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CCAC guidelines: Rats

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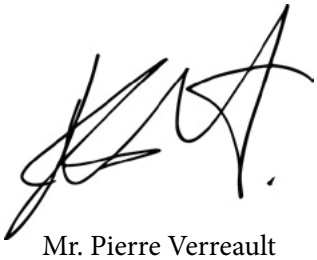
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TABLE OF CONTENTS

PREFACE 1

SUMMARY OF THE GUIDELINES LISTED IN THIS DOCUMENT 2

1. INTRODUCTION 6

1.1 Behavioural Biology 7
1.2 Senses 8
1.3 Anatomy and Physiology 8
1.4 Sources of Variation 9
1.4.1 Strains and Stocks 9
1.4.2 Individual Differences – Sex, Health Status, Microbiome 10
1.4.3 Effects of the Environment and Previous Experience 10

2. FACILITIES 11

2.1 Housing 11
2.1.1 Types of Cages 11
2.1.2 Special Purpose Housing 16
2.2 Surgical Facilities 17
2.3 Core Facilities for Generation of Genetically Modified Rats 17

3. FACILITY MANAGEMENT AND PERSONNEL 19

3.1 Managing the Environment 19
3.1.1 Lighting 19
3.1.2 Temperature and Relative Humidity 21
3.1.3 Air Quality and Ventilation 22
3.1.4 Sound and Vibration 22
3.2 Personnel 23

4. PROCUREMENT 24

4.1 Source 24
4.2 Documentation 24
4.3 Shipping and Receiving 25
4.4 Transportation 25
4.4.1 Shipping Conditions 25
4.4.2 Moving Rats Between Institutions 26
4.4.3 Moving Rats Within an Institution 26
4.5 Reception of Rats at an Institution 27

5. BREEDING 28

5.1 Record Keeping and Oversight 28
5.2 Identification of Breeding Colony Animals 29
5.3 Considerations for Breeding Management 29
5.3.1 Breeding Systems 30
5.3.2 Breeding Age 31
5.3.3 Weaning 32
5.3.4 Post-Partum Breeding 32
5.3.5 Cryopreservation 32
5.4 Factors Affecting Reproduction 33
5.4.1 Environmental Factors 33
5.4.2 Housing and Husbandry Influences 33
5.5 Health Issues 34
5.6 Genotyping 34

6. HUSBANDRY 35

6.1 Identification of Animals 35
6.2 Housing Management 35
6.2.1 Social Housing 35
6.2.2 Single Housing 36
6.3 Food, Water, and Bedding 37
6.3.1 Food 37
6.3.2 Water 38
6.3.3 Bedding and Nesting Material 38
6.4 Environmental Enrichment 39
6.5 Human Contact and Handling 39
6.6 Animal Observation 39
6.7 Cage Changing and Sanitation 40
6.8 Record Keeping 40

7. HANDLING AND RESTRAINT 41

7.1 Handling 41
7.2 Restraint 42

8. WELFARE ASSESSMENT 43

9. HEALTH AND DISEASE CONTROL 45

9.1 Disease Prevention 45
9.2 Health Monitoring and Disease Detection 46
9.3 Disease Management in the Event of an Infectious Outbreak 47

10. EXPERIMENTAL PROCEDURES 48

10.1 Animal Models 49
10.2 Administration of Substances 50
10.2.1 Injections 50
10.2.2 Oral Dosing 51
10.3 Collection of Body Fluids or Tissue 52
10.3.1 Blood Collection 52
10.3.2 Urine and Feces 53
10.4 Explants and Implants 53
10.5 Procedures for Genetically Modified Rats 54
10.5.1 Collecting Samples for Genotyping 54
10.5.2 Phenotyping 55
10.6 Imaging 55
10.7 Behavioural Studies 56
10.8 Food and Fluid Intake Regulation 56
10.9 Anesthesia and Analgesia 57
10.9.1 Anesthesia 57
10.9.2 Analgesia 58
10.10 Surgery 59
10.11 Monitoring and Post-Procedural Care 61
10.11.1 Monitoring 61
10.11.2 Post-Procedural Care 61

11. EUTHANASIA 63

11.1 Injection 63
11.2 Inhalation Techniques 64
11.3 Physical Methods 64
11.4 Other Methods 65
11.5 Euthanasia of Pre-Weaning Age Rats 65

12. END OF STUDY 66

12.1 Transfer of Rats Between Facilities or Protocols 66
12.2 Re-Homing 66
12.3 Disposal of Dead Rats 66

13. HUMAN HEALTH AND SAFETY 67

REFERENCES 69

APPENDIX 1
RESOURCES FOR INFORMATION ON RATS 88

APPENDIX 2
RAT HOUSING ASSESSMENT TOOL.....89

APPENDIX 3
METHODS OF IDENTIFICATION.....90

APPENDIX 4
INDICATORS THAT MAY BE USED TO ASSESS THE WELFARE OF RATS 92

GLOSSARY.....99

Rats

PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer-review organization responsible for setting and maintaining standards for the ethics and care of animals in science throughout Canada.

The *CCAC guidelines: Rats* provides information for investigators, animal care committees, facility managers, veterinarians, and animal care staff to help facilitate improvement in both the care given to rats and the manner in which experimental procedures are carried out.

CCAC guidelines are intended to provide assistance in the implementation of Russell and Burch's Three Rs (Replacement, Reduction and Refinement) principles for animals in science (Russell and Burch, 1959). The guidelines are based on expert peer advice and current interpretation of scientific evidence. They are intended to provide a framework for the implementation of evidence-based practices, which are constantly evolving. Implementation of evidence-based practices should result in continual improvement in animal welfare, and those involved with rats should keep abreast of the current literature.

For studies outside of Canada, Canadian investigators are subject to these guidelines as well as to the relevant legislation and regulations pertaining to animal care in the country where the study is conducted.

SUMMARY OF THE GUIDELINES LISTED IN THIS DOCUMENT

2. FACILITIES

Guideline 1:

The selection and purchase of caging that is suitable for the needs of the study and the welfare of the animals should be a collaborative decision involving investigators, animal care personnel, and other institutional parties.

Section 2.1.1 Types of Cages, p.11

Guideline 2:

Cages should be of a sufficient size and complexity to allow rats to be housed in appropriately sized groups and to perform behaviours important to their welfare.

Section 2.1.1.1 Cage Size, p.12

Guideline 3:

Cages should provide at least 800 cm^2 of floor space, and occupancy should be based on the minimum floor space required per animal.

Section 2.1.1.1.1 Cage Floor Area, p.13

Guideline 4:

Cages must allow for proper monitoring of the animals, preferably without disturbance, with consideration of research requirements.

Section 2.1.1.2 Cage Materials, p.14

Guideline 5:

Rats should be housed in cages with solid floors.

Section 2.1.1.3 Cage Floors, p.14

Guideline 6:

Structures for shelter should be provided.

Section 2.1.1.4.1 Shelters, p.15

Guideline 7:

Rats must only be housed in metabolic cages when necessary for the particular study, and for the shortest time possible.

Section 2.1.2.2 Metabolic Cages, p.16

3. FACILITY MANAGEMENT AND PERSONNEL

Guideline 8:

Laboratory management practices must aim to ensure the macroenvironment (room) and microenvironment (cage) maintain the health and welfare of both the animals and personnel, and provide consistency for research outcomes.

Section 3.1 Managing the Environment, p.19

Guideline 9:

Rats must be observed daily by trained personnel, with minimal disruption to the animals.

Section 3.2 Personnel, p.23

4. PROCUREMENT

Guideline 10:

The health status of the incoming rats should be reviewed before the animals are shipped.

Section 4.5 Reception of Rats at an Institution, p.27

5. BREEDING

Guideline 11:

Breeding colonies must be efficiently managed according to approved protocols, anticipated need, and the principles of the Three Rs.

p.28

6. HUSBANDRY

Guideline 12:

Rats should be group housed.

Section 6.2.1 Social Housing, p.35

Guideline 13:

Bedding and nesting material should be provided to allow rats the opportunity to dig, forage, and build nests.

Section 6.3.3 Bedding and Nesting Material, p.38

Guideline 14:

Cages should be changed at a frequency that maintains the intra-cage air quality and bedding within acceptable parameters, while recognizing the stress associated with cage cleaning and the potential impact on study data.

Section 6.7 Cage Changing and Sanitation, p.40

7. HANDLING AND RESTRAINT

Guideline 15:

Rats must be handled in a gentle manner that is safe for both the rats and the personnel.

p.41

8. WELFARE ASSESSMENT

Guideline 16:

All rats maintained in an animal facility should be subject to routine welfare assessments.

p.43

9. HEALTH AND DISEASE CONTROL

Guideline 17:

All rats should be included in an animal health program, irrespective of where they are housed.

p.45

Guideline 18:

Strategic measures for disease prevention should include a program for disease control and a system of regular monitoring and reporting for health assessment purposes.

Section 9.1 Disease Prevention, p.45

Guideline 19:

Standard operating procedures should be developed for assessing animal health, providing health care, and treatment of common health problems for the animals; these should be reassessed every three years to ensure relevance.

Section 9.2 Health Monitoring and Disease Detection, p.46

Guideline 20:

A management plan must be in place to deal with unanticipated disease outbreaks.

Section 9.3 Disease Management in the Event of an Infectious Outbreak, p.47

10. EXPERIMENTAL PROCEDURES

Guideline 21:

The least invasive method suited to the goals of the study must be used, with consideration of the potential impacts of the procedures on rats, including other rats in the room, and consideration of measures to reduce those impacts.

p.48

Guideline 22:

Endpoints must be developed and approved by the animal care committee prior to the commencement of the study, to minimize any negative impact of procedures on the animal.

p.49

Guideline 23:

Rats should be provided with analgesia for invasive procedures that are likely to be painful.

Section 10.9.2 Analgesia, p.58

Guideline 24:

Post-procedural care and monitoring must be planned based on the particular procedure and the individual needs of the animal, and adapted as necessary when unforeseen situations arise.

Section 10.11.1 Monitoring, p.61

11. EUTHANASIA

Guideline 25:

Euthanasia of rats must be carried out by competent personnel only, using the method best suited to the particular animals, their housing situation, and the impact on the study data.

p.63

1 INTRODUCTION

Throughout this document, the term 'should' is used to indicate an obligation, for which any exceptions must be justified to, and approved by, an animal care committee. The term 'must' is used for mandatory requirements.

Rats (of the genus *Rattus*) are a common species for animal-based science in Canada. The most recent CCAC animal data at publication reported rats as the fourth most common species, representing 5.5% of all animals involved in science (see the CCAC website for the most up-to-date information).

Rats are selected for many different types of studies; for example, cardiovascular and stroke studies, behavioural studies, neonatal and gestational studies, chronic pain studies, as well as studies involving imaging (e.g., cancer studies). They are commonly used in toxicology. While in recent years the numbers of rats involved in scientific studies has decreased, this may change with the introduction of new technologies permitting the modification of the rat genome.

There are a number of challenges that may be associated with rat-based studies, which include:

- gaps in the understanding of behavioural and environmental needs of rats;
- health problems of some strains (e.g., mammary tumours, chronic renal failure, and retinal degeneration);
- recognition, evaluation, and alleviation of pain, discomfort, and distress;
- technical difficulties for studies involving surgical procedures due to small animal size – rats are often chosen for studies involving surgical procedures as they are larger than mice; however, there are still the associated challenges of working with comparatively small animals;
- maintenance of aseptic technique for recovery surgery where there is limited technical support; and
- potential negative effects on animal welfare of transgenic rat models and other specialized disease models being generated.

As with any animal-based science, the scientific validity of any protocol involving rats must be established carefully, and the Three Rs of replacement, reduction and refinement (Russell and Burch, 1959) must guide decisions concerning experimental design and the care of the rats.

Replacement is an important consideration in planning any animal-based study. Consideration must also be given to reduction, to determine the fewest number of animals appropriate to provide valid information and statistical power, while still minimizing the welfare impact for each animal. Sample size calculations should be carried out and a biostatistician should be consulted when necessary.

The present guidelines focus primarily on refinement, both in terms of the care of rats in a facility, and of procedures carried out on rats as part of an animal-based protocol approved by an animal care committee. Animals living in an environment where facilities and practices are oriented toward the promotion of good

animal welfare are less likely to be stressed and more likely to exhibit normal behaviours and physiology (Poole, 1997; Garner et al., 2017), whereas unmanaged pain and distress can influence the reliability of experimental data (Jirkof, 2017).

The following sections provide a brief overview of the behavioural biology of importance to rat welfare (Section 1.1, "Behavioural Biology"), the sensory abilities of rats (Section 1.2, "Senses"), the particular anatomical and physiological characteristics of rats (Section 1.3, "Anatomy and Physiology") and potential inter-animal variations (Section 1.4, "Sources of Variation"), which form the basis of this guidelines document and have an impact on welfare considerations. It is important to consider the characteristics of the species, strain, and sex of the rats, as well as the specific characteristics of individuals, when considering the impact of a procedure or condition on the welfare of rats and on the research results.

1.1 BEHAVIOURAL BIOLOGY

Addressing the welfare of rats in the laboratory environment requires consideration of their natural behaviours (which will vary with the strain) and providing opportunity for those behaviours to be expressed where appropriate. Key behaviours that should be considered in developing appropriate plans for housing, husbandry and research are described below.

In their natural environment, rats are gregarious and live in varied groups, often with one dominant male and several females and younger males (Calhoun, 1963). Rats are social animals and there is evidence to suggest that they prefer to be housed with other rats (Patterson-Kane, 2002, 2004; Hurst et al., 1997). In the laboratory environment, rats show little aggression when housed in stable, single-sex groups, but mature males often engage in agonistic behaviours with other males when introduced to unfamiliar males (Blanchard and Blanchard, 1988), and when group housed with receptive females (Blanchard et al., 1988) (see Section 6.2.1, "Social Housing").

Rats will engage in a number of social activities, including grooming and allogrooming (Aldridge, 2004) and play, particularly young animals (Pellis and Pellis, 2004; Pinelli et al., 2017). They are primarily nocturnal or crepuscular (Antle and Mistlberger, 2004), tending to avoid open, bright spaces (Burn, 2008), and will spend time hiding and burrowing, particularly in the light phase (Boice, 1977; Calhoun, 1963; Makowska and Weary, 2016). Rats are sensitive to light levels and can discriminate light intensity down to 0.1 lux (Campbell and Messing, 1969).

Rats are exploratory in nature and engage in a variety of locomotory behaviours, including walking, running, jumping (Altman and Sudarshan, 1975), climbing (Foster et al., 2011), and stretching upright on their hind legs (Büttner, 1993; Makowska and Weary, 2009). Rats are omnivorous, foraging and feeding mainly during the night (Whishaw, 2004). Generally, rats will carry food by their teeth to a safe place before adopting a squatting posture and eating while holding the food in their forepaws (Lawlor, 2002).

The structure of the rats' environment plays a role in their ability to perform natural behaviours. Rats have shown a preference for shelters and material which they can manipulate (Manser et al., 1998; Patterson-Kane et al., 2001) or chew (Chmiel and Noonan, 1996). Rats usually sleep stretched out full length with their tail extended, but may sleep in a curled position when cold (Lawlor, 2002).

Normal rat behaviour also includes mating and other reproductive behaviours, including caring for young pups. Rats will build nests if given appropriate materials (Jegstrup, 2005; Manser et al., 1998), although in general, this behaviour only occurs if animals are given access to nesting materials starting at a young age

(Van Loo and Baumans, 2004). Some dams are very protective of their young and may bite when their nest is disturbed, particularly in the first week after birth.

The average litter size of rats in the wild is eight pups. At birth, rat pups are naked, blind, have limited hearing, and are completely dependent on the dam (altricial species). The pup's eyes begin to open, the ear canal increasingly opens, and they begin to eat bits of the dam's food at approximately two weeks of age. By three weeks of age, pups are foraging and feeding independently and are engaging in play with conspecifics. Play "fighting" (or rough-and-tumble play) peaks at approximately 4-6 weeks of age (Panksepp, 1981) and is important for establishing normal social behaviour and avoiding male-male aggression in adulthood (Pellis et al., 2010). The rearing environment can have an impact on the long-term development of rats (i.e., epigenetic effects, see Pryce and Feldon (2003)). Distress experienced by rat pups due to separation from the dam (for periods longer than 20 minutes) can result in heightened stress responses when those pups become adults (Gutman and Nemeroff, 2002).

While stereotypic behaviours are commonly observed in many species of laboratory rodents such as mice and gerbils, these behaviours are not generally observed in rats (Koolhaas, 2010). Although rare, facial barbering has been observed in some strains of laboratory rats (Bresnahan et al., 1983).

1.2 SENSES

Rats have highly developed senses of hearing, smell, and touch (Koolhaas, 2010). They use ultrasonic sounds for communication; for example, pups emit ultrasound to solicit care from the dam (Koolhaas, 2010). Ultrasonic vocalizations appear to be used to control aggression and mating (Gamble, 1982), and may be indicative of positive or negative affective states (Knutson et al., 2002). Olfactory cues can influence rats' behaviour and communication (Koolhaas, 2010). They respond to pheromones (Koolhaas, 2010) and use urine to mark territories (Brown, 1975; Doty, 1986; Manzo et al., 2002). Rats explore their environment with their nose, mouth and forepaws, and have extremely sensitive vibrissae (Burn, 2008). Rats may huddle in response to the need for olfactory or tactile stimuli and for thermoregulation (Alberts, 2007).

Light intensities common in human environments can cause retinal degeneration (Schlingmann et al., 1993a) and cataracts (Rao, 1991) in rats. Albino strains are most at risk due to their lack of melanin for protection (Schlingmann et al., 1993b). High light intensities can cause permanent visual damage in rats; light-induced damage has been found in pigmented rats at 950 lux (Williams et al., 1985), and in albino rats at levels above 130 lux (Semple-Rowland and Dawson, 1987).

For a thorough review of the literature on the rat's sensory perception, see Burn (2008).

1.3 ANATOMY AND PHYSIOLOGY

Anatomical and physiological characteristics of rats have important implications for their housing and care, and can impact research studies. These include continuously growing incisors, no gall bladder (McMaster, 1922), and a diffuse pancreas. Rats prevent overgrowth of their incisors through gnawing.

There can be significant differences in growth curves between sexes and strains that may also impact rat husbandry or the conduct of experimental procedures. In particular, there is a prolonged growth period for males.

Rats have a natural habit of coprophagy and have been shown to experience reduced efficiency in food and nutrient utilization if coprophagy is prevented (Cree et al., 1986). Deprivation of this activity among young rats may lead to malnutrition and abnormal eating behaviour when they are older (Novakova and Babicky, 1989).

See Appendix 1 for a list of resources for information on the anatomy and physiology of rats.

1.4 SOURCES OF VARIATION

As described below, particular aspects of the behaviour, anatomy, and physiology of a rat can vary with the animal's strain or stock, health status, sex, and age, as well as with the effects of any genetic modification and previous experiences.

1.4.1 Strains and Stocks

Laboratory rats are often divided into two distinct groups: outbred stocks and inbred strains. Outbred stocks are intentionally not bred with siblings or close relatives, as the purpose of an outbred stock is to maintain maximum heterozygosity. Outbred rats have a relatively long lifespan, are resistant to disease, and have high fecundity (MGI, 2013). An inbred rat strain originates from a single ancestral pair and has been mated brother to sister for 20 or more consecutive generations. The rats are then considered genetically identical and homozygous at almost all loci (MGI, 2013). However, genetic drift and other factors may result in notable diversity between the same strains obtained from different sources (Fahey et al., 2013; Hermsen et al., 2015; MGI, 2013).

Strains of rats may show a number of differences, including the following:

- visual acuity (Prusky et al., 2002);
- hormone profiles (Garland et al., 1987);
- lifespan (Pass and Freeth, 1993);
- responsiveness to anesthesia and analgesia (Avsaroglu et al., 2007);
- temperament (Hall, 1941) and agonistic behaviour;
- performance of natural behaviours (Benus et al., 1991);
- fecundity and age of sexual maturity (Wilkinson et al., 2000; Giknis et al., 2009);
- vocalization and hearing (Turner et al., 2003); and
- sensitivity to drugs and naturally occurring chemicals (Kacew and Festing, 1996).

Rats of different strains or rats that have undergone genetic modification may have different requirements for the environment in which they live. It is important to ensure that the strain is appropriate for the study and that the housing and care are aligned with the animal's requirements. For a review of various inbred rat strains, and the physiological attributes that may influence their environmental requirements, see the Mouse Genome Informatics Index of Major Rat Strains.

1.4.2 Individual Differences – Sex, Health Status, Microbiome

The importance of recognizing and understanding the implications of sex differences in research has been well established (McCarthy et al., 2012). McCarthy et al. (2012) and Becker et al. (2005) discuss considerations for identifying the nature and implications of sex differences in studies in order to improve study design and interpretation of results. While the estrous cycle can impact variability in some studies involving females, and sex differences may be more prominent during a particular stage of the estrous cycle (McCarthy et al., 2012), there are some studies where the estrous cycle is not a contributing variable that needs to be monitored (Dayton et al., 2017). Moreover, variability among males, which is influenced by factors such as dominance hierarchies, may also need to be considered (McCarthy et al., 2012).

The health status of rats has implications for their use in research and how they should be held within a facility. Health monitoring programs, as described in Section 9.2, "Health Monitoring and Disease Detection", and Mähler et al. (2014), are important in maintaining animals of a particular health status, as well as other potential disease states, including metabolic status (e.g., glucose and lipid levels in models of metabolic syndrome) and diabetes (Martin et al., 2010; Wong et al., 2016; Yokoi et al., 2013; Kurtz et al., 1989). It is also important to consider psychological well-being, since studies across various species have shown that stressors can affect physiology and susceptibility to disease, and these effects can be observed in the affected individual (Biondi and Zannino, 1997) and in their offspring (Meaney et al., 2007).

The gut microbiota of an animal affects its physiology and the onset of numerous diseases (Sekirov et al., 2016). The composition of the gut microbiota is influenced by factors such as genetics, age, gender, diet, housing, environment, antibiotic treatment, and stressful procedures, and has been linked to the variation in animal models among animal facilities and among suppliers (Turner, 2018).

1.4.3 Effects of the Environment and Previous Experience

Housing conditions have been demonstrated to have a number of effects on the physiology and behaviour of laboratory rats (see Prager et al., 2011; Simpson and Kelly, 2011; Würbel, 2001). In addition, housing conditions can affect male and female rats differently; for example, male and female rats have been found to respond differently to increased housing density, with male rats showing a greater stress response to overcrowding (Brown and Grunberg, 1995). Human contact can also be a factor, as rats that are handled frequently and gently (especially between 3-4 weeks of age) become easy to train (Maurer et al., 2008), and the related stress reductions may improve the validity of research.

Standardization is generally employed in research to minimize variability and maximize reproducibility, but even with apparent standardization of environmental conditions, animal behaviour can show considerable variation across experiments due to minor differences in treatment. Reproducibility may be improved by increased heterogeneity in environmental conditions, whereas standardized environments can compromise reproducibility by producing results that are specific to narrowly-defined environmental conditions (Richter et al., 2009). These potential effects should be carefully considered as part of the experimental design process.

Prior experiences can also influence the behaviour and physiology of laboratory rats, particularly during the perinatal period. Much of the in-strain variation seen in rats kept under the same environmental conditions is attributed to the effects of mothering and the early environment (i.e., epigenetics (Bredy et al., 2003; Champagne and Curley, 2009)).

2 FACILITIES

For general guidance on facilities, please see the *CCAC guidelines on: laboratory animal facilities – characteristics, design, and development* (CCAC, 2003). Additional guidelines and information of particular concern for rats is presented in this section.

2.1 HOUSING

Housing must confine the animals securely and safely, and ensure their welfare by permitting normal postural and behavioural adjustments. It should also allow rats the opportunity to perform behaviours important to their well-being. The various components of housing are discussed below (i.e., types of cages, cage size (floor area and vertical space), cage materials, cage floors and furnishings). Configuration of the living area should also be considered, with a view to accommodating nesting materials and cage furnishings. These elements must be considered together, rather than in isolation, with an awareness of how the needs of the rats may differ according to characteristics such as strain or stock, physiological state, age, and sex (note the Canadian Institutes of Health Research (CIHR)'s focus on involving both male and females in pre-clinical research (CIHR, 2015)). Housing can influence biological variables and should be taken into account in experimental design. However, changes which are beneficial to rat welfare and do not impact the physiology of the animal are not likely to impact research results (Pinelli et al., 2017).

2.1.1 Types of Cages

Guideline 1

The selection and purchase of caging that is suitable for the needs of the study and the welfare of the animals should be a collaborative decision involving investigators, animal care personnel, and other institutional parties.

Involvement of investigators, animal care personnel, and other institutional parties is critical to ensure that caging meets the needs of the research, meets the welfare standards of the institution, and can be properly used and maintained by the animal care personnel.

There are a variety of caging systems available (for a brief overview, see Howard et al. (2011), Voipio et al. (2011) and Smith and Baran (2013)). Multi-level cages are now available and are being shown to have welfare benefits (Wheeler et al., 2015). Before any new infrastructure is acquired, there should be extensive consultation among investigators or study directors carrying out rat-based studies and facility managers; input should also be sought from facility managers or directors from other institutions.

Factors to be considered in acquiring cages include:

- air quality – temperature, humidity, ammonia, carbon dioxide, particulates, volatile organic compounds, and pheromone levels within cages can be influenced by the rate and position of the air supply, air distribution, and movement of air within the cage (Rosenbaum et al., 2010);
- vibration – vibration from equipment associated with caging, as well as from other sources (e.g., construction), is an important consideration as it can disrupt a number of physiological systems (Krajnak et al., 2012) and is especially disruptive for breeding colonies (Norton et al., 2011) (see Section 3.1.4, "Sound and Vibration");
- noise – cage design features result in differences in noise levels, and it is important to minimize noise, including white noise, as it has been shown to negatively impact physiological systems in rats (Baldwin, 2007);
- ability to access animals – the configuration of cage components can affect the ability to handle the animals and consequently, affect the welfare of those animals;
- ability to monitor animals – this can be impacted by the colour and transparency of the cages, and the design of the rack;
- risk of ergonomic injury for personnel;
- ability to sanitize the cages and racks; and
- the need for barriers – to prevent animal infections and infestations from entering a unit from outside sources (i.e., biosecurity, which is achieved through exclusion barriers) and/or to prevent the escape of agents of disease from the animals in the unit to the outside (i.e., biosafety, which is achieved through inclusion barriers) (see the *Canadian Biosafety Standard* (CBS) (Government of Canada, 2015)).

2.1.1.1 Cage Size

Guideline 2

Cages should be of a sufficient size and complexity to allow rats to be housed in appropriately sized groups and to perform behaviours important to their welfare.

Positive welfare encompasses environmental comfort, freedom from pain and distress, freedom to express natural behaviours, and appropriate social interactions (CALAM, 2007; CCAC, 2017).

The choice of cage should support social housing of rats (see Section 6.2.1, "Social Housing"). Cage size will affect the number of rats that can be housed within the cage, and their ability to perform locomotory and exploratory behaviours, including play, as well as the capacity to add elements that can positively affect their welfare.

As institutions acquire new cages, they should use the performance standards indicated in Appendix 2 to be sure that the behavioural needs of the animals are addressed. Space allocations should be assessed and modified in accordance with the animal's health and opportunity to perform behaviours important to their welfare, keeping in mind the study objectives. Larger cages with furnishings that enhance welfare by providing opportunities for the animals to express additional natural behaviours are encouraged (see Section 8, "Welfare Assessment", for further information). One study with Wistar rats found a preference for larger

cages (Patterson-Kane, 2002), but preferences of individual animals may be influenced by the strain or stock, age, and previous experience, as well as the features of the cage itself. For example, another study found an interaction between cage size and environmental features, where rat stress responses were reduced in larger cages, but only when enrichment items were present (Foulkes, 2004).

If not properly planned for, the introduction of larger cages can have negative implications for the health and safety of personnel. Kerst (2003) provides guidance that can be used by institutions to find reasonable solutions to minimize these challenges. Ergonomics is not a justifiable reason to avoid following cage size recommendations.

2.1.1.1.1 Cage Floor Area

Guideline 3:

Cages should provide at least 800 cm^2 of floor space, and occupancy should be based on the minimum floor space required per animal.

Floor area should be sufficient to provide the animals with a comfortable resting area, including shelter, and allow the animals to express normal behaviours (both through available space and the inclusion of appropriate furnishings) (see FAWC, 1979). Normal behaviour includes, but is not limited to, social interaction (engaging in affiliative interactions and avoiding agonistic interactions), foraging, walking, running, jumping, fully stretching laterally, and play (for young rats). It is important to plan for the length of time the rats will be held to accommodate their requirements for increased space as they grow. This may include transferring rats to larger cages as they grow.

Floor area requirements vary with strain or stock, number of animals, age, familiarity with cage mates, and reproductive status (Arakawa, 2005; Koolhaas, 2010). Young animals require sufficient space for play to enable normal development of social behaviour (Koolhaas, 2010).

Sudden increases in group number and cage size can potentially cause welfare concerns and should be carefully monitored (Yildez et al., 2007).

Below are some indications of minimum standards; however, of much greater importance is the evaluation of whether the space provided permits the rats to carry out behaviours essential for their welfare, as outlined in Appendix 2.

Estimates for space requirements are based on the size of animals and the minimum space necessary for them to physically perform various behaviours that are relevant to their life stage. For example, it is estimated that a group of four 100 g weanlings would need a minimum of 900-1000 cm^2 to accommodate appropriate resources; however, a cage with a minimum of 1500 cm^2 would be needed to accommodate rough-and-tumble play behaviours, considered important for normal development (see Sections 1.1, "Behavioural Biology" and 6.2.1, "Social Housing"). Similarly, a pair of large 600 g male rats with body lengths of 26 cm would need a minimum of 1500 cm^2 to perform the basic series of rat behaviours (see Appendix 2).

2.1.1.1.2 Cage Height

The height of cages should accommodate typical postures of rats, including the ability to stretch vertically, which has been shown to be a common and important behaviour for rats (Büttner, 1993; Makowska and Weary, 2016). To determine the appropriate cage height, the length of the rat from the nose to the base of the rump should be estimated, and then approximately four centimetres added to account for the additional distance required for upright stretching with partial leg extension. For example, a fully grown male rat with a body length of 26 cm would require a cage height of 30 cm to accommodate appropriate rearing and stretching behaviour.

While young rats need to be able to stretch vertically, they must be able to reach food and water easily. Where cages are fitted with shelters, the height of the cage should allow rats to climb on top of the shelter to maximize the available space.

2.1.1.2 Cage Materials

Guideline 4:

Cages must allow for proper monitoring of the animals, preferably without disturbance, with consideration of research requirements.

As noted in Section 6.6, "Animal Observation", rats must be observed daily. While electronic monitoring systems are available for a variety of parameters, these systems have limitations and visual monitoring of the animals is still necessary.

Non-disposable cages must be made of materials that are easy to clean, non-toxic, non-absorbent, durable, resistant to heat and chemicals; they must also be escape and predator proof (Koehler et al., 2003). The advantages and disadvantages of different cage materials are described by Jennings et al. (1998). Cages should have rounded edges that facilitate sanitation and are less likely to injure the animals. Cage floors and walls should preferably be plastic, unless special-purpose cages are required.

Some synthetic materials release bioactive substances that may affect rats; for example, polycarbonate or polysulphone cages may expose rats to bisphenol A (BPA) via leaching, particularly with older cages (Howdeshell et al., 2003). This should be considered when replacing cages made of these materials.

Cage colour may have an impact on rats; for example, there is evidence that red-tinted cages affect the circadian rhythm of plasma corticosterone and so may have an impact on the general metabolism and physiology of rats (Dauchy et al., 2010).

2.1.1.3 Cage Floors

Guideline 5:

Rats should be housed in cages with solid floors.

Solid-bottom cages should be used unless there is clear scientific justification for the use of wire-bottom cages. Rats have been shown to prefer solid floors with bedding over wire-bottom floors, and will make considerable effort to access solid floors to rest (Blom et al., 1996; Manser et al., 1995; Manser et al., 1996).

During pregnancy, parturition, and lactation, breeding females must be kept on solid floors with bedding, as wire floors have been shown to negatively impact reproductive performance in terms of pre- and post-implantation losses, gestation index, litter index, number of littered pups, and mean pup weights (Deepananda, 2013; Bosque et al.,1994).

Wire-mesh floors have been shown to cause neuropathy and tactile hyperesthesia of the hind limbs, and may result in foot lesions after prolonged periods of six months or more (Harkness et al., 2010). Housing rats on wire floors for a period of two weeks has been shown to lead to increased blood pressure and heart rate, with the increase in blood pressure persisting even after the rat has been moved to a cage with a solid floor and bedding (Krohn et al., 2003). After rats were housed on wire floors for a week, the expression of genes encoding proteins involved in inflammatory or immune responses have been shown to be higher than for rats housed in solid-bottom cages (Uehara et al., 2012), which could have an impact on research data. In addition, for studies where it is necessary to prevent rats from consuming their feces, it should be noted that this is not completely prevented by the use of wire-bottomed floors. False bottoms can be added to solid bottom cages for a short period of time to allow for sample collection or if the experimental design needs to limit access to the bedding. This should be considered a temporary arrangement.

2.1.1.4 Cage Components

The provision of cage components should aim to address behaviours important to the rats' welfare. Added items can provide areas to hide, as well as expand the opportunity for activity by providing objects for the animals to walk around or through, stand on, hide inside of to eat, and gnaw. Increased activity helps to maintain a higher basal metabolism (which can slow), and possibly prevent the development of obesity (Turner et al., 2014).

The investigator or study director should be consulted prior to the addition of any items to rat cages. Items should be selected to meet the behavioural needs and drives of the species, and the impact of the items should be monitored and evaluated to ensure that they provide benefit to the animals and do not have any negative impacts on the animals or on the research.

2.1.1.4.1 Shelters

Guideline 6:
Structures for shelter should be provided.

A rat's use of a shelter can be influenced by the type of structure (Manser et al., 1998; Patterson-Kane et al., 2001; Patterson-Kane, 2003), as well as the strain or stock, physiological state, age, sex, and the housing conditions of the rat (Galef and Sorge, 2000). Shelters can address a variety of activities that are part of the rat's natural behavioural repertoire (e.g., Bradshaw and Poling, 1991; Jegstrup et al., 2005):

- withdrawal from light;
- control of microclimate (which aids in thermoregulation);

- escape from aggressive social interactions;
- thigmotactic avoidance of open spaces;
- nest building activity; and
- gnawing (depending on the materials structures are made from).

Rats that are provided with shelters show more exploratory behaviour and are less fearful (Townsend, 1997). Shelters or structures that allow rats to climb on the top enable greater use of vertical space, and rats will spend a considerable amount of time on top of the shelter or hiding structure during the dark period (Manser et al., 1998; Bradshaw and Poling, 1991). Rats have shown a preference for shelters with a roof and three walls that are large enough to accommodate at least two adult rats at once (Bradshaw and Poling, 1991). Tubes can also be used as shelters for small rats that can fit comfortably inside.

2.1.1.5 Feeders and Water Supply

Rats should have access to clean food and clean, fresh drinking water at all times. Food and water should be supplied such that it is easily accessible for all animals and contamination is minimized. Feeders and water systems should be designed to meet the safety requirements of the animals (e.g., no rough edges). For some rats that have restricted mobility (e.g., due to size, physiological state, age, or illness), it may be necessary to provide food and a water supplement at the cage floor level.

Food and water should be provided in a manner that allows rats to sit while eating and drinking (such as from hoppers where they can bite off small pieces of food). Degeneration of the hip joint can be a problem in young rats that have to stand on their hind legs to access food (Mihara and Hirano, 1998).

Water may be provided via a bottle, bag, or automated watering system. The best option should be chosen for the facility to ensure that animals always have access to water and the risk of cage flooding is minimized.

2.1.2 Special Purpose Housing

2.1.2.1 Pens

Pens or large cages, also referred to as 'parks', are used for colony housing or teaching, as well as in some research studies. This type of housing has large areas for rats to move horizontally and vertically, and results in improved welfare for the rats (Makowska and Weary, 2016), including increased physical fitness (Spangenberg et al., 2009). Pens or large cages may result in rats being more difficult to handle; however, habituation to people and handling will result in rats being similarly easy to handle in this type of housing (Lawlor, 2002).

2.1.2.2 Metabolic Cages

Guideline 7:

Rats must only be housed in metabolic cages when necessary for the particular study, and for the shortest time possible.

There are several aspects of metabolic cages which can have a negative impact on the welfare of rats. The key stressors for rats in metabolic cages are:

- single housing (see Section 6.2.2, "Single Housing");
- barren environment (see Section 6.4, "Environmental Enrichment"); and
- perforated or wire flooring (see Section 2.1.1.3, "Cage Floors").

All of these elements are associated with welfare concerns, which are described in the sections indicated. Where possible, alternatives to metabolic caging should be used, for example, sample collection in the home cage environment.

When the use of metabolic cages is justified, rats must only be held for the shortest time necessary. Changes in the physiology and behaviour of rats held in these cages may have an impact on study results (Barker et al., 2016; Whittaker et al., 2016). The negative impacts of holding rats in metabolic cages on their metabolism and behaviour may be influenced by the age of the animals, their sex (Barker et al., 2016), and the duration of exposure (Zymantiene et al., 2016), which should be taken into account in designing experiments using metabolic cages. Acclimatization can be done before the study begins so that it is not a sudden stressor at the onset of the study; however, the value of acclimatization will depend on the specific study. For studies where the rats will be held for a short time, adding time for acclimatization may not be effective and will extend the duration of the stress for the rats.

Where studies dictate that rats need to be held in metabolic cages for longer than 24 hours, animal comfort should be a consideration and the cage should include a resting platform and the means for the animals to establish a microclimate. Providing enrichment can improve the welfare of rats in metabolic cages; however, they will still be negatively impacted by the lack of social interaction, barren environment (Sørensen et al., 2008), and prevention of coprophagy.

2.2 SURGICAL FACILITIES

The *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (CCAC, 2003) requires a dedicated surgical suite or a dedicated area in a procedure room, separate from other activities. For rats, a dedicated surgical suite is preferable. Any recovery surgery should be carried out in a dedicated room. The dedicated room or area should be easily cleaned, with impervious surfaces that facilitate the control of contamination and maintenance of aseptic conditions. There should also be a separate surgical preparation area and a recovery area.

Waste scavenging equipment must be available where anesthetic is used. Biological safety cabinets should only be used when necessary. Measures should be taken to address the susceptibility of rats to hypothermia (see Section 10.10, "Surgery"). Any period of recovery from surgery should be conducted in a dedicated room or area.

2.3 CORE FACILITIES FOR GENERATION OF GENETICALLY MODIFIED RATS

The decision to invest in developing and using in-house rodent transgenic core facilities is complex and should take into account a number of factors. Among these considerations are availability of infrastructural resources, specialized human resources, geographical location, and requirements of the local scientific com-

munity. Institutions embarking on renovation, new construction, or program expansion should have building and housing infrastructure, as well as instrumentation and laboratory facilities either in place or planned to provide specific pathogen-free derivation facilities as well as barrier housing space.

Where dedicated core facilities for the generation of genetically modified rodents exist, they should include the necessary expertise and equipment to generate new lines efficiently. Where dedicated core facilities are not available, outsourcing of generation and preservation of rat lines should be considered. Both outsourcing and use of core facilities can help ensure that effective methods of producing animals are used, potentially reducing the numbers of animals involved.

Core facilities usually include facilities for cryopreservation (archiving). The freezing units should have backup power, liquid nitrogen sources, and an alarm system.

3 FACILITY MANAGEMENT AND PERSONNEL

3.1 MANAGING THE ENVIRONMENT

The *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (CCAC, 2003) should be consulted for general guidelines that are applicable to all species. This section provides additional considerations that are particular to rats.

Guideline 8:

Laboratory management practices must aim to ensure the macroenvironment (room) and microenvironment (cage) maintain the health and welfare of both the animals and personnel, and provide consistency for research outcomes.

Procedures for managing the environment will depend on the room layout and type of caging. Particular attention should be paid to maintaining an appropriate environment within cages.

3.1.1 Lighting

Appropriate light levels should take into account differences in strains or stocks (Borges et al., 1990; O'Steen and Donnelly, 1982), age (Pelosa et al., 2016), and previous experiences of the animals (Organisciak et al., 1998). Lighting that is too bright can cause retinal degeneration and cataracts (De Vera Mudry et al., 2013; Rao, 1991), as well as alter behaviour and reproduction (e.g., number of litters, litter size, and weight gain during gestation (Weihe et al., 1969)). Short bursts of light during the dark period should be avoided, since short, intense light exposures during the dark phase cause temporary retinal damage that is greater than when exposure occurs during the light phase (Organisciak et al., 2000).

Retinal degeneration has been, and continues to be, a confounding factor in research data (e.g., maze learning (Lindner et al., 1997; O'Steen et al., 1995) and phototoxicity studies (De Vera Mudry et al., 2013)). In addition, rats exposed to light at 400-500 lux have been used as models of stress, as they exhibit anxiety-related behaviours (Nathiya and Vanisree, 2010), and even brief exposure to bright light is aversive (Barker et al., 2010). Exploratory behaviour has been shown to be affected by light levels, and therefore even without damage to the eye, different levels of illumination can influence the results of behavioural studies (Garcia et al., 2005).

The room lighting specified in the *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (2003), i.e., 325 lux at 1 m above floor level, may be too bright for rodents in general, including rats (De Vera Mudry et al., 2013). In some cases, maintaining light levels below 325 lux may be appropriate when personnel are not performing tasks in the animal room. For breeding, Weihe et al. (1969) found that the number of rat litters produced was highest at a room light intensity of 250 lux, while the number of young per litter peaked at 60 lux. It is important to consider the effect of lighting on factors such as cycling, fertility, and fecundity.

Recent research has shown that chronic exposure to LED light emitting blue light (460 nm) is harmful for rats, necessitating a cautionary approach if using LED light as the primary source of lighting (i.e., consideration of wavelength and intensity) (Shang et al., 2014). Sorenson (2014) indicates that lack of UV light has a negative effect on welfare, and providing artificial full spectrum light at a low light intensity, combined with sufficient shelters or nesting options, is most suitable for rats.

Lighting should be consistent and not contribute to glare or variations in temperature throughout the room. Flickering lights should be corrected quickly, as they can be a potent source of stress for animals (Lalitha et al., 1988). The ballast of fluorescent lights also causes noise in the 50-60Hz range, which can interfere with electronic recordings and potentially disrupt breeding.

The light cycle is also important. The *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (2003) note that rats reproduce optimally using a diurnal cycle of 14-hours light and 10-hours dark; however, light cycles of 12:12 light:dark and 10:14 light:dark are also acceptable. Consistency in the diurnal cycle is often critical to reliable research results. Disruption of the dark phase, even by a very minimal amount of light, can disrupt circadian rhythms of endocrine metabolism and physiology (Dauchy et al., 2010). If making observations during the dark phase, red light (400-700nm) (Jennings et al., 1998) or sodium (589nm) lamps (McLennan and Taylor-Jeffs, 2004) should be used. However, some physiological parameters may be affected by red light (Dauchy et al., 2015).

The light level within the cage will vary with the type of caging, the provision of a shelter and/or nesting material, the front versus back of the cage (Weihe et al., 1969), the position of the cage on a rack (Rao, 1991), provision of shade over the top of the rack, tint or opacity of the cage walls, and the distribution of cages in a room. It is important to measure light levels in the room and cages once the equipment is in place, including at the top, middle, and bottom rows of the cages and inside cages at the front and back. This is very important to understand light exposure, and should be carried out periodically (with adjustment to lighting levels if necessary). The in-cage illumination level is the most important factor for rats, and if possible, should be kept in the range of 20 lux or lower.

Management practices to control light levels should be evaluated in the context of each study; for example, rotation of cages may not be practical where such movement will cause stress to the animals or result in mixing of dose groups for testing purposes. Management practices to control light levels within cages may include:

- rotating cage position on the rack;
- avoiding use of the top shelf of racks, or providing shade over the top shelves if the cage does not have a solid top;
- providing a shelter that the rat can choose to use to control light exposure;
- using red light or sodium lamps during the dark period for monitoring the active phase; and
- providing eye protection for the rats if it is necessary to use bright lights for particular procedures (e.g., bringing a few cages at a time into a procedure room and covering them with towels to occlude the light, or using opaque restraining tubes when handling the animals).

Additionally, investigators should be aware of situations that expose rats to different light levels (e.g., rats can be exposed to large differences in light levels when removed from their cages in transfer stations or when exposed to room lighting).

3.1.2 Temperature and Relative Humidity

Temperature and relative humidity should be considered together, as the combined effects of these two parameters influence the welfare of the animals. The most suitable temperature for rats is also affected by the air velocity, barometric pressure, the housing structure, and the effective radiant field, as well as the age, strain, and experience (e.g., thermal adaptation) of the individual rats (Romanovsky et al., 2002).

Facilities should have a system in place to monitor temperature and relative humidity, particularly within microisolator caging. The type and design of the caging, as well as the quality and quantity of bedding and nesting material, will affect the animal's ability to influence its own environment. Other factors that affect temperature and relative humidity include the number, age, type, and size of the animals, cage furnishings, the position of the cage on the rack, and the frequency of bedding changes.

Room temperature should be checked daily, or continuously monitored by a building automation system equipped with an alarm or notification system, and maintained in the range of 20-26 °C (Yamauchi et al., 1981), depending on the individual animals and the environmental conditions. Where possible, a temperature gradient should be achievable such that rats can select their preferred temperature, which may vary depending on the time of day (Hankenson et al., 2018; Gordon, 1994). If the temperature is too low, rats may exhibit excessive huddling and shivering (Vaillancourt et al., 2009), and if too high, rats may exhibit increased salivary secretion and excessive grooming behaviour (Yanase et al., 1991).

Temperature can affect the rat's reproductive performance (e.g., litter size, embryonic death, and impaired growth), and cause significant variation in food and water intake and in hematological and biochemical variables (Yamauchi et al., 1981). Additionally, gestating and lactating dams and pups up to 3-4 weeks old have reduced thermoregulatory ability and should be kept at temperatures of 24-26 °C (Clough, 1982; Knecht et al., 1980).

Temperature requirements may vary with some animals or experimental or husbandry procedures. For example, increased environmental temperatures are required for neonatal animals and hairless phenotypes, and for animals undergoing anesthesia and surgery, and during post-operative recovery. In some cases, this may best be addressed by raising the temperature in the microenvironment (e.g., by increasing the amount of nesting material or providing a water heating pad under the cage).

Sudden changes in temperature should be avoided. Fluctuations or extremes may result in behavioural, physiological, and morphological changes, which might negatively affect animal welfare and research results. For example, temperature extremes can lead to reduced weight gain (Jones et al., 1971). Appropriate nesting material should be provided to help avoid sudden intra-cage temperature changes.

Ideally, room relative humidity should be 40-60%, as stated in the *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (2003); however, 30-70% may be acceptable. The health status of the animals and the impact of humidity levels on animals undergoing experimental procedures are important considerations in determining the appropriate relative humidity level. Supplemental humidification should be provided where low relative humidity is a problem.

Health concerns for rats attributed to low relative humidity include middle-ear disease (for relative humidity of 10-12% (Lovejoy et al., 1994)) and ringtail in young rats (Harkness et al., 2010), which occurs under conditions of low relative humidity and low temperature. When relative humidity is high, the proliferation of bacteria and ammonia is enhanced (Broderson et al., 1976; Memarzadeh, 2005).

The health and comfort of personnel should also be taken into consideration.

3.1.3 Air Quality and Ventilation

As noted in the *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (CCAC, 2003), "the rate of air exchange within a room must be such that clean, fresh air is available to all animals and personnel at all times". See Section 12.3, "Heating, ventilation and air conditioning (HVAC)" of the *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (CCAC, 2003) for factors affecting room air quality. Intra-cage temperature, humidity, and ammonia levels are generally higher than room levels. The main components of air quality of concern in laboratory animal facilities are ammonia, carbon dioxide, particulates, and volatile organic compounds (CCAC, 2019). The most sensitive indicator of damage from chronic ammonia exposure in rats is histological changes in the nasal passages (Broderson et al., 1976), as observed post-mortem. While it has been suggested that maximum ammonia levels within cages should be 50 ppm (Allmann-Iselin, 2000), data from other rodent species indicate that exposure to ammonia levels of 25 ppm over a 7-day period resulted in lesions in nasal passages, suggesting that this level of ammonia is too high (Ferrechia et al., 2014; Mexas et al., 2015). Maintenance of acceptable air quality is generally accomplished by the frequency of cage changes, and in the case of individually ventilated cages, appropriate air exchange at the cage level. See Washington and Payton (2016) for details on the use of ammonia as a cage-change indicator.

3.1.4 Sound and Vibration

Ambient noise should be minimized as much as possible. Noise-generating equipment and noisy animals (e.g., dogs and nonhuman primates) should be located away from areas housing rats, and measures should be taken to mitigate any noise within the animal room. Turner et al., (2005) provide an overview of the many sources of noise in an animal facility, which fall into the three general categories of electronic equipment, maintenance equipment and activities, and the animals themselves. The hearing ability of rats and the effect of a particular noise on their welfare and/or the study results will vary among strains (Turner et al., 2005).

In general, rats are capable of hearing sound frequencies in the range of 0.25 to 80 kHz (Heffner et al., 1994, as cited by Burn, 2008); notably, rats are capable of hearing ultrasonic frequencies above the range of human hearing (maximum of ~20 kHz), and are sensitive to high frequency noises that humans are not able to detect. Ultrasonic vocalizations play an important role in rat social and sexual behaviour (Peterson, 1980); therefore, in addition to causing disturbance, extraneous noise in this range might interfere with their ability to communicate. The effects of noise stress in rats have been reviewed by Castelhano-Carlos and Baumans (2009) and Turner et al. (2005), and include changes in eating behaviour, weight, sleep patterns, hormone levels, and adrenal function, as well as changes to the gastrointestinal, immunological, reproductive, nervous, and cardiovascular systems. The effects of noise are complex and vary with both the intensity and duration of noise (Turner et al., 2005).

The person responsible for the animal facilities should establish a relationship with the institutional general facilities management and planning department to ensure that the animal facility is alerted to any new construction or renovations and can coordinate with the researchers to minimize the impact on the animals and on research activities.

Equipment and activities that generate large amounts of noise should be kept away from rats or have appropriate acoustical shielding. Ultrasonic signals, which come from a variety of sources (e.g., electronic equipment such as video monitors, metal pipes, and squeaky trolley wheels), should be minimized in rooms housing rats. Lighting fixtures (ballasts) that do not emit ultrasound should be used, and the fire alarm systems should operate at a frequency that is not disruptive to rats. Extraneous noises from sources such as

dripping taps should also be minimized, as should vibrations in rooms and cages. It is particularly important that rat breeding colonies be located as far away as possible from noise-generating equipment and noisy animals. Fertility and productivity of rats can be reduced by exposure to ultrasound (Zondek, 1964, as cited in Castelhano-Carlos and Baumans, 2009).

Although the *CCAC guidelines on: laboratory animal facilities – characteristics, design and development* (CCAC, 2003) states that in general, background noise may be useful in a laboratory animal facility to mask sudden noise and provide consistency, it appears not to be beneficial for rats (Baldwin, 2007; Krohn et al., 2011).

Sources of vibration within laboratory animal facilities include the room ventilation system, ventilated racks, and equipment related to husbandry or research activities, as well as activities occurring outside of the room (Norton et al., 2011). Vibrations can result in various physiological and pathological effects, and the impact of vibration caused by a particular source will depend on the species and age of the animal (Norton et al., 2011). In a study involving construction equipment, rats were found to experience more vibration than humans (Norton et al., 2011); therefore, measures should be taken to dampen all potential sources of vibration.

3.2 PERSONNEL

Guideline 9:

Rats must be observed daily by trained personnel, with minimal disruption to the animals.

Sufficient qualified animal care personnel are needed to ensure: 1) cages are cleaned, food and water are provided, and other husbandry requirements are addressed, as appropriate; and 2) animals are observed daily. All rats must be observed daily, seven days a week, by trained personnel who can recognize welfare concerns and health problems in rats and resolve them through institutional standard operating procedures, and who follow proper record keeping and reporting procedures to ensure the facility manager and veterinary team remain informed, and researchers are alerted to any changes.

Where welfare concerns are identified, any additional demands on personnel time for the implementation of appropriate mitigation strategies also need to be considered and accommodated.

All personnel should use appropriate practices that respect the welfare of the animals (e.g., not tapping on the cage and moving cages in a way that minimizes disturbance).

4 PROCUREMENT

The *CCAC guidelines on: procurement of animals used in science* (CCAC, 2007) should be consulted for general guidelines that are applicable to all species. This section provides additional considerations that are particular to rats.

4.1 SOURCE

Rats are obtained from a variety of sources, including commercial breeding companies, as well as individual laboratories sharing particular genetically modified strains or disease models. The necessary background data for the maintenance and record keeping for any imported genetically or surgically modified animals should be obtained before acquiring the animals.

There are many confounding issues that may prevent the use of pre-existing animal lines, including intellectual property protection, scientific barriers (e.g., assurance of the same site of insertion of the gene of interest), animal quality concerns, incorrect analysis, compromised background strains, health status, availability, and accessibility. However, investigators should take reasonable steps to ensure that they are not generating new genetically modified rat lines when scientifically adequate lines already exist that are accessible.

If rats have not been subject to major invasive procedures, they may be transferred to another study or protocol. The transfer of animals that have been surgically prepared (e.g., adrenalectomy, thymectomy or cannulation) by breeding institutions for inclusion in an animal-based protocol is also permitted. However, these animals should only be transferred when they are fit to travel.

4.2 DOCUMENTATION

An effective documentation system should be in place to provide information on the identity and welfare of rats, including health concerns and any special needs, to ensure their proper care and to inform decisions regarding any animal-based procedures. This information should accompany the rats and be accessible to all involved in their care and in the conduct of procedures.

Correct nomenclature should be used to identify the animals, especially in the case of genetically modified rats. For guidance, see the *Guidelines for Nomenclature of Mouse and Rat Strains*, published jointly by the International Committee on Standardized Genetic Nomenclature for Mice and the Rat Genome and Nomenclature Committee, and updated annually.

Genetically modified rats brought into an institution should have accompanying documentation, which should include:

- accurate and correct nomenclature of strain and genetic modification(s) in accordance with the *Guidelines for Nomenclature of Mouse and Rat Strains*;
- information on the phenotype of the animal, including welfare status, mitigation strategies for pain and/or distress, and humane endpoints;

- special conditions for husbandry and breeding (e.g., special requirements to address behavioural needs, nutritional needs, or sensitivity to temperature);
- the procedure used to distinguish genetically modified animals from wild-type animals; and
- archiving strategy (e.g., cryopreservation) or archive location if the line is archived off-site.

4.3 SHIPPING AND RECEIVING

Standard operating procedures and documentation are needed for moving animals within an institution, as well as for procuring animals from other institutions or suppliers. These standard operating procedures should describe procedures to protect the health and welfare of both the animals being transported and others they may come in contact with. Prior to accepting new genetically modified rats for breeding purposes, the animal care committee should ensure that the investigator has a plan in place to appropriately manage breeding, including how animals carrying the genotype of interest will be identified.

Transportation plans must be in place to manage any unanticipated delays. Facilities should develop a policy or standard operating procedure for performing a risk assessment and determining necessary precautions for transportation (see Section 5, "Transportation", in the *CCAC guidelines on: procurement of animals in science* (2007)). The policy or standard operating procedure should ensure the transport route is well planned in advance, with consideration to the ambient conditions. The assessment of risk should be based on the type, duration, and route of transportation, the weather, and the likelihood of delays. Short-duration movement of animals (< 1 hour) may not require a risk assessment if the animals can be returned to the original location should problems arise; however, even short transportation within an institution needs to be planned in advance (e.g., avoiding people and public elevators).

4.4 TRANSPORTATION

4.4.1 Shipping Conditions

As noted in Section 5, "Transportation", of the *CCAC guidelines on: procurement of animals in science* (2007), "[i]nstitutions, in consultation with animal users (particularly for protocols using nontraditional or field species), are responsible for selecting the method and timing of transportation of animals from the suppliers, and monitoring the transportation process."

Temperature limits should be specified and measures taken to protect rats from exposure to temperatures outside these limits (e.g., transporting rats at night during hot weather and providing sufficient bedding and nesting material for thermoregulation (Arts et al., 2014)). As a general guide, ambient temperature limits for transportation of rats should be 6-33 °C; however, other factors need to be taken into consideration, such as relative humidity, wind speed, and sun exposure (ILAR, 2006).

Rats must be transported in a safe, non-compressible container from which they cannot escape. The container should be a specifically designed transport box to contain all wastes, protect rats against potential contamination, prevent exposure of personnel and the public to allergens, zoonotic disease, or any hazards (e.g., biological, chemical, or radiation), and keep the animals out of view. Containers must permit adequate ventilation for the rats, even when stacked. Specifications for containers are covered in the *CCAC guidelines on: procurement of animals in science* (2007), and further details concerning the design and construction are given by ILAR (2006) and Swallow (2005).

When a divider is used, individual rats in a container must be identifiable in case the divider is breached. The divider should be constructed so as to prevent the rats from crossing to the other side. Whenever possible, sexually mature male and female rats or those of different ages or health status should be shipped in separate containers rather than separated by a divider within the same container.

Rats must have food and water prior to and during transportation. Rats lose more heat and calories and become dehydrated more quickly than larger animals; for this reason and the necessity of anticipating potential delays, the amount of food and water available during transport should be at least twice that required by the animals for the expected length of travel (CCAC, 2007). Gels are the preferred method for providing hydration during transport, and they should be provided to the rats the day before shipping to allow for acclimation. Water must not be placed in the animal container as it is likely to spill. If rats become wet, hypothermia may develop rapidly, depending on the external temperature.

The transport of cryopreserved embryos or sperm is a useful alternative when the well-being of live rats may be compromised by long periods of transport or when their health status is not compatible with transportation. This is particularly important for genetically modified rats. However, the receiving institutions must have facilities available to handle embryos and sperm appropriately. Those involved in the transport of rats should be aware of further refinements to transport methods, as they become available.

Institutions acquiring gametes should be aware that this material can be a source of contamination and introduce pathogens into a facility.

4.4.2 Moving Rats Between Institutions

Care should be taken not to ship rats that are already welfare compromised to the extent that they will experience distress as a result of transportation practices.

An American Association for Laboratory Animal Science (AALAS) and Federation of European Laboratory Animal Science Associations (FELASA) working group has developed a health monitoring report form that can be used to convey standard health information between institutions (Pritchett-Corning et al., 2014).

4.4.3 Moving Rats Within an Institution

Short-duration transport of rats (e.g., within an institution) should be in accordance with policies or standard operating procedures that emphasize good procedures for protection of both animals and people. Rats should be transported in a covered, closed, and secured cage or container. Home cages may be used, provided they are properly secured and the water bottle removed. Care should be taken to maintain the cage in a horizontal plane during transport to optimize ventilation and to minimize potential accidents, including escape. Personnel transporting rats should be aware that rats are sensitive to vibration and noise, and choose a method that reduces those elements. For example, placing a folded towel or drape under the cage will significantly diminish the vibration transferred between the cart and the cage (Hurst and Litwak, 2012).

If rats are to be moved between buildings, consideration must be given to the weather. Suitable precautions must be taken to protect the welfare of the animals if they are to be moved during extreme weather conditions (i.e., both winter and summer). Rats must not be left unattended in a non-secure area during transport and should be placed in a secure location and attended to as soon as possible upon arrival at their destination.

4.5 RECEPTION OF RATS AT AN INSTITUTION

Guideline 10:

The health status of the incoming rats should be reviewed before the animals are shipped.

Rats are obtained from a variety of sources, including breeding companies and individual laboratories sharing animals. However, as pointed out by Shek (2008), animals are imported from a variety of research institutions with variable biosecurity, husbandry, and health monitoring practices, resulting in the potential for parasite infestations and microbial infections that are largely eliminated by commercial specific pathogen-free rodent suppliers.

In addition to reviewing the health status of the animals in advance of their arrival, it is also important to obtain as much information as possible on the details of the husbandry and other rat-related practices of the source institution shipping the rats. This will assist in establishing quarantine conditions for the animals upon arrival.

Reception conditions should be described in a standard operating procedure and include procedures to be followed upon opening containers, such as:

- verifying that the animals received correspond to the order;
- decontaminating the exterior surfaces of non-disposable containers;
- opening the container in such a way as to prevent escape;
- preferentially opening the container and transferring rats to their cage under a laminar flow hood;
- handling the rats in such a way as to prevent contamination (e.g., not touching the rats with the hand that touched the exterior of the container);
- verifying that all animals have been removed from the transport container; and
- dealing with animals that are sick or dead on arrival.

Assessment of the condition of the animals upon receipt should be carried out according to the institution's standard operating procedure. A visual examination of the rats upon arrival is valuable to assess any need for immediate treatment (e.g., for dehydration, trauma). Observation of animals received from a shipper is also important to ensure that the new groupings of rats are compatible.

Animals brought into the facility must undergo a period of quarantine when required by the health status of the animals.

A period of acclimatization, which can run concurrently with the period of quarantine, is important to ensure any stress associated with transportation has been alleviated and the physiology of the animal has returned to a normal state. The length of time required will depend on the conditions of transport, age of the animals and the particular animals involved (e.g., animals with stress-sensitive genotypes may require a prolonged period of acclimation (Obernier and Baldwin, 2006; Capdevila et al., 2007; Arts et al., 2014)). During this period, the animals should be habituated to the method of food and water delivery and to the new environment. Animals should also be acclimated to study conditions and procedures that will be conducted while they are conscious.

5 BREEDING

Guideline 11

Breeding colonies must be efficiently managed according to approved protocols, anticipated need, and the principles of the Three Rs.

Prior to undertaking in-house breeding, careful consideration must be given to factors such as the necessity of the breeding program, the availability of suppliers, and the availability of appropriate space and personnel (see the *CCAC guidelines on: procurement of animals in science* (CCAC, 2007), Section 4.2, "In-house Breeding Colonies"). Large numbers of animals may be required in the maintenance of breeding colonies of genetically modified rats. Every animal from a breeding colony should be on a protocol and requires assessment of the phenotype for identifying genetic drift and animal welfare concerns (see Section 8, "Welfare Assessment").

Breeding protocols must be approved by the animal care committee. Any projected or expected effects of genetic modification on rat health must be considered, along with strategies to mitigate those effects. The breeding protocol should also include screening protocols so that the appropriate animals can be identified based on their genotype or phenotype. The facility manager or institutional veterinarian can be a resource for developing breeding protocols.

As soon as the genetically modified animal with the desired genetic modification or phenotype has been generated or acquired, a breeding strategy is required if there is a need to further propagate the particular line of animals. Well-established institutional guidelines for colony management and animal monitoring are necessary to ensure animal welfare and the quality of experimental data.

5.1 RECORD KEEPING AND OVERSIGHT

Proper record keeping is extremely important to ensure effective and efficient management of breeding colonies, including detection and spread of disease, and the reproducibility of research experiments (Casellas, 2011). Records must be kept and should include the following:

- the particular animals involved in the breeding program (e.g., source, date of birth, unique identifier (e.g., ear notch, tattoo), health status, genetic information, breeding history and productivity, previous care, and involvement in any procedures or studies);
- housing and husbandry requirements;
- details of expected and demonstrated phenotypes (including behaviours);
- details of diseases and treatments;
- breeding parameters, such as fertility, fecundity, morbidity, and mortality;
- intended assignment to studies; and
- criteria for retirement from breeding.

Cage cards are important as a quick visual reference, but additional records are necessary for managing breeding programs. Breeding colony cage cards should include date of birth for litters and projected weaning dates. Where there are numerous inbred strains in a facility or room, record keeping becomes complex and computerized systems are strongly recommended. Colony-management software can provide automatic notification of when animals require weaning and details of life histories. Tyeus (2006) describes the benefits of relational databases in managing breeding systems involving more than 100 animals over three generations.

Current systems for managing digital data and records are becoming more capable of interfacing between protocol review processes, grant officers, financial administrators, and human resource data management (i.e., personnel certification and training records). Efforts that incorporate management of digital records for rat colony management facilitate the integration of animal data into such systems. Digitally based colony management and the need for additional infrastructure or computer applications might be considered in the planning and building of new animal care resources.

Breeding logs must be available to the veterinary team, animal health personnel, and the animal care committee to ensure appropriate procedures are followed. Institutional measures for post-approval monitoring should be developed and applied to ensure approved protocols and standard operating procedures are followed and to assess their efficacy. This can be facilitated through shared access to digitized records.

Where investigators have approval to undertake breeding, they must demonstrate competence in managing breeding colonies (see *CCAC guidelines on: training of personnel working with animals in science* (CCAC, 2015)) and demonstrate that they (and their personnel) can keep complete records.

All animals in a breeding colony (including both the number born and the number weaned) must be documented within the institution and reported at least annually to the animal care committee. The number of animals born should be determined as soon as they can be counted without disturbance. These numbers should be recorded per line. Annual reports to the animal care committee as part of the protocol renewal should also include information on the breeding system, reproductive performance (including any sudden change that could indicate genetic contamination), morbidity/mortality (based on number born versus number weaned), criteria used for culling animals, and other factors related to the way the breeding program is managed to meet the requirements of the research while avoiding overproduction of animals.

5.2 IDENTIFICATION OF BREEDING COLONY ANIMALS

Procedures for the timely identification of genetically modified founders and offspring should be developed and submitted to the animal care committee as part of the breeding protocol. For further details on particular methods, see Dahlborn et al. (2013).

When rats will be involved in long-term breeding, they must be permanently identified (see Section 6.1, "Identification of Animals").

5.3 CONSIDERATIONS FOR BREEDING MANAGEMENT

As with all breeding of animals, careful attention must be paid to limiting the production of surplus animals; in many cases, overproduction can be avoided through more precise planning. Communication with investigators or study directors and facility managers is essential to ensure planning of animal numbers is well considered far in advance. Specific training for people involved in breeding colony management is encouraged.

Careful tracking of all animals (including the number born, the number weaned, and the number transferred to experiments) provides data for investigators and animal care committees to apply the Three Rs. It also avoids shortfalls in the numbers of animals required. Inefficient breeding results in more animals being needed to produce a given number of pups. Purpose-designed colony management software may be useful (Hetherington et al., 2000).

Strategies should be in place for any surplus animals that are produced (e.g., for training exercises, or tissue or blood collection that can be stored and made available). A registry can be developed to notify researchers of any surplus animals that may be available. See CCAC (2014) for best practices in facilitating the sharing of animals and animal tissues.

If litters are large (i.e., >10 pups), it is recommended that they be reduced (e.g., through cross-fostering or culling) to promote the health of both dam and litter.

Breeding animals should be carefully selected based on genetic characteristics and factors related to their history and health that may have an impact on breeding success and the requirements of the scientific studies. Strain differences in reproductive performance (Gill et al., 1979) and maternal behaviour (Poltyrev and Weinstock, 1999) should be taken into account. Breeding management should aim to minimize genetic contamination, and a program should be implemented to monitor and limit genetic drift.

Deleterious mutations are frequently expressed as impaired reproductive performance and if the mutation is not the subject of the approved research, these animals should not be bred further. However, mutations may be very subtle and contamination of the genome may have occurred several generations prior to noticing a change in phenotype; in some cases, control of the offspring according to phenotype may be insufficient and a genome scan is necessary to detect contamination (Zimmerman et al., 2000).

For outbred animals, founding populations should be large enough to ensure long-term genetic heterogeneity of breeding colonies. Nomura and Yonezawa (1996) provide a comparison of four systems of group mating that are utilized to avoid inbreeding.

Investigators are responsible for describing the phenotype for the specific strains they are working with, and sharing that information with the animal care committee and veterinarian. The investigator, animal care committee, and veterinarian should collaborate on the development of a phenotyping plan, which should be described in a protocol approved by the animal care committee.

Monitoring of animals for which the phenotype is lethal or anticipated to have severe negative effects on animal welfare must be undertaken, and should be a collaborative effort involving the investigator, animal care personnel, and veterinarian. Collaboration is important to ensure welfare indicators are identified early and efficiently, and that animals with valuable phenotypes are not euthanized before they are identified.

Appropriate biosecurity measures must be in place to prevent genetic or pathogenic contamination of rat lines.

5.3.1 Breeding Systems

Important considerations in determining whether monogamous or polygamous (harem) mating should be used include the strain or stock of rat (inbred or outbred), space availability, the requirement for postpartum breeding, the potential for young rats to be trampled by older rats (in the case of harem breeding), and the need to maintain a particular health status. The management and degree of control over the genetic

makeup of the animals for different breeding systems is described by Koolhass (2010) and Schwarz et al. (2010). The type of breeding system may influence the weight of weanlings (Allen et al., 2013). Investigators should work with animal health personnel and the veterinarian to devise the best breeding system strategy for the experiment, while minimizing surplus rats.

Monogamous pairing simplifies record keeping and allows for post-partum breeding, which maximizes the number of litters per female; however, this system requires a large number of males. For monogamous pairing, Allen et al. (2013) suggest there may be benefits to the offspring when the male remains in the cage.

Polygamous mating requires larger cages and more complex records. Pregnant females should be removed from harems prior to parturition and returned when young are weaned, as this leads to better milk production and mothering behaviour (Kohn and Clifford, 2002; Kohn et al., 2007; Pass and Freeth, 1993), avoids losses from excessive crowding and potential interference by the male (Wolfensohn and Lloyd, 2013), and prevents mixing of litters. A variation of polygamous mating is to rotate males among separately caged females on a weekly basis (Harkness et al., 2010).

For all breeding systems, steps must be taken to limit the production of surplus animals. This includes obtaining animal care committee approval for the breeding scheme, close monitoring of the reproductive success of the colony, and reporting the numbers of rats born and the numbers of rats weaned to the animal care committee. A standard operating procedure should be developed describing breeding schemes approved by the animal care committee and the care to be given to the breeding rats and their pups

Where short-term breeding is carried out (e.g., rats are bred in order to study the products of reproduction or reproductive behaviour) and the rat strain is not intended to be maintained, the use of breeding schemes may not be necessary. However, good breeding practices and record keeping should still be in place.

5.3.2 Breeding Age

Both male and female rats reach puberty at approximately 50-60 days; the actual timing depends on the strain or stock, diet, and other management factors (Baker, 1979). In females, the vagina opens at about 35-90 days, depending on the stock or strain; however, to reduce dystocia and other reproductive complications, breeding should be delayed until 65-110 days (depending on the strain), when females are approximately 250 g and males are approximately 300 g (Harkness et al., 2010).

It is important to keep good records of the reproductive performance of the animals, as breeders should be replaced according to reproductive history. Indicators used to determine when to replace breeders include litter size, pup weight, and numbers of pups born and pups weaned, which are all strain specific (Harkness et al., 2010; Murray and Parker, 2005), as well as the interval between litters and the general health status of the breeder. Inefficient breeding ultimately increases the number of animals that are needed to produce a given number of pups.

Females should be retired from breeding by approximately one year of age to ensure maximal reproductive success. Productivity, including size of litters and numbers weaned, will usually start to decline after this point (Niggeschulze and Kast, 1994). Male rats are generally retired from breeding no later than one year of age.

5.3.3 Weaning

In the animal facility, 12-16 pups per litter is not uncommon. Where litters are large, it is recommended that they be reduced to 8-10 pups to ensure the healthy growth of the pups (e.g., through cross-fostering or culling).

Pups should be weaned promptly to avoid overcrowding of cages. Pups should generally be weaned between 21-28 days of age, by which time they weigh 40-50 g (Harkness et al., 2010). While earlier weaning is possible, the pups should not be separated from the dam before 17 days of age (Kohn and Clifford, 2002). Weaning earlier than 21 days may result in higher anxiety levels for the pups (Ito et al., 2006). Weaning procedures should be included in standard operating procedures approved by the animal care committee to describe the care to be given to breeding rats and their pups.

For some genetically modified strains with developmental impairment, it may be best to leave the pups with the dam for at least 28 days, providing the cage is not overcrowded and the dam was not mated in the post-partum period. When planning for late weaning, post-partum mating is discouraged.

Newly weaned rats should be closely monitored to ensure they are able to access food and water (for example, by placing a longer sipper tube on the water bottles). Weanling rats should be segregated by sex by approximately 7 weeks of age to avoid precocious breeding.

5.3.4 Post-Partum Breeding

Post-partum estrus occurs in rats within 48 hours of giving birth. Matings at that time are likely to be less successful than standard matings (Harkness et al., 2010; Pass and Freeth, 1993), and failure to conceive at that time will delay breeding until 2-4 days after weaning (Harkness et al., 2010). Nevertheless, if post-partum mating is adopted, then the health of the dam must be carefully monitored (e.g., for weight loss, poor body condition, and mastitis).

The time from fertilization to birth may be lengthened by 3-7 days due to delayed implantation following post-partum breeding (Harkness et al., 2010). However, pups should be removed from the cage by 20 days of age to ensure the new litter is not disrupted by the older offspring.

5.3.5 Cryopreservation

Archiving technologies can lead to a reduction in numbers of animals. Archiving can be used to:

- provide backup in case of catastrophic loss of a breeding colony;
- overcome problems associated with loss of fertility due to aging;
- preserve a line not currently being studied;
- limit genetic drift (in particular, the loss of the transgene expression in subsequent breeding generations);
- repopulate a breeding colony;
- enable a line of genetically modified rats to be shared and thereby reduce the need for the line to be generated elsewhere; and
- facilitate sharing of a line where transportation would result in considerable stress for the animals.

Cryopreservation of gametes and embryos should be used to minimize genetic drift and reduce the risk of losing a line due to disease, human error in screening and managing breeding colonies, or catastrophic events. It can also be more cost-effective to cryopreserve lines that are not currently being used for research, rather than maintain live animals. See Prins (2011) for a description of methods of cryopreservation and recovery.

Services for cryopreservation are available. Where cryopreserved samples are maintained in-house, they should preferably be saved in at least two geographically separate locations to avoid loss of cryopreserved gametes or embryos.

5.4 FACTORS AFFECTING REPRODUCTION

5.4.1 Environmental Factors

Environmental stressors can negatively influence reproduction (e.g., disrupt the estrous cycle and testosterone/sperm production) and pup welfare. Possible environmental influences include:

- Temperature and relative humidity – fluctuations should be held to less than 1 °C, but the reproductive parameters of rats seem not to be affected through a range of constant temperatures between 12 °C and 28 °C; neonates are particularly sensitive to temperature fluctuations, and low relative humidity may cause ringtail in pups (Harkness et al., 2010).
- Lighting – lighting has been shown to influence breeding (rats reproduce optimally using a diurnal cycle of 14 hours light and 10 hours dark (see Section 3.1.1, "Lighting"). For example, continuous light disrupts the estrous cycle (Campbell and Schwartz, 1980; Maeda et al., 2000), litter frequency will decrease in the absence of a sufficient light period, and interruption of the dark period by low light levels may cause ovarian atrophy (Beys et al., 1995).
- Noise and vibration – noise can disrupt maternal behaviour, and breeding performance can be affected by ultrasonic noise (Zondek, 1964, as cited in Castelhano-Carlos and Baumans, 2009) and vibration (Fujinaga et al., 1992).

5.4.2 Housing and Husbandry Influences

Breeding performance may be influenced by the type of caging, bedding, and nesting materials, animal density, cleaning regime, and nutrition. Cage sizes and requirements for bedding and nesting materials are discussed in Section 2, "Facilities", and Section 6, "Husbandry".

Unless a study specifically requires otherwise, female rats must be kept on solid floors during pregnancy, parturition, and lactation (Deepananda, 2013). The animal care committee should review the scientific justification if solid floors cannot be used. Parturient females should be supplied with bedding, nesting material, and refuges that allow for control of light, temperature, and noise, and that provide tactile comfort.

Rats of various strains exhibit nest-building behaviour; for more information, see Jegstrup et al. (2005). Rats have shown a preference for paper nesting material, which they manipulate during the dark period and rest on during the light period (Jegstrup et al., 2005; Manser et al., 1998). Provision of nesting material is particularly important during breeding.

Cages should not be cleaned during the first few days after delivery, to avoid disturbing the pups. If it is necessary to clean or change cages during that period, some of the nesting and bedding material should be retained and transferred to the new cage (Harkness et al., 2010).

A well-balanced diet is required for appropriate reproductive performance (Halloran and DeLuca, 1980; Nelson and Evans, 1953).

5.5 HEALTH ISSUES

Health issues associated with parturition occasionally occur in rats. If health concerns do arise, the veterinarian must be consulted to ensure concerns are dealt with in a timely manner.

When new lines are being produced, investigators may not know the resulting phenotypes of the rats. In such cases, the investigators should report disruptive or maladaptive phenotypes to the veterinarian. The veterinarian must be involved in decisions regarding the care and welfare of those animals and the physiological and behavioural risks to the success of the colony.

5.6 GENOTYPING

Genotyping is important for determining the genetic makeup of rats that have been genetically modified and for monitoring a breeding colony for genetic drift. The genotyping methodology (which may include outsourcing to a specialist company) must be approved by the animal care committee and should be described in a standard operating procedure.

The choice of method for obtaining genetic material should be based on: 1) the aim to minimize pain and distress for the animals; 2) the amount of tissue needed, which will depend on the type of analysis required; and 3) whether a suitable tissue sample can be obtained from the method used for identification of the animal (see Section 6.1, "Identification of Animals"). For a review of welfare concerns and potential refinements for methods of genotyping, see Bonaparte et al. (2013).

6 HUSBANDRY

The *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017) should be consulted for general guidelines that are applicable to all species. This section provides additional considerations that are important for rats.

6.1 IDENTIFICATION OF ANIMALS

If only short-term identification is needed, possible methods include the use of non-toxic dyes, permanent markers, and fur clipping. Where permanent identification is necessary, methods include microchips/transponders, tattoos, ear tags, and ear punches. Toe clipping must not be used solely as a means of identification. The advantages and concerns for each of these methods are described in Appendix 3, "Methods of Identification".

Genetically modified rats should be identified according to standardized nomenclature, which should be included in documentation that is maintained and transferred with the animals.

6.2 HOUSING MANAGEMENT

Addressing the physical and behavioural needs of rats through housing includes providing opportunity for social contact and locomotor and exploratory behaviours, as outlined in Section 1.1, "Behavioural Biology". Space requirements for rats are discussed in Section 2.1.1.1, "Cage Size"; the cage size should support group housing of rats and the inclusion of a shelter.

6.2.1 Social Housing

Guideline 12

Rats should be group housed.

Isolation, particularly following weaning, has a strong impact on the behaviour of rats (Pellis, 2010; van den Berg et al., 1999a, 1999b; Von Frijtag et al., 2002). For young rats, social contact with other youngsters permits normal play behaviours and interactions that are important for normal behavioural development (Pellis, 2010), as well as for adequate growth and behavioural regulation in the long term.

Determining appropriate group size should take into consideration the strain, sex, size, and individual characteristics of the rats involved. For example, male and female rats have been shown to use cage structures differently and respond differently to crowding (Brown and Grunberg, 1995). Several studies have provided evidence for optimal group size for rats based on criteria such as:

- preference – e.g., Patterson-Kane (2004) found female rats showed a preference for social housing with a group size of 6, compared to single or pair housing, or groups of 4 or 12;

- behaviour – e.g., Spangenberg et al. (2009) found rats in groups of 4 or 8 exhibited greater social interactions and activity levels and less emotional reactivity than pair-housed rats; and
- response to procedures – e.g., Sharp et al. (2002) found rats housed in groups of 4 were less stressed and returned to normal more quickly following procedures than rats housed alone.

Rats housed in enriched environments in groups of at least 4 have been shown to be less fearful (Botelho et al., 2007; Patterson-Kane et al.,1999) and perform better on memory and learning tests (Patterson-Kane et al., 1999) than rats that are single housed or in standard pair-housing conditions. However, overcrowding can also lead to increased anxiety among rats (Botelho et al., 2007). Additionally, housing conditions can affect the social development of young rats, as development of different areas of the frontal cortex of juvenile rats has been shown to vary with the number of peers present and their play experiences (Bell, 2007; Pellis, 2010).

Rats should normally be grouped by 7 weeks of age to minimize aggression. Strategies for successful grouping of rats include providing shelters and monitoring to ensure social stability and detect behavioural and physiological abnormalities.

Some agonistic behaviour among rats in social housing, including threats and actual fighting, is considered normal (Pinelli et al., 2017). Minor resulting lacerations should not be grounds to abandon social housing, as this phase will normally pass. However, the animals should be closely monitored and extra attention should be given to ensure that appropriate cage resources are available, with the inclusion of visual barriers. Sound judgment, based on knowledge of rat behaviour (especially social play and aggressive behaviours and vocalizations), should be used to determine when fighting among rats requires intervention.

Stable groupings are encouraged. When animals are removed from a group, the time period should be as short as possible, and preferably less than 48 hours to maintain recognition of members of the group (Burman and Mendl, 2006). Any time remixing of animals occurs, animals should be carefully monitored in case of social disruption. Sudden increases in group and cage size should be avoided as they can potentially cause welfare concerns (Yildez et al., 2007).

For rats involved in studies requiring chronic implants with an externally accessible portion, group housing is valuable to allow social interaction and reduce stress; however, the animals should be separated initially following surgery until their wounds heal (Schwarz et al., 2010). Once reintroduced, they should be carefully monitored to ensure that there are no adverse interactions.

6.2.2 Single Housing

Single housing should only be permitted where there is strong scientific, welfare, or medical justification, and it should be for the shortest duration possible. In such cases, rats should have visual, auditory, and olfactory contact with other rats, where possible; however, this can prove difficult for rats housed in individually ventilated cages. For studies that have in the past required individual housing for the collection of individual measures, a divider with social holes can provide social stimulation in a paired-housing situation (Boggiano et al., 2008). Providing sufficient environmental resources within the cage is also key in addressing the welfare of these animals. For example, providing an opportunity to chew (e.g., nylabones or wooden blocks) helps lower stress levels in singly housed rats (Belz et al., 2003).

Studies have shown that single housing is associated with few differences in physiological and immunological indicators in comparison to group housing (Azar et al., 2011; Turner et al., 2014; Krügel et al., 2014);

however, single housing is associated with behavioural changes that suggest potential welfare impairment (e.g., Hurst et al.,1997; Pinelli et al., 2017). Therefore, while single housing may not have any impact on research data, it may have an impact on the welfare of the rats.

In particular, single housing of young rats post-weaning has been found to lead to abnormal gait (Roberts et al., 2001) and changes in endogenous opioids of the brain (Granholm et al., 2015). It also increases behaviours related to anxiety and fear in adulthood (Lukkes et al., 2009). The effects on social behaviour later in life are especially pronounced when young rats are isolated at 4-5 weeks old, which corresponds to the peak in social play performance (Hol et al., 1999; Von Frijtag et al., 2002). Play also affects brain development in that it contributes to dampening the emotional reaction to a novel, unpredicted situation (Pellis, 2010). Rats that are singly housed can benefit from appropriate social contact with people in a manner that mimics their natural play behaviour (Cloutier et al., 2013).

6.3 FOOD, WATER, AND BEDDING

6.3.1 Food

The diet should be adapted to the age and nutritional needs of the animals. Rats should receive at least 90% of their diet as a complete and balanced ration; treats should not comprise greater than 5-10% of their diet. Treats should be offered in a manner that allows observation of the animals, and can be used to encourage and reinforce behaviour.

Rats with ad libitum access to high energy food can become obese, which, combined with insufficient exercise, contributes to health problems and a shorter lifespan, as well as to changes in physiological processes and cognitive functions that can impact study data (Martin et al., 2010; Turner et al., 2014). If food is provided ad libitum, the diet composition is important and appropriate cage configurations and resources should also be provided to encourage activity. For example, providing food embedded in a wooden block can reduce food consumption and weight gain (Kemppinen et al., 2008), while ensuring incisor wear.

In general, to minimize contamination, food should not be sprinkled on bedding. However, there are many situations (e.g., post-surgery, weakened or limited mobility animals, or enrichment) where food can be provided at floor level.

Sufficient food should be added to hoppers to ensure that rats have access to clean food at all times. Prior to adding fresh food, the remaining food should be examined and any spoiled or soiled food should be discarded.

Any imported food must follow the feed regulations from the Canadian Food Inspection Agency. Pathogens (e.g., parvovirus and rotavirus) can potentially be introduced through food and bedding (Clifford and Watson, 2008; Watson, 2013). Irradiated food or autoclaved food that is intended for autoclaving should be used in specific pathogen-free facilities, although pathogens can still be introduced through this food. Where special diets are required, arrangements should be made in advance to ensure uninterrupted supply, and the criteria for storage and use should be well understood to ensure the stability of any dietary additives.

If food needs to be ground in-house, it should be prepared according to a standard operating procedure on preparation and maintenance. Where the prepared food is to include an experimental compound, the properties of the compound (e.g., lipophilicity) need to be considered in relation to the actual dose to be

delivered to the animal (see Hayes and Kruger, 2014; United States Food and Drug Administration *Redbook 2000: IV.B.1. General Guidelines for Designing and Conducting Toxicity Studies* (FDA, 2003)).

6.3.2 Water

The amount of water consumed by rats can vary widely, even within a strain. For example, daily ad libitum fluid consumption in three different Sprague-Dawley rat colonies was reported to range from 80 to 125 ml/kg body weight (Wells et al., 1993).

Rats may require specially treated water (e.g., acidified, chlorinated, reverse osmosis, autoclaved or UV irradiated) to prevent introduction of pathogens. Facility managers and investigators should be knowledgeable about the available options for providing water to rats and select the method best suited to the particular animals and study.

6.3.3 Bedding and Nesting Material

Guideline 13

Bedding and nesting material should be provided to allow rats the opportunity to dig, forage, and build nests.

Bedding is important for absorbing urine and feces and controlling ammonia levels. Together with nesting material, it enables rats to create a comfortable microenvironment. Studies have demonstrated that both male and female laboratory rats build nests, and that this behaviour is not specific to the periparturient period. The extent of nest building by rats has been shown to vary with strain (Jegstrup et al., 2005), early exposure to nesting materials (Van Loo and Baumans, 2004), and ambient temperature (Kinder, 1927).

Nesting materials and bedding must be non-toxic, not harmful if ingested, absorbent but not dehydrating for neonates, consist of particles that are suited to the needs of the rat (e.g., ease of manipulation), and produce a minimal amount of dust (Baumans, 2010). In addition, when the rats are involved in an experiment or study, the materials should not have an impact on the experiment or study. Rats show a preference for large fibrous particles that they can manipulate, such as shredded paper (Blom et al., 1996). However, some paper bedding can quickly become wet from urine, and it may be beneficial to combine it with other materials (Blom et al., 1996). Paper strips have also been shown to be a preferred material by rats for nest building (Manser et al., 1998).

It should be noted that pathogens (e.g., parvovirus and rotavirus) can potentially be introduced through bedding (Lindstrom et al., 2018).

See Section 6.7, "Cage Changing and Sanitation", for guidance on changing bedding and nesting material.

6.4 ENVIRONMENTAL ENRICHMENT

Important basic requirements that address the physical and behavioural needs of rats are discussed in other sections of this document, for example:

- cages should be of a sufficient size to allow rats to perform behaviours important to their welfare, and promote the conduct of additional behaviours that will improve their quality of life (Section 2.1.1.1, "Cage Size");
- shelters are standard features in rat housing, as they address behavioural needs and provide comfort for the animals (Section 2.1.1.4, "Cage Components"); and
- rats should be group-housed (Section 6.2, "Housing Management").

Additional elements that may enrich the animals' environment are listed below, but must be considered in the context of the individual animal and the research requirements:

- features to promote activity (e.g., structures to climb on or around, exercise wheels, swings, and hammocks);
- substrates for burrowing;
- structures to facilitate hiding or avoidance of people or other rats (e.g., tubes, dividers);
- objects to chew (e.g., nylabones, woodblocks) to help prevent overgrowth of teeth;
- variety in food (e.g., rat treats compliant with biosecurity standards); and
- opportunities for foraging.

Providing additional opportunity for exercise is an important component of environmental enrichment, as there is evidence of lowered metabolism in rats where activity is not encouraged (Turner et al., 2014).

6.5 HUMAN CONTACT AND HANDLING

Habituating rats to routine husbandry practices through gentle, progressional handling should be encouraged whenever possible, as it may have beneficial effects on the behaviour of the rats and reduce their fear of humans. Maurer et al. (2008) describe a habituation program for rats aimed at reducing their fear of human contact. The time frame for habituation may be influenced by such factors as strain and age of the animals.

Rats are nocturnal animals and therefore, routine observations and cage changing should be conducted quietly and efficiently to minimize disturbance of animals, especially when these activities are performed during the rat resting phase (i.e., in the light part of the diurnal cycle). Breeding colonies and experimental animals should be separated, whenever possible, to minimize the disturbance of other animals when particular rats are removed for procedures.

For further details on handling, including techniques for removing rats from cages, as well as restraint, see Section 7, "Handling and Restraint".

6.6 ANIMAL OBSERVATION

Daily observation of animals should include evaluation of both health and behaviour, although it is recognized that the full range of behaviours is best observed in the dark phase of the light cycle. See Section 8, "Welfare Assessment", for examples of indicators that can provide useful information on the welfare of rats.

Some aspects of Appendix 4, "Indicators that May be Used to Assess the Welfare of Rats", might be useful to incorporate into daily observations of rats.

6.7 CAGE CHANGING AND SANITATION

Guideline 14

Cages should be changed at a frequency that maintains the intra-cage air quality and bedding within acceptable parameters, while recognizing the stress associated with cage cleaning and the potential impact on study data.

Standard operating procedures should be developed for cage changing, with consideration of factors such as the cage size, numbers of animals, the type and amount of bedding being used, number of air changes per hour, and the health condition of the animals (e.g., diabetic rats). Ideally, both air quality monitoring and visual inspection should be used to assess the need to change cages. Based on these considerations, and to balance cleanliness without disturbing the cage environment too frequently, Burn et al. (2006) recommended static cages be changed weekly. Similar data for individually ventilated cages is not available. Although cage changing is a routine procedure, it has been shown to elicit physiological responses (Meller et al., 2011; Sharp et al., 2002) and behavioural responses (Cloutier et al., 2015), which may be altered for several hours after cage changing procedures have occurred (Cloutier et al., 2012).

Adequate sanitation should ensure the removal of infectious agents to prevent their spread within the facility (Compton and Macy, 2015), as well as the removal of urine and fecal waste to prevent excessive contact with rats and to minimize ammonia levels.

6.8 RECORD KEEPING

It is important that all records identified in the *CCAC guidelines: Husbandry of animals in science* (2017), Section 12, "Record Keeping", are maintained. In many cases, group records are kept for rats; however, individual records are necessary for animals that undergo treatment, procedures, or breeding. Requirements for breeding records are detailed in Section 5.1, "Record Keeping and Oversight", in this document.

7 HANDLING AND RESTRAINT

Guideline 15

Rats must be handled in a gentle manner that is safe for both the rats and the personnel.

Handling can have a significant impact on both the welfare of the rats and on experimental results. Handling of rats can cause a number of changes indicative of stress: elevation in heart rate and blood pressure (Azar et al., 2005) and increased corticosterone levels (Dobrakovová et al., 1993).

Attention must be given to ensuring appropriate methods are used and the personnel involved are competent in using the chosen methods. Rats should be habituated to handling whenever possible.

7.1 HANDLING

Animals should be habituated to gentle handling as soon as possible, as this will facilitate handling for cage cleaning and experimental procedures. Positive reinforcement training can also be used to reduce the stress of procedures (e.g., Leidinger et al., 2018).

Failure to handle young rats may negatively impact research-related outcomes, leading to impaired learning and exploratory behaviour, hippocampal dysfunction, and impaired adrenocortical responses to stressors (Pham et al., 1999). Rats should be habituated to handling prior to experimental procedures to avoid unintended impacts on research (Maurer et al., 2008).

Containers may be used to help support animals when lifting them out of a cage, but this should not replace habituation to handling. When using a container to lift rats out of a cage, animals should be moved one at a time. Containers should be sanitized regularly, and between cages when necessary, to avoid cross-contamination.

Rats should not be picked up by the tail, but rather by placing a hand over the rat's back, palm-side down, and moving a finger and thumb around the rat's chest area, just behind forelegs. The rat can then be gently grasped and lifted while supporting its hindquarters with the free hand.

Tickling rats in a playful manner has been shown to induce a positive affective state that appears to minimize negative welfare impacts due to restraint and minor procedures (Cloutier et al., 2018; Cloutier et al., 2015). Where rats have become accustomed to gentle handling, stroking appears to have a calming effect; however, rats that do not have prior handling experience will likely respond negatively (Brudzynski and Ociepa, 1992).

7.2 RESTRAINT

Rats should only be restrained if less stressful procedures cannot achieve suitable results. Any restraint should be for as short a period as possible, and a restraining device that is appropriately sized to the animal should be used. Rats should be monitored closely while being restrained.

Several types of mechanical restraining devices are available; the devices are designed for purposes such as injection and withdrawal of blood, short-term cannula collection of bodily fluids, and restraint (positioning) during surgery. Habituation to restraint devices and positive reinforcement techniques should be used as much as possible. Alternative procedures that can reduce the need for restraint (e.g., catheters as opposed to multiple blood draws or injections) are encouraged.

Animal handlers should use the least restraint necessary to safely and effectively perform required procedures. It is important that restraint be conducted by personnel competent in the particular method to minimize the duration and stress of restraint.

Machholz et al. (2012) provide a detailed description and video of manual restraint techniques for the administration of compounds to rats. Where a restraining device is to be used on a conscious rat, the rat should be habituated to the device prior to use and be constantly monitored while being restrained.

8 WELFARE ASSESSMENT

Guideline 16

All rats maintained in an animal facility should be subject to routine welfare assessments.

Where rats are involved in protocols, the assessment should be tailored to the particular strain of rats and the specifics of the study.

Welfare assessment is a necessary component of animal-based studies, both for ensuring a good quality of life for the animals within the constraints of the study, and for the quality of the scientific data. While all personnel involved with the animals have a role in collecting information for welfare assessments, primary responsibility for planning and documenting welfare assessment rests with the investigator in consultation with the veterinarian, as approved by the animal care committee.

When possible, investigators should refer to basic standard operating procedures approved by the animal care committee and adapt them to suit the specific rat strain.

There should be a plan to integrate information from the daily care of the animals and the use of any assessment tools to provide a measure of rat welfare at the individual and group level. The overall focus of any welfare assessment should be on mitigation strategies or endpoints, with the aim of improving the welfare of the animals (i.e., monitoring should be to benefit the animals, not just for the sake of monitoring).

Investigators should ensure proper documentation of the results of welfare assessment procedures and the identification (if appropriate) of any mitigation strategies to address welfare concerns; assessments and concerns should be reported to the animal care committee.

The development of a welfare assessment plan should be based on the characteristics of the rats to be assessed, with consideration of potential sources of variation such as strain or stock, sex, age, health status, and previous experience (see Section 1.4, "Sources of Variation"). Consideration should also be given to the entire lifespan of the animals in terms of the number, frequency, and invasiveness of manipulations they will experience.

As welfare assessment is an integral part of the daily care of the animals, various aspects of welfare assessment are included in other sections of this guidelines document:

- daily observations of the animals and their environment (i.e., visual observation of the animals, the cage, food, water, and other aspects of their housing) is covered in Section 6, "Husbandry", and in the *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017);
- the suitability of the physical and social environment of rats is addressed in Section 2, "Facilities", and Section 6.2, "Housing Management", in this document;
- details of health monitoring for rats are provided in Section 9, "Health and Disease Control"; and

- additional monitoring requirements for rats that have undergone surgery or other procedures are outlined in Section 10, "Experimental Procedures", and in the *CCAC guidelines: Husbandry of animals in science* (CCAC, 2017), Section 10.3, "Animal Care Monitoring in Relation to Research, Surgery and Anesthesia".

Welfare assessment should involve integration of information collected during the daily care of the animals and the conduct of any procedures, rather than a duplication of effort, with additional information from other sources incorporated where necessary. Welfare assessment is more comprehensive than routine checks of the animals, and assesses their quality of life. Some indicators of welfare can only be observed once negative states are quite advanced (e.g., poor coat condition), while others are much more sensitive to gradual changes in the welfare of the animals (e.g., attenuated growth rate). Welfare assessment also includes indicators of positive states, for example, vocalizations of 50 kHz have been shown to indicate a positive state (Brudzynski, 2007). Hawkins et al. (2011) have developed a guide to defining and implementing procedures for the welfare assessment of laboratory animals. While all animals need to be assessed, it is acceptable to only record information for animals where there is a welfare concern.

It is important to understand the particular strain or disease model and how manipulations will affect the welfare of the animals, before the study is initiated. When developing welfare assessment criteria, investigators should think critically about these factors. The current literature should be reviewed as a starting point for developing assessment tools for the particular situation.

Genetically modified founders and their offspring require welfare assessment to identify and document any special unanticipated welfare concerns and determine the need for additional monitoring or mitigation strategies. If a new line is to be generated, or a line that is not well characterized is used, the investigator is responsible for providing plans for assessing and monitoring the welfare of that line during the animals' lifetime (i.e., during experiments, breeding, and colony maintenance), bearing in mind that the level of monitoring required may change over time. Assessments of the welfare of the animals can be done in parallel with research on new genetically modified strains; any additional information provided by the results of welfare assessment should be communicated to those involved in the assessment and care of the animals.

A number of assessment tools have been developed, such as body condition scoring (Hickman and Swan, 2010) and the facial grimace scale (Oliver, 2014). Assessment tools will evolve and should be reassessed over time. Tools should be practical and should be validated (i.e., to ensure the assessment tool is reporting the situation reliably); each tool will have limitations (Oliver, 2014). Validation is best carried out by the research team in collaboration with animal care technicians and veterinary personnel. Standardizing the terms used to describe the condition of the animals will assist in the clear understanding of welfare issues. Examples of possible indicators are provided in Appendix 4, "Indicators that May be Used to Assess the Welfare of Rats".

9 HEALTH AND DISEASE CONTROL

Maintaining healthy animals is important to the welfare of those animals and to the quality of the scientific data.

Guideline 17

All rats should be included in an animal health program, irrespective of where they are housed.

Veterinary professionals must be engaged in the development of the health program, which should be approved by the animal care committee and overseen by people competent in evaluating the health of rats. The animal health program should include:

- prevention of conditions conducive to ill health, with prevention strategies suited to the health status of the animals and the intended studies;
- health monitoring and detection of latent disease by systematic evaluation of individual animals and the health status of each colony; and
- an emergency plan for the management of disease in the event of a suspected outbreak.

9.1 DISEASE PREVENTION

Guideline 18

Strategic measures for disease prevention should include a program for disease control and a system of regular monitoring and reporting for health assessment purposes.

Animals should be free of unwanted pathogens and clinical diseases. A veterinarian should be integral in developing standard operating procedures to limit the risk of introduction of disease into the facility, and should be available for consultation on all matters relating to the health of the animals (Mähler et al., 2014).

The disease prevention and control plan should address the following:

- procurement – rats coming from a supplier should have a recent satisfactory health report provided by the supplier (commercial or non-commercial), and undergo a thorough health assessment upon arrival (see Section 4, "Procurement");
- quarantine – newly arrived animals should be kept separate from other animals in the facility (see Section 4.5, "Reception of Rats at an Institution");
- facilities and their management – facilities, equipment, and management practices should be in place to prevent airborne, direct contact, or fomite transmission of microorganisms (e.g., ventilated cages), water contamination, pest infestations, and contaminants from external sources;

- husbandry – rats should be fed a high-quality diet (irradiated or autoclavable) and practices should be in place for effective sanitation, prevention of overcrowding, and regular bedding changes;
- biosecurity – standard operating procedures should limit access and detail the use of personal protective equipment and handling of animals under a biosafety cabinet; and
- temporary holding – plans should be in place for holding contaminated animals separate from other animals in the facility in the event of a disease outbreak, and should include a disease prevention strategy.

Although occurrence is rare, appropriate measures should also be implemented to prevent the spread of infectious agents from humans to animals, e.g., *Streptococcus pneumonia*, is potentially fatal to rats.

Biosecurity is an important component of a preventative health program. Quality assurance programs that focus on biosecurity procedures and health and environmental monitoring are important to maintaining animals of a particular health status (see Shek, 2008 and Rehg and Toth, 1988). Infectious agents may affect experimental data without causing disease. A routine and comprehensive health monitoring program is essential.

9.2 HEALTH MONITORING AND DISEASE DETECTION

Guideline 19

Standard operating procedures should be developed for assessing animal health, providing health care, and treatment of common health problems for the animals; these should be reassessed every three years to ensure relevance.

Standard operating procedures should be developed for routine health checks and welfare assessment for individual animals and for each colony, based on the strain and health status of the animals, the type of research, and the potential effects on other animals in the facility. Animal monitoring requirements for health and disease control will also depend on the length of time the rats are housed and the type of facility in which they are housed. Health monitoring programs may include the use of environmental monitoring (e.g., through exhaust plenum testing) or the use of sentinel or colony animals, depending on factors such as the strain, sex, age of the rats, and caging system. Evaluation procedures need to be determined (e.g., test intervals, selection of agents, and verification). It is important that testing methods and samples are specific to the disease of interest, and where possible, adhere to the Three Rs principle of reduction. See Mähler et al. (2014) for further information on health monitoring programs.

Quality assurance programs should be updated over time in response to the regional prevalence of diseases in the area. The literature should be reviewed for information on diseases affecting rats and procedures for their detection (e.g., Barthold et al., 2016). The use of molecular assays to test directly for pathogens may be used to replace sentinel animals, including polymerase chain reaction (PCR) testing of the environment through the exhaust air duct (Henderson et al., 2013; Jensen et al., 2013a; Zorn et al., 2017). This approach is strongly encouraged as it can result in a significant reduction in the numbers of animals maintained in the facility.

See Mähler et al. (2014) for more information on potential viruses, bacterial agents, and parasites, as well as considerations for monitoring.

There should be procedures in place to ensure any animal health concerns or other potential animal welfare issues are documented and communicated to the veterinarian in a timely manner.

9.3 DISEASE MANAGEMENT IN THE EVENT OF AN INFECTIOUS OUTBREAK

Guideline 20

A management plan must be in place to deal with unanticipated disease outbreaks.

A management plan must be developed to deal with serious disease outbreaks within the facility and from outside sources, and to prevent pathogen transmission and infection recurrence. Plans should include a communication strategy involving veterinarians, veterinary and animal care personnel, investigators, the facility manager, and the animal care committee. Access to quarantine facilities or a means of isolating the animals must be available.

For infectious disease outbreaks, the veterinarian must be consulted to ensure that the techniques employed will eradicate the pathogens. Typical procedures may include quarantining the room in which the disease is discovered and tracking and testing any animals that were recently moved from that "source" room (Mähler et al., 2014). Follow-up actions, such as treatment, depopulation, re-derivation, etc., will depend on the nature and extent of the outbreak, the health status of the animals, and the type of research (Mähler et al., 2014). If infected animals are to be euthanized, proper containment measures must be in place for handling and disposal of the animals and bedding, as well as decontamination of cages and rooms to prevent the spread of disease (Mähler et al., 2014).

Non-invasive procedures such as cross-fostering may be employed when appropriate, to eliminate some agents from a colony.

Re-derivation of rat strains to address contamination may involve invasive procedures such as harvesting embryos or sperm, and their transfer to a recipient (clean) female, or birth by hysterectomy under sterile conditions and cross-fostering to clean foster mothers. These measures may be necessary to avoid having to re-generate genetically modified animals, which would involve significantly greater numbers of animals. Re-derivation procedures can be outsourced to take advantage of external expertise.

Embryo transfer is more effective than caesarean re-derivation for production of pathogen-free pups. Embryos can be produced that are free of infectious viruses known to be transmissible vertically (from mother to pups); appropriate application of this re-derivation approach will lead to specific virus-free sero-negative recipients and re-derived pups (Mahabir et al., 2009).

Where surgery is used for embryo transfer, all requirements for surgery, anesthesia, and analgesia must be followed (see Section 10.11, "Anesthesia and Analgesia", and Section 10.12, "Surgery").

Cryopreservation should be considered to prevent the loss of a valuable strain (see Section 5.3.5, "Cryopreservation").

10 EXPERIMENTAL PROCEDURES

Guideline 21

The least invasive method suited to the goals of the study must be used, with consideration of the potential impacts of the procedures on rats, including other rats in the room, and consideration of measures to reduce those impacts.

The institutional veterinarian must review all protocols involving experimental procedures (see the *CALAM Standards of Veterinary Care*, 2007). For routine procedures, standard operating procedures approved by the animal care committee should be available to all personnel involved with the animals to ensure consistency of procedures and animal care. Where new procedures are proposed, standard operating procedures should be developed in consultation with an expert in the subject matter and stakeholder input (from researchers, safety officers, and animal care personnel) should be sought before the standard operating procedure is approved and implemented. Standard operating procedures should be reviewed regularly and updated as new information becomes available. All procedures should be documented and records should be kept in close proximity to housing or procedure areas and be accessible to the veterinary team, animal care committee, and the research team.

Institutions should have a policy or standard operating procedure on repeated procedures on animals, including rats. The frequency, the duration of intervals between procedures, and the total number of procedures that may be performed on the same rat during its lifetime must be considered. The standard operating procedure must take into account the invasiveness and pain and distress associated with those procedures and their impact on the welfare of the rat, both in the short and longer term (CCAC, 1998).

Procedures that adversely impact animals should be avoided where alternative methods are effective in achieving the study outcomes.

All procedures have the potential to cause pain and distress. Many seemingly routine procedures are more complicated when conducted on rats because of their small size. Procedures may only be performed by competent people that have been properly trained by personnel with appropriate expertise. Where possible, it is preferable to use the expertise of a veterinarian and experienced animal care personnel to carry out these procedures.

Investigators should be aware of the potential impact that procedures may have on other rats in the room and take measures to minimize these effects. There is evidence that rats respond to fear responses of other rats (i.e., vocalizations of 22 kHz) (Kim et al., 2010; Parsana et al., 2012). Additionally, some studies suggest that rats may be capable of displaying empathy and pro-social behaviour toward other rats (Bartal et al., 2011).

All rats in an experimental cohort should undergo habituation to the procedures and the habituation program should be applied consistently within the cohort. Investigators should plan in advance for the required time for habituation. The specific habituation protocol will depend on the procedures being performed and the experimental design (e.g., Otis et al., 2016; Toval et al., 2017).

As techniques advance, refinements will continue to evolve in many of these areas and investigators, veterinarians, and animal care committees should evaluate new evidence on refinements and consider their implementation (Smith and Hawkins, 2016).

Guideline 22

Endpoints must be developed and approved by the animal care committee prior to the commencement of the study, to minimize any negative impact of the procedures on the animal.

The *CCAC guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (CCAC, 1998) provides the following definition of an endpoint: "the point at which an experimental animal's pain and/or distress is terminated, minimized or reduced, by taking actions such as killing the animal humanely, terminating a painful procedure, or giving treatment to relieve pain and/or distress."

Investigators, in consultation with the veterinarian, must establish appropriate and study-specific endpoints (e.g., initiation of treatment, termination of a procedure, and euthanasia) and plans for monitoring. Key references relevant to the particular study should be consulted in determining the earliest practical endpoints. Where a rat model may be in development or new to a researcher, pilot studies should be performed to establish endpoints.

Appropriate monitoring frequency must be established based on the level of invasiveness of the protocol and expected clinical or other signs, as well as the progression of the condition of the animal, the animal model, and the individual animal (e.g., previous experience). Monitoring should be documented.

Where appropriate and in accordance with the level of invasiveness of the protocol, monitoring score sheets incorporating several parameters of assessment can be helpful in monitoring for endpoints. Monitoring for endpoints should be a cooperative effort involving investigators, veterinarians, and veterinary and animal care staff.

Animals experiencing pain or distress that cannot be relieved and that is not approved as part of the research protocol must be euthanized promptly.

10.1 ANIMAL MODELS

Investigators or study directors should decide whether rats are required for the study, and if so, which rat strain(s) provide the best model of the biological processes involved in their work, taking into account the special needs of the strain, the ethical or welfare considerations of working with the strain for a given experiment or study, and the strain availability.

Strains that have special requirements must not be obtained until measures are in place to care for them appropriately. Particular studies may need to be redesigned if those requirements could pose difficulty with maintaining the health and welfare of the animals or be intensified as a result of the experimental interventions. The measures required in these situations may include special or additional technical expertise and highly trained personnel.

10.2 ADMINISTRATION OF SUBSTANCES

Administration of substances requires careful planning to ensure the substance is delivered effectively and negative impacts on the welfare of the rats are minimized. Planning includes selection of an appropriate administration route and use of appropriate equipment; determination of the required volume, frequency of delivery, and properties of the substance and diluent/solvent (i.e., pH, viscosity, sterility, osmolality); and consideration of requirements for restraint (Turner et al., 2011a,b; Morton et al., 2001). Standard operating procedures should be developed and approved by the animal care committee, following consultation with the institutional veterinarian. When the side effects or safety of a substance are unknown, pilot studies should be conducted.

Examples of common methods of administration are described in the following sections. Less common methods should be researched and approved by an animal care committee before use.

As noted in Section 7, "Handling and Restraint", restraint can have impacts on both animal welfare and experimental results, and the least restraint necessary to safely and effectively perform the required procedures should be used. Investigators should evaluate restraining techniques described in the literature to ensure they are using the most suitable method. An example of a method that minimizes restraint for injection of substances in rats is described by Stuart and Robinson (2015).

10.2.1 Injections

Injection solutions must be sterile or at least filtered (0.22 micron filter) and maintained in a sterile container, as infectious agents could be present in solutions and affect the animal, the facility, or the study.

The procedures must only be conducted by people competent in the technique, using appropriate handling and restraint. Needles should be the smallest size suitable for the situation, keeping in mind that very small needles are easily dulled (for example, while withdrawing from a multi-use vial) and should not be reused. Substances to be administered should not be irritating.

For rats, acceptable routes for injecting substances include subcutaneous, intraperitoneal, and intravenous (see Newcastle University's *Procedures with Care* for descriptions of procedures for each of these routes). Intramuscular injections are less preferred because of the potential for pain and pathology (i.e., subsequent lameness and sloughing of skin and muscle). Intradermal injections are also discouraged because they are technically difficult to perform; however, if used, the volume injected must be very small (Turner et al., 2011a), with consideration of the particular rat involved, the specific protocol, and the particular site of the intradermal injection (Workman et al., 2010).

The tail vein is the blood vessel of choice for injection into an unanesthetized rat; however, intravenous injection requires training and competence. Warming the tail in warm water or using a heat lamp can improve visualization of the tail vein; however, both approaches should be performed with caution to avoid injury to the tail. In addition, cleaning the tail and using a head-mounted magnifier can improve visualization of the tail vein. Restraint devices are available that facilitate intravenous injections into the lateral tail vein (e.g., a cylinder of appropriate diameter for the rat, with an adjustable-length divider to hold the body and a slotted end for exteriorizing the tail); see Turner et al. (2011b). Intravenous injection into other sites under general anesthesia is also acceptable.

The injection site should be monitored for perivascular irritation (i.e., redness and swelling at the site; the vein may appear white), and if it occurs, veterinary advice should be sought.

For maximum volumes and more information about injections, Turner et al. (2011a) and Diehl et al. (2001) should be consulted. Where repeated injections are required over an extended period (hours to days), osmotic mini-pumps or intravenous catheters should be considered. When catheters or mini-pumps are used to administer substances, animals with chronic infusion of fluids should be monitored for signs of adverse effects, such as fluid overload and pulmonary edema (Turner et al., 2011a). Catheters and the catheter site should be checked and cleaned regularly to ensure proper functioning and to prevent infection (Turner et al., 2011b).

Suggested volumes for bolus and slow injections (single and repeat doses) and infusion for various sites are provided by Turner et al. (2011a) and Diehl et al. (2001), with a caution that the volumes tolerated by the animals will be influenced by the physiochemical properties of the substance that is administered. Whenever possible, the smallest practical volume should be administered.

10.2.2 Oral Dosing

Methods of oral dosing include adding the drug to the animal's regular food (including as a compounded medical diet), a food treat, or the drinking water, and oral gavage (Turner et al., 2011a). The method required will largely depend on the specifics of the study and whether the animals will accept food or water that has been treated. Where other methods can be used (e.g., adding compounds to food, or a small amount willingly consumed by the animal), they are preferred over oral gavage. In some cases, oral gavage may be necessary because of the specific dosing requirements or where the properties of the compound and its absorption may be affected by the presence of food. Veterinary advice should be sought in determining the most appropriate method for the study. Pilot studies are encouraged when dosing in food or when using other forms of compounding, to ensure the actual dose received by the rat is equivalent to that of studies using gavage.

For oral gavage, rats must be properly and securely restrained, and the procedure must only be performed by personnel who are trained and have demonstrated competency in the proper technique (see *CCAC guidelines on: training of personnel working with animals in science* (CCAC, 2015)). Animal stress and mortality related to the procedure are minimized when it is properly performed and the animal is habituated to restraint.

Gavage administration of substances is best performed by inserting a tube or long, bulbous-ended needle (feeding needle) of pre-measured length (the tube should extend from the tip of the nose to the last rib of the rat (Morton et al., 2001)) over the tongue into the distal esophagus where it enters the stomach. Gavage needles should be the correct length for the size of rat. There are advantages and disadvantages of using steel versus plastic gavage needles. Steel gavage needles, when not perfectly used, can do more damage to internal tissues than plastic gavage needles. Plastic gavage needles are less damaging and small, soft, flexible disposable needles can work well. However, some plastic gavage needles of a larger diameter can bend or be bitten and swallowed, in whole or part, by the rat. These complications of using plastic material can necessitate repeating gavages and thus involve more stress for the rat.

For habituation and tolerance of repeated oral dosing or gavage, gavage needles can be dipped in a solution, such as a sugar solution, that is pleasing to the rat. Such needle treatment also stimulates the swallowing reflex, which facilitates the gavage procedure (Travers and Norgren, 1986). However, consideration should be given to the potential effects of the dipping solution on rat physiology in the context of the experimental outcome.

Agents to be administered must be in suspension or solution at room to body temperature. The smallest volume possible should be administered, optimally 5 ml/kg (Turner et al., 2011a), and consideration should

be given to the particular rat involved (e.g., a pregnant rat has a reduced stomach size). Larger volumes can result in passive reflux if the stomach is overfilled or result in aspiration pneumonia (Turner et al., 2011a).

The conduct of gavage procedures might need special consideration when performed in barrier conditions, which often require working within the confines of a biological safety cabinet. Personnel working in barrier systems should adapt techniques to optimize ergonomics for themselves, while minimizing injury and stress to the rats.

10.3 COLLECTION OF BODY FLUIDS OR TISSUE

Standard operating procedures for the collection of body fluids or tissues should be developed and approved by the animal care committee before use. The institutional veterinarian should be consulted on experimental protocols.

10.3.1 Blood Collection

10.3.1.1 Survival Blood Collection

Because of the relatively small size of rats and the potential for stress from handling and invasive procedures, only personnel competent in the specific procedure on rats may perform procedures for the collection of blood or tissue. The least invasive method for the volume needed should be used, taking into account the amount of animal handling required and potential problems.

For a healthy rat, up to 10% of the total blood volume can generally be taken through a single sample, and 3-4 weeks should be allowed for recovery before further samples are taken (Morton et al., 1993). The total blood volume of rats will vary, but it is typically 5-7 ml/100 g for a healthy, mature rat (Morton et al., 1993). For obese animals, the percentage of total blood volume that can be taken in a single sample should be reduced to a maximum of approximately 7%, as the circulating blood volume to body weight ratio is less than for non-obese rats (Morton et al., 1993). It is important that the tissues at the blood sampling site be allowed to recover between sampling.

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) provides a decision tree that may be useful in determining the most appropriate technique for the size of sample required (Rat: Decision tree for blood sampling). Information on appropriate techniques is also available on their website (Blood Sampling: Rat), and in Parasuraman et al. (2010).

The effect of the sample site on study results must also be taken into consideration (Diehl et al., 2001). Additionally, stress associated with handling may impact blood parameters (e.g., levels of glucose, corticosteroid), providing further reason for proper habituation and handling. This emphasizes the importance of using the same method throughout a study and reporting the method used in subsequent publications.

Preferred sites for blood collection in rats include the lateral tail vein, the saphenous vein (Diehl et al., 2001; Sharma et al., 2014), and the jugular vein (Karim and Ali, 2009). As mentioned in Section 10.2.1, "Injections", heat may be required to improve visualization of veins; however, it should be applied with caution.

Following collection of blood, and before the animal is returned to the cage, there must be assurance that bleeding has stopped and homeostasis is being maintained. Fluid replacement should be implemented if necessary.

10.3.1.2 Terminal Blood Collection

Cardiac puncture is only acceptable as a terminal procedure after the rat has been euthanized or where the rat is under deep anesthesia prior to euthanasia. Blood collection from the abdominal aorta under deep anesthesia is also acceptable. Where euthanasia by decapitation has been approved by an animal care committee, trunk blood can be collected (see the NC3Rs webpage on blood sampling in rats).

Retro-orbital bleeding has been associated with negative animal welfare consequences and therefore should only be performed as a terminal procedure. The sample obtained from retro-orbital bleeding is not representative of venous blood, as it is a mixture of venous blood and tissue fluid (see the NC3Rs webpage on retro-orbital blood sampling in rats).

10.3.2 Urine and Feces

The least invasive method of collecting urine or feces that suits the requirements of the study should be used. Kurien et al. (2004) review various methods of urine collection, ranging from voluntary voiding with non-absorbent sand to the use of metabolic cages and catheterization or surgical methods. It is important to address the requirements of the study, in terms of obtaining samples that are free of contamination and of sufficient volume and/or obtained at a particular time, as well as the need to minimize any pain and distress experienced by the animals, both for the welfare of the animals and for the quality of the samples obtained. For guidance on metabolic cages, see Section 2.1.2.2, "Metabolic Cages".

To maintain consistency, consideration should be given to collection of fresh feces rather than random sampling of fecal pellets from cage bedding, which can lead to variability in the viability of any pathogens or other biological materials being assayed in the feces.

10.4 EXPLANTS AND IMPLANTS

Animal care and monitoring are critical to the successful use of explanted and implanted devices. Commonly used equipment to deliver substances to rats or record from rats includes superficial or surgically implanted catheters, cranial implants, vascular access ports, and infusion-delivery pumps (Turner et al., 2011b). Implantation of telemetry and other recording devices is also common.

Surgical insertion of devices poses a risk of post-operative infection, often due to the difficulty of sterilizing and handling the device. Devices act as a nidus for bacterial biofilms, and the exteriorization of such devices allows microorganism to track from the skin or device into internal tissues. To minimize these risks, all devices should be sterilized using standard techniques, including autoclaving, gas or chemical sterilization, or irradiation. If sterilization is not possible, the institutional veterinarian should be consulted for other options. Aseptic technique should be used during surgical insertion of any device and during device handling and manipulation.

The size or weight of a device and the site of attachment or insertion should be chosen to minimize any effects on the rat's ability to perform normal activities (Morton et al., 2003). If there is concern the device may affect the animal's ability to reach food or water, provisions should be made for easier access.

Where devices are exposed and can be damaged, monitoring must be sufficient to ensure that there is minimal risk to the animal. Devices must be designed and attached or inserted to minimize the risk of dislodgement, and caging should be designed or altered to minimize the risk of catching any part of the exposed

device. Rats should only be singly housed when there are significant welfare risks to housing them in groups. Caging can also be modified to provide some degree of protected contact when animals must be separated, for example, through the use of a cage divider as described in Section 6.2.2, "Single Housing".

As far as possible, environmental enrichment should continue to be provided to rats with devices to permit the expression of motivated behaviours (Lidster et al., 2016).

10.5 PROCEDURES FOR GENETICALLY MODIFIED RATS

The selection of methods to generate new genetically modified strains should be made with consideration of the Three Rs, for example, reducing the number of animals used in creating and maintaining each line and consideration of overall welfare impacts, as some methods are more efficient than others. Procedures for the generation of genetically modified animals should be reviewed by the animal care committee during protocol review, in keeping with the rapidly evolving nature of genetic modification and advances in research on animal welfare. Submission of protocols for renewal by the animal care committee should include a report from the investigator on the efficiency of the methods used to produce new strains.

Archiving technologies contribute to reduction and refinement; expertise in this area should be sought in the development of a new line.

10.5.1 Collecting Samples for Genotyping

The sampling method should be the least invasive method that can provide the quantity and quality of tissue required for the particular genotyping method being used. Ideally, it would also serve as a means of identifying the animal, thus minimizing the handling of animals and the number of procedures carried out on each animal (see Section 6.1, "Identification of Animals").

Low-invasiveness methods are available for collecting samples for genotyping; however, these methods are more prone to cross-contamination (Cinelli et al., 2007; Robinson et al., 2003) and may not be suitable for some studies:

- stool sampling (Cinelli et al., 2007);
- saliva and buccal cell sampling (Robinson et al., 2003); and
- hair sampling (Robinson et al., 2003).

Where these methods are not appropriate for a particular study, either ear biopsy, tail biopsy or blood sampling should be used (NIH, 2018). An ear biopsy (also referred to as ear punching) involves the use of an ear punch device or fine-tipped straight scissors to remove a small tissue sample (up to a maximum of 2 mm in diameter) from the periphery of the pinna where the tissue is thinnest. Ear biopsy causes less discomfort to the animals than tail biopsy and results in minimal bleeding; however, it is not always suitable for quantitative genotyping and cannot be performed on animals less than 14 days of age. Ear and tail biopsy should only be used once the animal is large enough to obtain a suitable tissue sample.

A tail biopsy for DNA analysis involves removing a tissue sample of less than 5 mm in length from the distal aspect of the tail (Diehl et al, 2001). Where tail biopsy is deemed necessary, consideration of anesthetics and analgesics must include assessment of the pain associated with the procedure, the effects of recovery from anesthesia, and the potential long-term effects. The use of anesthetics may provide less benefit to young rats

undergoing tail biopsy than to adult rats, and anesthesia and associated recovery time may increase anxiety and decrease activity levels for rats of all ages (see Section 10.11, "Anesthesia and Analgesia").

Toe clipping must only be used as a last resort, and only after careful consideration of less invasive alternatives and approval by the animal care committee. Toe clipping must only be carried out on rats younger than 8 days of age, and only one toe may be clipped (NIH, 2018).

10.5.2 Phenotyping

Some procedures that are acceptable for animals that have not undergone genetic modification may not be acceptable for genetically modified rats with altered phenotypes; those procedures may need to be modified or avoided when animals have compromised ability to respond to stress. This includes the choice of procedures for phenotyping.

Once the animals are phenotyped, any additional information related to animal welfare should be given to the animal care committee as soon as possible. Stable germ-line transmission does not necessarily mean that there is a stable phenotype or stable animal welfare, since phenotypes can change (e.g., be age dependent, have background effects, require homozygosity, or require breeding to other mutant lines). Appropriate monitoring is needed for the lifespan of the animal or when the genetic background is changed.

Investigators should take reasonable steps to publicize to the research community all available phenotypic and welfare information, along with strategies for mitigating problems with genetically modified rat lines.

Genetically modified rats may respond differently to drugs and food, as well as a number of experimental conditions, when compared to rats that have not undergone genetic modification. These changes in response may be the result of differences in the animal's metabolism and are particularly relevant for the use of anesthetics and to the use of the rats for testing new drugs or in toxicity studies.

10.6 IMAGING

For imaging, it is imperative that a plan be developed in consultation with a veterinarian. Although studies involving repeated imaging can reduce the number of animals required for a research study, the procedures create numerous occasions for animals to be stressed. Some of the factors to consider include: repeated injections; anesthesia; handling and transportation; experimental conditions (e.g., tumour burden or surgery); hypothermia; and fasting (Hildebrandt et al., 2008). All of these factors should be addressed in relation to both the welfare of the animals and the validity of the imaging results. In particular, given the significant impact of repeated anesthesia on the physiology of an animal, consideration should be given to the number of times and frequency of imaging. See Section 10.9, "Anesthesia and Analgesia", for particular considerations regarding the use of anesthetics. For serial imagery, it is particularly important that animals are monitored between imaging sessions.

The schedule for imaging should be developed based on the anticipated outcomes of an intervention or timeline of an age-dependent change and the welfare of the animals.

A number of imaging methods are available (see Oguz et al. (2013), Hildebrandt et al. (2008), Holzer et al. (2006), and Zhi et al. (2014)). The least invasive method suited to the goals of the study must be used.

Equipment should be thoroughly cleaned and disinfected between uses to minimize the possibility of cross-contamination, especially when sharing equipment.

10.7 BEHAVIOURAL STUDIES

A healthy animal with a good welfare status is critical to achieving a valid and interpretable outcome of any behavioural testing regime.

Aversive stimulation, deprivation, or restriction of resources should only be used when alternative methods are not effective. For example, altering water taste to make it less attractive while using unaltered water as a reward has been found to be an effective strategy for motivating task engagement in rats without restricting water access (Reinagel, 2018). Where possible, a reward strategy (e.g., highly preferred food) should be used to motivate an animal rather than using aversion. Motivational studies using electric shock, aversion stimuli such as puffs of air, food and water restriction, and/or water escape should be justified to the animal care committee and used in the least invasive fashion and for the shortest duration possible.

For consideration for various types of behavioural studies, see the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (NRC, 2003), Chapter 9, "Behavioral Studies".

Equipment should be thoroughly cleaned and disinfected between uses to minimize the possibility of cross-contamination, especially when sharing equipment.

10.8 FOOD AND FLUID INTAKE REGULATION

The use of food or fluid regulation in rats requires consultation with the veterinarian to determine the necessary level of regulation, the potential adverse consequences of the technique, and methods for assessing the health and welfare status of the animals (NRC, 2003). The procedure must be scientifically justified to the animal care committee, and endpoints (i.e., indicators for when to alter or terminate the procedure) to maintain the animals' health and welfare must be defined (NRC, 2003; CCAC, 1998) and approved by the animal care committee.

For new procedures, where possible, pilot studies should be carried out to determine whether or not food or water regulation is essential. If it is necessary, preference should be given to using the timing of food or water intake to motivate the animals. When reduction in the amount of food or water is necessary to achieve the study outcome, the least reduction should be used and for the shortest duration possible. Food intake regulation is preferred to fluid intake regulation, as it has less potential for adverse health consequences. Water must not be withheld if other methods can be used effectively. Calculations of the amount of restriction need to take into account the body weight of the animal, and for growing rats, body weight gain suppression should be factored into the overall body weight calculation. Food and water restriction should not exceed 24 hours and the animals must maintain their predetermined weight during the restriction. There should be documented monitoring of the weight of the rat, and this information should be available to the veterinary staff.

Food removal and water restriction can cause changes in the physiological and biochemical processes of the animal, which become more severe with longer duration of withdrawal (Claassen, 1994; Turner et al., 2001). Characteristics such as strain (including genetic modification), sex, age, housing density, reproductive status, and room temperature markedly affect fluid and food intake patterns. In rats, food and water intake are coupled with circadian rhythm, and this should also be taken into account to ensure rats consume the designated amount (Claassen, 1994). Any food removal should be carried out in the photophase (light phase) to correspond with the behaviour of the species. The effect of food restriction is greatest when it occurs in the dark phase, as the animals are most active during this period. Significant reductions in liver weight and gly-

cogen content, as well as increases in levels of glycerol, free fatty acids, and acetoacetate, have been measured after 3 hours of food removal in rats (Palou et al., 1981). For rodents, the duration of food removal for many outcomes such as a steady-state blood glucose and insulin level does not equate to human fasting duration. Some studies show that gastric content is reduced as much after 4-6 hours of food removal in rats as after 12 or 18 hours (Prior et al., 2012; Prior et al., 2009); however, in rats, there can still be food in the stomach 24 hours after initial food removal (Turner et al., 2001). Even then, food deprivation may not be complete due to coprophagy and consumption of bedding materials.

10.9 ANESTHESIA AND ANALGESIA

10.9.1 Anesthesia

Anesthesia should be used as a tool for procedures that are potentially stressful or painful. Decisions regarding when this is warranted should be made in consultation with the veterinarian and approved by the animal care committee.

Anesthetics can have profound effects on the physiology of animals, affecting both the welfare of the animals and experimental results (Flecknell, 2009). The use of anesthesia should be considered a significant procedure, and careful planning of the anesthetic regime, including management of complications, is a critical component of good experimental design (Flecknell, 2009). The need for planning includes the selection of drugs and dosages that are suited to the specific anatomy and physiology of the rats, careful monitoring, and appropriate care for the rats during and after the procedure.

Specific training in the administration of anesthetics is critical to success. Only personnel with appropriate training and demonstrated competency must be permitted to perform anesthesia (CCAC, 2015).

Anesthesia management must reflect phenotype and health status. The possibility of the genetic modification, experimental procedures, and disease model affecting the phenotype should be considered when planning anesthesia. Circadian rhythm should also be a consideration, as there is evidence that anesthesia disrupts circadian rhythm in rats (Kikuchi et al., 2013), resulting in changes in brain functioning. Precautions must be taken to ensure safe anesthesia. Ideally, animals undergoing anesthesia should be in good health. Rats have a gag reflex but are unable to vomit; therefore, it is not necessary to restrict food and water for a long period before anesthesia. However, withholding food and water intake for a brief period before anesthesia will ensure the oral cavity is clear.

Animals should be weighed and, if necessary, the drugs diluted accordingly to ensure accurate dosage. Because of potential differences in drug metabolism, a pilot study may be helpful to determine the appropriate effective dosage when anesthetizing a new strain. The anesthesia regime will need to be adjusted if the animal's health is altered by the experimental procedure or its phenotype.

Balanced anesthesia is an approach that combines smaller amounts of multiple drugs to target the many components of an anesthetic state: consciousness, analgesia, muscle relaxation, and alteration of autonomic reflexes (Grimm et al., 2015). Balanced anesthesia may eliminate adverse effects that could accompany a large dosage of a single drug (Ilkiw, 1999).

Inhalation anesthesia, using an anesthetic chamber for induction and an appropriate breathing system for maintenance with a precision vaporizer, provides rapid onset of, and recovery from, anesthesia, and the level of anesthetic can be accurately and rapidly adjusted during the procedure (Flecknell, 2009). Vaporizers should be calibrated regularly to ensure accurate delivery of anesthetic.

The type of anesthetic and concentration must be carefully considered, as aversive behaviour in rats has been shown to be greater for some anesthetic agents than others, and for higher concentrations (Leach et al., 2002; Makowska et al., 2009; Bertolus et al., 2015). There is also evidence that rats that have been exposed to anesthetics will try to avoid repeat exposure (Wong et al., 2013), suggesting that anesthesia is not an innocuous procedure and is a welfare concern that must be considered, particularly for longitudinal studies.

Repeat-bolus dosing to extend a surgical plane of anesthesia must be performed with great care. This technique should be justified over other techniques that provide for a more stable plane of anesthesia, such as inhalant anesthesia, as there is an increased risk of animals regaining consciousness during procedures or experiencing anesthetic overdose. Continuous intravenous infusion is also an option, but it must be used with caution to avoid fluid overload.

Vital signs should be monitored continuously and reflexes of animals under anesthesia should be checked frequently (see Section 10.11, "Monitoring and Post-Procedural Care").

Adequate oxygenation and ventilation, and the elimination of dead space are critical and must be considered even when injectable anesthetic drugs are used. Hypoxia can affect metabolism and possibly the validity of research data; prolonged hypoxia can result in corneal lesions (Turner and Albassam, 2005) or sudden, unexpected death. Oxygen can be administered through a face mask, although intubation is also possible in rats. Pulse oximeter monitors that are suitable for rats are available. Warm fluids should be administered to compensate for evaporation through open surgical wounds, the respiratory system, and blood loss, and to support blood pressure. Corneal lubrication must also be considered.

When available, anesthetic reversal agents should be administered at the end of the period requiring anesthesia to accelerate recovery. Adequate pain control should also be provided for painful procedures, independent of the anesthetic.

Animals must be monitored regularly through the recovery period and should not be left unattended until they are able to ambulate. An external heat source should be provided during and following anesthesia until animals are able to thermoregulate on their own (generally recognized as able to move about the cage rapidly on their own without external stimulation). As with anesthesia of any animal, care must be taken to prevent overheating and burns when heating devices are used. Other forms of supportive care should be provided, such as fluids and eye lubrication.

Inhalant anesthetics, such as isoflurane and sevoflurane, can be used safely for potentially painful procedures on neonatal rats.

10.9.2 Analgesia

Guideline 23

Rats should be provided with analgesia for invasive procedures that are likely to be painful.

Decisions not to provide analgesia when animals are likely to experience pain (e.g., invasive procedures or models of disease such as arthritis) must be approved by the animal care committee. It is important to consider that rats experiencing pain may not show clinical signs of pain or may express behavioural signs not

usually associated with pain. Rats must not be denied analgesic treatment for a procedure that is likely to be painful on the sole basis that they do not overtly demonstrate signs of pain.

The need for analgesia and the type of analgesia should be reviewed with the veterinarian prior to the development of pain from procedures or the development of pain from chronic conditions. Waite et al. (2015) found that some analgesics commonly given to rats to address post-surgical pain are insufficient. Pre-emptive analgesia should be considered for procedures likely to be painful, as the lack of control of acute pain will increase the possibility of chronic pain that is resistant to analgesics emerging later as a result of wind up (central sensitization).

It is important to learn how to recognize signs of pain in rats and to develop strategies to evaluate and treat individual animal pain as accurately as possible. Physiological and behavioural signs should be monitored (see Flecknell, 2009; 2017). Acute pain may be detected through changes in facial expression (Oliver et al., 2014; Leung et al., 2016). Specific signs, such as back arching and horizontal stretching followed by abdominal writhing, are also helpful indicators of pain and discomfort (Roughan and Flecknell, 2001). Where analgesia is withheld because of contraindication, there must be clear endpoints, established in advance, and approved by the animal care committee.

Many common analgesic agents are too concentrated for accurate dosing of rats and must be appropriately diluted with a sterile diluent. Multimodal analgesia (i.e., the combined application of several different analgesics, each with differing mechanisms of action) should be considered. This approach can provide more effective pain relief, as the onset of action for various analgesic agents are different (Flecknell, 2009) and they target different pain pathways or receptors (Gaynor, 2009). Categories of analgesic drugs include non-steroidal anti-inflammatories, opioids, and local anesthetics.

The duration and frequency of analgesic treatment will depend on the type of surgery and the state of the animal; longer-term analgesia may be necessary for invasive surgery. Sustained-release drug formulations are available and can help provide long-lasting analgesia without requiring stressful handling for re-injection (e.g., Foley et al., 2011).

The least invasive route of administering analgesics should be used, when available (Abelson et al., 2012).

10.10 SURGERY

Surgery involves major interventions (penetration of the body cavity) and other invasive procedures, such as stereotaxic surgery with implants and orthopaedic surgeries. Consideration of the invasiveness of the procedure, whether it is recovery or non-recovery surgery, and length of time to recovery, inform the requirements for the procedures and the measures to be taken to minimize negative effects on the welfare of the animals.

Major survival surgeries should take place in a dedicated surgical suite (or dedicated area of a procedure room), where aseptic conditions are maintained through the use of sterile instruments and suture material. Appropriate attire (sterile gloves, mask, etc.) should be worn by the surgeon to maintain aseptic technique, and gloves should be changed between surgeries on different rats. See Héon et al. (2006) for additional practices to improve aseptic technique.

Surgical instruments should be of an appropriate size, and magnifying devices (binocular microscope, surgical glasses (referred to as loupes) can be used as appropriate to ensure proper visualization. Ideally, a sterile pack of surgical instruments should be used on each animal. When this is not possible, instruments must be

wiped clean and disinfected using an appropriate method (e.g., immersion in cold sterilant and then rinsed; hot bead sterilization and then cooled before reuse). Each pack must be used on no more than five animals, with a sufficient number of packs available to accommodate this.

Before performing recovery surgery, personnel must complete appropriate training and demonstrate competency with all necessary techniques (CCAC, 2015). Tissue trauma and tension on tissues during surgery should be minimized to reduce the amount of post-operative pain experienced by the animal and to improve recovery.

Consultation with a veterinarian or veterinary technician can assist in choosing sterile suture material of the appropriate size and type for rats. Inappropriate material will delay healing and contribute to post-operative complications, including discomfort and wound chewing. Suture material and wound clips should be removed when healing is sufficient, generally 7-10 days after surgery.

Prior to surgery, the maximum amount of permitted blood loss should be established. The amount of blood loss should be minimized and monitored during surgery; if it exceeds the established maximum, the animal should not be permitted to recover.

Surgeries on severely immunocompromised rats or rats treated with hazardous agents should be carried out in biological safety cabinets.

Hair must be removed from the surgical incision site prior to surgery and an area extending beyond the site should be clipped and the skin prepared. However, the large surface area to body mass ratio of rats causes them to lose heat rapidly, making them very susceptible to intraoperative hypothermia (Taylor, 2007). To minimize this risk, the area shaved for surgery should be only as large as necessary for surgical access and to maintain asepsis. Solutions used for skin preparation should be applied to the surgical area with precision to minimize cooling effects (Skorupski et al., 2017). Finally, rats should be placed on a warm surface during surgery. Use of heating devices requires care to prevent overheating and burns, and the rat's temperature should be monitored.

To ensure an appropriate plane of anesthesia for invasive procedures and surgery, the pedal withdrawal reflex should be monitored regularly. Care should be taken to neither over nor under extend the leg while checking the pedal withdrawal reflex. The duration of the surgical plane of anesthesia is defined as the time between the loss of the pedal withdrawal reflex and reappearance of that reflex (Jaber et al., 2014). Alternatively, the tail can be gently pinched if the foot is not accessible.

Warm isotonic fluids should be administered to compensate for evaporation through open surgical wounds or the respiratory system, or blood loss. Warm isotonic fluids can also support blood pressure when surgeries last longer than 30 minutes, when there is significant blood loss during surgery, or when rats are otherwise likely to be debilitated.

Special consideration must be given to the small size of the animal and any potential monitoring difficulties that may be encountered due to the presence of surgical drapes or ancillary equipment, which may limit clinical observation. Strategies and equipment must be in place to ensure appropriate monitoring to avoid potential hypothermia, hypoglycemia, dehydration, and blood loss.

Detailed surgery logs must be kept by the investigators and be accessible to the veterinarian, animal care committee, and others, as needed. There should also be an indication of any procedures performed (and when they were performed) on the cage card for cage-side assessment of the rats.

10.11 MONITORING AND POST-PROCEDURAL CARE

10.11.1 Monitoring

Guideline 24

Post-procedural care and monitoring must be planned based on the particular procedure and the individual needs of the animal, and adapted as necessary when unforeseen situations arise.

Each animal should be monitored throughout the entire procedure, from the point when the procedure could impact the welfare of the animal, until complete recovery, and monitoring should be documented. Monitoring rats may prove more difficult than for other species due to their small size. However, monitoring of rats should be done with the same rigour as for other species. For rodents, changes in their welfare status can occur quickly, and monitoring may need to occur more frequently than for larger animals.

Investigators must obtain the approval of the animal care committee regarding acceptable monitoring schemes and documentation practices after consultation with the veterinarian and prior to starting studies. This includes relevant endpoints (i.e., intervention, treatment, and euthanasia). The stress caused by handling rats for monitoring (e.g., to determine body weight or temperature), should be taken into consideration when developing a monitoring plan. It should be noted that as studies are refined, monitoring schemes and documentation may need to change.

Monitoring includes physiological measures and observations (e.g., weight loss), behavioural indicators (e.g., how well the animal moves around its cage), and other indirect measures, such as facial expressions and body condition scores. Where appropriate, scoring sheets should be developed based on validated assessment measures and used to assess rat condition. Body-condition scoring and regular observation of physical and behavioural indicators of health can be useful elements of a monitoring scheme. A behaviour-based, post-operative pain scoring system has been developed by Roughan and Flecknell (2001; 2003). The Rat Grimace Scale, a system of coding facial expressions as a measure of spontaneous pain, is also useful in quantifying some post-procedural pain (Oliver et al., 2014; Leung et al., 2016).

It is very important that peri-procedural monitoring be a collaboration involving animal care personnel and the research team. Timing of monitoring is very important (e.g., animals should always be weighed at the same time of day), and depending on when procedures occur, plans may need to be made to monitor rats outside regular staffing hours and overnight. Personnel responsible for monitoring rats and documenting their condition must be competent in recognizing and interpreting rat-specific clinical signs and conditions, as well as rat pain and pain behaviours, and be aware of the documentation and reporting procedures, including emergency contact information. Clinical signs and conditions may vary depending on the type of procedure and age or sex of the animal. Animal care personnel should have information about the surgery in advance to assist in identification of departures from normal patterns of post-surgery recovery.

10.11.2 Post-Procedural Care

The plan for post-procedural care should be developed in consultation with the veterinarian. Analgesics should be a component of care following any procedure that could cause pain (see Section 10.9.2, "Analgesia"). However, additional nonpharmacological approaches should also be considered to address animal comfort.

The recovery cage should be placed in a warm environment, with low levels of noise and light, to promote recovery. Animals may be housed singly until they are ambulatory (Kohn et al., 2007) and then returned to their regular housing (see Section 6.2, "Housing Management"). Prolonged single housing of rats can add to the stress of procedures and interfere with recovery. Dry, soft and/or more absorbent bedding should be provided as necessary for additional comfort and to prevent bedding from sticking to surgical wounds.

If an animal's movement is restricted, food and water should be made accessible. Soft, palatable food should be provided to promote rapid recovery and a return to normal digestive function (e.g., post anesthesia). Any new food or food supplement to be provided should be introduced to the animal prior to the procedure so that the animal will be familiar with it. Post-procedural care must continue until complete recovery.

Where palliative care is needed, hydration of the animal is particularly important. Alternative water sources, such as hydrogels, may need to be used if the animal is unable to reach the water source. The animal must be monitored and replacement fluids given subcutaneously, intraperitoneally, or intravenously if necessary, under veterinary direction. If body weight is to be monitored daily, handling stress should be minimized (e.g., removing the animal from the cage in a small container). Additional consideration is needed to care for wounds, and the advice of the veterinarian should be sought.

11 EUTHANASIA

Guideline 25

Euthanasia of rats must be carried out by competent personnel only, using the method best suited to the particular animals, their housing situation, and the impact on the study data.

The general guiding principles outlined in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) are applicable to euthanasia of all animals in science. Additional considerations are discussed in a report of the second Newcastle meeting on laboratory animal euthanasia (Hawkins et al., 2016). This section provides additional information that is specific to euthanasia of rats.

For all methods of euthanasia performed on rats, the following are important requirements:

- personnel involved in the procedure must be trained and have their competency assessed with regard to performance of the procedure on rats and their ability to confirm the death of rats;
- equipment must be appropriately maintained and cleaned before use or reuse;
- stress caused by handling should be minimized;
- animals must not be mixed with incompatible animals prior to euthanasia;
- when using a gassing method, ideally, rats should be euthanized in their home cage;
- pups that are still nursing should not be removed from the dam until just prior to euthanasia (unless provided with thermal support); and
- any animals undergoing euthanasia must not be left unattended prior to confirmation of death.

11.1 INJECTION

As noted in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010), an overdose of an injectable anesthetic suitable for rats (generally defined as double the dose calculated to achieve anesthesia) is an acceptable method of euthanasia, providing death is confirmed either by close observation or by a secondary method. However, intraperitoneal administration of irritating substances (e.g., pentobarbital) may provoke pain, and has been shown to produce inconsistent outcomes in rats (Chisholm and Pang, 2016); hence, its use should be carefully evaluated and approved by the animal care committee. Combining lidocaine or another local anesthetic with pentobarbital may reduce the negative welfare effects (Khoo et al., 2018).

Secondary methods are applied following application of the primary method without allowing the animal to regain sensibility. Possible secondary methods listed in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) are exsanguination by blood draw or organ removal, cervical dislocation, decapitation, and opening of the chest. For rats, intracardiac injection of potassium chloride (KCl) and intravenous perfusion (e.g., formalin, paraformaldehyde) can also be considered potential secondary methods for unconscious or deeply anesthetized animals.

11.2 INHALATION TECHNIQUES

Inhalation techniques for euthanasia of rats have potential welfare concerns and must be carefully considered.

Overdose of an inhalation anesthetic agent (e.g., isoflurane or sevoflurane) is an effective method of euthanasia of rats, resulting in rapid induction and unconsciousness; however, the time to death can be prolonged and a second method to ensure death of the animal is recommended once the animal is deeply anesthetized and unconscious. See Section 11.1, "Injection", for possible secondary methods.

The *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010) provides information on the use of carbon dioxide (CO_2) during euthanasia, and recommends the use of inhalant anesthetics prior to CO_2 where practical. Exposing rats to carbon dioxide for euthanasia is likely to cause pain and distress (Leach et al., 2004; Moody et al., 2014). While exposure to inhalant anesthetics has been found to be aversive to rodents, isoflurane has been shown to be less aversive to rats than CO_2 (Makowska et al., 2009). However, aversion to isoflurane and sevoflurane can increase with repeated exposure (Bertolus et al., 2015; Wong et al., 2013), and this should be taken into consideration. There is currently a substantial amount of research being conducted in the area of inhalant techniques for euthanasia and it is important to carefully evaluate any new evidence that becomes available (e.g., Valentim et al., 2015; Boivin et al., 2017). Additionally, Baker and Hickman (2018) caution that there is the potential for observer bias in rating the experiences of animals exposed to inhalation anesthetics.

Gasses must be adequately scavenged to ensure operator safety.

As stated in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010), the CO_2 chamber must be flushed with air between groups of animals being euthanized. If the CO_2 in the chamber is not dissipated prior to use, the animals may experience concentrations of CO_2 that induce breathlessness and distress (see Djoufack-Momo et al. (2014) for information on dissipation of CO_2 from the chamber).

11.3 PHYSICAL METHODS

Physical methods are often used to avoid confounding variability introduced by euthanasia or sedative agents. For example, cervical dislocation and decapitation, when properly performed, are generally not associated with elevated cortisol or stress hormone levels.

If physical methods are required, they must only be conducted by individuals who are highly competent in the use of those methods on rats, and must be justified in the animal protocol and approved by the animal care committee. As noted in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010), cervical dislocation and decapitation are considered conditionally acceptable methods of euthanasia, due to the potential for severe pain and distress if the procedures are performed incorrectly. When possible, alternate methods of euthanasia should be selected.

If cervical dislocation is used, consideration should be given to using a sedative or anesthetic agent beforehand (Carbone et al., 2012). The size limit for cervical dislocation of rats is 200 g (AVMA, 2013). However, competent performance of manual cervical dislocation is dependent on adequate physical strength; operators should consider their personal limits and level of fatigue when determining whether performance is appropriate. For rats larger than 200 g or for large numbers of rats, a mechanical cervical dislocator should be used for better accuracy. Cervical dislocation requires a high level of skill, experience, and decisiveness, and should not be considered a routine method of euthanasia for rats.

Decapitation of rats requires that the animals are appropriately restrained. Standard operating procedures must be followed to ensure operator safety. For this technique, consideration should also be given to the prior use of a sedative or anesthetic. If sedation or anesthesia is contraindicated by the protocol, the use of plastic restraint devices can aid in animal positioning and help to prevent operator error.

11.4 OTHER METHODS

Some studies require the use of focused-beam microwave irradiation for euthanasia of rats to preserve metabolites *in vivo* for subsequent analysis. Providing the equipment is specifically designed for euthanasia of rodents in laboratory situations, and the operator is competent in the procedure, this method results in rapid loss of consciousness and death (AVMA, 2013). However, this method requires firm physical restraint and must be justified to and approved by the animal care committee before being used.

11.5 EUTHANASIA OF PRE-WEANING AGE RATS

Euthanasia of pre-weaning age rats requires special consideration, as described in Section 6, "Considerations Relating to Fetal and Neonatal Euthanasia", in the *CCAC guidelines on: euthanasia of animals used in science* (CCAC, 2010).

Once fetuses become conscious, there is the likelihood that pain perception develops rapidly. Therefore, when pregnant dams are euthanized, the method chosen should provide rapid cerebral anoxia to the fetus with minimal disturbance to the uterus, thus limiting arousal of the fetuses.

If fetuses are required for the study, it is preferable to euthanize them by physical methods, such as decapitation with scissors. Anesthesia followed by exsanguination can also be used. Intraplacental injection of pentobarbital can be used if studies require preservation of the fetal anatomy for histology, or if it is important to avoid hypoxia.

Neonates up to 14 days of age are best euthanized by decapitation. Between the age of 14 days and weaning, rats can be euthanized with isoflurane, followed by a secondary method.

Fetuses and neonates may be killed by rapid freezing in liquid nitrogen only if preceded by anesthesia (Artwohl et al., 2006).

12 END OF STUDY

12.1 TRANSFER OF RATS BETWEEN FACILITIES OR PROTOCOLS

For rats that are to be transferred to another institution at the end of a study, see Section 4, "Procurement", particularly with regard to regulations, documentation, and transportation. As mentioned, this applies to rats that have not been subject to major invasive procedures, and are fit to travel.

If rats are transferred to an institution that is not CCAC-certified, it is the responsibility of the institution sending the rats to ensure the animals will receive appropriate care.

12.2 RE-HOMING

Where permitted by regulatory authorities, institutions may release healthy research rats (not genetically modified rats) that are commonly accepted pet or companion species to individuals who have the knowledge and ability to provide adequate care to the animals. No genetically modified rats may be moved from research facilities to private premises. As with any other species, if rats are to be released to the care of an individual as companion animals, the institution should develop an appropriate policy describing the conditions that need to be fulfilled before release of the animal. Institutions should ensure those who will be adopting the rats are aware of the care required.

12.3 DISPOSAL OF DEAD RATS

Dead rats must be disposed of according to relevant federal, provincial (or territorial), and municipal regulations for the disposal of biological materials.

13 HUMAN HEALTH AND SAFETY

Institutions have occupational health and safety programs that are specifically tasked with addressing this topic through risk assessments. The responsibility of the animal care committee extends to ensuring there is an institutional occupational health and safety program in place so that any risks to human health and safety are properly assessed.

Those working with animals must follow institutional policies and standard operating procedures outlining appropriate measures of prevention and protection. They should seek professional knowledge on animal allergens and zoonotic diseases, as well as other risks or hazards that may be associated with a particular study (e.g., exposure to radiation, anesthetic gas, chemical hazards, and human cell lines).

Laboratory animal allergy, triggered by the presence of rodent proteins, can have long-term effects on the health of people working with rats (Palmberg et al., 2015). Measures to control exposure to animal allergens should use a risk-based approach (e.g., Westall et al., 2015). Practices to minimize exposure include:

- engineering controls – good facility design, adequate ventilation, appropriate air pressure gradients, working with rats in biosafety cabinets or ventilated hoods when possible, use of bedding disposal stations, and use of appropriate caging and bedding;
- administrative controls – work practices and training that help reduce duration of individual exposures (e.g., job rotation, good housekeeping, personal hygiene practices such as hand washing and showering); and
- personal protective equipment (PPE) – facility-specific clothing, gloves, hair bonnets, shoes or shoe covers, eye protection, and adequate respiratory protection (Harrison, 2001).

A comprehensive approach should be taken; for example, the use of biological safety cabinets alone does not provide sufficient protection against allergens when bedding is disturbed during cage-changing (Westall et al., 2015).

If rats are purchased from sources other than reputable suppliers (as noted in Section 4, "Procurement"), there can be increased risks to human health due to the potential presence of zoonotic microbes. Zoonotic agents that have been reported include *Streptobacillus moniliformis*, which causes rat bite fever (CDC, 2015), and *Leptospira sp*., which can lead to Leptospirosis in humans (CDC, 2014).

Rats may be deliberately infected with zoonotic agents if they are to be models of infectious disease as part of a research protocol, or infectious agents may inadvertently be introduced through contaminated biologics or cell lines injected into rats (Peterson, 2008). Personnel with known or unknown immunodeficiency or reduced immune competency may be at increased risk of infection with certain rat pathogens.

It is also important to address any risks of agents carried by humans infecting the animals (e.g., *Streptococcus pneumonia*), as noted in Section 9, "Health and Disease Control".

Where there are potential biosafety concerns (including the use of viral vectors for transmission of transgenes), the protocol should be forwarded to the institutional biosafety committee or officer for review, prior

to review by the animal care committee. The biosafety committee or officer will ensure that the organism is appropriately classified and an adequate risk assessment is undertaken to be able to ascertain the necessary conditions for housing and care of the animals and for their subsequent disposal.

People working with rats should take precautions against bites and scratches, as appropriate. In addition, caution should be taken when using needles or sharp instruments on rats, as their small size may increase the risk of personnel poking or cutting themselves.

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APPENDIX 1
RESOURCES FOR INFORMATION ON RATS

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APPENDIX 2
RAT HOUSING ASSESSMENT TOOL

The housing system must:

- allow for social housing;
- enable proper access to food and water;
- allow for proper sanitation; and
- be safe for the animals and for humans.

The housing system also needs to provide sufficient and proper space for each animal to promote their natural behaviours. This can be determined through an evaluation of the housing system, including the elements listed in the table below. Elements should be rated on a scale of 1-5, where a score of "1" indicates the worst possible state. This approach should give users a quick visual indication of the suitability of rat housing within the facility and indicate areas where improvements could be implemented.

Table 1 Rat Housing System Assessment Tool

HOUSING ELEMENTS	SCORE				
	1	2	3	4	5
Sufficient floor area and cage height to walk, run, jump, stretch; also play for juveniles					
Structures or materials to enable avoidance of open space and light					
Structures or materials to facilitate hiding and escape from aggressor					
Structures or materials to develop a microenvironment					
Appropriate type and amount of flooring substrate (bedding)					
Appropriate materials to facilitate nest building					
Foraging opportunity					
Gnawing opportunity					
Structures or materials for climbing					
Objects for rats to manipulate					

APPENDIX 3
METHODS OF IDENTIFICATION

The following table lists methods according to invasiveness, with the least invasive methods appearing first.

METHOD	BENEFITS	ADVERSE EFFECTS / DISADVANTAGES
Non-toxic dye or permanent marker	Low invasiveness Easy to apply and reapply, especially when dealing with a small number of animals May not require extensive restraint	Only visible for a short period of time (may be longer for markers specifically for animals) Loss of visibility of marks may result in misidentification, or require additional handling for reapplication Application of dye or marker can affect anxiety levels and response to human handling, which might have implications for experimental results (Burn et al., 2008)
Fur clipping or shaving	Low invasiveness Quick	Noise of clippers may cause fear or be irritating to the animal Shaving can cause skin irritation Must be repeated as often as the hair grows back May require more restraint than permanent marker
Microchip transponder	Quick, effective Some enable remote monitoring of physiological parameters	May require the animal to be anesthetized prior to implantation Can induce physiological and behavioural changes indicative of stress (adverse effects can be reduced by choosing an appropriate-sized chip) Requires a scanner; equipment specific to the transponder is required to retrieve the information Material needs to be appropriate for the research (e.g., non-metal if MRI or scanning procedures will be performed)

METHOD	BENEFITS	ADVERSE EFFECTS / DISADVANTAGES
Tattoo	More permanent than dyes For neonatal animals, the technique is somewhat permanent, minimally invasive, and can be clearly read throughout the animal's life	May fade or become illegible over time if operator is not well trained Tattoo needles must be maintained sharp and sterile Commercial tattoo kits should be used (not do-it-yourself tattooing needles) Anesthetics/analgesics should be considered, providing the stress of the restraint and anesthetic does not impose a greater welfare concern than the tattooing procedure
Ear buttons	Less potential for loss or being ripped off than ear tags	
Tags	Moderate invasiveness Quick procedure Ease for any personnel to identify individual animals (i.e., highly visible, does not require specialized knowledge of coding systems)	Possible inflammation and tissue damage Can be lost or ripped off Size, shape, and placement of the tag is essential to the health and welfare of the animal and to prevent loss of tags Tag material must be appropriate for the research (e.g., non-metal if MRI or scanning procedures will be performed) Local anesthetics and analgesics should be considered; however, the welfare concerns associated with additional restraint of the animals prior to tagging may outweigh the benefits
Ear notch	Low to moderate invasiveness Quick procedure Tissue can also be used for genotyping	Requires training to ensure notching system is legible and the procedure is done properly with no bleeding May impact auditory communication Ears can be damaged by fighting and ear tearing can render the identification code impossible to read Tissue may grow back, closing the notch The corresponding identification system must be known to the user Anesthetics and analgesics should be considered; however, the welfare concerns associated with additional restraint of the animals prior to ear notching may outweigh the benefits

APPENDIX 4
INDICATORS THAT MAY BE USED TO ASSESS THE WELFARE OF RATS

Welfare assessment programs can include a combination of animal, resource, and management-based indicators. While animal-based indicators are necessary to make direct conclusions about the welfare state of an individual animal, information about resource provision and management strategies can also be useful for identifying potential welfare risks. Many animal-based measures require regular assessment over time so that alterations from normal can be detected. Assessments performed during the dark phase (i.e., the active phase) may be more effective at detecting subtle alterations in behaviour in rats since they are nocturnal. Appropriate training of individuals that are responsible for assessing these indicators is necessary to ensure that assessment is accurate and consistent. The animal-based indicators included in this appendix vary in terms of the level of scientific validation that they have undergone. Regardless of prior validation, indicators should be assessed on an ongoing basis by animal care staff and investigators to ensure they are effective and appropriate for the given application. For a general discussion of factors to consider when developing a welfare assessment program, see Hawkins et al., 2011. Where available, a sample of references has been provided for further guidance. This document is not intended to provide a comprehensive review on all indicators, but rather provides potential indicators that institutions can draw from and add to.

Table 1 Assessing Whether Environments are Appropriate for Individual Animals, Using Resource-Based Measures

INDICATOR	COMMENTS	FURTHER INFORMATION
Environment allows physical performance of important natural behaviours	Assessing the animal-environment match to determine whether all animals in the cage have sufficient space to perform important natural behaviours based on their size and activity level Consider potential for ambulating, turning, full upright and horizontal stretching, avoiding agonistic encounters, rough-and-tumble play in juveniles, and nursing postures in lactating dams	Section 1.1, "Behavioural Biology" Section 2.1, "Housing"
Provision of appropriate housing and husbandry	Basic features including bedding and nesting materials that are clean, dry, and provided in sufficient amounts; shelter; clean and accessible food and water; social contact Enhanced welfare through enrichment items that encourage natural behaviours such as gnawing, foraging, playing, object manipulation	Section 6.3, "Food, Water, and Bedding" Section 6.4, "Environmental Enrichment" Section 6.7, "Cage Changing and Sanitation"

INDICATOR	COMMENTS	FURTHER INFORMATION
Presence of negative environmental features that might impair welfare	Audible or ultrasonic noise, or the presence of equipment known to emit such noise Vibration, particularly in ventilated caging Excessive or inconsistent temperature and humidity Excessive and inescapable light levels within the cage Poor air quality, particularly due to ammonia	Section 3.1, "Managing the Environment"

Table 2 General Animal-Based Indicators of Possible Stress, Illness, Pain, or Discomfort in Rats

INDICATOR	COMMENTS	FURTHER INFORMATION
Altered food and water intake	Normally, poor welfare is associated with reduced intake as compared to normal intake for individuals, based on life stage and experimental details Sometimes increased consumption occurs with particular health-related disorders (e.g., increased water intake with diabetes)	Faraday, 2002 Flecknell and Liles, 1991 Hart, 1988 Liles and Flecknell, 1993 Monteiro et al., 1989
Weight changes	Normally, poor welfare is associated with a weight decrease, or lower gain than expected in growing animals Sometimes weight gain occurs in barren environments where opportunities for activity are restricted Body condition scoring can also be performed	Faraday, 2002 Hickman and Swan, 2010 Liles and Flecknell, 1993 Monteiro et al., 1989
Altered posture	Arched back with lowered head and front paws tucked under the body is associated with pain, discomfort, illness, and sometimes, stress	Hart, 1988
Altered grooming behaviour	Grooming behaviour in response to conditions involving poor welfare can be either increased or decreased, depending on the context; for example, grooming can be elicited in response to a wound, or decreased when pain is severe and mobility is restricted; similar complexity can occur with responses to stressors	Abbott et al., 1995 Fernandez-Teruel and Estanislau, 2016 Hart, 1988 Song et al., 2016
Coat condition	Coat should be clean and smooth; can assess for greasiness and discolouration (potentially indicating lack of grooming) and piloerection	Hart, 1988

INDICATOR	COMMENTS	FURTHER INFORMATION
Chromodacryorrhea (e.g., porphyrin staining)	Reddish brown staining underneath the eyes and around the nose; can spread throughout the body through grooming Can be subjectively scored	Mason et al., 2004
Damage to the fur or skin	Determine whether damage is self-inflicted, inflicted by agonistic encounters with cage mates, or due to infection or environmental injury	
Abnormal repetitive behaviours (e.g., stereotypies)	Laboratory rats rarely show locomotor or oral repetitive behaviours that are commonly seen in other rodent species Can be scored in terms of frequency or duration, and may precede specific activities	Kelley, 2001
Altered social behaviour	Self-isolation Agonistic behaviour towards cage mates, when not previously observed Potential for automated assessment	Hart, 1988 Peters et al., 2016 Wood et al., 2003
Altered activity levels	Activity levels that are markedly increased or decreased in comparison to normal Assess frequency and duration of locomotion and other normal behaviours Potential for assessment via automated systems using infrared beams, instrumented running wheels, or telemetry implants	Flecknell and Liles, 1991 Hart, 1988 Liles and Flecknell, 1993
Partially closed, sunken, or dull eyes		
Altered interactions with humans	Avoidance or agonistic behaviour towards human handlers, when not previously observed Assess latency to approach, possibly incorporate treats or 'tickling'	Section 6.5, "Human Contact and Handling" Section 7, "Handling and Restraint"
Altered physiological parameters	Examples include heart rate, respiratory rate, temperature, glucocorticoids Increased or decreased in comparison to normal	
20-kHz vocalizations	Associated with avoidance and contexts related to negative affective states	Brudzynski, 2013 Wohr and Schwarting, 2013

Table 3 General Animal-Based Indicators of Possible Neutral or Positive Welfare States in Rats

INDICATOR	COMMENTS	FURTHER INFORMATION
Any indicator	General references on topic	Boissy et al., 2007 Yeates and Main, 2008
Exploratory behaviours	Running, jumping, climbing, sniffing, stretching	Section 1.1, "Behavioural Biology"
Grooming	Self-grooming and allogrooming with cage mates Assess through observation of behaviour, or of coat condition	
Play behaviour	Object manipulation and rough-and-tumble play with cage mates and human handlers	Lampe et al., 2017
50-kHz vocalizations	Associated with rewarding contexts such as positive social interactions	Brudzynski, 2013 Wohr, 2018 Wohr and Schwarting, 2013

Table 4 Indicators That May be Useful for Assessing Welfare in Specific Contexts (Although Some Could be Adapted for Other Uses with Proper Assessment)

INDICATOR	COMMENTS	FURTHER INFORMATION
Facial grimace scale	Pain assessment, with validation for various applications	Leung et al., 2016 Oliver et al., 2014 Sotocinal et al., 2011 https://www.nc3rs.org.uk/rat-grimace-scale
Composite pain score	Pain assessment, validated for abdominal surgeries	Roughan and Flecknell, 2000, 2003, 2004, 2006
Burrowing task	Pain assessment, validated for inflammatory and neuropathic pain models	Andrews et al., 2012 Wodarski et al., 2016
Gait score	Pain assessment for lameness Potential for automated assessment	Gabriel et al., 2007 Lakes and Allen, 2016
Cornering behaviour	Observed in lactating females with pups, potentially avoidance-related	Gaskill and Pritchett-Corning, 2015

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GLOSSARY

Abnormal behaviours – actions performed by an animal that are not part of the behavioural repertoire of that species in the wild.

Affective state – refers to the mental state of an animal that leads to subjective experiences and physiological and behavioural changes in the body.

Analgesia – decrease in response to noxious stimuli.

Anesthesia – a state caused by an external agent leading to loss of sensation and motor function.

Barrier– a means of separating areas of an animal facility to reduce or minimize cross-contamination; barriers are commonly used to separate animals of different or unknown health statuses.

Bedding – material spread on the bottom of a cage, pen, stall, etc. for the purpose of providing comfort to the animals and keeping them dry; also referred to as substrate.

Cage components – temporary or permanent additions to an animal's enclosure that address its needs or enrich the environment.

Conspecifics – animals belonging to the same species.

Discomfort – a mild form of distress.

Distress – a state where the animal must devote substantial effort or resources to the adaptive response to challenges emanating from the environmental situation; it is associated with invasive or restrictive procedures conducted on an animal, or other conditions which significantly compromise the welfare of an animal, which may or may not be associated with pain.

Ear punch – the removal of a piece of an animal's ear (generally a notch or small hole), which can be used for identification, and the tissue that is removed can be used for genotyping.

Endpoint – predetermined criteria for intervening in a procedure to terminate, minimize, or reduce an animal's pain and/or distress, which takes into account the welfare of the animal (welfare endpoint) and the goal of the experiment (scientific endpoint).

Environmental enrichment – enhancements to an animal's environment that go beyond meeting its basic species-specific needs and further improve overall quality of life.

Experimental design – the process of planning a study to ensure the correct number of animals consistent with the scientific objectives, to use methods to reduce subjective bias, and to employ appropriate statistical analysis.

Genetically modified – a deliberate modification of the genome (the material responsible for inherited characteristics).

Genotyping – a process used to determine differences in the genetic makeup (genotype) of an individual animal by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.

Homeostasis – the process of internal regulation by which biological systems tend to maintain stability while adjusting to conditions that are optimal for survival.

Husbandry – all aspects of the care and management of animals in facilities: laboratory, farm, and aquatic (these guidelines do not include care of animals in the field).

Metabolic cage – individual housing for animals to permit the easy measurement of food and fluid intake and collection of urine and feces.

Pain – an aversive, sensory experience associated with actual or potential tissue damage.

Personal protective equipment – garments or equipment designed to protect personnel from injury, infection, or allergic reaction when working with animals; potential hazards include physical injury (bites, scratches, etc.), biohazards, and airborne particulate matter.

Phenotype – refers to the observable physical properties of an organism; these include the organism's appearance, development, and behaviour.

Play – voluntary interaction of animals with objects or other animals for purposes other than meeting their needs for survival or reproduction, which results in positive welfare.

Quarantine – confinement of animals which may carry an infectious disease, for a specified period to allow for evaluation.

Quality of life – the welfare of the animal throughout its entire lifespan.

Refinement – the modification of husbandry or experimental procedures to minimize pain and distress.

Sedatives – drugs which reduce an animal's agitation.

Sentinel animal – a specific pathogen-free (SPF) animal known to be susceptible to an infectious agent that is placed in the area suspected of being contaminated, for example in a new shipment of laboratory animals under quarantine; the sentinel animal is then tested for infection or development of antibodies to the infectious agent.

Specific pathogen-free – a designation used to describe the health status of animals for which a specific list of potentially infectious organisms have been tested for and not found.

Standard operating procedure – written document that describes in step-by-step detail how a procedure should be carried out.

Stereotypies – repetitive or unvarying behaviours that appear to have no purpose.

Stress – a state caused by factors external to an animal that displace homeostasis; stress can be beneficial (e.g., in triggering a flight response if the animal is threatened, thus helping it to cope with changes in its environment); however, prolonged stress can cause changes to an animal's endocrine system, leaving it less able to cope with its environment.

Three Rs – Replacement, Reduction and Refinement in animal-based science, as first explained by Russell and Burch in *Principles of Humane Experimental Technique* (1959).

Welfare – the physical health and mental well-being of the animal.