats vary in CO_2 sensitivity. I propose that to understand individual variation in rat CO_2 sensitivity, several factors must be taken into account.

In the euthanasia literature, outcome measures have been almost exclusively limited to active defence responses, while in other disciplines immobility is almost always examined and active defence responses are rarely considered. Failure to report the absence of a change may be attributable to omissions in what was measured, rather than a lack of response per se. Thus the first objective of Chapter 2 was to examine both passive and active defence responses during CO_2 forced exposure. Consistent variation in rat responses to CO_2 could be reflective of personality differences in behavioral strategies in response to threatening stimuli; some rats may consistently express active while others passive strategies. The second objective of Chapter 2 was to determine if behavioural responses are consistent within individuals and across aversive stimuli. The third objective of Chapter 2 was to assess consistency in individual differences in aversion to CO_2 in aversion-avoidance and approach-avoidance tests, as evidence of variability in rat responses to CO_2 in motivational tests suggests variation in CO_2 sensitivity. In humans, sensitivity to CO_2 is consistent within individuals. In rats, individual differences in sensitivity could be reflected in consistent behavioural responses across time, regardless of type of defence behaviour expressed (i.e. active and passive responses), and situational differences (e.g. forced exposure and aversion tests). Hence the fourth objective of Chapter 2 was to determine how individual differences in rat responses to CO_2 relate between situations (aversion tests and forced exposure).

In Chapter 3, I explore the stability and consistency of rat individual differences in aversion to CO_2 in approach-avoidance across multiple exposures. In addition, approach-avoidance tests rely on the strength of rat motivation to approach or avoid paired stimulus (sweet rewards and CO_2, respectively). Hence, individual differences in aversion to CO_2 could be masked by variation in the value of the paired stimulus. Thus the second objective of Chapter 3 was to independently evaluate individual differences in rat motivation to access sweet rewards, as another potential predictor of individual differences in response to CO_2 exposure. In Chapter 3 I also assessed variability in CO_2 aversion through use of the regulatory focus theory as variation in the strength of promotion and prevention focus could be predictive of CO_2 aversion. The third objective of Chapter 3 was to determine if regulatory focus theory can be used to predict individual thresholds of aversion to CO_2 in approach-avoidance tests.

Individual differences in rat aversion to low concentrations of CO_2 may be driven by variation in the onset of feelings of anxiety. In Chapter 4, I assessed the effects of the benzodiazepine midazolam on rat individual thresholds of aversion to CO_2.

Chapter 2: Consistent individual differences in rat responses to CO_2

Carbon dioxide (CO_2) is a widely used but controversial method of killing laboratory rodents (Hawkins et al., 2016). Guidelines and regulations commonly accept this agent as a 'humane' killing method (e.g. Charbonneau et al., 2010; European Union, 2010; Leary et al., 2013), implying that animals should not experience high arousal negative emotions during exposure, including pain, fear, distress, or anxiety.

Here we refer to emotional responses as objectively observable behavioural, physiological and brain responses to stimuli (Damasio, 2004). Emotions in animals are often inferred from behavioral responses during forced exposure to a noxious agent. The frequency, duration and intensity of rat active defense responses (e.g. increased locomotion, rearing, and the attempts to escape the cage, etc.) have been interpreted as signs of a negative emotional experience during CO_2 exposure (e.g. Blackshaw et al., 1988; Britt, 1987; Coenen et al., 1995; Niel and Weary, 2006). Choice and between-motivation tests, which are based on the animal's motivation to approach desired and avoid undesired states (see Fraser and Duncan, 1998), have also been used to assess rat emotions elicited by CO_2. Choice tests provide rats with two mutually exclusive conditions (e.g. a chamber pre-filled with high CO_2% and low CO_2% pre-filled chamber), such that the amount of time animals spend in each condition is indicative of preference (e.g. Leach et al., 2002). Between-motivation tests compare aversion to CO_2 with motivation to approach or avoid a stimulus thought to elicit positive or negative emotions, respectively. For example, in aversion-avoidance tests the cost of avoiding CO_2 is exposure to an aversive brightly light chamber (e.g. Wong et al., 2013), and in approach-avoidance tests the cost of avoiding CO_2 is loss of a sweet food reward (e.g. Niel and Weary, 2007).

Choice tests have shown that rats prefer (total time in the chamber between 36 and 51 s) to be exposed to <1% CO_2 than to be exposed to 25.5% CO_2 (total time in the chamber around 2.1 s) or 50.8% CO_2 (total time in the chamber around 0.7 s; Leach et al., 2002, 2004; Niel and Weary, 2007; Wong et al., 2013). Research using between-motivation tests has consistently shown that rats find CO_2 aversive and that they are motivated to avoid CO_2 concentrations between 14% and 18% (e.g. Kirkden et al., 2008; Niel and Weary, 2007, Niel et al., 2008 a; Niel et al., 2008 b), well below the concentrations required to render animals recumbent (approximately 33% CO_2; Niel and Weary, 2006). The results of forced exposure tests have been less consistent. Some studies have found behavioral responses in rats exposed to CO_2 (e.g. Coenen et al., 1995; Niel and Weary, 2006; Niel et al., 2008 b; Makowska and Weary, 2012), but others have reported little or no response (e.g. Blackshaw et al., 1988; Burkholder et al., 2010; Smith and Harrap, 1997). Results from choice and between-motivation tests indicate that CO_2 elicits negative states which rats are motivated to avoid, indicating that CO_2 is not a humane killing method for rats, but the lack of agreement between studies using forced exposure tests may help perpetuate the use of this method. Indeed, this lack of consistency is cited in recent reviews supporting the use of CO_2 as a humane killing method (Boivin et al., 2017; Valentim et al., 2016).

Research examining CO_2 as a euthanasia agent has only considered active defense responses to forced exposure, but rats also show passive responses (freezing/immobility; Blanchard et al., 1990; De Boer and Koolhaas, 2003), and these responses have been the focus of research on the use of CO_2 as an anxiogenic (e.g. Johnson et al., 2005a, b; Winter et al., 2017). As some of the previous euthanasia research may have failed to find effects because only active responses were considered, the first aim of our study was to examine both passive and active

defense responses during CO_2 gradual-fill forced exposure. We predicted that when exposed to CO_2, rat passive and active defense responses would increase from baseline more than when exposed to Oxygen (O_2) as a control.

A number of studies have reported between-rat variation in response to gradual-fill CO_2. For example, previous studies from our research group found that the frequency of escape behaviors ranged between individuals from zero to 34 (Niel and Weary, 2006), and that about 50% of the rats tested showed increased locomotion (Niel et al., 2008 a). Smith and Harrap (1997) found that about 20% of rats climbed or moved around the perimeter of the cage in response to CO_2 exposure. Leach and colleagues (2002, 2004), using choice tests, reported high inter-individual variability in responses. Aversion to CO_2 is also variable among rats. For example, one study using aversion-avoidance testing found that the time to avoid CO_2 varied among rats from 7 to 48 s (Wong et al., 2013), and a study using approach-avoidance found that the concentration of CO_2 avoided varied from 11% to 18.6% (Niel et al., 2008 a).

Evidence of variability in rat responses to CO_2 in motivational tests suggests variation in CO_2 sensitivity. It has been well documented that humans vary in their emotional responses to CO_2. For example, following a double inhalation between 9 and 35% CO_2, approximately 50% of healthy humans experience anxiety (Griez et al., 2007), and a single inhalation of 35% CO_2 elicits panic in between 43 and 94% of patients with panic disorder (PD; for a review, see Colasanti et al., 2012). Heightened sensitivity to CO_2 in humans may be associated with a false suffocation alarm (i.e. an inappropriate activation of systems that monitor suffocation; Klein, 1993).

Personality differences – extensively documented in different animal taxa (Gosling, 2001) – may account for variation in rat responses to CO_2 in a situation-dependent manner. We

define personality following Réale et al. (2007) as individual differences consistent across time and contexts. Variation in rat responses to CO_2 may reflect different behavioral strategies. For example, de Boer and Koolhaas (2003) found that some rats attempted to bury a prod that delivered shocks, but others moved away from the prod and remained immobile. If consistent within rat, this variation between rats could be related to more general personality differences in how individuals respond to threatening stimuli. The second aim of our study was to determine if rats consistently vary in behavioral strategies when exposed to CO_2. If variation in response to CO_2 is reflective of individual differences in response to threatening stimuli in general, we expected that responses to CO_2 would be related to those to fox scent, and that passive and active defense responses would be consistent within and between stimuli.

Human variation in CO_2 sensitivity is consistent between repeated exposures (e.g. Coryell and Arndt, 1999; Poma et al., 2005; Verburg et al., 1998). In rats, individual differences in sensitivity could be reflected in consistent behavioural responses across time, regardless of the type of defence behaviour expressed (i.e. active and passive responses), and situational (e.g. forced exposure and aversion tests). Thus, the third aim of our study was to assess rat variation in CO_2 sensitivity. Our hypothesis was that variation in rat responses to CO_2 is reflective of CO_2 sensitivity, and we predicted that rat responses to CO_2 would be consistent within and between aversion-avoidance, approach-avoidance, and forced exposure tests.

2.1 Methodology

2.1.1 Subjects and housing

We used twelve female Sprague-Dawley rats, all obtained as surplus stock from the University of British Columbia. Rats were not part of any experimental procedure prior to this study. One animal showed signs of ill health, was treated with an anti-inflammatory and was not used in the tests. Rats were individually marked with a permanent marker (Ketchum Manufacturing Inc., ON, Canada), and housed in groups of three in two polycarbonate cages (Lab Products, Inc. DE, USA) connected by a red tinted polycarbonate tube (7.6 cm diameter, 15 cm long), to provide rats with more home-cage space. One cage was smaller (20 x 45 x 24 cm) and contained food (Rat Diet PMI 5012, Lab Diets, Land O'Lakes, Inc., MN, USA), tap water and bedding material (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA), while the other cage was bigger (20 x 50 x 40 cm) and contained bedding material (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA), a PVC tube, and a cardboard box. All cages and bedding were replaced once a week on Thursday after 1700 h, to reduce the risk that any effects from cage-changing (which can last several hours (36)) affected our results. All animals had ad libitum access to food (Rat Diet PMI 5012, Lab Diets, Land O'Lakes, Inc., MN, USA) and tap water and received daily treats (oats and shredded coconut). Rats were kept under reverse lighting (dark period from 0800 h to 2000 h). Temperature and humidity were controlled and averaged (mean ± standard deviation) 24 ± 0.6 °C and 52 ± 5.8 %, respectively. Rats were 9 months old and weighed 403 ± 54 g at the end of the study.

2.1.2 Handling and experimental room

All rats were habituated to handling during a 10-day period before experiments started. In all experiments, each rat was tested only once per day. Tests were performed between 0900 h and 1700 h, and each rat was tested at similar times within and across all experiments. All tests were performed in an experimental room with a ventilation rate of 12 room air changes per h with a wireless controlled lighting system programmed to deliver light at 615 nm (red light; Philips HUE Personal Wireless Lighting BR30 LED, Koninklijke Philips, AMS, Netherlands). The oxygen analyzer was kept on during all habituation and testing sessions. For all experiments, habituation, and training, rats were individually transported into the experimental room in a transport cage covered with black plastic. Once in the experimental room, rats were left in the transport cage undisturbed for 5 min. Subjects were isolated from cage-mates for a maximum of 40 min per day.

2.1.3 Experiment 1: forced exposure

2.1.3.1 Apparatus

Forced exposure tests were performed in plastic cages (20 x 45 x 24 cm) with bedding (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA), covered with an acrylic glass lid that contained a gas inlet, a gas-sampling hole, two air outlets (covered with a mesh), and a metallic tea ball attached between the air outlets (Fig 2.1 a).

CO_2 was delivered from compressed gas cylinders (Praxair, BC, Canada), through a clear vinyl tube inserted in the gas inlets. Gas flow was regulated using a flow meter (CO_2: Western Medica, OH, USA). A wall-mounted outlet (Amico Corporation, ON, Canada) delivered O_2 through a clear vinyl tube inserted in the gas inlets; flow was regulated with a flow meter

integrated in an anesthetic machine (VetEquip, Inc., CA, USA), with no anesthetic used. The sampling tube was attached to an oxygen analyzer (Series 200, Alpha Omega Instrument Corporation, RI, USA).

2.1.3.2 Experimental design

Twelve rats were exposed during four consecutive days, once to each of three treatments: CO_2 gradual-fill (18.5% chamber vol. min^{-1}), oxygen (O_2; 3.5 L min^{-1}) gradual-fill (as a control), and fox scent (as a passive response eliciting stimulus; TMT at 5 µl at 3.87 µmol, Scotts Miracle-Gro Company, OH, USA) (Wallace and Rosen, 2000). As a part of another study, rats were also exposed to a bleach treatment (2 ml; The Clorox Company, CA, USA; results are not reported further but experimental procedures are provided in Appendix A: Bleach treatment). Order of exposure was allocated using three 4x4 Latin squares (four rats and four treatments: CO_2, O_2, fox scent and bleach). Three days later, the same twelve rats were re-exposed to the treatments, allocating treatment order in three different 4x4 Latin squares (a timeline of the experiments is presented in Appendix B: Experiments timeline).

2.1.3.3 Testing procedure

Rats were individually placed in the experimental cage covered with the baseline lid, and remained there for 5 min. The lid was then replaced with the experimental lid. For the fox scent treatment, the tea ball containing filter paper with 5 µl of fox scent was attached to the experimental lid. For CO_2 and O_2 treatments, the tea ball attached to the experimental lid was empty. After the experimental lid was in place the gas flow started. Tests were stopped when CO_2 reached 25% in the experimental cage, after 120 s in O_2 tests and after 15 min in the fox

scent treatment. After this last treatment no tests were performed in the room for at least 20 min to allow the ventilation system to make a minimum of four complete room air changes.

After each test, the experimental cage and lids were cleaned with Quatricide (Pharmacal Research Laboratories, Naugatuck, CT, USA), rinsed with water, cleaned with ethanol, and bedding was replaced. All forced exposure tests were performed under red light.

2.1.3.4 Behavioral observations

All forced exposure tests were video recorded. The videos were divided into baseline (60 s before any test) and initial response periods (first 60 s of the test). In the fox scent treatment during re-exposure, two animals were excluded because of lost video. For all treatments, videos were scored using Solomon (Solomon coder Version beta 15.11.19). A trained observer, blind to rat identity and treatment, recorded active and passive behavioral responses (Table 2.1). To estimate inter-observer reliability, another independent observer, again blind to treatment scored 20 of the videos. Inter-observer reliability was assessed using Pearson correlation tests following Martin and Bateson (2007; rearing: $r = 0.91$, line-crossing: $r = 0.77$, immobility time: $r = 0.99$, bedding manipulation: $r = 0.76$; lid-pushing was too rare to assess).

Table 2.1 Description of active and passive behavioral responses of rats during forced exposure.

Type of response	Behavior	Description
Active	Rearing	Raising the upper body on the hind limbs, in a vertical position with both front

Type of response	Behavior	Description
		paws off the ground (frequency)
	Line-crossing	Horizontal locomotor activity that results in the rat's forepaws crossing a line that divides the length of the chamber in half (frequency)
	Lid-pushing	Push at the cage lid with the nose or front paws (frequency)
	Bedding manipulation	Displacement (pushing, shoveling, flicking, or digging) of bedding material with front and/or back paws (frequency)
Passive	Immobility time	Absence of movement, except for small and slow lateral movements of the head between frames.

Type of response	Behavior	Description
		Behavior measured as time(s) spent immobile

2.1.4 Experiment 2: aversion-avoidance

2.1.4.1 Apparatus

The aversion-avoidance apparatus consisted of an acrylic glass light-dark box consisting of two compartments (14 x 27 x 30 cm each), connected by a smaller buffer compartment (10 x 14 x 30 cm). The light compartment was covered with white plastic, and illuminated by two bulbs placed above the lid. The bulbs provided a light intensity of 1650 lux, measured at the bottom of the compartment. The dark compartment was covered with opaque black plastic. All compartments contained bedding (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA). Doorways of the buffer compartment were covered with plastic flaps. The light-dark box was covered with an acrylic glass lid. The lid contained a gas inlet in the middle of each compartment, a gas-sampling hole, and a scavenger tube attached to a hole in the middle of the buffer compartment. The portion of the lid corresponding to the dark compartment was covered with opaque black plastic (Fig 2.1 b).

Air was regulated using a flow meter (Dwyer instruments, Inc., NI, USA), and delivered from a compressed gas cylinder through a clear vinyl tube inserted in the gas inlets. CO_2 was regulated and delivered as described for Experiment 1.

2.1.4.2 Habituation and training

Rats were habituated to the light-dark box over four consecutive days. Each subject was placed in the light compartment of the apparatus and left to explore for 30 min. On Day 1, rats were placed in the apparatus under red light. From Day 2 onwards, the light level was 1650 lux in the light compartment. On the third and fourth day, airflow (3.5 L min^{-1}) was delivered in both compartments.

2.1.4.3 Experimental design

The same rats tested in Experiment 1 were use in this experiment. Rats were exposed twice to CO_2 (19% chamber vol. min^{-1}) during two consecutive days (see Appendix B: Experiments timeline).

2.1.4.4 Testing procedure

Rats were individually placed in the bright compartment of the dark-light box and left for 30 min to explore the apparatus with airflow delivered to both compartments. All subjects settled down in the dark compartment for at least 10 min by the end of the 30-min period. CO_2 flow was then started in the dark compartment. The test stopped when the rat moved from the dark to the light compartment (i.e. shoulders crossed from the buffer compartment to the light compartment); the latency to leave the dark chamber was recorded as the dependent variable. The dark-light box was cleaned with Quatricide, rinsed with water, and the bedding replaced after each test.

2.1.5 Experiment 3: approach-avoidance

2.1.5.1 Apparatus

The approach-avoidance apparatus consisted of each rat's bigger home cage placed 20 cm higher (top cage) than a smaller bottom cage (20 x 45 x 24 cm). A transparent acrylic glass tube (10 cm diameter, and 45 cm length), with cleats to prevent slipping, connected the two cages. An acrylic glass sliding door (10 x 10 cm) was attached between the connection tube and the top cage. Both cages contained bedding (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA). The bottom cage was covered with an acrylic glass lid that contained two air outlets, a gas inlet, and a gas sampling tube in the middle of the cage (Fig 2.1 c). CO_2 and O_2 were delivered and regulated following Experiment 1.

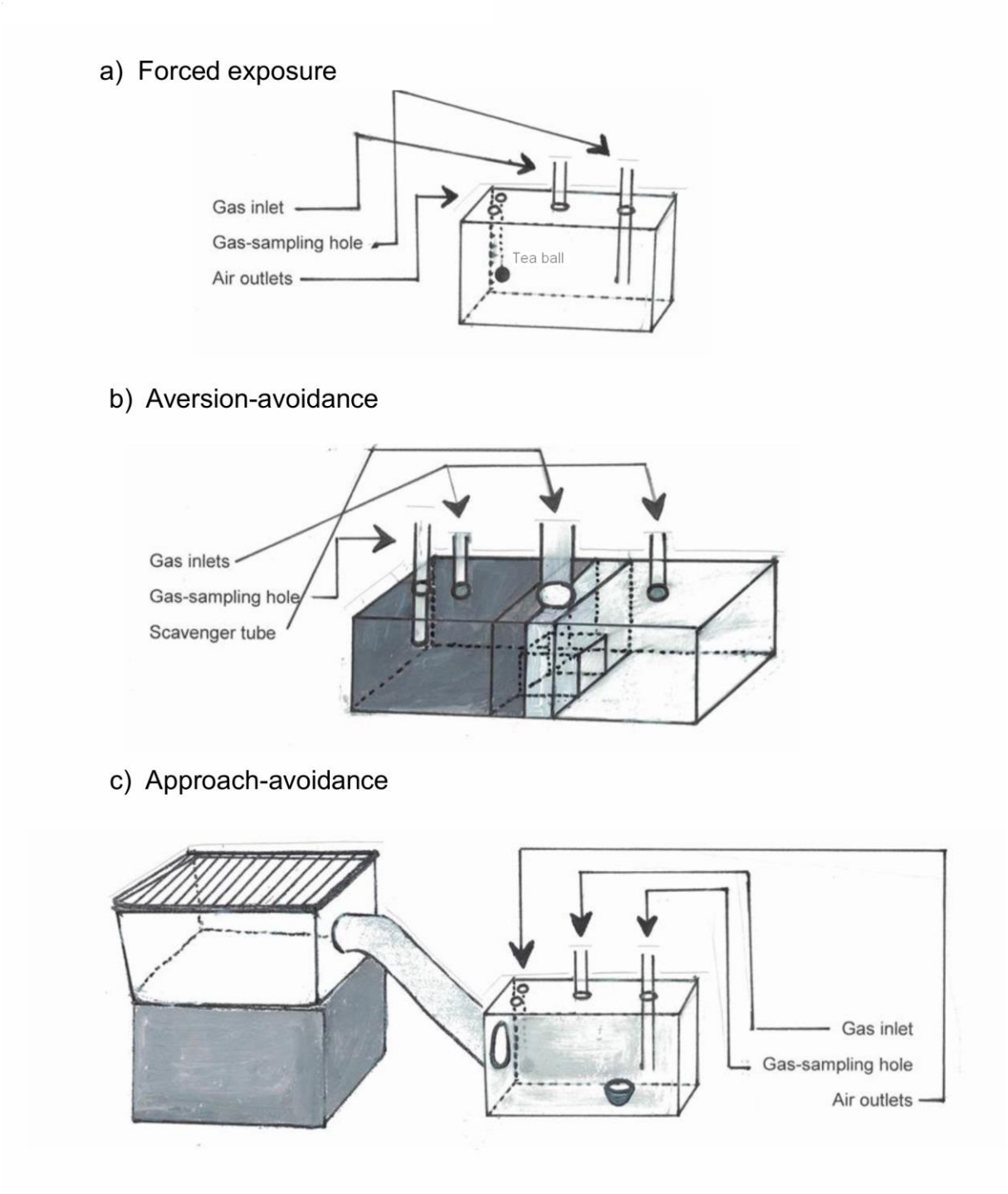


Figure 2.1 Experimental apparatus. Apparatus used in the a) forced exposure, b) aversion-avoidance, and c) approach-avoidance experiments

2.1.5.2 Habituation and training

Rats were trained for approach-avoidance testing for 12 days. Each rat was placed in the top cage of the apparatus and was able to move freely throughout for 5 min. After this period, if the rat was in the bottom cage, it was encouraged to return to the top cage with a reward (one Cheerio; Honey Nut Cheerios TM, General Mills Inc., MN, USA). The rat was kept in the top cage for 2 min by closing the sliding door, and 20 Cheerios were placed in the bottom cage. The sliding door was then opened and the rat was allowed to descend to the bottom cage and eat the Cheerios; as soon as the rat returned to the top cage the sliding door was again closed. O_2 (3.5 L min^{-1}) was introduced into the bottom cage as soon as the rat started eating.

2.1.5.3 Experimental design

The same rats were tested as those used in Experiments 1 and 2. Rats were exposed twice to CO_2 (18.5% chamber vol. min^{-1}).

2.1.5.4 Testing procedure

Rats were introduced into the top cage of the approach-avoidance apparatus and allowed to explore the apparatus for 5 min. Rats were then encouraged to return to the top cage (if not already there) using a Cheerio as a treat, and the door was closed. After 2 min, twenty Cheerios were placed in the bottom cage, and the rat was allowed to descend. Gradual-fill of CO_2 began as soon as the rats started eating the Cheerios. The test stopped once the rat left the bottom cage (i.e. shoulders crossed into the connecting tube); latency to leave the bottom chamber was recorded as the dependent variable. After each test, the bottom cage was cleaned with Quatricide, rinsed with water, and the bedding was replaced.

2.1.6 Assessment of CO_2 concentrations

To describe the changes in CO_2 concentration during the gradual-fill procedure, nine trials were conducted in both the aversion- and approach-avoidance cages with no animals present. CO_2 was introduced into the aversion-avoidance apparatus at a flow rate of 19% chamber vol. min^{-1}. In the approach-avoidance apparatus CO_2 was introduced at 18.5% chamber vol. min^{-1}. The oxygen analyzer, attached to the gas sampling tube (Fig 2.1 b, c), was video recorded during the filling process (5 min). Changes in O_2 were used to estimate CO_2 concentration at each time point using the formula CO_2 (t = x) = 100 – ((O_2 (t = x) * 100) / O_2 (t = 0).

2.1.7 Data analysis

All analyses were conducted with R (R Development Core Team, Version 3.4.1) and RStudio (RStudio, Inc., Version 1.0.136). Results are reported as means ± standard errors.

2.1.7.1 Experiment 1: forced exposure

To compare rat responses between the three different treatments (i.e. CO_2, O_2 and fox scent), we used Linear Mixed Models. The response variables were rearing, line-crossing and immobility time, all expressed as change from pre-exposure baseline. In the models we included treatment, exposure number (exposure and re-exposure) and previous exposure to bleach (the day before the test; 0 = yes, 1 = no) as fixed factors, time of the day (h) as a covariate, and the interaction between treatment and exposure number, previous exposure to bleach and time of the day. We also included rat identity nested within cage as random intercept. The significance of

the random intercept was assessed though the likelihood ratio test (LRT). Tukey post hoc tests were used to explore significant effects. Normality of the residuals was visually assessed.

To assess consistency of rat responses between exposures within treatment, we used Pearson correlation (CO_2: rearing and line-crossing; fox scent: immobility time) or Kendall rank correlation with normal approximation and continuity correction for ties if responses were not normally distributed (CO_2: immobility time; fox scent: rearing and line-crossing). Consistency between treatments is not reported due to low consistency within fox scent treatment.

2.1.7.2 Experiments 2 and 3: aversion- and approach-avoidance

To explore variability in the strength of aversion to CO_2 within each aversion test, two Linear Mixed Models were used with the response variable latency to avoid CO_2. The models included exposure (exposure vs. re-exposure) as a fixed factor, time of the day as a covariate, and rat identity nested within cage as random intercept. We evaluated the significance of the random intercept though LRT. Normality of the residuals was visually assessed.

Consistency within aversion- and approach-avoidance tests was assessed using Pearson correlation. The average latency to avoid CO_2 per rat in each test was used to analyze the relationship between aversion- and approach-avoidance tests using Pearson correlation. Within rat, the average rearing during CO_2 forced exposure (for exposure and re-exposure; as these were found to be consistent) was compared with the average latency to avoid CO_2 in aversion- and approach-avoidance tests (for exposure and re-exposure; again consistent), using Pearson correlation.

2.2 Results

2.2.1 Active and passive responses during forced exposure

Lid-pushing was rare; one rat pushed four times during the first exposure to CO_2. Bedding manipulation was observed in one trial during baseline testing, and in 6 trials during the first exposure (2 rats for CO_2, 3 rats for O_2, and 1 rat for fox scent); the frequency of manipulation within test ranged between 1 and 6. These variables were not further analyzed.

We found a tendency for an interaction between treatment (i.e. CO_2, O_2, and fox scent) and exposure number (i.e. exposure and re-exposure) for rearing behavior ($F = 3.05$, $df = 2, 42$, $p = 0.06$). Post hoc analysis showed that the change in rearing (from baseline) tended to be greater during exposure and was significantly greater during re-exposure with CO_2 than with O_2 (exposure: $p = 0.07$; re-exposure: $p < 0.001$). Rearing behavior was greater during CO_2 exposure and re-exposure than during fox treatment (exposure: $p < 0.01$; re-exposure: $p < 0.0001$). No differences were detected between O_2 and fox scent treatments for the change in rearing from baseline during exposure and re-exposure (Fig 2.2 a). We found no effects of time of the day ($F = 0.61$, $df = 1,42$, $p = 0.44$) and previous exposure to bleach ($F = 0.66$, $df = 1,42$, $p = 0.42$), and no evidence for an interaction between treatment and these variables (time of the day: $F = 0.25$, $df = 2,42$, $p = 0.78$; previous exposure to bleach: $F = 0.09$, $df = 2,42$, $p = 0.92$). Cage and rat identity nested in cage accounted for little of the variation in this behavior (cage: ~0% of the variation; Likelihood Ratio Test: $LR < 0.0001$, $p \sim 1$; rat identity nested in cage: 5.5% of the variation, $LR = 0.36$, $p = 0.55$).

The effect of treatment was significant for line-crossing behavior ($F = 30.11$, $df = 2, 42$, $p < 0.001$), with no effect of exposure number ($F = 2.41$, $df = 1,42$, $p = 0.13$), time of the day ($F = 0.51$, $df = 1,42$, $p = 0.48$) or previous exposure to bleach ($F = 0.54$, $df = 1,42$, $p = 0.47$). There

was no interaction between treatment and exposure number ($F = 0.35$, $df = 2,421$, $p = 0.70$), time of the day ($F = 0.37$, $df = 2,42$, $p = 0.69$), or previous exposure to bleach ($F = 1.18$, $df = 2,42$, $p = 0.32$). The change in line-crossing (from baseline) was greater for CO_2 than during O_2 or fox scent ($p < 0.0001$ and $p < 0.0001$, respectively), with no difference between O_2 and fox scent treatments (Fig 2.2 b). The random intercept accounted for little variation in line-crossing (cage number: 2.8% of the variation, $LR = 0.23$, $p = 0.63$; rat identity nested in cage: ~0% of the variation, $LR < 0.0001$, $p \sim 1$).

We found a significant interaction between treatment and exposure number ($F = 4.11$, $df = 2,42$, $p < 0.05$). Post hoc analysis showed that the change in immobility was no different between treatments during exposure (Fig 2.2 c). During re-exposure, rats showed less increase in immobility during CO_2 than during O_2 and fox scent treatments ($p < 0.01$ and $p < 0.001$, respectively). We found a significant interaction of treatment and time of the day on immobility ($F = 4.93$, $df = 2,42$, $p < 0.05$). For CO_2 and O_2 treatments, change in immobility as a function of time of the day was not significant (CO_2: $\beta = 1.53$, $t = 1.05$, $df = 42$, $p = 0.30$; O_2: $\beta = -2.60$, $t = -1.86$, $df = 42$, $p = 0.07$); during fox scent treatment, immobility time decreased with time of the day ($\beta = -4.16$, $t = -2.56$, $p < 0.05$). The effect of previous bleach exposure was not significant ($F = 0.21$, $df = 1,42$, $p = 0.65$) and we found no interaction between treatment and previous bleach exposure ($F = 0.17$, $df = 2,42$, $p = 0.84$). The random intercept accounted for little variation in this response (cage number: 12% of the variation; Likelihood Ratio Test: $LR = 2.9$, $p = 0.15$; rat identity nested in cage: ~0% of the variation, $LR < 0.0001$, $p \sim 1$).

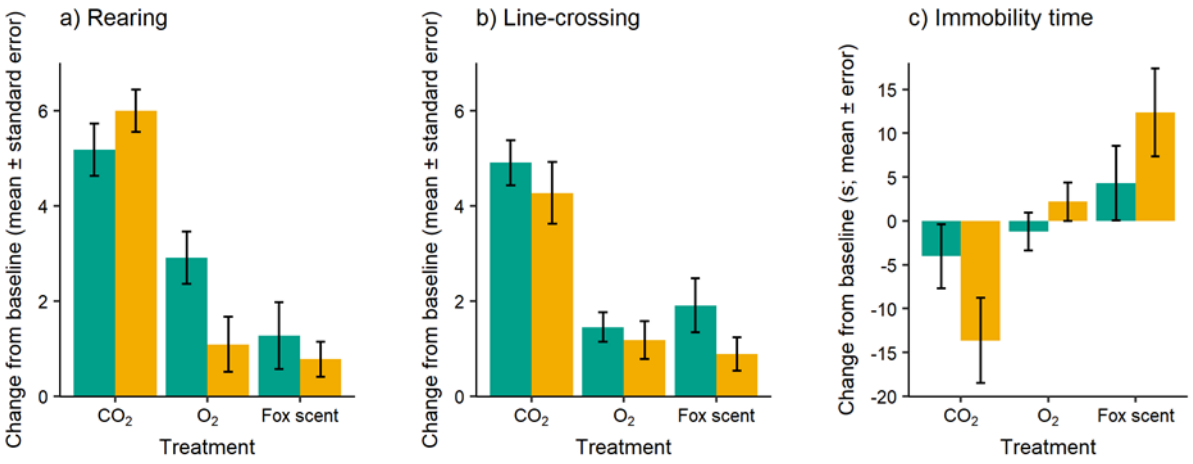


Figure 2.2 Responses to forced exposure. Rat behavior during exposure (green bar) and re-exposure (orange bar); n = 11 rats for all conditions except for n=9 rats fox scent re- exposure.

2.2.2 Within- and between-treatment consistency in active and passive responses

Rats were individually consistent in their rearing responses across two exposures to CO_2 (Pearson correlation test: r = 0.62, df = 9, $p < 0.05$), but line-crossing and immobility time were not consistent (line-crossing: r = -0.13, df = 9, p = 0.71; immobility time Kendall rank test: tau = -0.17, p = 0.58). We found little evidence of consistency for rearing, line-crossing and immobility time within fox scent treatment (rearing: tau = 0.10, p = 0.82; line-crossing: tau = -0.37, p = 0.25; immobility time: r = 0.52, df = 7, p = 0.15).

2.2.3 Consistency in the strength of aversion to CO_2

During the last O_2 training trial in the approach-avoidance task, rats left the cage after 237 ± 27 s. All rats avoided CO_2 before any signs of ataxia in the aversion- and approach-avoidance tests.

During the first exposure in the aversion-avoidance test, latency to avoid CO_2 ranged between 17 and 60 s (35 ± 4 s), which corresponds to approximately 8 and 22% CO_2 (15 ± 1% CO_2). During re-exposure, latency to avoid CO_2 ranged between 11 and 70 s (33 ± 6 s), corresponding to approximately 5 and 25% CO2 (14 ± 2% CO_2). Exposure and time of the day had no effect on the latency to avoid CO_2 in the aversion-avoidance test (exposure: $F = 0.62$, $df = 1, 9$, $p = 0.45$; time of the day: $F = 1.24$, $df = 1,9$, $p = 0.29$).

For the approach-avoidance test, latency to avoid CO_2 ranged between 11 and 54 s (23 ± 4) during the first exposure and between 9 and 47 s (28 ± 4 s) during the second exposure. These latencies correspond to approximately 4 and 19% CO_2 (9 ± 2% CO_2) during the first exposure, and 3 and 17% CO_2 (11 ± 1% CO_2) during re-exposure. No effect of repeated exposure or time of the day was detected on the latency to avoid CO_2 (exposure: $F = 2.52$, $df = 1,9$, $p = 0.15$; time of the day: $F = 0.14$, $df = 1,9$, $p = 0.72$).

Cage was accounted for little variation in the latency to avoid CO_2 in aversion- (15%) and approach-avoidance (~0%) tests (aversion-avoidance: $LR = 0.11$, $p = 0.74$; approach-avoidance: $LR < 0.0001$, $p \sim 1$). Rat identity nested within cage explained 73% ($LR = 13.52$, $p < 0.001$) and 66% ($LR = 6.22$, $p < 0.05$) of the variation in the latency to avoid CO_2 in the aversion- and approach-avoidance tests, respectively. Within aversion tests, the latency to avoid CO_2 was consistent (aversion-avoidance: $r = 0.88$, $df = 9$, $p < 0.001$; approach-avoidance: $r = 0.69$, $df = 9$, $p = 0.02$; Figs 2.3 a and 2.3 b). However, aversion to CO_2 was not correlated between aversion- and approach-avoidance tests ($r = 0.29$, $df = 9$, $p = 0.38$; Fig 2.3 c).

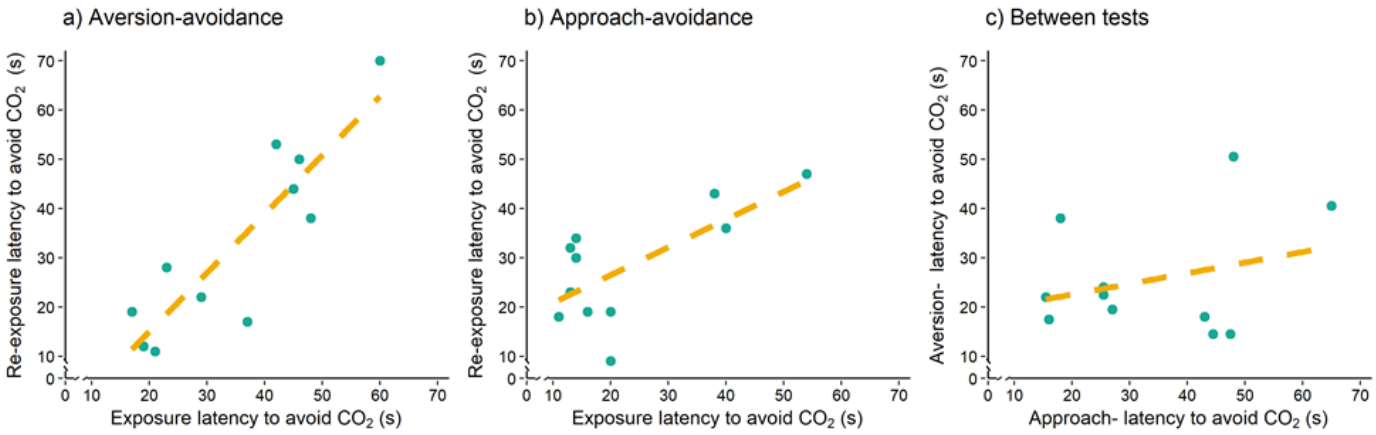


Figure 2.3 Within aversion tests consistency. Within-tests consistency between exposure and re-exposure on the latency to avoid CO_2 in a) aversion-avoidance (n = 11 rats); b) approach-avoidance (n = 11 rats) and c) average latency to avoid CO_2 between aversion- and approach-avoidance tests (n = 11 rats).

2.2.4 Responses to forced exposure and strength of aversion to CO_2

Average rearing during forced exposure to CO_2 was negatively correlated with latency to avoid CO_2 in the aversion-avoidance test ($r = -0.62$, $df = 9$, $p = 0.04$; Fig 2.4 a). There was less evidence of a negative relationship between rearing and latency to avoid CO_2 in the approach-avoidance test ($r = -0.49$, $df = 9$, $p = 0.13$; Fig 2.4 b).

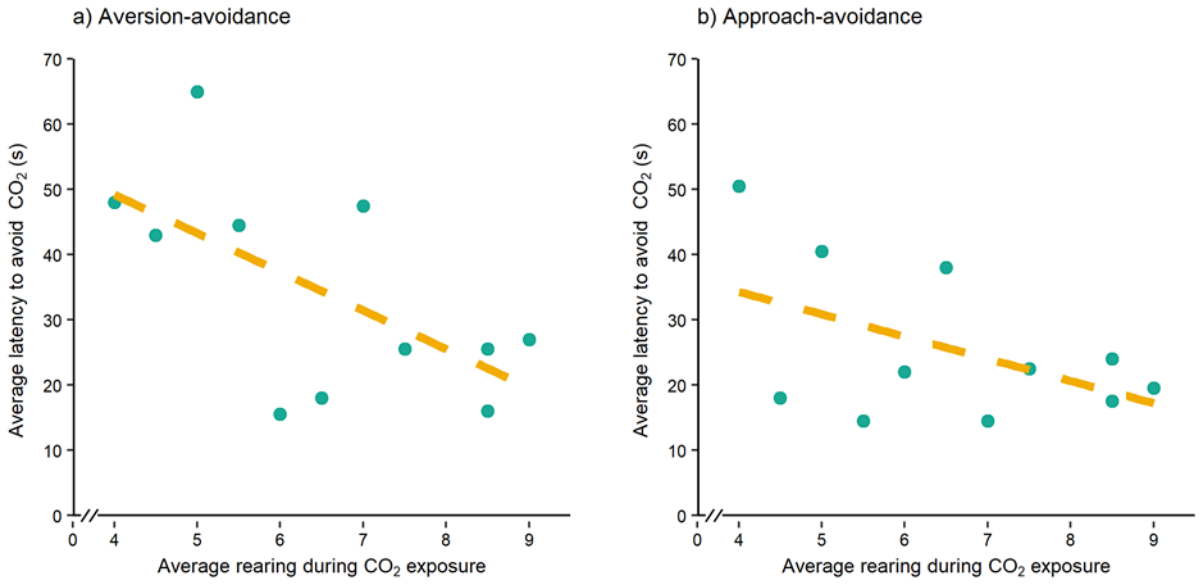


Figure 2.4 Forced exposure and strength of aversion. Relationship between the average frequency of rearing during forced exposure to CO_2 and the average latency to avoid CO_2 in the a) aversion-avoidance (n = 11 rats) and b) approach-avoidance tests (n = 11 rats).

2.3 Discussion

2.3.1 Active and passive responses during forced exposure

In agreement with another study using similar flow rates (~ 17% CO_2 chamber vol. min^{-1}) and air exposure as a control treatment (Niel and Weary, 2006), we found that the change from baseline in rearing and locomotion was higher with CO_2 than the O_2 tratment. The change in locomotion (line-crossing) from baseline was approximately 3.5 and 3 times higher during CO_2 exposure and re-exposure, respectively, compared to O_2 treatment; this change from baseline was 3 times greater than during fox scent exposure and re-exposure. The change from baseline for rearing was approximately 2 and 5 times higher during CO_2 exposure and re-exposure,

respectively, compared to O_2 treatment; this change from baseline was 4 and 5 times greater than during fox scent exposure and re-exposure, respectively.

We found no evidence of increased immobility during CO_2 exposure, and the change from baseline in this measure was lower with CO_2 than with O_2 and fox scent treatments during re-exposure. The lack of increase in passive responses during CO_2 exposure may be due to the strain used in this experiment. Winter et al. (2017) reported that when exposed to 10% static CO_2, Long Evans responded with higher immobility times than Wistar and Sprague Dawley rats. However, within the same strain variation between studies still exists. Sprague Dawley rats exposed to the CO_2 challenge (rapidly increasing concentration stabilizing at 20% CO_2 after 5 min), increased active but not passive responses in one study (Hickman et al., 2016) but showed a decreased response in others (Johnson et al., 2005a, b). Strain differences in responses to fox scent have also been reported. Sprague Dawley and Long Evans rats increased immobility when exposed to fox scent, and this response is greater than that of Wistar rats (Fendt and Endres, 2008; Rosen, West and Donley, 2006). In the current study we found an increase in active responses in Sprague Dawley rats, but other authors using the same strain reported an absence of active responses to CO_2 exposure (Burkholder et al., 2010; Smith and Harrap, 1997). We suggest that strain differences may be important but are unlikely to explain all of the between study differences in active and passive responses.

CO_2 concentration and the possibility of avoiding exposure might also influence responses. The type and intensity of rat defensive behaviors expressed when confronting threatening stimuli is plastic, sensitive to specific features of the stimuli, and situation-dependent. For example, the behavioral responses of rats vary in intensity depending on predator scent concentration (Wallace and Rosen, 2000), but if provided with the opportunity, rats will

actively avoid the scent (McGregor et al., 2002; Vernet-Maury et al., 1992). Passive responses were reported for rats exposed to 10% static CO_2 (Winter et al., 2017). Using the 20% CO_2 challenge, Johnson et al. (2005a, b) report that rats froze when CO_2 concentrations reached around 15%. For rats exposed to a medium flow rate of CO_2, the peak of active responses occurred at around 20% CO_2 (Niel and Weary, 2006). Another study found that when rats were provided the opportunity to escape (in an approach-avoidance experiment) they tolerated 10% static CO_2 for around 5 min and consumed all available food rewards, but at 15% CO_2 rats remained 46 s and consumed only a few of the available food rewards (Niel and Weary, 2007). Other studies have found that when exposed to medium flow rates, rats avoided an average of between 15 to 18.4% CO_2 (Niel and Weary, 2007; Niel et al., 2008 b). It is plausible that at lower inescapable concentrations (between 10 to 15% CO_2) CO_2 elicits freezing, but if an escape route is provided rats will tolerate similar CO_2 concentrations if motivated to do so. However, higher CO_2 concentrations (over 18% CO_2) appear to elicit active responses and are always avoided by rats.

2.3.2 Within- and between-treatment consistency in active and passive responses

We found that rearing was consistent between the first and second forced exposures to CO_2, but not between exposures to fox scent treatment. Rats increased rearing from baseline, indicating that rearing during test was an avoidance-motivated behavior (for a review, see Lever et al., 2006). In addition, previous work using similar flow rates has shown that during the first 20 s of gradual-fill, CO_2 concentrations at the bottom of the cage tended to be 7 % higher than at the top of the cage (Niel and Weary, 2006). Hence, consistency in rearing responses between

exposures suggests that rat motivation to avoid CO_2 during forced exposure, rather than variation in their motivation to explore the cage.

In the current study, forced exposure to CO_2 and fox scent failed to elicit consistent passive responses in rats. Fox scent consisted of the compound TMT which is found in fox feces (Vernet-Maury et al., 1984). Although it has been previously reported that rats respond to TMT with immobility (e.g. Wallace and Rosen, 2000; Endres et al., 2005; Keßler et al., 2012), some studies have found a lack of a response (e.g. Day et al., 2004; Staples and McGregor, 2006). The different factors that could account for the absence of passive responses during forced exposure to fox scent have been reviewed by Fendt and Endres (2008). Since variation in rat coping strategies is characterized by active and passive responses (De Boer and Koolhaas, 2003), the lack of consistency in passive responses during CO_2 exposure suggests that variation in rat responses during CO_2 exposure do not represent general differences in how individuals cope with threatening stimuli; this conclusion is tempered by the lack of consistency in passive responses during the fox scent treatment.

2.3.3 Consistency in the strength of aversion to CO2

In the current study, rats avoided on average 9 and 11% CO_2 in the approach-avoidance test during exposure and re-exposure, respectively. These concentrations are lower than those reported in previous studies using similar flow rates. For example, using medium flow rates (between 15 and 20% CO_2 cage volume min^{-1}) rats avoided on average between 14 and 18% CO_2 (Kirkden et al., 2008; Niel and Weary, 2007; Niel et al., 2008 a). It is possible that tolerance to CO_2 increases with exposure and experience in these tests. When rats were repeatedly exposed to CO_2 medium flow rates in the approach-avoidance test, the average tolerance to CO_2 increased

from ~14% CO_2 in the first three trials, to 18% CO_2 the last exposure (Niel et al., 2008 b). Humans habituate to CO_2, reducing chemoreceptor sensitivity (McMahon et al., 2002) and anxiety (Van den Hout et al.,1987), and increasing the threshold for the onset of air hunger and respiratory response (Li et al., 2006). However, our results also differ from those obtained from naïve rats (~15% CO_2; Niel et al., 2008 b). The rats tested in the current study had previous experience of forced CO_2 exposure. This forced exposure may have affected their willingness to tolerate the gas in the later tests. It has been shown that acute (over 35% static CO_2) exposure to CO_2 produces conditioning which resists extinction in rats (Mongeluzi et al., 2003). It is worth noting that in the current study during O_2 training rats also left the bottom cage earlier than reported in previous studies where training was done with air (between 62 and 74 s earlier; Niel et al., 2008 a; Niel et al., 2008 b). We suggest that the high-oxygen environment created by O_2 flow (as opposed to airflow) was aversive; rats are able to discriminate between different above atmospheric concentrations of O_2 (Arieli, 1990).

Individual variability in strength of aversion to CO_2 may indicate variation in CO_2 sensitivity. Previous studies using approach-avoidance testing have reported between individual variability in CO_2 aversion (Kirkden et al., 2008; Niel et al., 2008 a). We found that rat identity was an important source of variation in CO_2 thresholds of aversion for the aversion- and the approach-avoidance tests. Within each aversion test, latency to avoid CO_2 was consistent between two exposures, and active defense responses during CO_2 forced exposure were associated with latency to avoid CO_2 in the aversion tests.

In the current study we found no evidence of consistency between aversion assessed through approach-avoidance and aversion-avoidance. These results indicate that aside from CO_2 sensitivity, other factors may influence variation in rat aversion to CO_2. There are a number of

situational-elicited individual differences that might account for this variation. In both aversion tests, it was assumed that all rats were strongly motivated to approach or avoid the paired stimuli (sweet rewards and a brightly lit chamber, respectively) used to assess the strength of aversion to CO_2. Food deprived rats are motivated to avoid light exposure (1650 lux) even at the cost of losing a food reward (Barker et al., 2010). In addition, even without food deprivation, rats are highly motivated to approach sweet rewards (Kirkden et al., 2008). However, individual rats vary in their motivation to approach and avoid these paired stimuli. Rats consistently vary in light aversion (Whishaw, 1974) and in their motivation to work for sucrose (Brennan et al., 2001; De Sousa et al., 1998; Tõnissaar et al., 2006). Between-subject variation in aversion- and approach-avoidance tests is likely influenced by motivational differences in addition to CO_2 sensitivity.

2.3.4 Responses to forced exposure and strength of aversion to CO2

In the current study we found that rearing during CO_2 forced exposure was negatively correlated to the latency to avoid CO_2 in the aversion-avoidance test. Consistency in rat responses to CO_2 within testing situations, and between forced exposure and aversion-avoidance tests, provide evidence of rat variation in CO_2 sensitivity. In humans, individuals differ in the type and intensity of the responses when inhaling CO_2 (Colasanti et al., 2008, 2012). For example, anxiety was experienced by 60% of healthy humans during prolonged inhalation of low CO_2 concentrations (7% CO_2 during 20 min), and this experience was consistent between exposures (Poma et al., 2005). Feelings of immobility and desire to flee were experienced by 13% and 20% of healthy individuals, respectively, during shorter exposure to medium CO_2 concentrations (20 s exposure to 20% CO_2; Schmidt et al., 2008). Panic attacks are experienced

by healthy individuals following a double inhalation of 35% CO_2 (Leibold et al., 2013), but panic attacks and anxiety are consistently elicited with a single inhalation of 35% CO_2 in panic disorder patients (Verburg et al., 1998).

2.4 Conclusions

Rats varied consistently in their responsiveness to CO_2 exposure. If these responses relate to the animal's affective states, then the emotional experience when killed with CO_2 may also vary among rats. Overall, our results indicate that variation in rat responses to CO_2 exposure is situation-specific and relate to variation in CO_2 sensitivity. CO_2 concentrations well below those necessary to induce unconsciousness were aversive to all rats, indicating that CO_2 exposure compromises rat welfare even for the least sensitive rats.

Chapter 3: Individual differences in rat sensitivity to CO_2

People report feelings of fear, anxiety, dyspnea and panic during CO_2 inhalation (for a review see Colasanti et al., 2012). This emotional response to CO_2 inhalation is known to vary among individuals. With a double inhalation of 35% CO_2, 68% of healthy humans experience panic attacks (PAs; Leibold et al., 2013), and 43 to 94% of panic disorder (PD) patients experience PAs after a single inhalation of 35% CO_2 (see Colasanti et al., 2012). When inhaling 35% CO_2, the anxiety experienced by healthy people, and the PAs experienced by PD patients, are highly consistent between exposures (Coryell and Arndt, 1999; Verburg et al., 1998). This between subject variation, consistent through repeated exposures, is known as CO_2 sensitivity.

Rats respond to CO_2 exposure with defence behaviours (e.g. Chisholm et al., 2013; Niel and Weary, 2006; Winter et al., 2017), and are motivated to avoid this agent (e.g. Niel, Kirkden, and Weary, 2008; Wong, Makowska, and Weary, 2013; Kirkden et al., 2008). Rats have also been used as translational models for understanding the underlying mechanisms of the emotional response to CO_2 inhalation (Johnson et al., 2015; Leibold et al., 2016).

Rat behavioural responses to CO_2 are highly variable. For example, during forced (i.e. unavoidable) exposure to CO_2 "escape attempts" were reported to range between 0 to 34 among rats (Niel and Weary, 2006), with 50% of the rats showing an increase in locomotion (Niel, Kirkden, and Weary, 2008), and 20% of the rats moving around the cage perimeter (Smith and Harrap, 1997). Aversion to CO_2 is also variable among rats. For example, in one study the latency to avoid CO_2 varied between individuals from 7 s to 48 s in an aversion-avoidance setting (in which the cost of avoiding the CO_2 delivered in a dark compartment was escaping to a CO_2-free compartment that was brightly lit; Wong, Makowska, and Weary, 2013). In an

approach-avoidance setting (in which the cost of escaping to a CO_2-free compartment was the loss of sweet rewards), the threshold of aversion ranged between 11 to 18.6% CO_2 between rats (Kirkden et al., 2008; Niel, Kirkden and Weary, 2008). In more recent work we found that variation in rat behaviour was consistent between two exposures to CO_2, in each of three testing situations (i.e. forced exposure, aversion- and approach-avoidance), and we have found that rats that consistently showed higher responses during CO_2 forced exposure were consistently less tolerant to CO_2 when tested in aversion-avoidance (Chapter 2). These results suggest that variation in rat responses to CO_2 is linked to consistent individual differences in CO_2 sensitivity; i.e. like humans, rats may vary in the emotional experience elicited by CO_2. The first aim of the current study was to determine whether individual differences in rat aversion to CO_2 in approach-avoidance tests are stable and consistent through multiple exposures.

Individual differences in aversion to CO_2 could be caused by behavioural differences elicited by testing contingencies specific to the approach-avoidance setting. In approach-avoidance tests, exposure to CO_2 is paired with access to sweet rewards that rats are motivated to approach (Kirkden et al., 2008). An underlying but untested assumption is that the strength of motivation to approach the sweet rewards is similar among rats. However, motivation for sucrose is known to consistently vary among rats (Brennan et al., 2001; DeSousa et al., 1998; Tõnissaar et al., 2006), so it is possible that variation in rat aversion to CO_2 in approach-avoidance tests is due to individual variability in motivation for the sweet rewards. Thus the second aim of our current study was to assess if individual differences in rat responses to CO_2 in an approach-avoidance test are associated with variation in sweet reward motivation.

Following regulatory focus theory (Higgins, 1997), variation in rat behaviour in approach-avoidance could be related to individual differences in the strength of promotion and

prevention motivations. Individuals focused on promotion are more motivated to approach gains and are more sensitive to their presence or absence; individuals focused on prevention are more motivated and sensitive to safety related incentives (e.g. Shah and Higgins, 1997). Promotion and prevention motivations are independent; hence individuals can be high or low in promotion or prevention motivations, or both (e.g. Franks Higgins and Champagne, 2014). These motivational foci are consistent over time in rats (Franks, Higgins and Champagne, 2012; Franks Higgins and Champagne, 2014). Approach-avoidance tests involve both gain (sweet rewards) and safety (CO_2-free cage) incentives. Individual differences in promotion and prevention motivations could account for variation in CO_2 thresholds of aversion. Promotion focused individuals may tolerate CO_2 concentrations to maximize gains, and prevention focused individuals may seek non-threatening CO_2 concentrations to maximize safety. Thus the final aim of this study was to determine if individual differences in regulatory focus are related to variation in CO_2 aversion in approach-avoidance.

3.1 Methodology

3.1.1 Subjects and housing

Twelve female Sprague-Dawley rats were used in this study. Rats were obtained as surplus stock from the University of British Columbia. Rats were 9 months old and weighed 435 ± 65 g (mean ± standard deviation) at the beginning of the experiments. All subjects were marked with a permanent animal marker (Ketchum Manufacturing Inc., ON, Canada) and housed in groups of three on a 12 h light/dark cycle at controlled temperature and humidity (21 ± 0.4 °C and 52 ± 11 %, respectively). The housing system consisted of two cages (20 cm x 50 cm x 40 cm) connected by a red tinted polycarbonate tube (7.6 cm diameter, 15 cm long). Both

cages contained bedding (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA) and environmental enrichment (e.g. cardboard boxes, hammocks, PVC pipes, and shredded paper towels). Rats were provided ad libitum food (Rat Diet PMI 5012, Lab Diets, Land O'Lakes, Inc., MN, USA) and tap water. Rats had daily access to a playpen for 30 min. The playpen was a highly enriched large cage, following the design of the semi-naturalistic cages described by Makowska and Weary (Appendix C: rat playpens).

3.1.2 Handling and transport

Rats used in the current study were habituated to handling during the week before experiments started (Appendix D: agency-based handling and transport). For all sessions, rats were individually transported in a cage covered with black plastic. All habituation, training and test trials were conducted during the light cycle between 900 h and 1700 h. Each rat was habituated, trained, or tested only once per day at similar hours each day across all tests, and only separated from cage-mates for a maximum of 40 min per day. Test cages and the apparatus were cleaned with a combination of water and isopropanol (70%), and bedding was replaced before the beginning of next session.

3.1.3 Experiment 1: repeatability of aversion to CO_2

3.1.3.1 Apparatus

An approach-avoidance apparatus was used to assess repeatability in aversion to CO_2 through multiple exposures. The apparatus consisted of a bigger top cage (one of the rat home cages) elevated 20 cm higher than a smaller bottom cage. Both cages contained bedding. The top cage was connected to the bottom cage by a transparent acrylic glass tube. The inside of the tube

contained plastic cleats to prevent rats from slipping. A plastic sliding door was attached to the entrance of the connecting tube at the top cage end. The top cage was covered with a wire lid, and the bottom cage was covered with a clear acrylic glass lid with two scavenging outlets and a gas inlet (Figure 3.1 a).

Two flow meters (CO_2: Western Medica, OH, USA; air: Dwyer instruments, Inc., NI, USA) were used to regulate gas flow. Gases (air and CO_2) were delivered from compressed gas cylinders (Praxair, BC, Canada), through a clear vinyl tube inserted in the gas inlet.

3.1.3.2 Habituation, training and testing procedures

Subjects were previously trained in approach-avoidance for another study. Rats were re-trained to eat 20 sweet rewards (Cheerio; Honey Nut Cheerios TM, General Mills Inc., MN, USA) in the bottom cage of the apparatus. At the beginning of each training session, the subject was placed in the top cage and was allowed to explore the apparatus for 5 min, with air (4 L min^{-1}) flowing into the bottom cage at all times. The experimenter then tapped her fingers on the side of the cage, and gave the rat one sweet reward in the top cage, and closed the sliding door to block access to the bottom cage. The sliding door remained closed for 60 s; during this time, 20 sweet rewards were placed in a dish in the bottom cage. The sliding door was then opened and the rat was able to descend into the bottom cage to consume the sweet rewards. The training session ended as soon as the rat left the bottom cage (i.e. shoulders crossed into the tube when exiting the bottom cage). A rat was considered to have met the training criterion when, during three consecutive training trials, it stayed in the bottom cage for 5 min or finished all 20 sweet rewards, whichever occurred first.

Once trained, rats were repeatedly exposed to CO_2 gradual-fill (20% CO_2 cage vol. min^{-1}) in the approach-avoidance apparatus. We ran one control test (air flow of 4 L min^{-1}) after every two CO_2 exposures. During CO_2 tests, the air flow was substituted for CO_2 as soon as the rat started eating the sweet rewards. The test trials ended as soon as the rat left the bottom cage. Latency (s) to exit the bottom cage was recorded by direct observations. If a rat failed to stay for 5 min or eat all 20 sweet rewards in a control test, the previous CO_2 test trial was excluded and the rat was re-trained until the learning criterion was met before continuing in CO_2 tests.

3.1.3.3 Assessment of CO_2 concentrations

With no animal present, twelve CO_2 flow trials were conducted in the approach-avoidance apparatus to assess changes in CO_2 concentration during gradual-fill (18.5% CO_2 chamber vol. min^{-1}). A clear plastic sampling tube, connected to an oxygen analyzer (Series 200, Alpha Omega Instrument Corporation, RI, USA), was introduced to the cage though an inlet placed in the middle of the acrylic glass lid. The oxygen analyzer readings were video recorded during the filling process (5 min). At each time point (every 0.2 s), CO_2 concentrations were estimated from changes in oxygen concentrations using the formula $CO_{2\,(t = x)} = 100 - ([O_{2\,(t = x)} * 100] / O_{2\,(t = 0)}$.

3.1.4 Experiment 2: sweet reward motivation

3.1.4.1 Apparatus

A modified approach-avoidance apparatus was used for this test. During baseline, the apparatus remained the same as described for the approach-avoidance test. During test sessions, the bottom cage was replaced with a new test cage measuring 20 cm x 45 cm x 24 cm. The test

cage contained two ice cube trays with 12 holes each, and was filled with ~ 1 cm of autoclaved sand, and covered with a wire lid (Figure 3.1 b).

3.1.4.2 Training and testing procedure

During sweet reward motivation tests, rats were required to search and dig rewards hidden under a layer of sand; rewards were more dispersed across each consecutive test. Rats were habituated once and tested three times for sweet reward motivation in the modified approach-avoidance apparatus. At the beginning of each session, the subject was placed into the top cage, and the rat could freely move between the top and bottom cages for 5 min. The rat was then called to receive one sweet reward in the top cage, and the sliding door was closed, keeping the rat in the top cage for 60 s. During this period the baseline bottom cage was replaced with the test cage. The test cage contained 20 sweet rewards placed in the ice tray holes and hidden underneath a layer of sand. One sweet reward was left on top of the sand in the middle of the cage. The rat was then allowed to descend to the bottom test cage to search for and consume the rewards. The session ended if the rat left the test cage (i.e. shoulders crossed into the tube exiting the bottom cage) without carrying a sweet reward, or if the subject had left the cage carrying a sweet reward but did not return to the test cage immediately (~ 3 s) once the sweet reward was consumed.

For the training trial, the sweet rewards were distributed in 6 consecutive reward holes of the ice tray, with 3 to 4 sweet rewards per hole. For rats that consumed fewer than 15 sweet rewards during their training trial (n = 5 rats), training was repeated a second time. In first test trial, the sweet rewards were distributed into 9 reward holes, separated by empty holes, with 2 to 3 sweet rewards per hole. In the second test trial, sweet rewards were evenly distributed

throughout the tray with only one sweet reward per reward hole and at least one empty hole between each reward. In the third test trial, the sweet rewards were randomly distributed throughout the tray at coordinates obtained from a random number generator with a maximum of 2 rewards per hole.

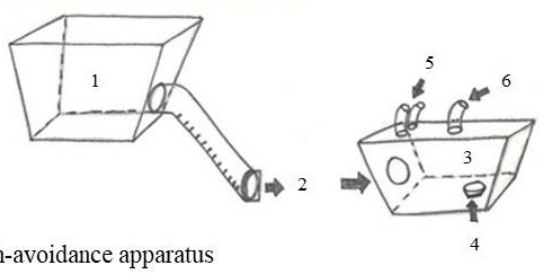
Any rewards remaining were counted at the end of the test. All tests were video recorded and scored using Boris software (Version 7.0.9; Friard and Gamba, 2016). A trained observer, blind to rat identity and test number, scored the videos for the number of sweet rewards consumed and searching time between each consecutive reward found (s). Inter-observer reliability was estimated from 10 videos scored by the trained observer and another independent observer (number of sweet rewards consumed: $r = 0.99$; searching time: $r = 0.99$).

3.1.5 Experiment 3: regulatory focus

3.1.5.1 Apparatus

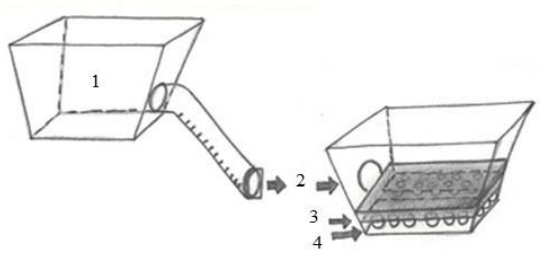
Following Franks et al. (2012), a modified open field arena was used for regulatory focus profiling. The modified open field arena was made of white acrylic glass and contained two smaller acrylic glass boxes placed against the center of two adjacent walls of the arena (treat and dark locations; Figure 3.1c). The arena was illuminated with red light and white light that provided an average light intensity of 82 ± 1.6 lux (mean ± standard deviation) at the center of the arena floor.

a. Approach-avoidance apparatus



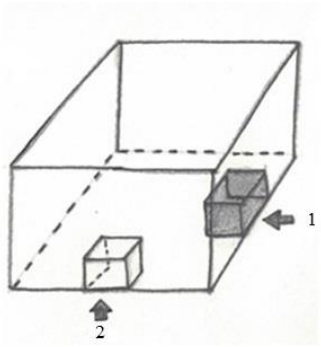
1. Top cage
2. Connecting tube
3. Bottom cage
4. Plate with cheerios
5. Scavenging outlets
6. Gas inlet

b. Modified approach-avoidance apparatus



1. Top cage
2. Connecting tube
3. Sand cover
4. Ice cube trays

c. Modified open field apparatus



1. Dark location
2. Treat location

Figure 3.1 Experimental apparatus. a) Approach-avoidance apparatus used to assess aversion to CO_2, measurements were: the top cage 20 cm x 50 cm x 40 cm, bottom cage 20 cm x 45 cm x 24 cm, connecting tube 10 cm diameter x 45 cm long, and plastic sliding door 10 cm x 10 cm. b) Modified approach-avoidance apparatus used to evaluate motivation for sweet rewards, the test cage measured 20 cm x 45 cm x 24 cm and the ice cube trays 32 cm x 12 cm x 4 cm. c) Modified open field arena used to assess promotion and prevention motivation focus, the arena was made of white acrylic glass (100 cm x 100 cm x 61 cm) and contained two smaller acrylic glass boxes (10 cm3) placed against the center of two adjacent walls of the arena (treat and dark locations).

3.1.5.2 Habituation and testing procedure

Rats were habituated twice and tested twice in the modified open field arena (for order and length of the four experiments, see Appendix E). Before each habituation and test trial, a variety of food rewards (20 Cheerios: Honey Nut Cheerios TM, General Mills Inc., MN, USA; 10 sunflower seeds: Raw Sunflower Seeds, Western Family, Overwaitea Food Group LP., BC, Canada; 1 yogurt drop: Drops Yogurt Flavoured Treats, Living World, Hagen Inc., QC, Canada; 2 peanut M&Ms: Mars Canada Inc., ON, Canada; 20 peanuts: Peanuts Roasted in the Shell, Western Family, Overwaitea Food Group LP., BC, Canada) were placed inside of one of the small boxes (treat location) inside the arena. Rewards were not exclusively sweet to control for rat motivation for sweet rewards. The other small box was left empty (dark location). At the beginning of each trial, the subject was introduced to the modified open field in the farthest corner from and equidistant to the treat and dark locations. Rats were left in the modified open field for 10 min; if the rat approached the dark location (within one body length) the light would turn off for 30 s or until the rat left the dark location, whichever occurred first.

All tests were video recorded. A trained observer, blind to rat identity and test number, scored for the amount of time rats spent within 20 cm of the treat and dark locations (s), using Boris software. Another independent observer scored 4 videos to estimate inter-observer reliability (treat location time: $r = 0.99$; dark location time: $r = 0.99$).

3.1.6 Data analysis

Analyses were carried out with R (R Development Core Team, Version 3.4.1) and RStudio (RStudio, Inc., Version 1.0.136). The model residuals and data were visually assessed for normality. Results are reported as means ± standard errors.

3.1.6.1 Experiment 1: repeatability of aversion to CO_2

To assess changes in aversion to CO_2 through repeated exposures, we used a Linear Mixed Model (with compound symmetrical correlation structure) with the response variable latency to avoid CO_2 in approach-avoidance tests, exposure number as a fixed factor, and rat identity as random intercept. We compared the fit of this model to models with different variance-covariance structures (i.e. autoregressive and autoregressive with heterogeneous variances; Pinheiro and Bates, 2006). The model with compound symmetrical correlation structure had the best fit (i.e. the lowest Akaike's Information Criterion values; data not reported). The significance of the random intercept was evaluated though the likelihood ratio test (LRT). Repeatability (R) of latency to avoid CO_2 across exposures was estimated following Nakagawa and Schielzeth (2010). CO_2 concentrations avoided by rats in each test were estimated using the average CO_2 value corresponding to the time point at which rats exited the cage (i.e. latency to avoid CO_2).

3.1.6.2 Experiment 2: sweet reward motivation

To assess individual differences in sweet reward motivation, the total number of rewards consumed, and the total searching time were included as response variables in two Linear Mixed Models. In the models, test number was included as fixed factor and rat identity as a random

intercept. LRTs were used to assess the significance of the random intercept, and repeatability (R) across tests was assessed.

We then estimated the average number of sweet rewards consumed and total searching time per rat across tests. The relationship between the two measures of rat motivation for sweet rewards and the average latency to avoid CO_2 in approach-avoidance tests was assessed using Kendall rank correlation.

3.1.6.3 Experiment 3: regulatory focus

To assess consistency in promotion (and prevention) focus, we used Pearson correlation to examine the percentage of time spent in the treat (and dark) location in the two test trials. Then, for each rat, we estimated the average percentage of test time spent in the treat (and dark) location across the two tests. We used Kendall rank correlation to assess the relationship between promotion (and prevention) focus and the average latency to avoid CO_2.

3.1.6.4 Sample size

Three rats failed to meet training criterion after six training trails in approach-avoidance. The remaining nine rats were tested with CO_2; hence the sample size for Experiment 1 was nine rats. Due to repeated failure to meet training criterion during control trials (four consecutive tests), not all rats were tested with CO_2 for the same number of exposures (4 exposures: n = 1 rat, 7 exposures: n = 1 rat, 9 exposures: n = 5 rats, 10 exposures: n = 2 rats). For experiments 2 and 3 we tested 11 rats – one of the rats tested in approach-avoidance showed aversion to handling (i.e. failed to follow handling procedures) and was excluded from these experiments. To assess the relationship between aversion to CO_2, sweet reward motivation and regulatory focus, the sample

size was eight rats. Each rat was assigned an identification number (rat 1 to 12) that was maintained across all experiments.

3.2 Results

3.2.1 Experiment 1: repeatability of aversion to CO_2

During control trials, rats left the cage after 5.6 ± 0.2 min and ate all 20 Cheerios. In all test trials, rats avoided CO_2 before recumbency. We found no evidence that the latency to avoid CO_2 differed between repeated exposures ($F = 1.06$, $df = 9, 58$, $p = 0.41$; n = 9 rats). Rat identity explained 40% of the variation (Likelihood ratio test: $LR = 13.80$, $p < 0.001$; n = 9 rats) in the latency to avoid CO_2. Latency to avoid CO_2 was repeatable across exposures ($R = 0.44$, $p < 0.0001$; n = 9 rats; Figure 3.2). The average CO_2 concentrations avoided ranged between 4.5 and 15% among rats.

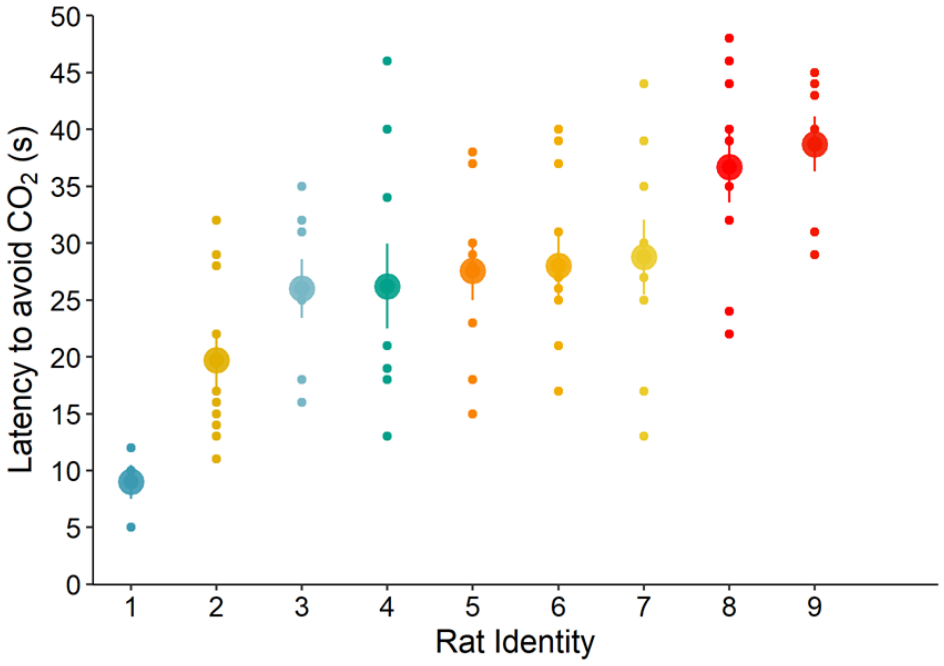


Figure 3.2 Individual differences in aversion to CO_2, arranged from the least to the most tolerant rat. Dots represent the average and error bars correspond to the standard error of the latency (s) to avoid CO_2 across repeated exposure by each subject (n = 9 rats). Latencies corresponded to 4.5, 8.8, 10.9, 11.0, 11.4, 11.6, 11.8, 14.3, 15% CO_2. Rats corresponding to numbers 10, 11 and 12 failed to meet the training criterion hence are not represented in the figure.

3.2.2 Experiment 2: sweet reward motivation

Test number did not affect rewards consumed or total searching time (sweet rewards consumed: F = 0.33, df = 2, 20, p = 0.72; searching time: F = 0.19, df = 2, 20, p = 0.83; n = 11 rats; Figure 3.3). Rats spent on average 174 ± 17.2 s searching for rewards, and consumed on average 14.2 ± 1.04 sweet rewards. The random intercept (rat identity) explained 56% (LRT = 9.9, p < 0.01, n = 9 rats) and 60% (LRT = 11.61, p < 0.001, n = 9 rats) of the variation in searching time and rewards consumed, respectively. Searching time and sweet rewards

consumed were repeatable (searching time: R = 0.56, p < 0.0001; sweet rewards consumed: R = 0.60, p < 0.001; n = 9 rats).

Average latency to avoid CO_2 was not related to searching time (tau = 0.07, p = 0.9; n = 8 rats) or rewards consumed (tau = 0.14, p = 0.71; n = 8 rats) in sweet reward motivation tests.

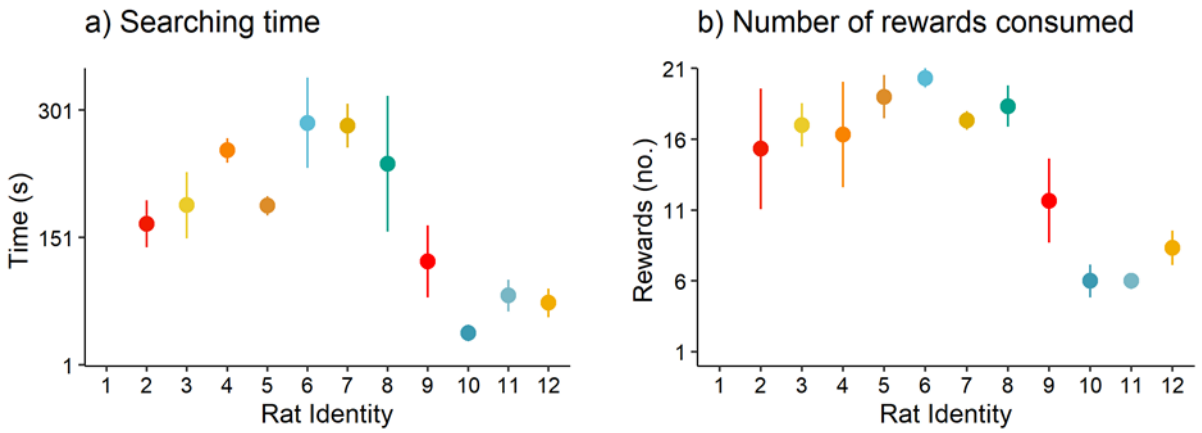


Figure 3.3 Individual differences in sweet reward motivation. Panels shows individual rat (n = 11) mean (± SE) a) searching time, and b) rewards consumed, across three sweet reward motivation tests. Rat identity follows that shown in Figure 3.2. Rats 10 to 12 failed to meet training criterion in approach-avoidance, and rat 1 was excluded due to aversion to handling.

3.2.3 Experiment 3: regulatory focus

Rats spent on average 31% and 51% of the test time in the treat and dark locations, respectively. Across the two test trials, rats consistently varied in the percentage of time spent in these locations (treat: r = 0.80, p < 0.01; dark: r = 0.81, p < 0.01; n = 11 rats; Figure 3.4). The latency to avoid CO_2 was not related to the percentage of time spent in the treat (tau = -0.36, p = 0.27, n = 8 rats) or dark locations (tau = 0.29, p = 0.39, n = 8 rats).

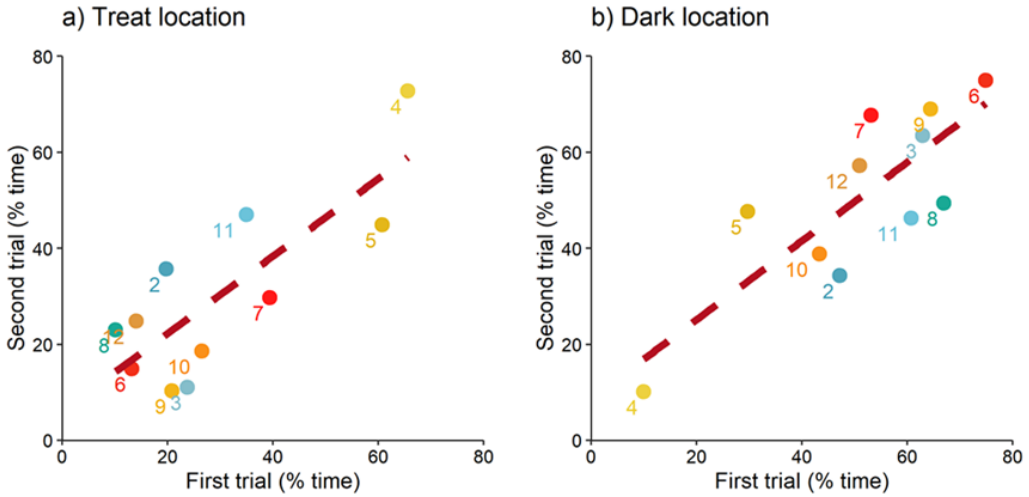


Figure 3.4 Regulatory focus consistency for individual rats (n = 11) tested in two tests. Results are shown separately for measures of a) treat location time (promotion motivation), and b) dark location time (prevention motivation). Numbers represent rat identity following that shown in previous figures.

3.3 Discussion

In agreement with previous studies by our research group (Chapter 2), we found that rat aversion to CO_2 consistently varied among rats, ranging between 4.5 and 15% CO_2. To our knowledge, the current study is the first to show that rat individual thresholds of aversion to CO_2 are stable and highly repeatable across repeated exposures (R = 0.44: the average repeatability estimates across behaviors and among taxa has been shown to be of 0.37; Bell et al., 2009).

Previous studies have reported high between-individual variation in rat responses to CO_2 during forced exposure (Niel and Weary, 2006; Niel, Kirkden and Weary, 2008; Smith and Harrap, 1997) and choice (Leach et al., 2002, 2004) and aversion tests (Kirkden et al., 2008; Niel, Stewart and Weary, 2008; Wong, Makowska and Weary, 2013). Nonetheless, in these studies, the source of this variation was not explained. Rats defence behaviours are plastic, varying with environmental familiarity (i.e. habituation; e.g. Fernandes and File, 1996; Romero

and Chen, 2004; Treit, Menard and Royan, 1993), situational contingencies (for example, threat proximity and possibility to escape; Eilam, 2005; Blanchard, Fukunaga and Blanchard, 1976; McGregor et al., 2002), and with specific conditions prior or during testing (Archer, 1973; Davis and Pérusse, 1988; Eilam, 2003; Lapin, 1995; Pritchard, van Kempen and Zimmerberg, 2013; Walsh and Cummins, 1976). Behavioural plasticity could account for the between-individual variation in rat responses to CO_2 previously reported. Individual thresholds of aversion to CO_2 did not change over repeated exposures, indicating that this response is less sensitive to habituation.

Behaviours consistent across time and contexts are often referred to as personality traits. These individual differences are more or less permanent characteristics that distinguish individuals from one another (see Dingemanse et al., 2010; Réale et al., 2007). For example, within the same strain, the degree to which rats explore novel environments is consistent between four and eight months of age (Cavigelli and McClintock, 2003). Individual differences in this behavioural trait are heritable; two rat lines, originated from individuals that differed in active avoidance acquisition (i.e. Roman high avoidance and Roman low avoidance), consistently differ in their degree of exploration of novel environments (see Steimer et al., 1997). Our results showed that rat thresholds of aversion to CO_2 vary among individuals and are highly repeatable, and can be considered a lasting characteristic of the individual (i.e. personality trait).

In the current study we tested individual differences in motivation to access a sweet reward using a modified approach-avoidance apparatus. Traditionally, rat motivation is measured through a progressive ratio schedule, in which the cost of gaining a reward is increased. Animals continue to invest as the required effort increases, until the cost is higher than the value of the reward (Hodos, 1961). In the current study, rat motivation was assessed in an experimental

setting similar to that used to assess differences in foraging behaviour in wild rodents (e.g. Kotler and Brown, 1990; Smith, 1995). The individual's motivation to engage in a goal directed behaviour, like searching for food, is affected by expectancy and value (Wigfield and Eccles, 2000; Tolman, 1955). Motivation is expected to be higher as the likelihood of success and the value of the resources increase. When foraging in a patch, animals experience a decrease in the rate at which resources are found as they consume the available items (diminishing returns); this decrease imposes a trade-off between investing time searching for resources or leaving the patch (marginal value theorem; Charnov, 1976). In this experimental setting rats searched and dug to gain access to sweet rewards that were hidden in a layer of sand, with rewards being progressively more dispersed across tests. The assumption was that rats would invest time searching and digging to gain access to the rewards until the required effort surpassed the value of the reward.

We found that rat motivation for sweet rewards varied among individuals. For example one rat invested on average 5 min searching and consumed all rewards, and another one consumed on average 6 rewards and spent less than 40 s searching. We also found that motivation for sweet rewards was highly repeatable and not affected by repeated testing. These results align with what has been reported in the literature; rat preference and motivation for sucrose is a stable personality trait (Sills and Vaccarino, 1996; Tõnissaar et al., 2006) that does not change with repeated testing (Tõnissaar et al., 2006). High sucrose consumers ingest over two times more sucrose than low consumers (DeSousa et al., 2000, 1998; Gosnell, 2000), but among-individual variation in sucrose preference is not related to variation in food consumption (DeSousa et al., 2000). Under a progressive ratio schedule, high consumers work harder to earn sucrose than do low consumers (Brennan et al., 2001).

Individual differences in rat motivation for sweet rewards were not related to aversion to CO_2. However, it is important to note that rats that showed low motivation for sweet rewards typically failed to meet the training criterion for the approach-avoidance test. This result suggests that a bias of approach-avoidance tests is that only reward-motivated rats are likely to be selected.

In the regulatory focus experiment of the current study rats spent similar time in the treat location, but spent less time in the dark location than reported by Franks and colleagues (2012). Rats consistently varied in their motivation to approach gains (promotion motivation) and pursue darkness (prevention motivation). These results correspond to those previously reported using the same experimental setting. For example, rats that consistently pursued darkness in the modified open field apparatus, consistently spent more time burying a noxious object (Franks, Higgins and Champagne, 2012). High prevention motivated rats avoid risk, but also may approach potential threats in order to maintain safety. In the current study we found no relationship between individual differences in the strength of promotion or the strength of prevention motivation and rat aversion to CO_2. Thus our results indicate that personality differences in regulatory focus are not related to aversion to CO_2 in approach-avoidance.

Human variation in the felt experience due to CO_2 inhalation is well documented. The increase in feelings of anxiety is eight times higher in individuals that are responsive to CO_2, than that of non-responsive individuals (Poma et al., 2005). The feelings of anxiety and experiences of panic due to ~ 7% CO_2 inhalation are consistent between repeated inhalations (Poma et al., 2005; Roberson-Nay et al., 2017). Vulnerability to CO_2-induced anxiety and panic increases in people diagnosed with panic disorder (Gorman et al., 2001; Monkul et al., 2010) and individuals with a first-degree relative diagnosed with panic disorder (Coryell and Arndt, 1999;

Perna et al., 1999; van Beek and Griez, 2000). Thus, human CO_2 sensitivity involves stable individual differences in the emotional response to CO_2. We found that individual differences in rat thresholds of aversion to CO_2 were stable and consistent, and not related to sweet reward motivation or the strength of promotion and prevention motivations. It is likely that individual differences in the emotional responses are the underlying cause of among-rat variation in aversion to CO_2 (i.e. CO_2 sensitivity), indicating that some rats experience a higher emotional response when exposed to CO_2. It is important to note that the specific negative emotions experienced by rats (e.g. fear, anxiety, dyspnea or panic), and how these change with increasing concentrations of CO_2, is still unknown.

3.4 Conclusion

Variation in rat aversion to CO_2 was repeatable across multiple exposures but was not related to individual differences in motivation for sweet rewards, promotion or prevention foci. These results indicate that individual differences in aversion to CO_2 reflects variation in CO_2 sensitivity.

Chapter 4: Variation in the onset of CO_2-induced anxiety

Carbon dioxide (CO_2) is one of the most used methods to kill laboratory rats (Hawkins et al., 2016), but mounting evidence indicates that CO_2 elicits negative affective states. Rats are highly motivated to avoid CO_2 in aversion tests (e.g. Leach et al., 2002, 2004; Niel and Weary, 2007; Niel, Kirkden, and Weary, 2008; Niel, Stewart, and Weary, 2008; Wong, Makowska, and Weary, 2013; Kirkden et al., 2008), and when exposed to this agent these animals express a wide range of defence behaviours – e.g. rearing, pushing the cage lid, increased locomotion, vocalizations and freezing (e.g. Chisholm et al., 2013; Niel and Weary, 2006; Winter et al., 2017). Recent work, described in Chapters 2 and 3 of this thesis, indicates that rats vary in CO_2 sensitivity and that aversion to CO_2 consistently varies among individuals across repeated exposures.

Carbon dioxide inhalation is widely used in human research to induce feelings of anxiety, fear and panic (see Esquivel et al., 2010; Liu et al., 2019). Humans vary in CO_2 sensitivity, with panic disorder patients being sensitive to even low concentrations (Gorman et al., 2001; Kent et al., 2001; Monkul et al., 2010). In healthy volunteers, self-reported feelings of fear, anxiety and panic increase with CO_2 concentration (Griez et al., 2007; Leibold et al., 2013). It has been proposed that human CO_2 sensitivity is mediated by the GABAergic system (Bailey and Nutt, 2008). Healthy subjects and panic disorder patients that are pre-treated with benzodiazepines (thus increasing GABAA receptor functioning) experience less fear, anxiety and panic resulting from CO2 inhalation (Bailey et al., 2007; Nardi et al., 1999). In rats, exposure to higher CO2 doses enhance anxiety-like behaviours in the Vogel conflict and social interaction tests; effects

that are counteracted by the administration of benzodiazepines (Concas et al., 1993; Cuccheddu et al., 1995; Johnson et al., 2015; Sanna et al., 1992).

The aim of this study was to assess the effects of midazolam on individual thresholds of aversion to CO2 in rats. We hypothesized that rat aversion to CO_2 is caused by feelings of anxiety, and predicted that aversion to CO_2 would decrease when rats were pre-treated with midazolam. We further hypothesized that individual differences in rat CO_2 sensitivity are driven by variation in the onset of feelings of anxiety, and predicted that an increase in CO_2 tolerance due to midazolam treatment would reduce individual differences in the threshold of aversion.

4.1 Methodology

4.1.1 Subjects and housing

We used eight 16-month-old female Sprague-Dawley rats that, in an effort to reduce the total number of animals used, were transferred from another study (obtained from the University of British Columbia surplus stock). Two rats reached humane end points (due to mammary tumor development) and were euthanized before the study was completed. The remaining rats (n = 6) average 642 ± 46 g (mean ± standard deviation). Rats were marked with a permanent marker (Ketchum Manufacturing Inc., ON, Canada) for individual identification.

Rats were housed in pairs in a caging system consisting of two cages (20 cm x 50 cm x 40 cm) connected by a red tinted polycarbonate tube (7.6 cm diameter, 15 cm long). The caging system contained bedding (1/4 inch PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA) and environmental enrichment (e.g. cardboard boxes, hammocks, PVC pipes, and shredded paper towels). Animals were kept on a 12-h light/dark cycle, under controlled temperature and humidity (22 ± 0.15 °C and 57 ± 0.44 %, respectively). Rats were provided with

ad libitum food (Rat Diet PMI 5012, Lab Diets, Land O'Lakes, Inc., MN, USA) and tap water, and provided 30 min of daily access to a large enriched cage (following Makowska and Weary 2016a, b; Appendix C: rat playpens).

4.1.2 Handling and transport

Rats were habituated to handling and transport for 6 months before the study (following, Appendix D: agency-based handling and transport). All trials were performed in an experimental room during the light cycle between 900 h and 1700 h; a cage covered with black plastic was used to transport animals. Subjects were habituated, trained or tested only once per day at similar hours each day. Rats were isolated from cage-mates for a maximum of 40 min per day during habituation, training or testing. Before the beginning of each trial, the apparatus was cleaned with Quatricide (Pharmacal Research Laboratories, Naugatuck, CT, USA).

4.1.3 Experimental design

Rats used in the current experiment were repeatedly exposed to CO_2 in the approach-avoidance apparatus and so were highly habituated to both the agent and the apparatus. To reduce potential carry over effects from the drug, rats were exposed to CO_2 gradual-fill (20% CO_2 cage vol. min^{-1}) three times for the control treatment and three times for the midazolam treatment. One air exposure (air flow of 4 L min^{-1}) was run between every CO_2 trial, providing data for three control and three midazolam air trials. Two days before the first exposure to CO_2 rats were tested in an open field and an elevated plus maze under both treatment conditions (Figure 4.1). The anxiolytic effects of benzodiazepines are inconsistently detected when assessed in the open field test (for a review, see Prut and Belzung, 2003). Hence, the open field test was

used to assess any effects of midazolam on locomotion, and the elevated plus maze was used to assess anxiolytic effects.

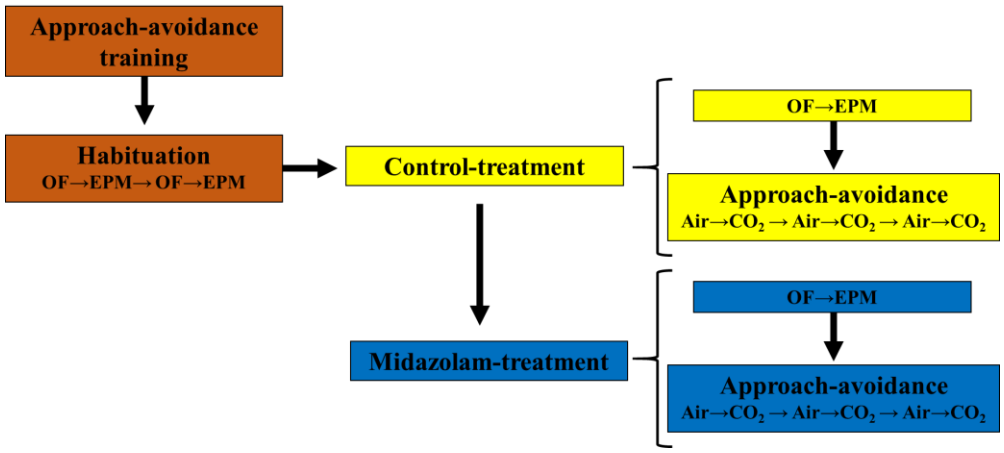


Figure 4.1 Testing order. Rats (n = 6) were trained in approach-avoidance and habituated in the open field (OF) and elevated plus maze (EPM). For control and midazolam treatments rats were tested in the open field, elevated plus maze and the approach-avoidance apparatus.

4.1.4 Midazolam administration

Midazolam (5 mg/ml, Sandoz, Boucherville, Qc, Canada) was mixed with 1 ml of vanilla pudding (Vanilla Flavored Pudding Cup, Western Family, Overwaitea Food Group LP, BC, Canada) and administered orally at 0.375 mg/kg (Dielenberg, Arnold and McGregor, 1999) 30 min before testing. For the control treatment, rats received 1 ml of untreated vanilla pudding, also 30 min before testing.

4.1.5 Locomotor effect

4.1.5.1 Apparatus

The open field consisted of a white acrylic glass arena (100 cm long x 100 cm wide x 61 cm high) placed on wooden base (52 cm high). The arena was visually divided into 25 squares (20 cm x 20 cm; defined by black lines on the floor) to quantify movement (Appendix F: open field arena and elevated plus maze).

4.1.5.2 Habituation, training and testing procedures

To control for changes in locomotion due to habituation (Romero and Chen, 2004; Wehrmeister et al., 2010), rats were exposed to the open field arena twice before testing (Figure 4.1). We tested rats once in the control treatment and once in the midazolam treatment. The rat was placed in the center of the open field arena at the beginning of each trial (see Appendix D). Trials lasted 5 min and rats could move freely within the arena during this time. Video recordings were scored (using Boris software, Version 7.0.9; Friard and Gamba, 2016) by observers blind to rat identity and treatment for frequency of line-crossing (i.e. rat's shoulders and head crossing any line that divided the floor of the arena). To measure interobserver reliability, 50% of the trials were rescored by an independent observer; the two sets of scores were highly related ($r = 0.99$).

4.1.6 Anxiolytic effect

4.1.6.1 Apparatus

An elevated plus maze was used to measure the anxiolytic effects of midazolam. The apparatus was made of two open and two closed black acrylic glass arms (each arm 50 cm long

and 10 cm wide; closed arms each had two walls 61 cm high) arranged in a cross shape with a square (10 cm x 10) cm in the center, and placed on a wooden base (52 cm high; Appendix F: open field arena and elevated plus maze).

4.1.6.2 Habituation, training and testing

Open arm behaviour in the elevated plus maze is known to change from the first to second exposure (i.e. one-trial-tolerance), but not between the second and subsequent exposures (Fernandes and File, 1996; Treit, Menard and Royan, 1993; Wehrmeister et al., 2010). Thus we exposed rats twice to the elevated plus maze prior to the experiment, and then retested rats once in each treatment condition (Figure 4.1). Trials lasted 5 min; at the beginning of each trial subjects were placed at the center of the elevated plus maze and were left to explore the apparatus (see Appendix D). Fecal boli were counted at the end of each trial. Behaviours were scored from video. Again, interobserver reliability was assessed by rescoring 50% of the trials by an independent observer, and again scores were highly consistent (time in the open arms: r = 0.82; open arms entries: r = 0.83).

4.1.7 Aversion to CO_2

4.1.7.1 Apparatus

To assess the effect of midazolam on aversion to CO_2 we used an approach-avoidance apparatus. The approach-avoidance apparatus consisted of a top cage (one of the rat's home cages) placed 20 cm above a bottom cage (20 cm x 45 cm x 24 cm). Both cages contained bedding. Cages were connected by a transparent acrylic glass tube (10 cm diameter, 45 cm long), with cleats on the inside for traction. The connecting tube contained a plastic sliding door (10 cm

x 10 cm) at the top cage entrance. The lid for the top cage was made of wire, and the bottom cage lid was made of clear acrylic glass with two scavenging outlets and a gas inlet (Figure 4.2).

CO_2 and air were delivered from compressed gas cylinders (Praxair, BC, Canada), and the gas flow was regulated through flow meters (CO_2: Western Medica, OH, USA; air: Dwyer instruments, Inc., NI, USA).

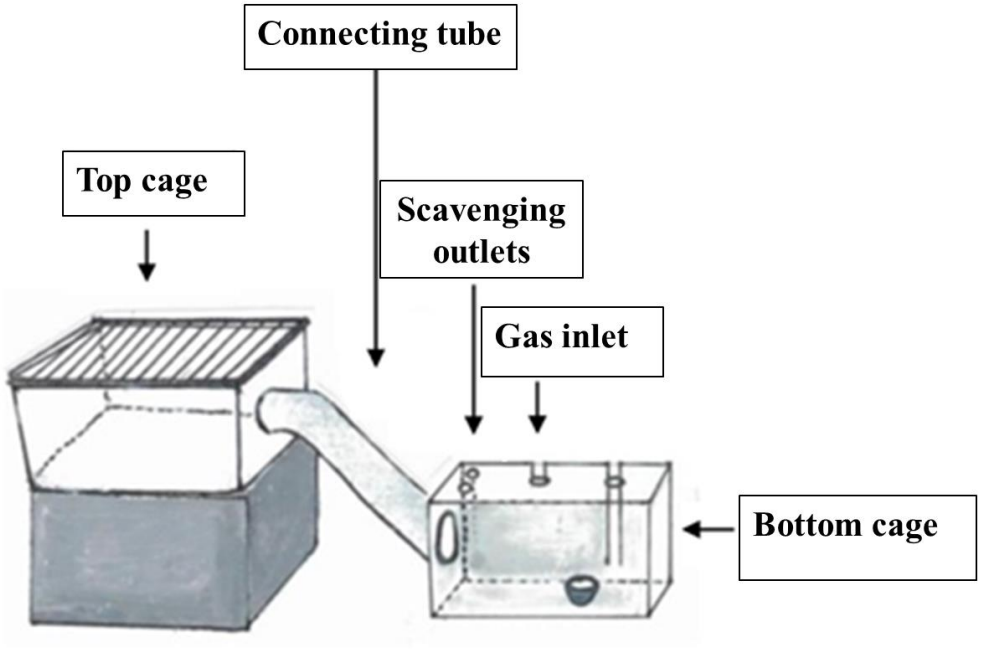


Figure 4.2 Approach-avoidance apparatus used to measure rat aversion to CO_2

4.1.7.2 Habituation, training and testing procedures

Rats had been trained in the approach-avoidance apparatus for another study (in which they had been repeatedly exposed to CO_2 in approach-avoidance testing). At the beginning of the current study these rats were re-trained to go down the tube of the apparatus to enter the bottom

cage and eat 20 sweet rewards (Cheerio; Honey Nut Cheerios TM, General Mills Inc., MN, USA) in the presence of air flow (4 L min^{-1}). First, we placed a rat in the top cage of the apparatus and allowed it to explore for 5 min. Then, we delivered a sweet reward in the top cage and closed the sliding door while the rat ate the reward, blocking access to the bottom cage. We placed 20 sweet rewards in a dish in the bottom cage. After 60 s, we opened the sliding door allowing the rat to descend into the bottom cage to consume the sweet rewards. As soon as the rat's shoulders crossed into the tube to exit the bottom cage the training session ended; rats were not allowed to return to the bottom cage. Rats were considered to have met the taining criterion if they stayed in the bottom cage for 5 min or consumed all 20 sweet rewards for three consecutive training trials.

Once trained, rats were exposed to CO_2 in the approach-avoidance apparatus. For CO_2 trials, we substituted the flow of air for CO_2 as soon as the rat started eating the rewards. We measured the latency (s) to exit the bottom cage and the number of rewards consumed by direct observation.

4.1.7.3 Assessment of CO_2 concentrations

We ran twelve CO_2 flow trials in the approach-avoidance apparatus to estimate CO_2 concentrations during gradual-fill (18.5% CO_2 chamber vol. min^{-1}). No animal was present during these trials. Measurements were made with an oxygen analyzer (Series 200, Alpha Omega Instrument Corporation, RI, USA) attached to a clear plastic sampling tube introduced the cage through an inlet. We estimated changes in CO_2 concentrations every 0.2 s from the readings of oxygen concentrations using the formula $CO_{2\,(t = x)} = 100 - ([O_{2\,(t = x)} * 100] / O_{2\,(t = 0)}$.

4.1.8 Data analysis

Analyses were carried out with R (R Development Core Team, Version 3.4.1) and RStudio (RStudio, Inc., Version 1.0.136). Normality of the residuals and differences of matched pairs were visually assessed. Results are reported as mean ± standard error.

4.1.8.1 Locomotor effects

We estimated the rate of line crossing per second and then compared treatments using a paired t-test.

4.1.8.2 Anxiolytic effects

Treatment differences in the time spent in the open arms of the elevated plus maze and the number of open arm entries were tested with paired t-tests.

4.1.8.3 Aversion to CO_2

Response variables (latency to leave the bottom chamber during CO_2 and air trials, and the number of rewards eaten during CO_2 trials) were analyzed with linear mixed models. The models included treatment (control and midazolam) as fixed factor, exposure number (1st, 2nd and 3rd within each treatment) as a covariate, the interaction between treatment and exposure number, and rat identity as random intercept. For CO_2 trials, we also estimated CO_2 concentration at the time when rats exited the bottom chamber. Concentrations were estimated using the average CO_2 concentration at each time point (measured every 0.2 s) during the 12 CO_2 flow trials.

For each rat in each treatment, we estimated the average (from the three trials) latency to leave the bottom chamber and the number of rewards eaten during CO_2 trials. Consistency of individual differences in the average latency to leave the bottom chamber and number of rewards eaten between treatments were assessed with Pearson correlation tests.

4.2 Results

4.2.1 Locomotor effects

Control and midazolam treatments did not differ in the rate of line-crossing (control: 0.2 ± 0.06 crossings s^{-1}; midazolam: 0.4 ± 0.10 crossings s^{-1}; $t = -2.06$, $df = 5$, $p = 0.09$).

4.2.2 Anxiolytic effects

No rat produced fecal boli in the elevated plus maze. Rats spent more time in the open arms in the midazolam treatment (23 ± 4.1 s) compared to the control (13 ± 3.9 s; $t = -2.70$, $df = 5$, $p < 0.05$). The number of open arm entries did not differ between control (2.3 ± 0.56 entries) and midazolam treatments (4.0 ± 0.86 entries; $t = -1.89$, $df = 5$, $p = 0.12$).

4.2.3 Aversion to CO_2

During training (with air) rats left the bottom cage after 364 ± 15 s and ate all 20 rewards. During test sessions with air, we found an interaction between exposure number and treatment on latency to exit the cage ($F = 5.87$, $df = 1,27$, $p < 0.05$). The average latency to leave the bottom cage with midazolam treatment was 391 ± 28 s and 420 ± 27 s with control. In the control treatment, latency to exit the cage decreased with exposure number ($\beta = -20.25$, standard error = 9.13, $t = -2.22$, $df = 11$, $p = 0.05$). In the midazolam treatment there was no evidence for a

change in latency to exit the cage as function of exposure number ($\beta = 10.33$, standard error = 9.14, $t = 1.13$, $df = 11$, $p = 0.28$). Again, rats ate all 20 sweet rewards (in both treatments) when exposed to air.

We found an effect of treatment on the latency to avoid CO_2 and number of rewards consumed (latency to avoid CO_2: $F = 21.59$, $df = 1,25$, $p < 0.001$; rewards consumed: $F = 14.55$, $df = 1,25$, $p < 0.001$). Rats tolerated CO_2 for longer and consumed more rewards with midazolam-treatment than with control (Figure 4.3). The CO_2 level when the rats left the chamber (i.e. the average latency to avoid CO_2) was $10.7 \pm 1.14\%$ CO_2 during control and to $15.5 \pm 1.41\%$ CO_2 during midazolam-treatment. Exposure number and its interaction with treatment did not affect the latency to avoid CO_2 (exposure number: $F = 0.1$, $df = 1,25$, $p = 0.75$; interaction between exposure number and treatment: $F < 0.001$, $df = 1,25$, $p = 0.98$) or the rewards consumed (exposure number: $F = 0.53$, $df = 1,25$, $p = 0.47$; interaction between exposure number and treatment: $F = 0.14$, $df = 1,25$, $p = 0.71$).

Individual differences in the latency to avoid CO_2 were consistent across the two treatments (Pearson correlation test: $r = 0.83$, $df = 4$, $p < 0.05$; Figure 4.3 a). Rat aversion to CO_2 ranged between 6.2 and 13.6 % CO_2 among rats during control and between 10.9 and 19.3 % CO_2 during midazolam trials. Number of rewards consumed tended to be consistent between the two treatments ($r = 0.78$, $df = 4$, $p = 0.07$; Figure 4.3 b).

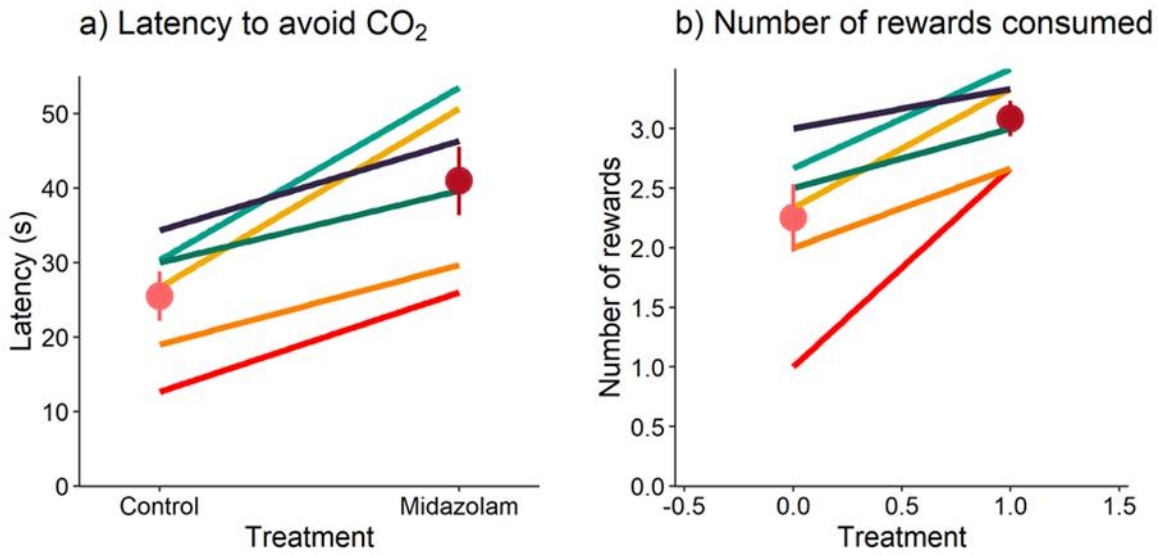


Figure 4.3 Effect of midazolam on rat aversion to CO_2. Rat responses showing treatment effects and consistency in individual rat responses between control- and midazolam-treatment (each line corresponds to an individual rat; n = 6 rats; dots and error bars represent the mean ± standard error). a) Latency to avoid CO_2 and b) number of rewards consumed.

4.3 Discussion

4.3.1 Locomotor and anxiolytic effects

We found no effect of midazolam on locomotion in the open field, indicating that midazolam at the dose provided in this study did not impair locomotor activity; this result is consistent with previous work showing low doses do not interfere with normal activity in rats (Dielenberg, Arnold and McGregor, 1999; Miao et al., 2014; McGregor et al., 2004). Studies have shown a dose dependent effect of midazolam on activity (Yerbury and Cooper, 1987); doses in excess of 1 mg/kg can reduce locomotion (King, Bouton and Musty, 1987) and doses in excess of 10 mg/kg can induce anaesthesia (Pain et al., 1997; Kissin, Brown and Bradley, 1992).

Previous studies have shown that midazolam increases open arm exploration in the elevated plus maze (Bertoglio and Carobrez, 2002; Miao et al., 2014; Ramos et al., 2008; Salonen, Onaivi and Maze, 1992), reduces defensive burying (Treit, 1990), predator odor avoidance (Dielenberg, Arnold and McGregor, 1999; McGregor et al., 2004), and freezing due to place conditioning (Miao et al., 2014). In the current study, pre-treated rats spent more time in the open arms of the elevated plus maze, adding to the existing evidence that midazolam has an anxiolytic effect.

In addition, we conclude that oral administration of 0.375 mg/kg midazolam decreases anxiety without impairing motor function. The pharmacodynamics of this drug show no differences between oral and intravenous administration. Midazolam is absorbed rapidly when administered orally (reaching peak plasma concentration 5 to 15 min after administration) with a systemic availability and metabolic clearance of 45% and 27 min ($t_{1/2}$), respectively (Mandema et al., 1991). In the current study, all rats rapidly and willingly consumed the pudding mixed with midazolam, without the need for handling, restraint, or injection, procedures that induce stress in rats (Balcombe, Barnard and Sandusky, 2004; Andrews, Zharkovsky and File, 1992; Gruen et al., 1995; Vinkers et al., 2009), and can alter responses in behavioural tests (e.g. Davis and Pérusse, 1988; Lapin, 1995; Pritchard, van Kempen and Zimmerberg, 2013).

4.3.2 Aversion to CO_2

It is reasonable to conclude that that the increased CO_2 tolerance was due to the anxiolytic effect of midazolam, and not factors such as habituation, impaired locomotion and increased food motivation. When treated with midazolam, rats showed a 45% increase in tolerance of CO_2 (tolerance increased from 10.7 to 15.5% CO_2). It is unlikely that order accounts

for these results, given that we found no within-treatment effect of exposure order on aversion to CO_2, and that rats used in the current study, were familiar with CO_2 exposure and approach-avoidance testing. Familiarity with CO_2 and the testing environment likely reduced within-individual variation in responses (see Biro, 2012). Previous studies using the same experimental setting (i.e. approach-avoidance testing with similar flow rates of CO_2) showed that tolerance of CO_2 does not increase with consecutive exposures (between 2 and up to 10; Chapters 2 and 3). Hence we argue that the observed increase in tolerance to CO_2 was due to midazolam and not habituation.

Midazolam did not alter normal activity in the open field test; hence, is unlikely this drug impaired locomotion and in this way reduced avoidance behaviour. Moreover, during air trials all rats eventually exited the bottom cage in every test.

It has been reported that benzodiazepines increase food palatability and intake (for a review, see Berridge and Peciña, 1995), so it is possible that rat motivation to consume the sweet rewards increased with midazolam. However, the effect of midazolam on sucrose consumption is dose dependent; midazolam affects sucrose consumption at doses greater than 3.0 mg/kg but has negligible effects at doses similar to that used in the current study (Shimura, Kamada and Yamamoto, 2002). In addition, rat aversion to CO_2 in approach-avoidance tests is not related to food motivation (Kirkden et al., 2008). Since midazolam also reduced evidence of anxiety in the elevated plus maze, it is reasonable to conclude that the increased CO_2 tolerance was due to the anxiolytic effect of midazolam. Future work should consider the use of motivation trade-offs that are not food related, for example, the use of the light-dark apparatus.

Aversion to CO_2 is likely driven by feelings of anxiety. Rats tolerated an average of 10.7% CO_2 during control trials. Similar concentrations of CO_2 elicit feelings of anxiety in

humans. When inhaling 7.5% CO_2 healthy humans show an increase in escape responses (i.e. request to stop the test) and feelings related to anxiety (e.g. alertness, anxiety, fear, feel like leaving the room, feeling paralysed, tense, irritable, nervous, worried; Bailey et al., 2005; Poma et al., 2014), but panic responses are rare at this concentration. Gorman and colleagues (2001) reported a panic rate of 5% in healthy people when inhaling 7% CO_2 for 20 min. In contrast, a single inhalation of 35% CO_2 results in panic in 23 to 41% of healthy volunteers (Monkul et al., 2010; Poma et al., 2014; van Beek and Griez, 2000; Verburg et al., 1998). Inhalation of lower concentrations (~7% CO_2) elicits feelings similar to those experienced by people with generalized anxiety disorder (Bailey et al., 2007; Bailey et al., 2009; Diaper et al., 2012; Poma et al., 2014); whereas, the emotional experience felt at higher concentrations (35% CO_2) resembles naturally occurring panic attacks (Schruers and Griez, 2004). When inhaling 7.5% CO_2, healthy individuals pretreated with the benzodiazepine lorazepam experience fewer feelings related to anxiety (Diaper et al., 2012; Bailey et al., 2007). Pre-treatment with the benzodiazepine alprazolam – an anti-panic drug – reduced feelings and somatic symptoms associated with panic elicited by 7 and 35% CO_2 inhalation (Bailey et al., 2009; Poma et al., 2014). In the current study, providing midazolam before CO_2 exposure increased the average threshold of aversion to 15.5% CO_2. This increase in tolerance indicates that rat aversion to lower concentrations of CO_2 is elicited by feelings of anxiety, and that these feelings are reduced by midazolam.

It is important to note that all rats still avoided CO_2 at concentrations far lower than those needed to induce unconsciousness. This result suggests that higher concentrations of CO_2 evoke emotional experiences (e.g. air hunger or panic) that are not sensitive to the anxiolytic effect of this dose of midazolam.

It has been shown that thresholds of aversion vary among rats, ranging between 5.6 and 18.3% CO_2 in one study (Chapter 2) and between 4.5 and 15% in another (Chapter 3). In agreement with these results, we found that during control tests the threshold of aversion ranged from 6.2 to 13.6% CO_2 among rats. In contrast, the CO_2 concentrations avoided when rats were treated with midazolam ranged between 10.9 and 19.3%. Individual differences in CO_2 aversion were consistent within rats across treatments. Variation in rat CO_2 responsiveness has been linked to the activity of neurons involved in the mediation of anxiety and panic experiences (i.e. orexin neurons in the lateral hypothalamus; Johnson et al., 2012; Monfils, et al., 2019). These results suggest that individual differences in rat CO_2 sensitivity are due to differences in the onset of feelings of anxiety.

4.4 Conclusion

Midazolam treatment reduced rat anxiety without affecting locomotor activity, and increased individual rat thresholds of aversion to CO_2. These results indicate that rat aversion to CO_2 is driven by feelings of anxiety, with an onset that varies among individuals. Even with midazolam treatment all rats avoided CO_2 before loss of consciousness, indicating that even with this refinement CO_2 will induce negative affective states.

Chapter 5: General conclusions and discussion

5.1 Thesis findings

Several studies have reported that the behaviour of rats exposed to CO_2 varies between individuals. Individual differences in the behavioural responses to CO_2 could indicate that the emotions experienced by rats during gas exposure also vary between individuals (CO_2 sensitivity). In this thesis, I propose that inferences regarding rat emotions elicited by CO_2 can be draw by understanding this individual variation. To investigate between individual differences in rat CO_2 sensitivity, I explored different proximal explanations that could account for the observed variation.

In Chapter 2, I assessed whether variation between individuals could be a reflection of the type of behaviours measured (passive or active responses), rather than any intrinsic differences in the experience of the animals. My results indicate that gradually increasing concentrations of CO_2 do not elicit passive responses in rats. One previous study did find that rats respond with passive behaviours, but in this case used a forced exposure paradigm and a static concentration of 10% CO_2 (Winter et al., 2017). Another study that exposed rats to the same concentrations of CO_2, but provided rats with opportunity to escape exposure (approach-avoidance test), found that rats tolerated exposure to CO_2 for around 5 min (or enough time to consume all available food rewards; Niel and Weary, 2007). I found that rats increased active responses from baseline when exposed to CO_2 gradual fill, a result that aligns with previous studies using this method (Niel and Weary, 2006; Niel et al., 2008 b). As discussed in Chapter 2, it appears that rat behavioural responses to CO_2 are plastic. Immobility may be elicited at static lower concentrations of CO_2, but when concentrations of CO_2 are gradually increased, active

behaviours may represent the adaptive response. I conclude that it is unlikely that the source of the reported behavioural variation is due to a lack of measurement of passive responses.

One of the main findings of this thesis is that individual rat identity can account for much of the variation in behavioural responses to CO_2 exposure. In this thesis, the individual rat explained between 40 and 73% of variation in responses. In Chapter 2, I showed that within each of three experimental settings (i.e. forced exposure, aversion- and approach-avoidance tests) rat responses to CO_2 gradual fill were highly consistent across two exposures. Individuals that showed a higher response to CO_2 during forced exposure were also more averse to CO_2 during aversion-avoidance testing. Results from the experiments conducted in Chapter 3 indicated that rat aversion to CO_2 in approach-avoidance was highly repeatable and stable across multiple exposures. In Chapter 4, I found that individual differences in rat aversion to CO_2 were consistent even when rats were pre-treated with an anxiolytic. Overall, these results indicate that individual differences in the behavioural responses to CO_2 represent a personality trait – i.e. consistent individual differences across time and contexts (see Dingemanse et al., 2010; Réale et al., 2007).

Consistent individual differences in rat response to CO_2 could reflect variation in other personality traits. In Chapter 2, I assessed whether individual differences in coping strategies relate to variation in rat responses to CO_2. It has been shown that when exposed to a prod that delivers shocks, rats adopt two different strategies: some consistently express passive responses while others actively burry the prod (De Boer and Koolhaas, 2003; Koolhaas et al., 1999). In Chapter 2, I found that when forced exposed to CO_2 and fox scent (TMT), rats do not consistently show passive responses. Lack of rat passive responses to TMT is consistent with the results of earlier work (e.g. Day et al., 2004; Staples and McGregor, 2006). Active responses

during CO_2 forced exposure were consistent across exposures, but no consistency in these responses was found during fox scent exposure. Since the dichotomy between active and passive responses characterizes rat coping strategies (De Boer and Koolhaas, 2003; Koolhaas et al., 1999), the lack of consistency in passive responses to CO_2 suggests that the individual differences in responses to CO_2 are related to variation in how individuals cope with threatening stimuli in general, but rather are specific to CO_2.

In Chapter 3, I evaluated whether individual differences in aversion to CO_2 could be related to variation in motivation-related personality traits: sweet reward motivation, and the strength of promotion and prevention motivations. Using a similar experimental setting to that used to assess differences in foraging behaviour in wild rodents (e.g. Kotler and Brown, 1990; Smith, 1995), I detected consistent individual differences in rat motivation to access sweet rewards, a personality trait has been previously reported in the literature (Brennan et al., 2001; DeSousa et al., 2000, 1998; Sills and Vaccarino, 1996; Tõnissaar et al., 2006). I also was able to identify individual differences in rat motivation to approach gains (promotion motivation) and pursue darkness (prevention motivation), consistent with the results reported by Franks and colleagues (2012, 2014). While individual variation in these motivation-related personality traits was consistent across trials, variation in aversion to CO_2 was not related to individual differences in rat motivation for sweet rewards, or the strength of prevention and promotion focus.

In Chapter 4, I investigated the type of emotional experience that could drive the broad variation in rat thresholds of aversion to CO_2 found in the previous Chapters (individual thresholds of aversion to CO_2 ranged between 5.6 and 18.3% CO_2 in Chapter 2, and between 4.5 and 15% CO_2 in Chapter 3). If aversion to CO_2 is caused by feelings of anxiety, I predicted that the administration of an anxiolytic would increase tolerance to CO_2. When rats were treated with

an anxiolytic, they showed more exploration of the open arms of an elevated plus maze and increased tolerance to CO_2 – from 10.7% CO_2 during control to 15.5% CO_2 during midazolam-treatment. These results indicate that when exposed to CO_2 concentrations similar to those that elicit feelings of anxiety in humans, rats also experience anxiety. Individual differences in CO_2 aversion were consistent between control and treatment with the anxiolytic; this indicates that variation in rat CO_2 sensitivity is related to differences in the onset of anxiety.

Collectively, results from this thesis indicate that rats vary in CO_2 sensitivity, with some rats consistently finding CO_2 exposure more aversive than others, and these results suggest that individual differences in sensitivity are linked to variation in the onset of CO_2-induced anxiety. In the following sections, I provide alternative proximal explanations for the variation in rat responses to CO_2, then discuss the contribution of this thesis to the field of animal welfare, future research directions, and limitations of the thesis.

5.2 Alternative personality traits

In this thesis I assessed whether individual differences in coping strategies, sweet reward motivation and prevention and promotion motivation could explain the variation in rat responses to CO_2. In the following section, I will present other situational-elicited personality traits that could explain individual differences in rat aversion to CO_2.

5.2.1 Individual differences in approach and avoidance motivation (bold/shy)

Individual differences in approach and avoidance motivation have been described as personality dimensions in several taxa (for a review, see Jones and Gosling, 2013). Individuals that are highly approach-oriented are more motivated to approach positive end-states. Individuals

that are highly avoidance-oriented are more motivated to avoid negative end-states (Eliot and Thrash, 2002). Rats vary consistently among individuals in approach and avoidance motivation; often referred to as exploration, novelty seeking, risk taking, or boldness/shyness, neophobia, and threat avoidance (Jones and Gosling, 2013). For example, among-rat variation in the degree of exploration of novel environments has been shown to be highly repeatable (Rebouças and Schmidek, 1997), consistent between four and eight months of age (Cavigelli and McClintock, 2003), consistent between testing situations, and heritable (Steimer et al., 1997a). Following Réale et al. (2007), boldness/shyness can be distinguished from novelty-seeking (or exploration/avoidance), as boldness refers to animal's behavioural response to a non-novel risky environment or stimulus. In aversion tests, rats are trained or habituated to the testing apparatus, hence individual differences during testing are less likely related to novelty seeking. Consistent individual differences in aversion to CO_2 could be explained by variation in approach and avoidance motivation, specifically related to boldness/shyness. Given the propensity of bold individuals to approach risky situations, these individuals may be tolerating higher CO_2 concentrations. As discussed in Chapter 3, when using approach-avoidance tests rats can avoid CO_2 by approaching the safety of the CO_2-free cage, making it challenging to identify clear predictions based on motivation using an unitary view of the hedonic principle. I argue that the regulatory focus approach better fits the situational contingencies of approach-avoidance testing.

5.2.2 Interactive effect between the value of the reward and the value of the threat

In Chapter 3, I explored individual differences in sweet reward, promotion and prevention motivations, as this could be driving variation in aversion to CO_2 in approach-avoidance tests. Results from Chapter 3 indicated no relationship between these traits and

individual differences in aversion to CO_2. It is important to note that during exposure to CO_2 gradual fill in approach-avoidance, it is possible for the value of the reward and the value of the threat (CO_2) to vary with time within a single trial; the value of rewards may decrease as rats consume more while CO_2 concentrations are gradually rising. Future research could assess individual differences considering this interactive effect, perhaps by assessing individual differences in rat responses to static CO_2 concentrations against variation in sweet reward, promotion and prevention motivations.

5.2.3 Individual differences in optimism and pessimism

As discussed in Chapter 3, expectancy and value of the emotion-eliciting stimulus affect the animal's motivation to pursue a desired end-state (Wigfield and Eccles, 2000; Tolman, 1955). Hence, not only individual variation in the value of the paired stimuli (sweet rewards and light exposure), but also variation in rat expectations regarding the attainability of the paired stimuli, could be related to aversion to CO_2 in aversion- and approach-avoidance tests. Optimism and pessimism relate to expectations for success at approaching or avoiding desired end-states; optimistic individuals have more favourable expectations than do pessimist individuals (for a review, see Carver and Scheier, 2014). In rats, individual differences in pessimism and optimism are a stable trait (Rygula et al., 2013, 2015). In approach-avoidance tests, optimistic rats may have more positive expectations regarding the attainability of the sweet rewards and hence be more willing to tolerate higher concentrations of CO_2 than pessimistic rats. Individual differences in optimism and pessimism could also explain why, in choice tests, some rats leave a CO_2 pre-filled chamber and never return while some others return to the CO_2 chamber (Leach et

al., 2002, 2004). However, I will argue that it is unlikely that differences in optimism and pessimism explain variation in rat responses to CO_2.

There is some overlap between the conceptions of optimism-pessimism and promotion-prevention focus. In humans, an optimistic perspective has been associated with more promotion-motivated individuals, whereas pessimistic perspectives are more prevention motivated (Hazlett and Molden, 2011; but see Higgins, 1997). Similarly, there is evidence of overlap in rats. Rygula and colleagues (2015) profiled rats as optimistic or pessimistic, based on the consistency of rat's favourable or unfavourable expectations following an ambiguous tone. Then, individual differences in rat motivation to press a lever to gain a reward, and to press a lever to prevent punishment were assessed. I argue that motivation measured through lever pressing to obtain a reward represents an approach-promotion, while approach-prevention is represented by lever pressing to prevent punishment (see Chapter 1, Figure 1.1). Results from this study showed that optimistic rats were more motivated to press the lever associated with sucrose gain; showing that optimistic rats were more promotion focused. However, optimistic and pessimistic rats showed no differences in lever pressing associated with prevention of punishments, leading the authors to conclude that the punishment incentive was low. An alternative interpretation is that since the prevention goal was achieved by approach, optimistic and pessimistic rats were equally successful in approaching prevention of punishment. High promotion and prevention focused individuals are more successful at achieving goals when the situation matches their strategic inclination: high promotion focused individuals perform better when the situation is framed in terms of approaching matches to desired end-states, and when the situation is framed as avoiding mismatches to desired end-states high prevention focused individuals are more successful (e.g. Shah et al., 1998). It is likely that in approach-avoidance

testing, trait optimism and pessimism overlap with the rat strength of motivation to approach gains or maintain safety, as in this testing situation rats can successfully match their motivational foci with their strategic inclination.

In summary, I argue that individual differences in rat responses to CO_2 are not due to alternative situational-elicited personality traits. This interpretation is supported by the fact that, across all experimental settings from this thesis, variation in rat responses to CO_2 was stable and consistent.

5.3 Significance of this thesis for animal welfare

The results of this thesis indicate that rat behavioural responses to CO_2 are indicative of the animal's emotional experience and show that rats likely feel anxiety when exposed to low CO_2 concentrations. To establish this, I have focused on the individual experience rather than only measures of central tendency. My results reinforce the importance of assessing affective states at the level of the individual (discussed by Fraser, 2009) and indicate that accounting for individual differences may allow for a better understanding of experimental results, including when assessing animal welfare (Manteca and Deag, 1993).

This thesis provides support for previous studies that deemed CO_2 for killing rats as incompatible with the definition of euthanasia (e.g. Chisholm et at., 2013; Conlee et al., 2005; Niel and Weary, 2006; Niel et al., 2008a, 2008b; Leach et al., 2002, 2004). In addition, individual differences in rat CO_2 sensitivity could be used as a model for the assessment of felt emotions in animals, a core aspect of the animals overall welfare (see Fraser et al., 1997; Mellor, 2016; Weary et al., 2017).

In Chapter 1 (section 1.1.2), I presented an operational definition of emotions and the combination of evidence that could strengthen inferences regarding the consciously felt emotions in animals. Results from this thesis provide evidence for variation in the emotional experience of rats due to CO_2 exposure. As reviewed in Chapter 1 (sections 1.2.4 and 1.2.5), physiological and neurobiological responses concomitant to the behavioural responses (e.g. Johnson et al., 2012, 2015; Ziemann et al., 2009) could be measured. In addition, and as discussed by Weary and colleagues (2017), future studies could assess individual differences in behaviours indicative of the maintenance of attention or awareness.

Throughout this thesis, I used functional homology to link rat and human felt emotions during CO_2 inhalation. Although such inferences require caution, the results of my research show the value of this approach. This thesis supports that conclusion that rats differ individually in CO_2 sensitivity. There is considerable research in human CO_2 sensitivity, and results from this thesis suggest that there is opportunity to study this parallel between species (further discussed in the following section).

Finally, in this thesis I showed how drugs that reduce anxiety due to CO_2 inhalation in humans also reduced aversion in rats, illustrating one way in which inferences regarding the animal's emotional experiences can be drawn.

5.4 Future research directions

I found that variation in the onset of CO_2-elicited anxiety drives individual differences in aversion to CO_2, but all rats avoided CO_2 at concentrations well below those required to render rats unconscious (~33% CO_2; Niel and Weary, 2006). In humans, the feelings experienced when inhaling CO_2 differ between individuals in type and intensity. Inhalation of 7% CO_2 elicits

feelings of anxiety in 60% of individuals (Poma et al., 2005), whereas panic is rarely elicited at this concentration (5% of individuals; Gorman et al, 2001). At higher concentrations of CO_2 (35% CO_2) between 23 to 41% of individuals experience panic (Monkul et al., 2001; Poma et al., 2014; van Beek and Griez, 2000; Verburg et al., 1998). Future research could assess whether rats experience panic at higher concentrations, and if this experience varies between individuals.

In the paragraphs that follow I describe tests, CO_2 concentrations, delivery methods, and drugs treatments that, alone or in combination, could be useful for further assessment of individual differences in rat CO_2 sensitivity.

Individual differences in rat CO_2 sensitivity could be investigated by assessing rat emotional experience when CO_2 in no longer present. Place conditioning/preference testing could be used; previous work on rats has shown that exposure to CO_2 concentrations induces conditioning that increases with concentration, and the degree of behavioural conditioning and extinction is related to severity of the experience (Mongeluzi et al., 2003). Using a two-chamber apparatus, similar to the aversion-avoidance apparatus used in this thesis (Chapter 2, Figure 2.1 b), exposure to CO_2 (e.g. static at different concentrations, with or without drug treatments; further discussed in this section) could be paired with one of the chambers. Then, avoidance or preference of chambers could be assessed as indicative of the rat previous experience when inhaling CO_2 (e.g. Pain et al., 1997). Alternatively, the chamber could also contain a distinctive odor (e.g. vanilla; following Mongeluzi et al., 2003). Another alternative would be to use judgement bias testing. Closely related to trait pessimism and optimism discussed earlier in this Chapter, judgement bias could be used to assess rat emotional experience to CO_2 exposure. Exposure to stressful or painful stimuli has been shown to reduce expectations of success (e.g. Neave et al., 2013; Rygula et al., 2013). Rats could be trained to associate one cue with a

positive outcome and another cue with a negative outcome. Rat judgement bias could be assessed with the presentation of ambiguous cues after acute exposure to high concentrations of CO_2, or during prolonged exposure to low CO_2 concentrations (further discussed later in this section).

In this thesis I assessed rat responses to gradually increasing concentrations of CO_2 and found that CO_2 concentrations that evoke anxiety in rats vary among individuals, a phenomenon also reported in the human literature. I suggest that to make better approximations using functional homology, further research could use static CO_2 at 7% and 35%; these concentrations have been validated in humans to assess anxiety and panic, respectively (e.g. Bailey et al., 2007, 2009; Nardi et al., 2006; Perna et al., 2003; Schmidt et al., 2005). One example of a study comparing rodents and humans using the same CO_2 concentrations is that by Leibold and colleagues (2016). These authors used static 9% CO_2 concentrations to compare behavioural, respiratory and cardiovascular responses of mice and humans. This study found that mice avoided the central areas of an open field, and showed immobility responses and produced fecal boli during 9% CO_2 exposure, and humans reported an increase in feelings of fear and panic due to 9% CO_2 inhalation. Inhalation of 9% CO_2 induced bradycardia in both species. The authors concluded that mice reactivity to CO_2 can be used as model for humans. I suggest also that the opposite is also true; i.e. that human reports can be used to better understand the emotional experience of rodents during CO_2 exposure.

For future work I encourage assessing the effects of benzodiazepines such as alprazolam and clonazepam. Alprazolam reduces CO_2-induced panic feelings and somatic symptoms in humans exposed to 7 and 35% CO_2 (Bailey et al., 2009; Poma et al., 2014). Following an inhalation of 35% CO_2, the panic rate of panic disorder patients pre-treated with clonazepam was

reduced from 83% to between 14 and 33% (Nardi et al., 1999, 2000; Valença et al., 2002). Niel and Weary (2007) found that, to consume all sweet rewards, rats tolerated static concentrations ranging from <1% to 10% CO_2. When concentrations between 15% and 20% CO_2 were used, tolerance to CO_2 decreased and the variability between rats was more evident. At 15% CO_2 one rat did not consume any rewards, and at 20% CO_2 one rat did not enter the bottom cage and only two rats consumed rewards. Future studies could, for example, evaluate the effect of alprazolam and clonazepam using approach-avoidance tests, measuring aversion to static 35% CO_2.

5.5 Thesis limitations

In this thesis, I used sample sizes that ranged between 6 and 11 rats. These small sample sizes could explain the failure to detect some relationships and differences (for a review, see Button et al., 2013). I was, however, able to detect some clear treatment effects – for example, between force exposure to CO_2 and fox scent (Chapter 2), and between control and anxiolytic treatments (Chapter 4), and I found consistency in behavioural responses across all experiments suggesting that these experiments were powered sufficiently for these aspects of the study. With any study there is merit in constructive replication, and I encourage further work using a larger sample.

This thesis included multiple tests within each study, increasing the risk of type I error (see Cabin and Mitchell, 2000). My approach to reducing this risk was to focus on (and provide statistical tests for) only the specific relationships for which I had strong predictions. An alternative approach would be to omit inferential analyses and report correlation coefficients descriptively (e.g. Meagher et al., 2016).

In Chapters 2 and 3, I assessed individual differences in rat responses to CO_2 prior to personality profiling. Consistency across studies and the detection of personality traits that correspond to those published in other studies, indicate that an order effect of experiments was unlikely to bias these results. However, I encourage future research to profile personality rat traits a priori, divide individuals as to their trait characteristic (e.g. passive versus active responders, high versus low sweet reward motivated, high prevention or high promotion), and then assess the relationship between their trait characteristic and individual differences in response to CO_2. This approach has been used to determine the relationship between personality traits in rats (e.g. Boersma et al., 2010; DeSousa et al., 1998; Gosnell, 2000; Rygula, 2015).

The experimental design employed in Chapter 4 intentionally confounded order and treatment. To reduce the risk of order effects I used animals that were highly habituated to CO_2 and the test apparatus, and I tested for order effects within treatment. That said, I encourage future studies to employ an A-B-A (return to baseline) design to further account for order effects.

To ensure that the behavioural response (avoidance) was related to the aversive stimuli rather than the experience of a flow, approach-avoidance tests required rats to meet a training criterion. In the current study this was that rats stayed in the bottom cage for at least 5 min or consumed all available sweet rewards while air flow was delivered into the cage. As discussed in Chapter 3, rats that did not meet this criterion were less sweet reward motivated, indicating a selection bias inherent in approach-avoidance testing. I suggest the use of alternative incentives to help overcome this bias.

5.6 Conclusions

Studies assessing the humaneness of CO_2 for rat euthanasia have been carried on for over 30 years, sometimes with conflicting conclusions. In a survey carried on by our research group in the spring of 2019 (unpublished data), we found that approximately 50% of respondents reported that they use CO_2 to kill laboratory rodents, even though the large majority were unsure or ambivalent as to whether this is method was appropriate. A better understanding of rat emotions induced by CO_2 may help people better understand the experiences of these animals during exposure, and why responses may vary among individuals.

When exposed to CO_2 rats experience variable emotional states among individuals. At lower CO_2 concentrations rats likely experience feelings of anxiety. As concentration rises some individuals may show a higher behavioural response; these individuals are likely experiencing an earlier onset of feelings of anxiety. As CO_2 concentrations further increase even the less CO_2 sensitive rats likely experience negative emotions.

Further research is needed to elucidate the type of emotions that drive variation at higher concentrations of CO_2, including air hunger, panic, pain or some combination thereof. Even without this future research, the results of the current thesis and past studies support the conclusion that CO_2 exposure compromises rat welfare even for the least sensitive rats.

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Appendices

Appendix A Bleach treatment of Chapter 2

Experimental design

All rats tested in Experiment 1 were forced exposed to bleach (2 ml; The Clorox Company, CA, USA. Order of exposure was allocated using three 4x4 Latin squares (considering the three treatments of the main experiment, four rats and four treatments per Latin square). Three days later, the same rats were re-exposed to bleach, allocating treatment order again in three 4x4 Latin squares. Each rat was tested only once a day. Tests were performed between 900 h and 1700 h, and each rat was tested at similar hours within and across all experiments.

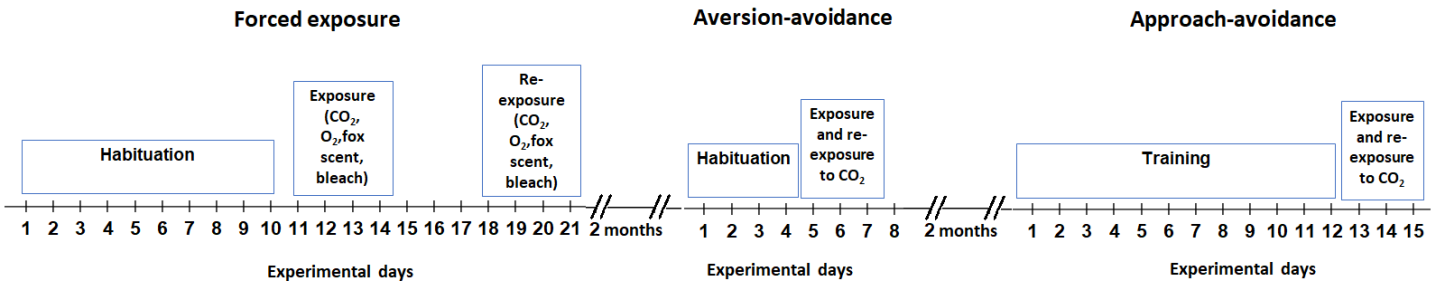
Testing procedure

Rats were individually placed in the experimental cage covered with the baseline lid, and remained in it for 5 min (baseline). The lid was then replaced with the experimental lid. The tea ball attached to the experimental lid contained a cotton ball soaked with 2 ml of bleach. Tests lasted 15 min.

Appendix B Experiments timeline of Chapter 2

Timeline of the three experiments performed in the current study

All rats were tested in each of the three experiments (i.e. Forced exposure, Aversion-avoidance and Approach-avoidance) in the same order and timeline (presented below)



Appendix C Rat playpens of Chapters 3 and 4

Methodology

After each habituation, training and experimental trial session, subjects were introduced to a 'playpen' (see below) and left to explore for 30 min. Subjects were introduced to the playpen with their cage-mates. After 30 min, rats were removed from the playpen agency-based handling and transport as described in Appendix D) and re-introduced into the home-cage.

Cage specifications

Playpens were large (91 x 64 x 125 cm) wired cages (Figure A; Critter Nation™ double unit with stand, MidWest Homes for Pets, Muncie, IN, USA). The upper section of the cage contained diverse enrichment materials: hammock, PVC pipes, bedding material (PBP with Enrichment Bedding, Biofresh, Absorption Corp, WA, USA), and a container (13 x 16 x 17.5 cm) filled to a depth of ~5 cm with room temperature tap water (changed weekly). The bottom section of the cage contained a plastic bin (60 x 60 x 30 cm) filled with a mixture of autoclaved soil and sand (3:1). We sprayed the burrowing substrate regularly with tap water from a spray bottle to prevent drying. Enrichment materials and the soil mixture were added as needed.

Figure A. Rat playpen.

Appendix D Agency-based handling and transport of Chapters 3 and 4

We used an agency-based method (i.e. rats were allowed to be agentic in this procedure) to place and retrieve rats in and out of the different apparatus and playpen.

Methodology

At the beginning of the session, rats were signalled (by gently tapping the side of the cage) and provided with a food reward once upon entering one of the cages of the system. Once all rats entered the cage to receive a reward, the tube connecting the cages was removed and the cage was placed on a table or cart.

The lid of the cage was gently removed, and a transport cage was placed next to the home cage. Rats were left to explore, both the table and the transport cage. Then, the rat to be tested was signalled to enter the transport cage in the same manner as described above. If a non-subject rat attempted to hop into the transport cage, the experimenter gently placed a hand between the rat and the cage to prevent entering. The home cage and transport cage were then covered with lids. The rat to be tested was then transported into the experimental room. The remaining cage-mates were left inside the home cage.

Experimental room procedure

Once in the experimental room, the transport cage was placed next to the apparatus (i.e. approach-avoidance, open field arena, or elevated plus maze), the lid of the transport cage was removed, and the experimenter waited until the subject voluntarily entered the apparatus.

Once the session was complete, the experimenter signalled the subject and waited until it returned to the transport cage. The transport cage was covered with its lid and the rat was transported back to the housing room.

Once the rat was brought into the housing room, the transport cage was again placed on the table next to the home cage. The lids of the transport cage and home cage were removed, and the rat was left to hop back to its home cage. The home cage was closed after the rat entered the home cage. The home cage brought back into the rack re-connected with to the tube leading to the second compartment of the home cage.

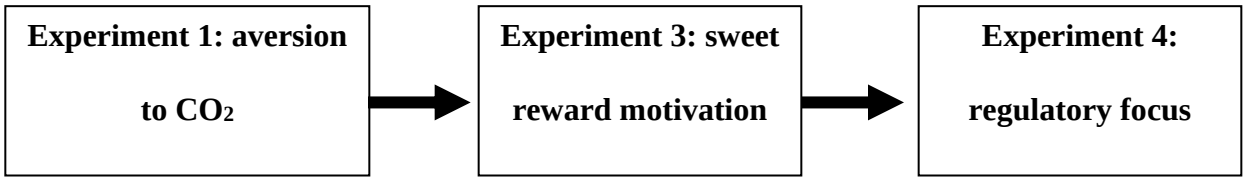
Playpen procedure

The transport cage was placed next the opened upper door of the playpen and the lid of the playpen was removed. The experimenter waited until the rats hopped out into the playpen and closed the playpen door. After 30 min, the experimenter opened the upper door of the playpen and placed the transport cage next to it. Rats were signalled to hop into the transport cage. If a rat failed to hop into the transport cage after ~ 5 min, the transport cage was placed inside of the playpen, and the experimenter waited ~ 2 min until the rat entered the transport cage. If rats did not hop into the transport cage placed inside the playpen, the researcher gently and slowly guided the rat towards a PVC tube using one hand, while with the other closing the opposite entrance of the tube. Once the rat was in the PVC tube, the tube was placed into the transport cage. The transport cage was covered by its lid and brought back to the table. Once in the table, the lids of the transport cage and home cage were removed, and the rat was left to hop back to its home cage. The home cage was closed after the rat enter the home cage. The home cage brought back into the rack and the connection tube was placed again.

Appendix E Experiments timeline of Chapter 3

Timeline of the three experiments performed in the current study

Rats were tested in three experiments (i.e. aversion to CO_2, sweet reward motivation and regulatory focus) in the following order (presented below)



Appendix F Open field arena and elevated plus maze

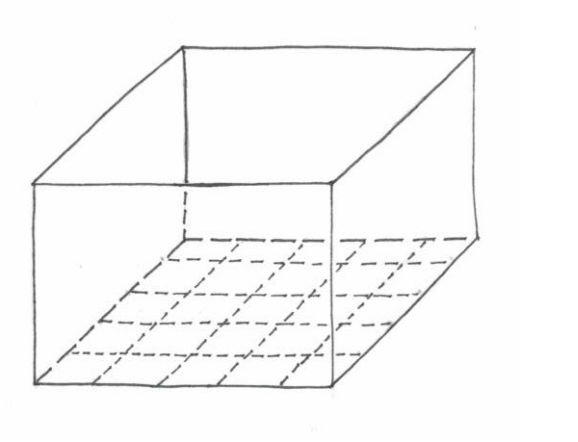


Figure B. Open field arena

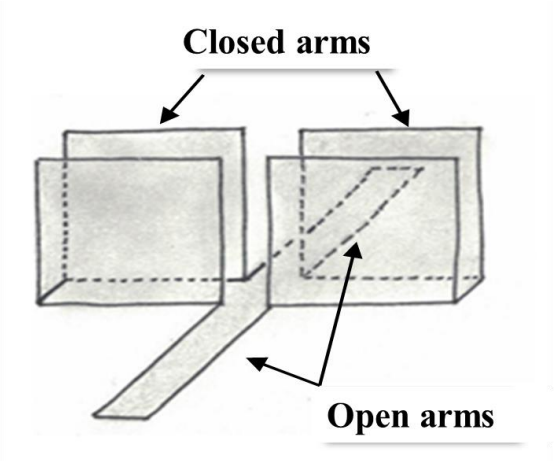


Figure C. Elevated plus maze apparatus