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Effects of a complex semi-natural cage system on Sprague Dawley rat welfare using behaviour, faecal glucocorticoid metabolites and selected organ weights as indicators.

By

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DECLARATION

I declare that this dissertation has not previously been submitted for consideration at this University or any other academic institution of learning, and that it is the result of my own research, except where the contributions of others are acknowledged.

Richard Mavunganidze

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ABSTRACT

The primary objective of using animals for research is to collect reliable data that is reproducible and translatable to the intended species. Sometimes, it is difficult to obtain reliable data because the animals are exposed to stress from experimental manipulation, or from housing environments that may not meet their species-specific requirements. This issue is especially important in rodents, which are housed in cages that are structurally different from their natural environments as most rodent housing systems are designed on economic and ergonomic factors with little consideration to the environmental needs of animals. One approach towards improving rodent housing environments is to include environmental enrichment. One idea of enriching laboratory rat cages is adding physical structures to their enclosures to create complex environments that mimic their natural habitats. For this study, we designed a complex caging system (semi-natural cages) furnished with different types of enrichment items together with increased cage space to determine if this could promote species-specific behaviour as a means of reducing stress and improving laboratory animal welfare. The study utilised previously described methods for welfare monitoring in animals namely change in home cage behaviour, monitoring of faecal glucocorticoid metabolites and monitoring changes in body weights and selected organ weights. Twenty-four female Sprague-Dawley rats were randomly allocated to either semi-natural or standard cages (each cage housing four rats), and evaluated weekly for six weeks. Behaviour data was collected via date-stamped video footage that was randomly scored using scanning and focal methods. A competitive enzyme immunoassay was used to determine the faecal glucocorticoid metabolite concentrations. The results show that animals in the semi-natural cage expressed normal rat behaviour, showed increased natural locomotory activity and were leaner than those in standard cages; characteristics that define healthier animals with improved welfare. An unexpected finding in the study was elevated faecal steroid concentration in the animals in the semi-natural cages, which will require further investigation. Basing on the outcomes of this study, we recommend semi-natural cage housing when room space and the study design allow for their use.

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LIST OF ABBREVIATIONS

AEC: University of Pretoria Animal Ethics committee

ANOVA: One-way Analysis of Variance

EE: Environmental Enrichment

EIA: Enzyme Immunoassay

FCM: Faecal Corticosterone Metabolites

HPA: Hypothalamic-pituitary-adrenal Axis

IVC: Individually ventilated cages

LAS: Laboratory Animal Science

OVARU: Onderstepoort Veterinary Animal Research Unit

PVC: Polyvinyl Chloride

REC: Faculty of Veterinary Science Research Ethics Committee, University of Pretoria

SAM: Sympathetic Adreno-medullary

SAVP: South African Vaccine Producers

SPSS: Statistical Product and Services Solution

Chapter 1: INTRODUCTION

1.1 Background

Animal research frequently has an impact on animal welfare, either because animals are intentionally subjected to stress, during experimental and husbandry manipulation, or because the animals are subjected to housing conditions of animal facilities that are different from their natural habitats. Animal housing is crucial in maintaining the quality of laboratory animal research, because animals are restricted to these environments. Unfortunately, most laboratory animal housing systems are limiting in the sense that animals are not free to perform species-specific behaviours due to structural designs and enclosures that differ from their natural habitats, (Sherwin, 2004; Weary and Robbins, 2019) with space being the most important limiting factor in most laboratory animal facilities, as housing systems need to be economic and ergonomic due to overall costs associated with housing viz. space needs to be adequately managed. Not surprising, sacrifices have to be made, with the result that little consideration goes into the environmental needs of research animals. The latter is especially important in rodents, as they are housed in cages that are structurally different from their natural environments. In their natural environments as an example, rats live in large social groups with well-established dominance hierarchies in areas with plenty of ground cover (Boice, 1977).

Over the years much work has been done to improve housing conditions for laboratory rodents (Olsson and Dahlborn, 2002; Makowska and Weary, 2016; Makowska *et al*., 2019), with emphasis towards standardising experiments and not necessarily to improve the welfare of the animals. Nonetheless, these studies recognized potential welfare problems associated with behavioural restriction in conventional (standard) laboratory rodent cages and recommended improvements to housing environments and further studies to assess effects of cage modifications. According to the studies, ideal housing for rodents should provide them with structures to hide and explore, material to build nests, and should enable social contact.

Housing environments that deprive animals of their natural behaviour, negatively affects their emotional state (Boissy *et al*., 2007), and prolonged exposures to such environments can lead to compromised animal welfare (Beausoleil and Mellor, 2015; Mellor, 2016). Animals in poor welfare states have altered psychological and physiological status and are likely to influence research data by for example falsifying animal behaviour and altering physiological parameters such as glucocorticoid and glucose levels. The issue is also a major ethical

concern, because falsified experimental results are not reproducible and not translatable, waste animal lives and impede progress in the fields of animal research, while all along wasting research funds (Kappel *et al*., 2017). Poor animal welfare may in some instances emotionally affect animal facility staff who want to promote a culture of care and may in extreme cases evolve into a psychosocial work hazard.

One approach towards improving laboratory rodent housing environments is environmental enrichment, which in simpler terms means modifications to their environment that result in significant welfare improvement. Environmental enrichment encompasses structural modifications to supply animals with complex environments that meet their species-specific needs while also giving animals some control over their surroundings; social housing that can stimulate numerous positive effects on the animals; and occupational modifications such as positive reinforcement to stimulate problem-solving, motor skills, and coordination on the part of the animal. Despite its widespread acceptance by many regulators and guidelines, the concept of 'environmental enrichment' has presented challenges to animal research in several ways. Its implementation lacks standardisation because the concept is applied differently and sometimes loosely by different groups of scientists (Hessler, 1999). To neuroscientists, environmental enrichment is considered as an experimental variable, whose effects are studied on the progression of diseases, neurodevelopment, and brain activity (Ratuski and Weary, 2022). Animal welfare scientists and other researchers outside neuroscience, conceptualise enrichment as modifications intended to enhance animal welfare (Olsson and Dahlborn, 2002). Also, most enrichment studies focus on adding modifications to animal enclosures without enlarging them, sometimes reducing the cage space available for the animals (Patterson-Kane, 2002), forgetting that cage space is an essential factor in the wellbeing of animals and an important confounding variable of animal research. Again, the guidelines for the environmental enrichment of laboratory rat cages are different to those of pet rat cages (Neville, Hunter, *et al*., 2022). An important follow-up argument is why welfare guidelines for the conspecifics should differ depending on animal use or the purpose for which they are kept. It is not surprising that the differences could only be due to practical considerations like maximising space utilisation (animal space economics) and ensuring that the animals are easy to check.

Since environmental enrichment can in some instances cause harm to animals, welfare indicators should always be measured, when implementing environmental enrichment. For the current study, we compared the home cage behaviour, growth parameters and faecal

corticosterone metabolite (FCM) measurements as welfare indicators of rats housed in cages enriched with semi-natural environments to their counterparts in standard cages commonly used in rodent facilities. The standard cages were polycarbonate cages with bedding, nesting material and a shelter. The semi-natural enriched cages were more complex caging systems furnished with different types of enrichment items and increased cage space.

1.2 Study aim

To compare the indicators of welfare and stress in rats housed in groups in semi-natural enriched cages to a separate group housed in standard cages.

1.3 Objectives

The study objectives were as follows:

1. To compare the cage behaviour of rats housed in standard cages and semi-natural cages.
2. To compare FCM measurements of rats housed in standard cages and semi-natural cages.
3. To compare animal weight and selected organ weights of rats housed in standard cages and semi-natural cages.

1.4 Benefits that arose from the study

The following benefits arose from the study:

1. Addition of literature on the enrichment and housing of rats.

1.5 Hypothesis

Animals housed in semi-natural cages will not show differences in cage behaviour, growth parameters and FCM measurements when compared to their counterparts in standard cages.

Chapter 2: LITERATURE REVIEW

2.1 The laboratory rat

Rats (*Rattus norvegicus*) are among the most widely utilised small research animals. They are second only to mice and zebrafish in terms of numbers (Canadian Council on Animal Care, 2018). Relatively short life cycles and gestation periods, docile demeanour, and easy accessibility of strains and stocks with known health statuses and genetic make-ups are some of the attributes that make them excellent research models. The availability of technologies to manipulate their genotypes to form a wide range of animal subtypes with various diseases or conditions also gives rats an edge over other species as research candidates. Not surprising rats have been used in behavioural, neurological, nutritional, and endocrinology studies for many decades (Blanchard *et al*., 2001). They are also standard model species for toxicological, teratological, and carcinogenesis testing (Flatland *et al*., 2018). Their size and availability of substantial literature on their biology makes them an ideal animal model for surgery.

Despite rats being adopted for research and even undergoing genetic modification, rats remain nocturnal animals with a well-defined biological clock (Fox *et al*., 2015). They remain a social species, which is linked to the natural habitat where they live in social groups of up to hundreds of animals with defined dominance hierarchies (Hurst *et al*., 1996). Rats are also nest builders within burrows which they dig (Manser *et al*., 1998). As a result, housing systems that facilitate performance of these behaviours have been recommended in laboratory environments to fulfil the rats' behavioural need (Makowska and Weary, 2016). In the absence of the said activities abnormalities can result, as seen in young rats deprived of play time at critical ages which develop abnormal sexual and social behaviours (Vanderschuren *et al*., 1997). Generally, there are two types of rodent housing systems with the first being research housing and the other domestic housing. Besides being tools for scientific enquiry, some laboratory rat strains such as Sprague Dawley and Long-Evans rats are often kept as pets, to which they readily adapt being sentient individuals. In these homes, the rats are typically housed in complex caging systems furnished with different types of enrichment items and perform behaviour such as burrowing, foraging and exploration, as is normally observed in their natural habitat. Ideally the pet rat cages are enriched with multiple tiers/levels, digging opportunities, multiple feeding and drinking points, opportunities to exercise, refuge areas, sufficient horizontal and vertical space and suitable bedding and

nesting substrates (Neville, Hunter, *et al*., 2022). In contrast, research rats are housed in simple polycarbonate shoebox-shaped cages with or without limited environmental enrichment items such as one or two polyvinyl chloride (PVC) tunnels and nesting material, and the environments are not as complex as in the house pet cages. Since rats are naturally prey species, they require complex environments to for example escape and hide from potential predator encounters. They rarely get these opportunities from standard laboratory cages; hence, it is not astonishing to find laboratory-housed rats with signs of impaired welfare. Interestingly, while many would argue that pet and research rats show different behaviour based on their breeding and genetic backgrounds, this is not the case. This has been demonstrated with laboratory (Albino) rats being shown to exhibit similar species-specific behaviour when placed in similar complex environments as pet rats, (Modlinska *et al*., 2015), albeit with different quality, thereby indicating that laboratory rats have retained some of their wild characteristics, resulting in discussion on the need for enrichment for rats housed under laboratory settings. Understanding the influences of environmental enrichment on the wellbeing of animals requires some background knowledge of the different concepts of animal welfare and a few of them are discussed below.

2.2 Animal welfare concepts

Animal welfare is a complex concept influenced largely by personal values and ethics. Whilst people agree that providing animals with good welfare is the correct thing to do, debates begin when it comes to welfare assessment. The reason is that scientists hold multiple notions of animal welfare when it comes to its assessment (Robbins *et al*., 2018), and focusing on one concept rather than the other can lead to different conclusions about animal wellbeing (Weary and Robbins, 2019). Of the multiple concepts available, a few are widely accepted by scientists. The first one is a popular theory stating that animal welfare is based on the subjective experiences of the animal as dictated by the person doing the welfare assessment (Balcombe, 2009). According to this ideology known as welfare hedonism, good wellbeing should consist solely of the presence of pleasure and the absence of pain experienced by an individual. It is assumed that, when an animal experiences negative emotions, its welfare diminishes but when it experiences positive emotions, its welfare is enhanced (Weary and Robbins, 2019). Another popular theory by Fraser and co-workers (1997) states that ideal animal welfare should lie at the intersection of three ethical concerns commonly raised regarding the welfare of animals referred to as the three domains namely affective states, biological functioning and natural living as displayed in figure 1.

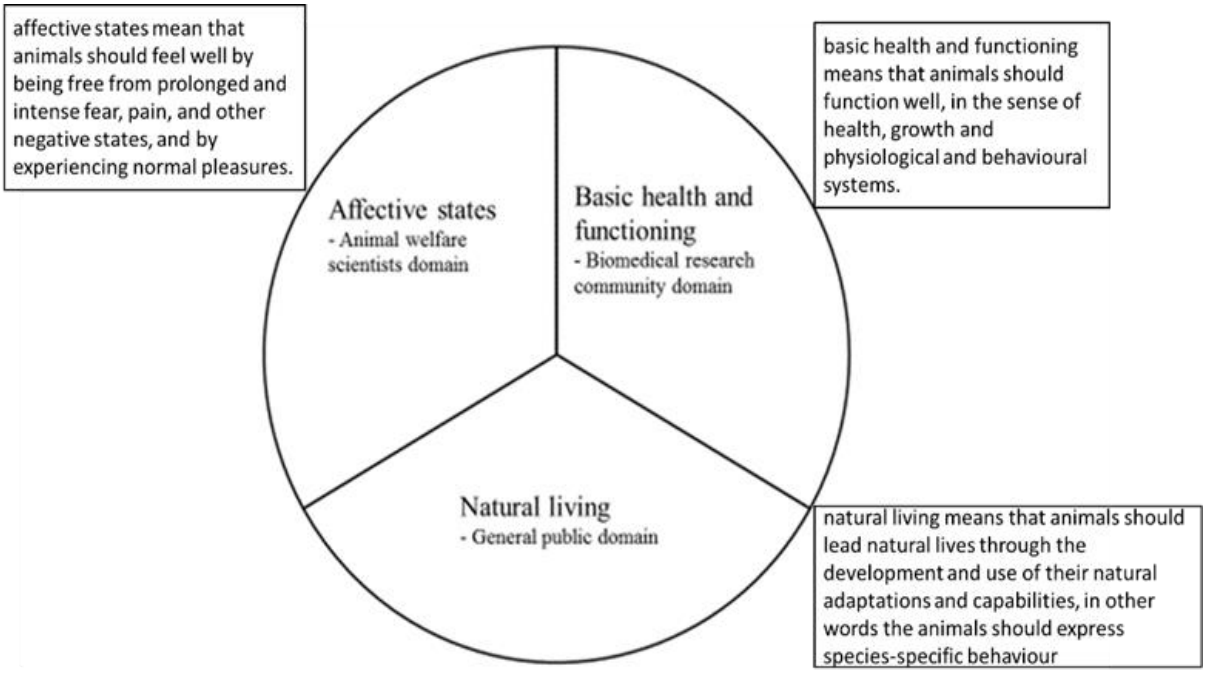

Figure 1. Animal welfare conceptual framework shows the three domains of animal welfare namely affective states (being free from fear, distress, and other negative emotions), biological functioning (optimal health, growth and physiological functioning), and natural living (animals should be free to express species-specific behaviour) (Fraser *et al.*, 1997).

However, different stakeholders give priority to different domains during welfare assessment. The biomedical research community place emphasis on biological functioning (Martin *et al*., 2010), whilst animal welfare scientists are polarised towards the animals' affective states (Robbins *et al*., 2018), and the general public prioritizes the natural living of the animals (Schuppli *et al*., 2014). The three domains are however interrelated in that the affective states of an animal are products of the biological functioning of that animal and may cause the animal to exhibit stereotypic behaviours (Hemsworth *et al*., 2015; Makowska and Weary, 2020).

2.2.1 The stress perspective

Rodents have developed a set of physiological and behavioural strategies collectively called the stress response that enables them to deal with stressors in their natural environments. During the stress response process, endogenous mediators from the body lead to the physiologic and behavioural outcomes depending on the magnitude and duration of the stressor (Rowland and Toth, 2019). Of the outcomes, the behavioural changes are the first to

appear and are the most significant (Blanchard *et al.*, 2001). Behavioural strategies include the ultrasound and olfactory communications between animals (Akyazi and Eraslan, 2014), as well as the escape and hiding episodes into burrows and shelters (Makowska and Weary, 2016). Studies have also revealed the transmission of stress between stressed and unstressed rats housed together (Gordon, 1990, 2012; Akyazi and Eraslan, 2014; Maloney *et al.*, 2014). According to the studies, ultrasound noises and olfactory cues from stressed animals are perceived by conspecifics that in turn exhibit necessary behavioural responses such as escaping and hiding. Housing environments that deprive rats from expressing these behavioural responses are likely to exacerbate the stress situation whilst those that allow the rats to express the behaviours ameliorate the situation.

2.2.2 The Good Life concept

Animals are said to have a "good life" if their standard of living is above the legal requirements for good welfare and the animals are experiencing positive emotions such as pleasure (Edgar *et al.*, 2013). The concept requires the animals to be healthy and to have what they want (desire fulfilment), to express their natural behavioural repertoire (natural living), and to actively engage with their surroundings (pleasant mental states). Even though the concept looks straightforward, problems arise in trying to quantify the indicators of good life because animals experience some realms of pleasure outside of human knowledge so applying human definitions of pleasure is limiting. Some behavioural scientists advocate for allowing the animals to live a natural life, in order to afford them a good life (Weary and Robbins, 2019; Makowska and Weary, 2020), a task that is difficult with laboratory animal housing systems. Behaviours associated with natural environments such as social interaction, reproductive activity, play, self-grooming, and exploration may then provide evidence of positive emotional experiences, and animal pleasure.

2.3 Welfare indicators for laboratory animal housing

Although the concepts of animal welfare differ, they have a common goal of ensuring that the wellbeing of animals is good; a goal realizable if the idea behind the different concepts is translated into practical perspectives. Animals in good welfare states should be free from stress, lead natural lives and function well in terms of physiological and behavioural systems, as indicated by valid welfare assessment tools. One of the initial tasks in attempts to quantify the wellbeing of animals is defining the reason for the welfare assessment. Possible reasons applicable to laboratory rodents include, routine day-to-day welfare monitoring, assessing

how animal welfare changes after environmental manipulation, assessing preferences or strength of motivation for specific resources, or assessing an animal specifically for pain after a research procedure. The next step is selecting the assessment tools suitable for each situation and setting values of normality (Dawkins, 2004). There is no single assessment tool applicable to assess welfare on its own, rather a collection of behavioural, biochemical, and physiological measures is used. The challenge with using this plethora of measures is integrating them to provide an accurate picture of an animal's welfare and deciding, which measures to prioritize over others, because some measures are more reliable or better than others are. The measures chosen should be practical, robust and adequate for capturing all the facets of animal welfare (mental and physical) (Beausoleil and Mellor, 2015).

2.3.1 Changes in behaviour

A comprehensive measure of animal wellbeing based on behaviour can be obtained by qualitative behaviour assessment, using an ethogram. Animal activities are tracked over time, and each spectrum of behaviour can be classified into predefined activities using the ethogram. The animals are observed in their natural environment without being moved or handled, as this can cause stress. An animal experiencing negative affective states will behave differently from the one unstressed or experiencing positive affective states (Bateson, 1991; Lawrence *et al*., 2019). Understanding the species' normal behavioural repertoire is essential for distinguishing normal from abnormal behaviour. The animals' moods at the time of observations can affect behaviour assessment, for example, an animal experiencing a negative emotional state, may be observed as displaying behaviour typical of an animal enjoying a positive emotional state if the behaviour assessments are confounded by mood changing stimuli. Other behavioural observation methods such as the open field and preference tests and their modifications can be used to assess the wellbeing of laboratory rodents. They test the animals' exploratory behaviour (Gob *et al.*, 1987) and environmental preferences (Blom *et al*., 1995). The principle behind these tests is that stressed and unstressed animals will react or respond differently to the tests. The emergence test and elevated maze test are two other observation tests, evaluating the animals' exploratory behaviour and environmental preferences (Krohn *et al*., 2001).

2.3.2 Faecal hormone metabolite measurements

Rodents respond to stressors by increasing their glucocorticoid levels via the hypothalamic-pituitary-adrenal Axis (HPA) and, the hormone can be used to predict their well-being

(Sheriff *et al*., 2010). Corticosterone is the primary glucocorticoid in rats and can be sampled from blood, urine, saliva, integumentary structures and faeces. Corticosterone is essential for the mobilization of stored energy as the animal prepares to escape or react to a perceived stressor or threat. Measurement of corticosterone levels, especially from plasma as a stress indicator has some drawbacks (Peckett *et al*., 2011). The blood sampling procedure is the major cause. Manual restraint and the presence of personnel around the animal during blood sampling may result in higher mean plasma corticosterone concentrations than values obtained from undisturbed animals (Burke *et al*., 2000). The same is true for saliva samples, and these animal handling stress induced elevations are due to readily available corticosterone sources rather than *de novo* hormone synthesis and secretion.

The liver metabolizes glucocorticoids, and the metabolites are excreted in urine and faeces where they can therefore be measured. This technique of measuring glucocorticoid metabolite in excreta has become a popular non-invasive tool for hormonal analysis because sampling is simple and does not involve animal separation or handling. The technique avoids disrupting natural animal behaviour and stressing the animal during sample collection. The method uses a small faecal sample, with 1g being adequate in most cases (Palme *et al*., 2013). Unlike plasma samples, which represent a snapshot of circulating hormones at the time of sampling, faecal samples contain a build-up of metabolized hormones over time (Sheriff *et al*., 2010). Even though the extent of the elevations of faecal metabolites is much lower compared to the corresponding steroid plasma concentrations, the method is sufficiently sensitive to detect stressor-induced changes. (Bamberg *et al*., 2001).

2.3.3 Physical Health and Performance

An overview of the wellbeing of animals can be determined from their health status and performance. This statement is based on the assertion that a physically healthy animal is perceived to be enjoying good welfare. Parameters namely body condition score and animal weight and weights of internal organs such as thymus and adrenal glands are useful indicators of animal welfare, as they react to chronic exposures to unpleasant environments (Spangenberg et al., 2005; Konkle et al., 2010). Normally stressed animals will lose weight or show signs of delayed weight gain or both. Prolonged exposures to stressful conditions or unpleasant environments cause adrenal hypertrophy and increased weight of the adrenal glands (Marashi *et al*., 2003) and reduced thymus gland weight (Reber *et al*., 2007). However, the use of physical health and growth parameters as a welfare measure does not

provide evidence about mental well-being of animals and one cannot conclude that the animals are experiencing positive affective states from such data. Despite these caveats, the parameters find application in welfare assessment as adjuncts to other methods.

2.3.4 Other methods of welfare assessment

- Another promising avenue of studying rodent wellbeing is analysis of vocalizations as a welfare assessment tool (Hinchcliffe *et al*., 2022). Animal vocal expressions are physiologically linked to their emotions and their interpretation can determine how well animals are coping with their surroundings. An example is the 22 kHz ultrasonic alarm call in rats associated with negative experiences (Litvin *et al.,* 2007). Increased recognition of the importance of positive animal emotional state as an indicator of good welfare, has led to more research on vocalizations that imply positive emotions. The most popular and extensively researched of these is the 50 kHz call in rats associated with positive affective states such as play behaviour (Panksepp and Burgdorf, 2000), food consumption and mating (Wöhr *et al*., 2008).
- Observations of the cage with or without animals may indirectly give evidence of the animals' welfare. For instance, evidence of unconsumed food, diarrhoeic faeces, haemorrhage or other secretions inside the cage enclosure may indicate something abnormal about the animals and should prompt further examination of the animals to look for signs that point to the affected individual (Krohn et al., 2001).
- New technologies like thermal imaging or infrared thermography are becoming popular as tools to remotely monitor animal wellbeing (Pereira *et al*., 2018). They have found application in the measurement of respiratory rate and heart rate, and monitoring of thermoregulation, circulation and perfusion dynamics as well as observing behaviour in research animals. The technology records radiation naturally emitted from the body and converts it to a temperature map, or to display images of temperature distribution (Pereira *et al.*, 2014). The thermal images are reproduced in colour to reveal the surface heat transfer and blood flow (Pereira *et al*., 2018). Unlike other imaging technologies standardizing the positioning of the camera (angle and distance) is necessary and is sometimes problematic because the animals cannot be restrained.

Technology companies have developed novel computer software such as the artificial intelligence software with the capability to unobtrusively and non-invasively record animals in terms of their location, position and behaviour (Congdon *et al.,* 2022). This computer technology is applicable to different branches of animal research such as animal

welfare science and comparative evolution and cognition as a tool to record behavioural data. The technology provides surveillance of animals wild or domestic regarding their location and species.

2.4 Ways of improving animal welfare

Improving animal welfare requires efforts from all relevant stakeholders involved in animal use and care, from caretakers to the management of animal facilities. Personnel responsible for the care and scientific use of animals should easily raise any concerns on expectations around management of animal welfare. Competent authorities can also become involved through improving the animal welfare regulatory framework (Rault *et al.*, 2022)

Improving animal welfare follows a stepwise process that includes recognising that bad animal welfare is occurring, followed by identifying the likely source of bad welfare and then taking some remedial actions to improve welfare. The remedial actions often include removal of the cause, refinement of procedures, administration of medication or control of extraneous variables, depending on the identified cause of bad welfare (Olsson and Dahlborn, 2002). Remedial actions are usually followed by a re-assessment of the animals' welfare status to ascertain if they were successful in improving animal welfare. Environmental enrichment is also an important component of refinement, which, in addition to improving animal welfare, has the added benefit of making animals easier to handle (Hutchinson *et al.*, 2005).

2.5 Enrichment in Laboratory Housing

2.5.1 Laboratory housing systems as enrichment.

The history of the use of laboratory rodent caging systems date back to the 1920s when rats were housed in glass containers as well as wooden or metal cages designed by individual researchers (Henrique Franco, 2013). Back then, there were no standard requirements for cage dimensions and individual researchers designed cages to suit their situational needs. Several changes and modifications have been made to the rodent caging system before finally arriving at the commonly used commercial polycarbonate shoebox cages in 1953 (Hessler, 1999), which have become the industry standard. According to current animal care guidelines of various countries, polycarbonate cages housing two or three rats must be at least 180-200 mm in height and 82 500-92 000 mm^2 as floor area (Wheeler *et al.*, 2015). However, the height sometimes restricts adult rats from making bipedal stances as they explore the cage environment (Makowska and Weary, 2016). It is important to note that current cages were

designed based more on the traditional considerations viz. existing practices, professional judgment, hygiene, economics and ergonomics rather than animal welfare considerations (Makowska *et al*., 2019). Of the different factors important in the cage's design, the size of the enclosure, an important factor in how well a cage accommodates the animal's welfare, has not received sufficient attention. As a result, laboratory rats have access to smaller space compared with their free-living counterparts. Some present-day researchers and animal welfare stakeholders advocate for the addition of enrichment structures into the cages to substitute space related behaviour with other positive behaviour that could result from interaction with said enrichment structures. The traditional methods recommended for laboratory rodent environmental enrichment include social housing, providing foraging opportunities, nesting material, shelters and other structural items, such as climbing platforms, as well as providing pelleted feed and play objects (table 1). Generally, the enrichment methods can be classified into structural, social and occupational enrichments depending on the incentives provided to the animals to improve their lives (Newberry, 1995; Olsson and Dahlborn, 2002).

- **Structural enrichments**: Structural enrichments include any physical objects and parameters of the animal's enclosure, which make-up the microenvironment of the animal. For rodents the structures include the cage, feed and water systems, bedding and nesting materials, running wheels and other toys and hiding shelters or tunnels (Newberry, 1995). There are debates over the use of play objects with others saying they are non-beneficial as rats have not been observed playing with the objects (Fox *et al*., 2015). The structural enrichment items should be appropriate for the species, should not endanger the animals, and should not interfere with the caretakers' daily routines such as experimental procedures, observations, and cage cleaning.
- **Social enrichments**: This category refers to group housing of social species. Its benefits have been described and cannot be overstated because the evidence is clear that single housing has numerous negative effects on social species while social housing, presents positive effects on them (Krohn *et al*., 2011; Krügel *et al*., 2014) . Social housing should be managed properly by grouping compatible conspecifics and closely monitoring the grouped animals for compatibility. In situations where physical contact is not possible, at least visual and olfactory contact can be maintained between the animals. Group-housed rats are often observed laying together or grooming conspecifics, and such rats show

reduced to no stereotypic behaviours (Hurst *et al*., 1999), which often develop when essential needs of an animal have not been fulfilled.

- **Occupational enrichment**: Occupational enrichment although not common in rodent housing stimulates problem solving, motor skills, and coordination on the part of the animal, and examples include puzzle feeders or positive reinforcement. Positive reinforcement is common in animal research and involves interaction of the animals and the caretakers (Marashi *et al*., 2003).

2.5.2 Debates around the use of environmental enrichment.

It is now well accepted that enriching the laboratory animal cages prevents stereotypic behaviour and reduces stress levels in animals, resulting in better research subjects, although some scientists still argue that enrichments disrupt standardisation, and reduce the replicability of animal experiments. Regardless, most animal use and care guidelines and some regulatory authorities have recognised the value of environmental enrichment and adopted the concept (National Research Council, 2011). According to the guidelines, animals held in enclosures should be cared for in a way that promotes their health and natural behaviour (Spangenberg *et al.,* 2005). Despite this widespread acceptance, the term "environmental enrichment" is still a misnomer in several ways. Firstly, rather than standard baseline requirements, the term is used to describe optional extra provisions offered to the animals, and these extra provisions are not standardised. As an example, some scientists regard addition of nesting material as enrichment (Kulesskaya *et al.*, 2011; Sheba R *et al*., 2016), while others regard it as a baseline cage requirement to standard cages (Latham and Mason, 2010), or as their conventional or non-enriched condition (Khoo *et al*., 2020; Ratuski and Weary, 2022). Secondly, different groups of animal users apply the term differently (Rasyidah Ismail *et al*., 2021). The term is sometimes used for any changes made available to the husbandry of animals, without considering the benefits of the changes to the animals (Olsson and Dahlborn, 2002). Such use of the term makes it meaningless, and promotes the provision of enrichments that do not benefit the animals.

Table 1; Summary of commonly recommended (beneficial) rodent environmental enrichment (EE) listing their goals, requirements and associated risks. Additional requirements refer to actions to consider to make the EE effective. Risks of EE refer to potential adverse effects that may arise with the use of the EE and/or situations that make the EE ineffective, both causing the EE to become non-beneficial (Ratuski and Weary, 2022).

Goals of EE	**Recommended EE**	**Risks of EE**	**Additional Requirements**
Enabling animals to express species-specific behaviours.	-Increasing cage height and play space. -Nesting material -Foraging opportunity -Social housing -Addition of climbing structures. -Addition of shelters	-Wasting resources and time on unnecessary enrichments not needed by the animals. -Risk of unacceptable increases in variability. -Potential for injury to animals. -EE may have a negative impact on the health or safety of animals. EE may result in significant additional work for personnel and become costly.	-Personnel should be knowledgeable about rodent behaviour. -EE should satisfy sanitary requirements. -Animal inspection should not be hampered by EE.
Improving psychological well-being by creating conditions "that will improve the welfare of the animals and reduce boredom".	-Addition of gnawing items. -Social housing -Pelleted diet	-May create aggression encounters.	
Promoting typical brain functioning.	-Regularly rotating EE for novelty.	-Concerns about variability.	-The accessibility of resources

		-The benefits of EE may depend on species, age, or gender.	-Practicality in terms of for example space availability.
Creating opportunities for rewarding behaviours.	-Cognitive training	-Enrichments may interfere with experimental design or outcome -The benefits of EE may depend on species, age, or gender	-Some enrichments require qualified personnel or adequate availability of space to implement
Making the lives of animals more enjoyable and interesting	-Background music to muffle the effects of sudden noise -incorporating a shelf within the cage	-Researchers' reluctance -Expenses	
Giving animals a degree of choice of activities and some control over their surroundings	-Increasing cage height and play space space -Nesting material -Foraging opportunity -Addition of climbing structures -Addition of shelters	-Wasting resources and time on unnecessary enrichments not needed by the animals -risk of unacceptable increases in variability -Potential for injury to animals. -EE may negatively impact health or safety of animals. -some EE may increase personnel workloads and become expensive.	-Personnel should be knowledgeable about rodent behavior. -EE should satisfy sanitary requirements. -Animal inspection should not be hampered by EE.
Correcting behavioural problems such as stereotypies, aggression and over-grooming	Foraging opportunities: -Food particles in the bedding - combining food and other substrates in a dish	-Laboratory cages are restrictive in size	-EE must be species appropriate

	-Providing access to whole food pellets or different types of food -incorporating multi levels within the cage -Nesting material -Addition of climbing structures -Addition of shelters		
Improving animal health or biological functioning and increasing the animal's ability to cope with stress of captivity	-Access to running wheels -Addition of shelters -Nesting material -Foraging opportunities -addition of climbing structures	-Risk of reducing available cage space -EE may jeopardize the health and safety of animals.	-To draw animal welfare conclusions, preference studies must be combined with motivational strength studies. -Personnel should be knowledgeable about rodent behaviour.

2.5.3 Shortcomings of Enrichment

Some enrichments have proven to be of no benefit or sometimes harmful or stressful to the animals, as evidenced by the provision of mouse shelters. Research has revealed that provision of shelters to males of some mouse strains result in increased aggressive behaviour (Haemisch *et al*., 1994). Given these risks, Würbell and Garner (2007) have proposed a classification of environmental enrichment into non-beneficial or pseudo-enrichment, conditionally beneficial enrichment, and beneficial enrichment. The following are some examples to explain the classification:

- **Pseudo-enrichment**: Refers to enrichment that does not benefit the animal in anyway. While it can be argued that toys and devices allow for better interactions with the animal's environment, consideration also needs to be given to the physical nature of the object in use. Some environmental enrichment items being space-occupying objects, when added into cages can render their beneficial effects useless as it can further reduce cage space, and affect the ambient temperature of the cage microenvironment. Marbles also induce stress in mice and are commonly used as a stressor in mouse tests of anxiety (Würbell and Garner 2007).
- **Conditional beneficial enrichment**: Refers to enrichments that are beneficial to some animals or beneficial to animals under some circumstances. An example is provision of adequate ventilation to mice. Although it decreases ammonia levels, and benefits the animal's physical health, mice can find the airflow aversive (Newberry, 1995).
- **Beneficial enrichment**: Refers to enrichment items that result in animals with better states of welfare at all times. A good example is nesting material (Olsson and Dahlborn, 2002).

Thus, whether or not specific enrichment enhances the well-being of animals is an empirical question that requires proper behavioural assessment. As mentioned earlier space occupying enrichments can affect the ambient temperature of the cage microenvironment. The follow up question always asked is "at what room temperatures should rodents be housed, in enriched cages". It is difficult to answer this question because different physiological functions require different optimal environmental temperatures and some physiological responses occur only within specific temperature ranges. For example, the ideal ambient temperature for comfortable sleep is far too warm for strenuous exercise. The preferred ambient temperature for rats is 30 °C, which is above the standard temperature of 22 ±2 °C recommended by

various guidelines for standard housing systems (Hankenson *et al*., 2018). Studies have revealed that the cages are actually warmer and fall within the rats' preferred temperature ranges (Gordon, 1990, 2012; Maloney *et al*., 2014), and that adding enrichment structures to the cages translates to further increasing the micro environmental temperature in the cages, making it difficult to determine the ambient temperatures that are optimal for the animal models. Providing a temperature gradient in the rodent housing, and allowing the animals to select their preferred conditions and self-thermoregulate has been described as a shortcut to determining thermal ranges that benefits specific animal models and housing conditions, a task that has proved to be complicated (Gordon, 1990). Adopting the pet rat cage design can be used as a cost-effective way of providing this temperature gradient to rodent cages, because the cage has enough space for the animals to select their preferred ambient temperature. The cage system has adequate space to fit complex enrichment structures such as burrowing substrate, without compromising the microenvironment. Although the pet rat caging system has been documented to work well with pet rats in terms of animal welfare (Neville, Hunter, *et al*., 2022; Neville, Mounty, *et al*., 2022) its relevance to the laboratory environment needs to be investigated, because some enrichments have proved to be beneficial in some situations and non-beneficial or even harmful in others.

Chapter 3: MATERIALS AND METHODS.

3.1 Animal ethics statement

The study was approved by both the University of Pretoria Faculty of Veterinary Science Research Ethics (REC) and University of Pretoria Animal Ethics (AEC) committees (Protocol Number REC022-20).

3.2 General animal housing and husbandry

Twenty-four female Sprague-Dawley rats aged 12 weeks at the time of arrival, were used for this investigation. The number of animals was determined from previous published work in the same field (Abou-Ismail *et al.*, 2010) and G-power calculations. The rats were sourced from South African Vaccine Producers (SAVP) and housed at Onderstepoort Veterinary Animal Research Unit (OVARU) under controlled temperature (22 ± 2ºC), humidity (45 ± 20%), and light/dark (12hr/12hr) cycle. During the acclimation period, the rats were pair housed in cages, similar to those used by the breeders (1500U Eurostandard type IV S) on wood shavings for bedding. Irrespective of the cage type, all rats were provided with *ad libitum* access to Epol rodent cubes (Epol®) (see addendum 1 of the feed composition) and reverse osmosis water, and were checked daily. Cage changing was conducted once every week.

3.3 Experimental treatments

After an acclimation period of fourteen days, the rats were randomly allocated to either semi-natural or standard cages (each cage housing four rats), and housed for six consecutive weeks:

i. The standard cages were 1290D eurostandard type III cages made of polycarbonate and measuring 425 x 266 x 155 mm (L x W x H) manufactured by Techniplast (Décines-Charpieu, France). Each cage contained wood shavings as bedding, one Polyvinyl chloride pipe, and two pieces of egg tray paper for enrichment.
i. The semi-natural cages were custom modified rabbit cages at OVARU made of galvanized wire bars, measuring 640 x 450 x 450 mm (L x W x H). The semi-natural cage was provided with burrowing substrate (black earth and compost), nesting material, two polyvinyl chloride pipes, one nest box, two climbing structures, gnawing objects, and enlarged the cage height and enclosure size; items that enable rodents to express behaviours such as burrowing, foraging, climbing, and exploration as is normally

observed in their natural habitats (figure 2). Only cage material and enrichment items that are cheap and readily available from local pet shops were considered. The larger size of the cage enclosure allowed for housing of larger social groups of up to eight rats meaning that 96 rats can be housed in twelve semi-natural cages on a single cage rack 1820 x 640 x 1620 mm (L x W x H) (Figure 3). The same number of rats cannot fit to two cage racks of 20 standard cages (1500U Eurostandard type IV S) 1300 x 450 x 1550 mm (L x W x H) that house two rats per cage.

Figure 2. Picture combo (A to C) of the semi-natural cage and enrichment items provided for the study. Picture A shows the size differences between the standard cage and the semi-natural cage. Pictures B and C show the various enrichment items provided, namely burrowing substrate, nesting material, two polyvinyl chloride pipes, a climbing structure a horizontal rope across the cage and gnawing objects.

Figure 3. The cage rack used to hold the semi-natural cages. Although the individual cages are large, they saved laboratory space by housing more rats in one cage, such that one cage rack of 12 cages can house 90 rats.

3.4 Data collection

3.4.1 Home cage observations

The rats were recorded for the eight weeks in their home cages using a digital video recorder (Hikvision DVR – Model:DS-9016HFI-S [Hikvision corporate solutions, Alberton, South Africa] and Samsung camera – Model: SHC721AP [Samsung Electronics, Johannesburg, South Africa]) to gather information about the behaviour they exhibited in the two types of cages. For behaviour scoring, eight video footages (observation sessions) of two hours' length after every one-hour interval for a complete 24-hour period were scored for the counts of selected behaviour using the scan and focal sampling methods (Martin and Bateson, 1993). Evaluations were carried out once weekly by the same person throughout the study. For both

sampling methods the observed behaviours were manually recorded onto the respective forms shown in addendum 3.

Scan sampling: Each footage (cage) was scanned for approximately 10 seconds once every ten minutes throughout each observation session (2 hours), and animals scored as active if they were observed moving around the cage, grooming, eating and other forms of activities) or inactive if they were observed lying or sitting in one place (Table 2 and addendum 4). Each observation session lasted 2 hours, and yielded 12 scans, thus a total of 96 scans were observed per cage per day (eight observation sessions) or per observation week.

Focal sampling: The video footages (cages) were continuously sampled for five minutes every fifteen minutes to record counts of behaviour of interest. Each cage was observed eight times every observation session (2 hours), and observed behaviour counts recorded manually onto the the forms (addendum 3) . A selection of behaviours of interest are shown in the ethogram in table 2 and addendum 4.

Table 2. Ethogram of behaviours scored, and their definitions

Behaviour	**Description**
Active behaviour	Refers to rats engaging in behaviours such as feeding and drinking, non-intake maintenance, movement activities, exploratory and enrichment directed behaviour, and social interactions.
Inactive behaviour	Refers to rats sleep or awake but non-active.
Intake behaviour	Rat feeding, eating faeces or anything in the cage such as bedding material and drinking water
Non-intake and exploratory behaviour	Rat self-grooming, and standing upright on two hind legs (stretching and yawning), moving and/or climbing the cage enclosure and sniffing.
Enrichment-directed behaviour	Rat climbing and manipulating the enrichment objects and bedding material. Also includes rat inside the enrichment structure
Social interaction	Rat engaging in allo-grooming and aggression behaviours, either on the giving or receiving side

Two-observation sessions of two hours length were randomly selected and used for inter- and intra-observer reliability testing to remove bias. For intra-observer reliability testing the footages were scored as described above by the primary observer for three times every other day and the consistency of the behaviour counts compared. For inter-observer reliability testing the observation sessions were observed and scored by two other selected observers and the results compared with that of the primary observer.

3.4.2 Faecal sample collection

Faecal samples were collected from the animals once every week for six weeks at roughly the same time (09:30hrs) in the morning. The samples were collected into labelled containers and frozen at -21 °C within 30 minutes of collection (to avoid hormone degradation) until hormone extraction. Individual animals were removed from their cages and briefly handled for 2-3 minutes to stimulate release of a faecal bolus.

3.4.3 Faecal corticosterone metabolite extraction

Faecal corticosterone metabolite extraction was done in the Endocrine Research Laboratory at the Mammal Research Institute, University of Pretoria according to a method developed by Palme and Möstl (1997). The frozen faecal samples were freeze dried for two days at -50 ºC and 0.96 mbar using a lyophilizer (CHRIST Alpha 1-2LD plus), and then ground with a pestle and mortar, and sifted through a mesh strainer to separate faecal powder and debris. A 0.10 g aliquot from each sample was transferred into a test tube with 3 ml of 80 % (v/v) ethanol. The mixture in each test tube was centrifuged at 2 500-x g for 10 min after being vortexed for 15 min. An aliquot of the supernatant (1.5 ml) was diluted (1:10) with assay buffer and transferred into a microcentrifuge tube and frozen at -21 ºC until the time for corticosterone metabolite determination.

3.4.4 Corticosterone metabolite determination

The concentration of corticosterone metabolites in the faeces was determined using a competitive enzyme immunoassay (EIA) method (Touma et al, 2003). Plates were coated with anti-rabbit-IgG raised against 5a-pregnane-3b,11b,21-triol-20-one coupled with bovine serum albumin. In short, samples (50 µl) were incubated in duplicate with 5a-pregnane-3b,11b,21-triol-20-one (100 µl) and antibody (100 µl) at 4 °C overnight. After incubation, plates were rinsed four times with Tween 20 90.02%) (Merck, Germany) and blotted dry. Subsequently 250 µl streptavidin horseradish peroxidase conjugate (¼ 4.2mU, Boehringer,

Germany) was added and plates were then left at 4 °C in the dark on stirring platform for 45 min. Hereafter, plates were washed with 250 µl tetramethylbenzidine (¼ 69.4 nmol/well; Fluka Austria) and incubated for an additional 45 min at 4 °C. The final enzymatic reaction was stopped by the addition of 50 µl/well sulphuric acid (2mol). Absorbance was measured at 450 nM (reference filter: 620 nm) on an automatic plate reader (DigiScan, Austria). The final hormone concentrations were determined by the following equation:

FCM [µg/g DW] = [20 × Amount of Ethanol (ml) × Dilution Factor × EIA Mean] / [Faecal Weight (g) × 1000000].

3.4.5 Animal weights

Rats were weighed weekly using a calibrated balance (Jadever scale model JWE-3K S/N W34911T0026). At the conclusion of the six weeks period, the animals were euthanized using an overdose of isoflurane. The carcasses were dissected and brain, thymus gland, spleen and adrenal gland collected and weighed using Soehnle Professional Scale 9437 S/N.

3.5 Statistical analysis

SPSS (version 28 for Windows) was used for all statistical analyses. To determine the suitability of parametric tests, the data set was checked for normality and variance homogeneity using the Shapiro-Wilk test. Data sets that did not meet the parametric test requirements were transformed; if the data still did not follow a normal distribution after transformation, equivalent non-parametric tests were used. The Mann-Whitney U test was used to determine the significance of the differences between observations. While differences over time within groups were not expected, a repeated measures ANOVA was used to evaluate data determining the duration and frequency of activities. The weights of selected organs were expressed as relative weights of body weight, and the significance of differences between rats from the two housing systems were determined using an independent t-test. The Kruskal-Wallis test was used to determine inter-observer reliability.

Chapter 4: RESULTS

The costs of the cage modifications and the enrichment items implemented (R990.00 per cage) were much lower compared to the price of standard cages (R1545.00 per cage). Again the cages were easy to sanitise, did not influence the physical health of the animals, and did not interfere with animal checks. Weight of the cages was the only disadvantage we noted with the semi-natural cages (comments from caregivers). The cages weighed 9kg, which made the cage changing process a challenging task requiring more hands compared to standard cages. The weight was specifically attributed to the type of steel bars used to make the cages.

4.1 Behavioural observations

While all observers recorded similar counts in most behaviours, there were a few instances where the second observer tended to underestimate the counts of intake behaviour in semi-natural cages and non-intake behaviours in the standard cages, although the differences in the counts were not statistically significant, $\chi^2(2) = 0.971$, $p = 0.615$ and $\chi^2(2) = 0.766$, $p = 0.682$ respectively. Mean rank counts for intake behaviour were 8.30 for the principal observer, 6.50 for the second observer and 9.20 for the third observer. Mean rank counts for social interaction behaviour were 8.50 for the principal observer, 6.60 for the second observer and 8.90 for the third observer. The third observer overestimated the counts of enrichment directed behaviour in the standard cage, but the differences in the counts were not statistically significant $\chi^2(2) = 3.558$, $p = 0.169$, with mean rank counts of 6.40 principal observer, 6.60 second observer and 11.00 the third observer. For all recordings used for interobserver reliability testing, the principal observer was always consistent with either all or at least one of the two observers, therefore we can generally conclude that there were consistencies amongst the observers.

Based on the subjective assessment of the principal observer, generally animals in semi-natural enriched cages were more active than in standard cages as shown in the simple bar graphs of weekly mean counts of behaviours observed in figures 4, 5 and 6. The bar graphs also show that the animals were most active during the dark period of the light/dark cycle and that the observations did not follow any trends within the group over time. These subjective observations were consistent with statistical analysis (table 3). Semi-natural cages recorded statistically significantly higher counts of enrichment directed ($U = 617$, $p < 0.001$) and non-intake ($U = 1908.5$, $p < 0,001$) behaviours which included bedding material manipulation,

self-grooming and cage exploratory behaviour than the standard cages. Although the counts of intake (U = 2776, p = 0.89) and social interaction (U = 2255, p = 0.034) behaviours were higher in standard cages than semi-natural cages, the differences were not statistically significant. Social interaction behaviours included activities such as allogrooming given and received, aggression and dominance-oriented bullying.

Table 3. Summary of behavioural data mean counts and test statistics

Behaviour	**Cage type**	**Mean counts**	***U* statistic**	***P* value**
Enrichment-directed	Semi-natural cage	104.77	617.5	<.001
	Standard cage	46.23		
Intake	Standard cage	75.99	2776	.890
	Semi-natural cage	75.01		
Non-intake activity	Semi-natural cage	87.55	1908.5	<.001
	Standard cage	63.45		
Social interaction	Standard cage	82.93	2255.5	.034
	Semi-natural cage	68.07		

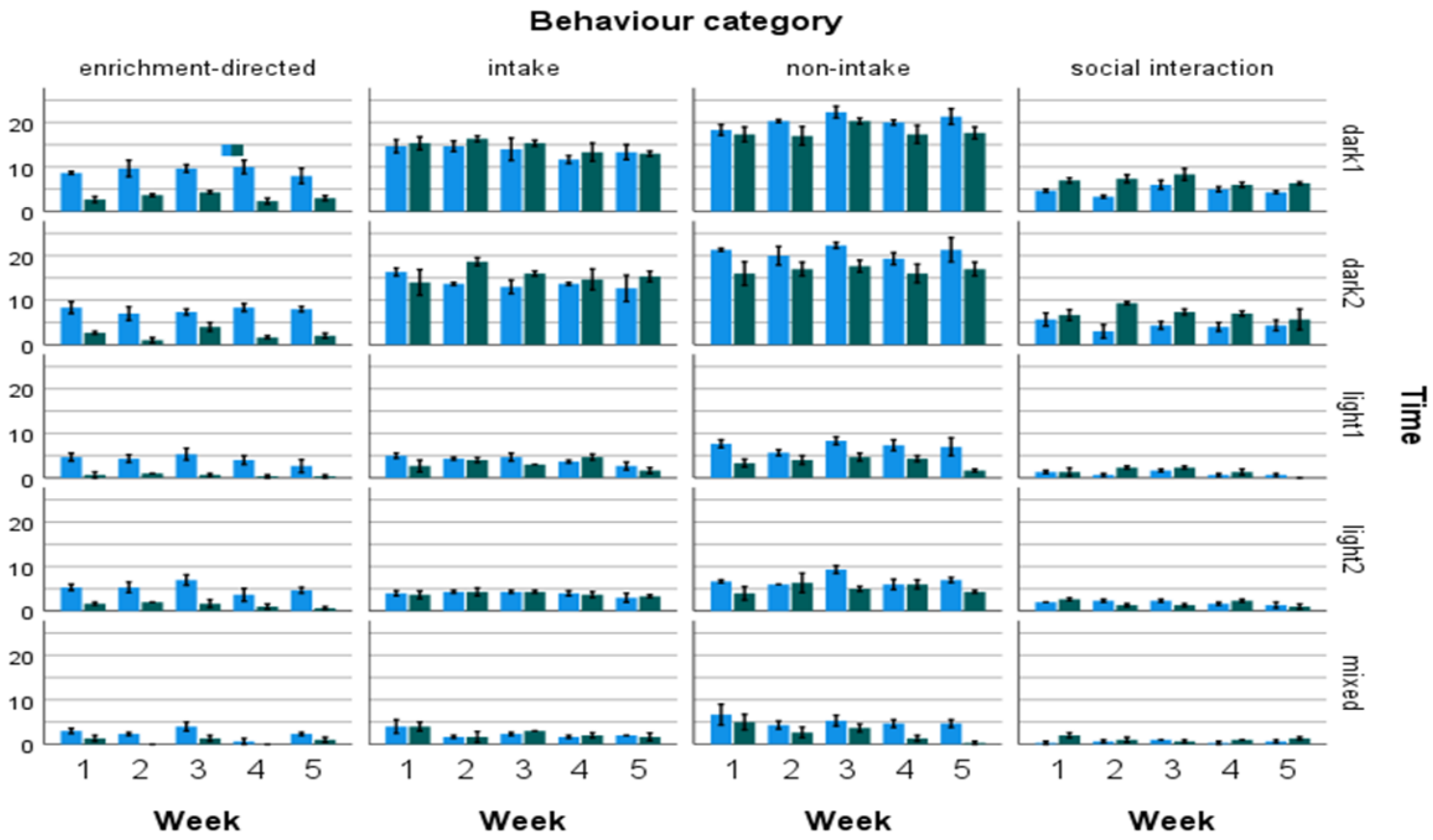

Figure 4. Simple bar combo showing weekly mean counts of behaviour scores by cage type. Blue represents the semi-natural cages and green represents the standard cages. Dark, light and mixed refers to the five 2 hour periods of the dark/light cycle when the observations were done, precisely two 2-hour periods during the dark cycle (dark 1 and dark 2), two 2-hour periods during the light cycle (light 1 and light 2) and one 2-hour period during transition between dark and light (mixed). Error bars: 95% CI ± 1SE.

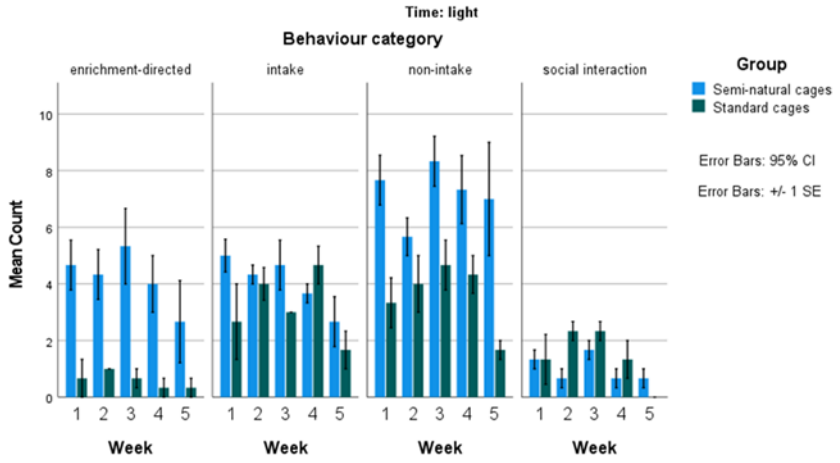

Figure 5. Simple bar graph of weekly mean counts of behaviour scores by cage type during light cycle of housing

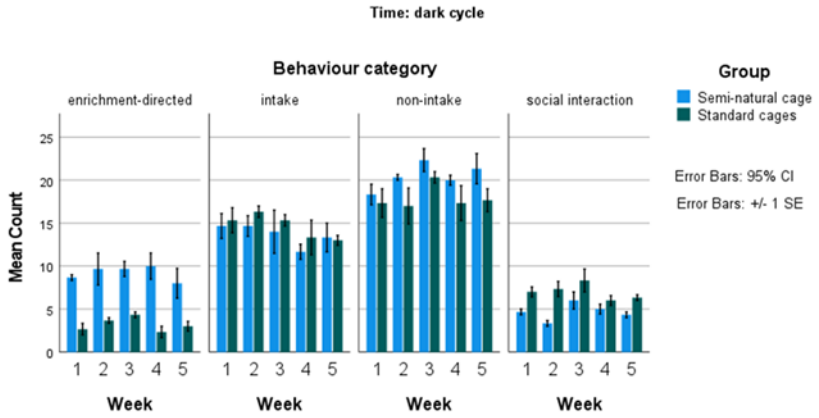

Figure 6. Simple bar graph of weekly mean counts of behaviour scores by cage type during dark cycle

4.2 Physical Health and Performance

Rats housed in semi natural cages had statistically significantly lower body weights (253±14.6g) at the end of the six week sampling period compared to rats in standard cages (265±18g), $t(16)=1.564$, $p=0.922$. The standard cage housed rats weighed 5%, or 12g, more than rats in semi-natural cages. Enriched and standard cage housed rats showed 10% and 15% increases in mean body weight, respectively, over the entire treatment phase (figure 7).

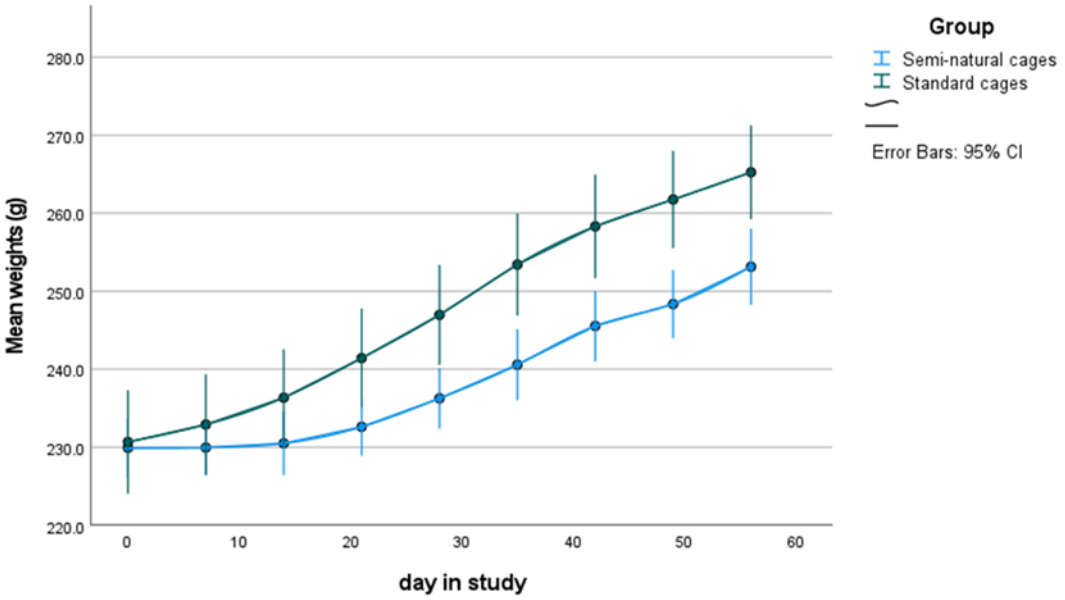

Figure 7. Scatter Plot of rats' weights (g) by day in study by Group

Weights of selected organs were expressed as absolute and relative weights (table 4 and figure 8). The absolute weights of the thymus glands (p = 0.949) and spleen (p = 0.425) of rats housed in standard cages were greater than those of rats in semi natural enriched cages, whilst the adrenal glands (p = 0.024) and brain (p = 0.864) absolute weights of rats housed in standard cages were smaller than those of rats in semi natural cages, but the weight differences were not statistically significant. However, no differences in weights of all organs were observed when they were expressed as relative weights of total body mass.

Table 4. The absolute and relative weights of selected organs of rats ± SE.

Housing	**brain**	**thymus gland**	**spleen**	**adrenal glands**
Standard cage absolute weight (g)	1.64±0.05	0.27±0.02	0.64±0.04	0.09±0.005
Semi-natural cage absolute weight (g)	1.65±0.03	0.27±0.02	0.59±0.02	0.11±0.006
Standard cage relative weight (g)	$6.279x10^{-3}$±0.00	$1.009x10^{-3}$±0.00	$2.357x10^{-3}$±0.00	$3.72x10^{-3}$±0.00
Semi-natural cage relative weight (g)	$6.263x10^{-3}$±0.00	$9.95x10^{-4}$±0.00	$2.388x10^{-3}$±0.00	$3.92x10^{-3}$±0.00

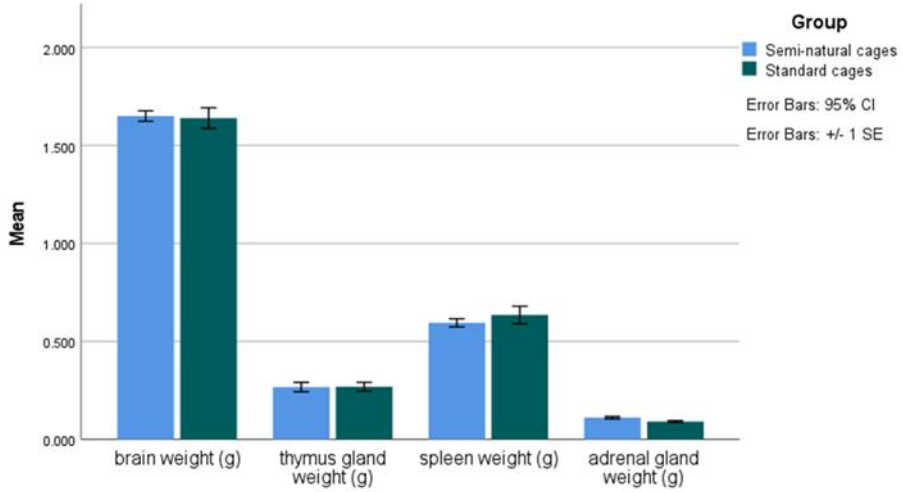

Figure 8. Simple bar of mean weights of brain, thymus gland, spleen and adrenal glands (g).

4.3 Faecal corticosterone metabolite (FCM) concentrations

The mean FCM concentrations of rats housed in the two cages types are shown in figure 9 and table 5. Rats housed in standard cages recorded high mean FCM concentrations for baseline values and during first week of the study (4.88± 0.877 and 6.59±2.614 µg/g DW), although the differences were not statistically significant (p=0.736 and p=0.303 respectively). This trend was followed by consistently high mean FCM concentrations in semi-natural cages for the rest of the study. The difference between the means were not statistically significant accept for the fifth week where the p-value was 0.034 (table 5). The results show increases of means FCM concentrations in both cage types compared to the baseline values, although the increase was not consistent overtime.

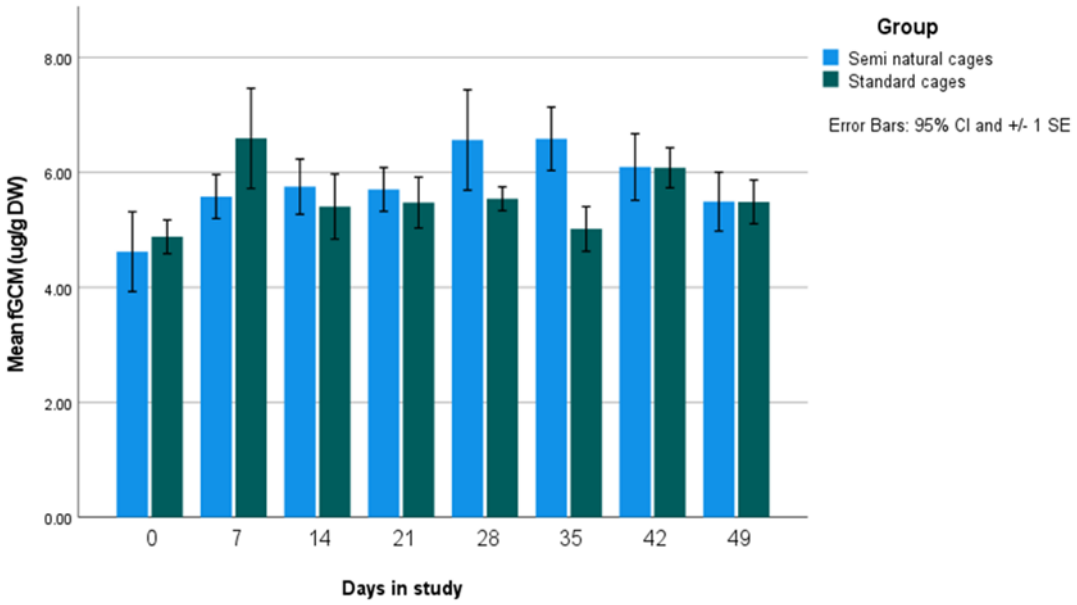

Figure 9. Simple Bar graph of the weekly mean FCM concentrations (µg/g DW) of rats housed in semi-natural and standard cages.

Table 5: Summary of results for descriptive statistics and *t* test for equality of means of faecal corticosterone concentrations of rats housed in semi natural cages and in standard cages.

Week in study	Group (cage type)	Mean FCM concentration (µg/g DW)	Standard deviation	T statistic	Degrees of freedom	p-value
Week 0 (baseline	Semi-natural cage	4.6222	2.08220	-0.344	16	0.736
concentrations)	Standard cage	4.8811	0.87713			
Week 1	Semi-natural cage	5.5800	1.14840	-1.065	16	0.303
	Standard cage	6.5933	2.61430			
Week 2	Semi-natural cage	5.7522	1.44233	0.466	16	0.648
	Standard cage	5.4067	1.69636			
Week 3	Semi-natural cage	5.7044	1.14369	0.392	16	0.700
	Standard cage	5.4756	1.32898			
Week 4	Semi-natural cage	6.5644	2.62215	1.138	16	0.272
	Standard cage	5.5422	0.62030			
Week 5	Semi-natural cage	6.5856	1.65487	2.324	16	0.034
	Standard cage	5.0178	1.16549			
Week 6	Semi-natural cage	6.0944	1.73368	0.020	16	0.984
	Standard cage	6.0811	1.04718			

Chapter 5: DISCUSSION

5.1 Introduction

This study had the aim of comparing the welfare conditions of female Sprague-Dawley rats housed in semi-natural enriched cages to those in standard cages. The idea was conceptualised from mounting evidence that standard cages deprive laboratory rats from expressing their species-specific behaviour (Modlinska *et al.,* 2015). Low cost complex caging system similar to pet rat cages were designed. The cages were furnished with different types of enrichment items that enable rodents to express behaviours such as burrowing, foraging, climbing, and exploration as is normally observed in their natural habitats. The belief was providing the rats with an enriched environment that mimic their natural habitat as found in pet cages would stimulate expression of species-specific behaviour, and promote good animal welfare. The enrichments added included increased cage height to allow for upright standing and climbing behaviours and adequate cage space for play and to enable addition of enrichment items. We added burrowing substrate (deep litter) to facilitate burrowing, and nesting material and shelters to facilitate hiding and to prevent or correct problems such as stereotype behaviour, aggression and over-grooming. Collectively the enrichments created a complex environment to promote thigmotactic behaviour and foraging opportunities, behaviours known to improve health or biological functioning, promote typical brain functioning and to enhance animals' capability to cope with captivity's stressors and challenges. Although the semi natural cage rack occupies larger space, it is economical for studies utilising large number of animals in saving much needed laboratory space. The larger size of the cage enclosure allows for housing of larger social groups of up to eight rats, hence a single cage rack has the capacity to house more rats (96 rats) compared to two cage racks of 20 standard cages (1500U Eurostandard type IV S) that house two rats per cage. The evaluation was undertaken knowing that some enrichment have proven to be harmful or stressful to the animals, as evidenced for example by the provision of mouse shelters which stimulate aggression related behaviour in male mice (Haemisch *et al*., 1994). Another adverse consequence that was considered in this study was the potential of enrichment to disrupt standardisation and reduce the precision and replicability of animal experiments.

5.2 Behavioural observations

The lower levels of social interaction behaviour observed in semi-natural enriched cages compared to standard cages may have been due to the lack of physical structures and play

space in standard cages. This implied that the chance of two or more rats coming into direct contact increased, attracting more interaction behaviour. On the other hand, provision of enrichment structures and larger enclosure space in semi-natural cages provided rats with an opportunity to escape allo-grooming and aggression episodes. Similar results have been previously reported by other scientists from both laboratory and pet rat studies, although data from pet rat studies are based on surveys rather than empirical studies (Abou-Ismail, 2011; Hutchinson *et al*., 2012; Neville *et al*., 2021; Neville, Mounty, *et al*., 2022; Tallent *et al*., 2018). In a study to determine the influence of environmental enrichment on social interaction of Sprague-Dawley rats Abou-Ismail (2011), reported statistically significantly lower levels of aggression and allo-grooming bouts in enriched cages compared to barren cages. In the study 48 rats where group housed in either cages enriched with several physical structures such as gnawing sticks, shelters, climbing structures (ladders and ropes) and toys (crawl and wood balls) or barren cages for 6 weeks. They attributed the lower levels of aggression and allo-grooming bouts in the enriched cages to the availability of physical structures, which provided the rats with opportunities to escape and hide from the encounters. Tallent and co-workers (2018) reported decreased aggression when they investigated the effects of adding physical barriers into mouse standard cages on aggressive behaviour. The physical barriers mimicked the mice's natural habitats without having to compromise cage area. Neville and colleagues' (2022) survey of United Kingdom pet rat owners to investigate welfare revealed common pet rat behaviours such as burrowing, foraging, exploration, and climbing, which standard laboratory cages and poorer pet rat housing restrict rats from expressing. They suggested modifications of the standard laboratory rat cage to facilitate similar behaviours. Prior to this, they had revealed differences in enrichment standards or requirements for pet and laboratory rat cages, in studies to develop guidelines for housing pet rats (Neville *et al*., 2021). Although these studies had notable limitations they again suggested elevating the laboratory housing standards.

We can also say the rats in semi-natural cages experienced improved welfare because the enrichment allowed them to satisfy their behavioural needs and exercise control over the environment. The rats in semi-natural cages retreated into shelters, climbed on structures provided and explored the various resources in the cage, behaviours that were not observed in standard cages. Rats in standard cages were frequently observed under the food hopper, an indication that they required some sort of shelter to hide. We are not sure if these rats were retreating or hiding from threats or if it was just a behavioural need or if these were

thermoregulatory responses. Feeding or drinking cage mates frequently disturbed these rats underneath the food hoppers and waterspouts, resulting in increased bouts of social interaction behaviour in the standard cages. The observation of rats under the food hopper and waterspouts is consistent with the sentiments by Abou-Ismail and Mahboub (2011), that laboratory rats are thigmotactic (edge-users), and utilise the majority of their inactive periods by laying in contact with physical structures such as surrounding walls or enrichment items in their environment. With rats being prey animals, said thigmotactic behaviour reduces the potential time that the rats have to look out for predator attacks and improves their ability to exert control over their environment (Simpson and Kelly, 2011).

The results revealed increased intake behaviour in the standard cages compared to the semi-natural cages. The lack of alternative activities in standard cages may have promoted this increased feeding and drinking behaviour. Whilst rats in semi-natural cages spent time exploring and engaging the various resources provided, those in standard cages could not do much more than just eating, drinking and sleeping. The scenario can be likened to the situation in zoos where animal enclosures are way too small compared to their natural habitat. For example the smallest wild territories of elephants are typically 60 to 100 times bigger than their zoo enclosures, because African elephants can roam over 10 000 km² in their natural habitat (Jacobs *et al*., 2022). Also in the wild, food resources for primates are spatially distributed and some have seasonal availability in a patchy fashion such that the animals have to move long distances in search of food, a behaviour deprived by space in captivity (Schwitzer and Kaumanns, 2003). The small enclosures in captivity (animal facilities and zoos) prevent animals from roaming long distances, natural foraging and freely interacting with conspecifics and their environment. As a result the animals end up just eating and sleeping out of boredom and may become obese.

5.3 Physical Health and Performance

Low body weight gains observed in semi-natural cages could be attributed to the increased physical activity of rats. This assumption is based on the availability of a combination of enriched environment and a larger area in the semi-natural cages. Both opportunities which are favourable to locomotory activity of rats and were absent in the standard cages. Similar results have been previously reported in enriched cages (Skalicky and Viidik, 1999; Augustsson *et al*., 2002; Spangenberg *et al*., 2005; Konkle *et al*., 2010). Suzuki and Machida (1995) revealed that even slightly increased levels of activity were efficient in preventing

excessive weight gain in rats. Increased intake behaviours observed in rats housed in standard cages also contributed to the higher body weight indices compared to rats housed in semi-natural cages. Standard cages commonly encountered in vivaria often lead to overweight animals due to *ad libitum* feeding and restricted opportunities for physical activity, which may affect both animal welfare and experimental outcome. Unfortunately, we did not measure feed intake and cannot make a conclusive statement that the difference in the weight indices was due to differences in exercise or in feed intake, or both. Spangenberg and co-workers (2005) measured feed intake and reported a weight difference of 14% between rats held in standard cages and rats held in large enriched cages with playpens after four weeks. Their feed intake was the same for the two cage types. Our findings of a weight difference of 5%, or 12g, between the two cage types also agreed with those of Skalicky and Viidik, (1999) who reported a weight difference of 9% between Sprague Dawley rats in enriched and standard cages. Pang and Hannan, (2013), also reported beneficial effects of physical activity in animal models of brain disorders such as improving cognition, delaying disease progression and enhancing cellular plasticity. Reports by Chamove, (1989), Tsai and colleagues (2003) and Abou-Ismail (2011) further support that increased physical activity results in healthier animals. They reported elevated body weight and weight gain of rats housed in enriched standard cages deprived of additional space compared to barren standard cages. It is likely that the addition of enrichment structures to the standard caging systems further decreased the enclosure area limiting the cage space for the rats to move around and exercise.

Whilst differences were observed in absolute weights, no differences in relative weights of all organs were found in our study. The result may indicate that animals in semi-natural cages were leaner (sign of good health) than those in standard cages, an area that will require investigation by comparing body fat. These results are similar to those from previous studies (Augustsson *et al*., 2002; Spangenberg *et al.,* 2005; Konkle *et al*., 2010). In their four week study to investigate the effects of pen housing on physical activity and fitness of Sprague Dawley rats Spangenberg and colleagues (2005) reported no differences in relative organ weights between enriched and standard cage housed rats. Earlier on Augustsson and co-workers (2002) had reported heavier hearts in enriched cages and no differences in other organ weights after housing Sprague Dawley rats in cages enriched with play pens for 10 weeks and attributed this to enlarged heart muscle as a result of increased physical activity

5.4 FCM measurements

Rats housed in standard cages recorded high FCM measurements during the first week, followed by consistently high concentrations in semi-natural cages for the rest of the study. The high FCM measurements observed in standard cages although not statistically significant was expected and can be attributed to the acute stress associated with regrouping and increasing the housing density of the rats (from two to four in our case). Even though group housing of rodents positively contributes to their welfare, it initially causes stress via confrontational encounters as the animals establish dominant-subordinate hierarchies (Rowland and Toth, 2019). We assumed that provision of enrichment created opportunities for rats in semi-natural cages to escape these adversarial encounters as evidenced by the low FCM measurements observed during the first week although this require further investigation. The consistently high FCM concentrations observed in semi-natural cages for the rest of the study may be attributable to the environmental enrichments we implemented, again another area that will require further investigation. Studies have reported an association between environmental enrichment and hypothalamic-pituitary-adrenal (HPA) and sympatho-adreno-medullary (SAM) axes activation in rodents (Augustsson *et al.*, 2002; Konkle *et al*., 2010; Rowland and Toth, 2019; Akalestou *et al*., 2020). This HPA and SAM activation in environmental enrichment animals do not indicate a bad experience but rather prepares the animals to engage with the novel environments. Precisely the HPA system in this situation has a physiological effect of releasing energy for physical activity by increasing blood glucose through gluconeogenesis. Augustsson and colleagues (2002) also reported higher plasma corticosterone and urine corticosterone/creatinine ratio in enriched cages compared to standard cages and attributed the increased values to higher activity levels in enriched cages. Their enriched cages where standard cages provided with play pens (3150cm² per rat). Konkle and colleagues (2010) in their work to compare the effects of rearing rats in standard cages and enriched cages on corticosterone levels following restraint stress, reported higher baseline plasma corticosterone levels on the sixth week in enriched cages. They housed both Sprague Dawley and Long Evans in either standard cages or three storey commercial ferret cages enriched with a running wheel, climbing rope, cloth hammock and several toys, for six weeks. One limitation of our study was that the oestrous cycle was not recorded in the rats. The nadir and peak values of corticosterone of female rats vary with the oestrous cycle. Precisely corticosterone concentrations are low during oestrus and rise progressively from metestrus to proestrus, such that the concentrations at proestrus can be two or three-fold

compared to those at oestrus or metestrus (Cavigelli *et al*., 2005). Other limitations to this study that will also need consideration in future studies include use of a single age group, weighting the feed/determining feed consumption, and weighting the heart. This could assist in correlating the data with other published data.

5.5 General conclusion

Collectively the results show that animals in semi-natural cages assessed in this study expressed normal rat behaviour and showed increased natural locomotory activity. These characteristics are known to result in healthier animals with improved welfare. The findings support the need for re-evaluation of the current laboratory rat housing standards to provide better environments in terms of animal welfare. We also recommend use of semi-natural cage housing when room space and the study parameters allow for their use. An unexpected finding in the study was elevated faecal steroid concentration in the animals in the semi-natural cages, which will require further investigation.

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ADDENDUMS

Addendum 1; Ethical clearances

Faculty of Veterinary Science
Animal Ethics Committee

11 January 2021

Approval Certificate
New Application

AEC Reference No.: REC022-20
Title: A comparison of home cage behaviour, growth parameters and faecal glucocorticoid metabolites of Sprague Dawley rats group housed in semi-naturalistic and convectional standard cages.

Researcher: Dr R Mavunganidze

Student's Supervisor: Prof V Naidoo

Dear Dr R Mavunganidze,

The **New Application** as supported by documents received between 2020-08-17 and 2020-12-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-12-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Sprague Dawley Rats	24
Samples Faecal sample collection	 Weekly (including acclimation period)
Home cage observations	Digital Video Recorder

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-01-11.

3. Please remember to use your protocol number (REC022-20) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.

6. As part of your approval, the committee requires that you record **a short video footage** of major animal procedures approved in your study. The committee may request them for monitoring purposes at any later point.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Dr Heike Lutermann
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 04 November 2020

CONDITIONALLY APPROVAL

Ethics Reference No **REC022-20**

Protocol Title **A comparison of home cage behaviour, growth parameters and faecal glucocorticoid metabolites of Sprague Dawley rats group housed in seminaturalistic and convectional standard cages.**

Principal Investigator Dr R Mavunganidze
Supervisors Prof V Naidoo

Dear Dr R Mavunganidze,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC022-20) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTE: Conditionally approved (pending obtaining other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN

Chairperson: Research Ethics Committee

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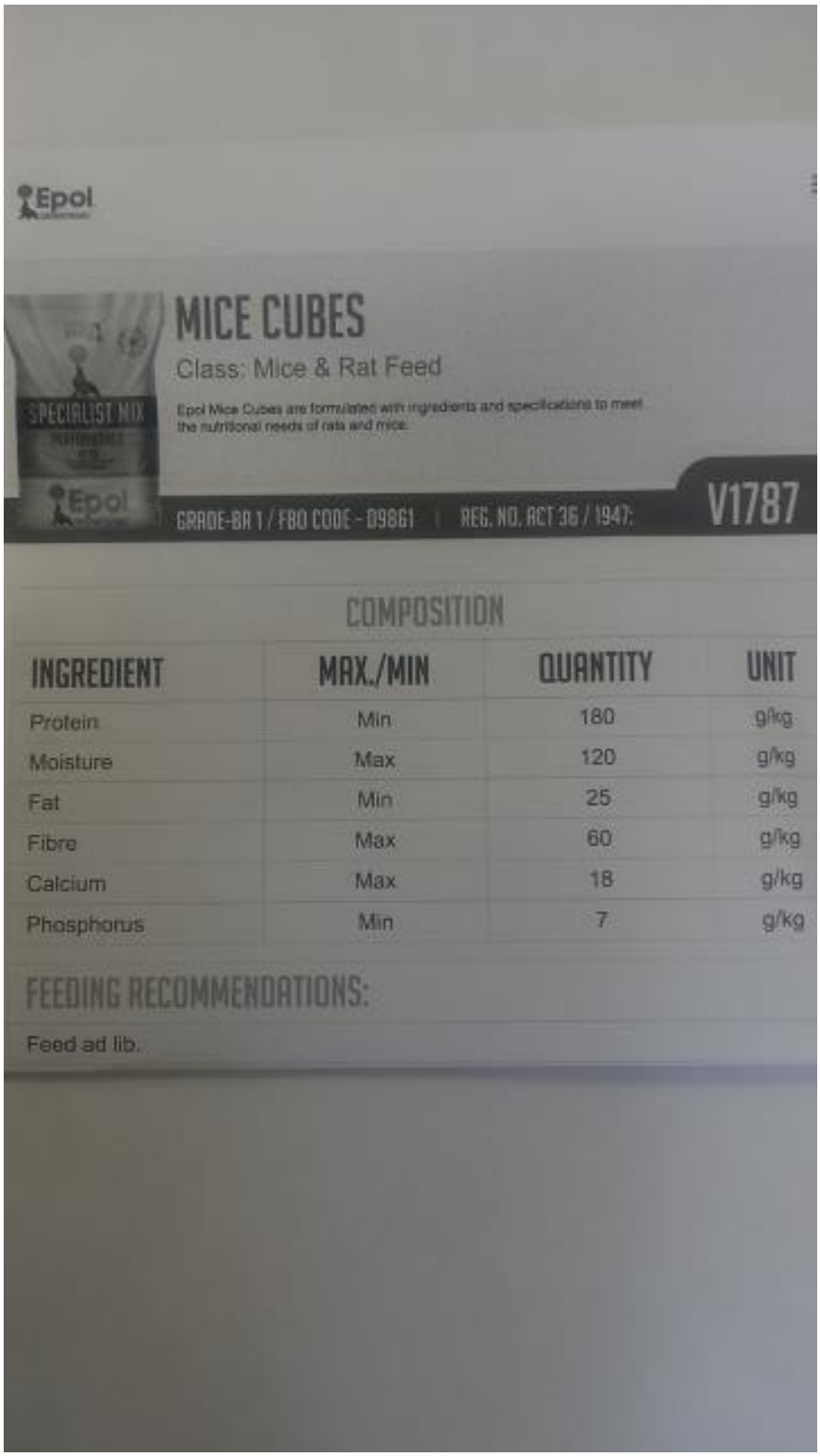
Addendum 2; Feed composition



Epol

MICE CUBES

Class: Mice & Rat Feed

Epol Mice Cubes are formulated with ingredients and specifications to meet the nutritional needs of rats and mice.

GRADE-BR 1 / FBO CODE - 09861 | REG. NO. ACT 36 / 1947: V1787

COMPOSITION

INGREDIENT	MAX./MIN	QUANTITY	UNIT
Protein	Min	180	g/kg
Moisture	Max	120	g/kg
Fat	Min	25	g/kg
Fibre	Max	60	g/kg
Calcium	Max	18	g/kg
Phosphorus	Min	7	g/kg

FEEDING RECOMMENDATIONS:

Feed ad lib.

Addendum 3; Behaviour recording forms

Data recording sheet for focal observations REC 022-20

Date:

Cage number:

Observation number and time:

Time interval	Behaviour code	Number of animals engaged	duration	Frequency of behaviour	Comments

Data recording sheet for scan observations REC022-20

Date:

Cage number:

Scan number and time:

	Scanning time intervals (minutes) to show number of animals engaged in a behaviour state											
	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100-110	110-120
Behaviour codes	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
	Scanning time intervals (minutes) to show number of animals engaged in a behaviour state											
	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100-110	110-120
Behaviour codes	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
	Scanning time intervals (minutes) to show number of animals engaged in a behaviour state											
	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100-110	110-120
Behaviour codes	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												

Foot notes (comments):

Addendum 4; Ethograms

Ethogram for behavioural components recorded by scan sampling REC022-20

Behavioural category		**Behavioural component**	**Description**	**Code**
A	Active	-Feeding and drinking -Non-intake maintenance -Movement activities -Exploratory and enrichment directed behaviour	-Eating food and drinking water -Self-grooming and pandiculation (stretching and yawning) -Movement and/or climbing the cage lid -Sniffing cage wall, cage top and air outside the cage and manipulating the enrichment objects.	**A**
B	Inactive	-Sleep or awake non-active	-Lying unalert with both eyes closed/open. Stationary and sitting in one place.	**B**

Ethogram for specific behavioural components recorded by focal sampling REC022-20

Component		**Description**	**Code**
C	Intake behaviour	-Feeding, eating faeces and drinking	C
D	Non-intake and explaratory behaviour	-Self-grooming, and pandiculation (stretching and yawning) -Movement and/or climbing the cage lid -Sniffing cage wall, cage top, and sniffing air outside the cage.	D
E	Enrichment-directed behaviour	-Chewing, climbing and manipulating the enrichment objects. -Bedding material manipulation behaviours such as digging and pushing or pulling bedding material. The animal tries to bury its body, or part of it, as to be, partly inside the bedding material, or completely under it	E
F	Social interaction	-Allo-grooming given and received, aggression, biting given and chasing given or defence, biting received and chasing received	F

Addendum 5; Corticosterone metabolite determination procedure

ENZYME IMMUNO ASSAY

1) General:

We use a competitive enzyme immunoassay method to measure steroid concentrations in faecal extracts.

2) Enzyme Immunoassay, day 1:

Defrost and dilute faecal extracts according to assay requirements.

Defrost standard stock, quality controls (QCs), antibody, biotin-labelled steroid, and IgG coated 96-well microtiter plate.

Prepare serial dilutions of the standard stock with assay buffer as specified in the protocol.

Dilute antibody and biotin-labelled steroid with assay buffer as specified in the protocol.

Wash the IgG coated 96-well plate 4 times with 300 µl washing solution per well, pat dry.

Add 50 µl assay buffer, standards, QCs and diluted sample extracts into the respective wells as specified in the protocol.

Add 50 µl biotin-labelled steroid solution to every well.

Add 50 µl antibody solution to every well except for the blanks, add 50 µl assay buffer to the blanks.

Cover 96-well plate with cling wrap, mix contents gently without spilling, store at 4°C overnight.

3) Enzyme Immunoassay, day 2:

Defrost streptavidin-POD (20 µl aliquot), and mix with 16 ml cold assay buffer.

Discard contents of the 96-well plate, and wash as described above, pat dry.

Add 150 µl streptavidin-POD solution to every well, and cover the plate with cling wrap.

Incubate the 96-well plate at 4°C with gentle shaking for 45 min.

Discard contents of the 96-well plate, wash as described above, pat dry.

Mix 250 µl TMB with 17 ml cold substrate using solution directly before use.

Add 150 µl substrate solution to every well, and cover the plate with cling wrap.

Incubate the 96-well plate at 4°C with gentle shaking until the maximal optical density is ~ 1.0.

Stop the enzyme reaction by adding 50 µl H_2SO_4 (2M) per well, mix gently.

Read the plate at 450 nm and 630 nm, using the software provided (Gen5).